UNIVERSITY OF WARWICK
DEPARTMENT OF BIOLOGICAL SCIENCES

STUDIES OF THE HYDROXYLASE COMPONENT OF SOLUBLE METHANE MONOOXYGENASE FROM *Methylococcus capsulatus* (Bath)

A thesis submitted in partial fulfilment of the requirements for the degree of D. Phil.

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To my husband yongfeng
and my son lingxi

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SUMMARY

Methane monooxygenase catalyses NAD(P)H_2- and O_2-dependent oxidation of methane to methanol and this reaction initiates the metabolic pathway that supplies the total carbon and energy needs of methanotrophic bacteria. The soluble methane monooxygenase isolated from *Methylococcus capsulatus* (Bath) is comprised of three proteins: a hydroxylase (*Mr.* 250 kDa), a reductase (*Mr.* 38.6 kDa) and a regulator protein (named protein B, *Mr.* 16 kDa), all three of which are required for enzymatic activity. The hydroxylase is comprised of three polypeptides (α, 60.6 kDa; β, 45.0 kDa; γ, 19.8 kDa) of $\alpha_2\beta_2\gamma_2$ stoichiometry and contains a non-heme binuclear iron active centre responsible for methane hydroxylation and a wide variorty of hydrocarbon oxidations. The reductase transfers electrons from NADH to the hydroxylase, and protein B appears to have several regulatory activity.

Characterisation of oxygen donor for soluble methane monooxygenase has shown that oxygen is necessary for sMMO-catalysed oxygenation but the high oxygen concentrations result in a decrease of sMMO activity since toxic oxygen species, superoxide anion or peroxide ion, appeared in the reaction system under high oxygen concentration conditions. Characterisation of alternative donors, NO and N_2O, demonstrated that they are not suitable for sMMO. Also, the designed experiments to encourage sMMO to catalyse radical recombination reactions were not successful either using non-natural donors or mimicing the conditions used for chemical oxidative coupling. These studies may indicated that upgrading of methane by the native sMMO complex would be impossible.

Hydrogen peroxide can replace protein B, the reductase, oxygen and NADH in activation of the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath) during catalysis of the oxidation of hydrocarbons. Hydrogen peroxide activation of the hydroxylase occurs at the active site iron atoms. The O atom derived from H_2O_2 is transferred to the substrates and decomposition of peroxide to O_2 does not occur in the reaction. The homolytic cleavage of Fe bound O-O- pathway is favoured in the H_2O_2-driven system and hydroxyl radicals may be involved in the reaction cycle. Protein B may not only be in controlling electron flow in the sMMO system, but may also be connected with O_2 binding to the active site.

Proteolysis of the hydroxylase showed that the hydroxylase could be degraded by chymotrypsin and trypsin, but chymotrypsin could greatly degrade the protein resulting in a loss of protein activity. However, even when using a high concentration of trypsin to cleave the hydroxylase, a high percentage of the catalytic activity was still observed. Proteolysis of the hydroxylase by chymotrypsin or trypsin demonstrated that the iron atoms in the hydroxylase active site played an important role in the oxidation of hydrocarbons and that the certain structure of the hydroxylase protein were all necessary for enzyme activity.

Chemical modification of the hydroxylase was also studied and the results showed that the hydroxylase protein thermostability could be raised by using crosslinking reagent, polyoxyethylene bis(imidazolyl carbonyl) and that the the PEG-modified hydroxylase could show activity in several organic solvents when H_2O_2 was used to provide oxygen and electron equivalents.

ABBREVIATIONS

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
CAT	catalase
DNase	deoxyribonuclease I
DTT	D,L-dithiothreitol
EDTA	ethylenediaminetertraacitic acid
ε	extinction coefficient
FPLC	fast protein liquid chromatography
GC	gas chromatography
Gif system (Gif, GO, GoAgg, GoChAgg)	G is for Gif-sur-Yvette; O is for Orsay; Agg is for Aggieland, Texas A&M, Ch is for Chernogolovka, Russia.
Gln	glytamine
Glu	glutamic acid
Gly	glycine
His	histidine
HPLC	high performance liquid chromatography
Ile	isoleucine
KO_2	potassium superoxide
Leu	leucine
Lys	lysine
Met	methionine
MOPS	3-(N-morphlino)propane sulfonic acid
NADH	diphosphopyridine nucleotide
PAGE	polyacrylamide gel electrophoresis
PEG	methoxypolyethylene glycol
PhIO	iodosobenzene
pMMO	particaule methane monooxygenase

PMSF	phenylmethylsuphonyl fluoride
Phe	phenylalanine
Pro	proline
Ser	serine
SDS	sodium dodecyl sulphate
sMMO	soluble methane monooxygenase
SOD	superoxide dismutase
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
t.l.c.	thin layer chromatography
TNBS	2,4,6-trinitrobenzenesulfonic acid
Tris	Tris(hdroxymethyl)aminomethane
Val	valine

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CHAPTER 1
INTRODUCTION

1.1 Methane conversion and activation

Methane is the major constituent (up to 97%) of natural gas. It is well known that natural gas is found in many different areas of the world and is an abundant resource with world reserves in excess of 10^{14} m^3 (Burch and Parkyns, 1992). The chemical industry uses about 7% of the annual world consumption of natural gas and the remainder is used as fuel. At the current rate of use these reserves will last for 60 years compared with 30 years for crude oil (Burch and Parkyns, 1992). Newly discovered sources of crude oil, which currently provides nearly 98% of basic organic chemical materials and 70% energy fuel, are on the decline. If methane could be used efficiently, it could replace projected dwindling petroleum reserves in the 21st century. Research emphasis, therefore, has now been directed at the utilisation of natural gas and the activation of methane, in particular as a source of chemicals and fuels. The current reserves of natural gas are considered as a large under-utilised energy resource because a large proportion of these reserves are remotely located from high densities of consumers. This situation, coupled with the gaseous state and low boiling point of methane (-161.5°C), makes this resource too expensive to transport. An obvious solution is the conversion of methane into efficiently transportable liquid materials, such as methanol or liquid hydrocarbons, at the remote site before transportation. Also, vast quantities of natural gas are currently flared, particularly at locations where the gas is associated with crude oil, hence, chemical conversion could be both economically and environmentally beneficial.

Methane is a very unreactive molecule, as demonstrated by the high C-H bond strength [D(C-H) = 438.8 KJ/mol], high ionisation potential (12.5 eV), low proton affinity (4.4 eV), and low acidity (pKa = 48) of the molecule. The conversion of methane into more valuable chemical products (Cover and Peterson, 1988; Green and Ramanathan, 1988), e.g. methanol and ethane, is not a new research topic. A large amount of pioneering research has been done in the 1920s and 1930s in which the direct conversion of methane and the partial oxidation reaction were investigated as a high pressure gas phase reaction (see review by Gesser et al., 1985). Here, Figure 1-1 briefly describes the chemical processes for the production of base, intermediate and fine chemicals from methane (Keim, 1986). In a few processes methane is directly converted into desired chemicals, such as halomethanes, hydrogen cyanide, acetylene, carbon black and carbon disulfide, in which methane is activated either at very high temperatures or by the halogen radicals. Most chemicals derived from methane are indirectly converted via a syngas process. Such processes have been developed, but current technology is based on the high-temperature (700-900°C), energy-intensive conversion of methane and water to carbon monoxide and hydrogen (eq 1-1) which is not a thermodynamically efficient reaction.

$$CH_4 + H_2O \xrightarrow[700\text{-}900^{\circ}C,\ 1\text{-}25\ bar]{15\text{-}20\%\ Ni\ catalyst\ on\ Al_2O_3\ or\ SiO_2} CO + 3H_2 \qquad (1\text{-}1)$$

This reaction is very endothermic (ΔG°_{1000K} = -27.1 KJ/mole, ΔH°_{1000K} = 225.1 KJ/mole), and as such the requirement of complex equipment is essential to perform the reaction. Another disadvantage is that the ratio of H_2/CO in the resulting gaseous mixture is too high to carry on the production of oxygenates *in situ*. So that several other catalytic steps are required to effect its conversion into methanol, for example:

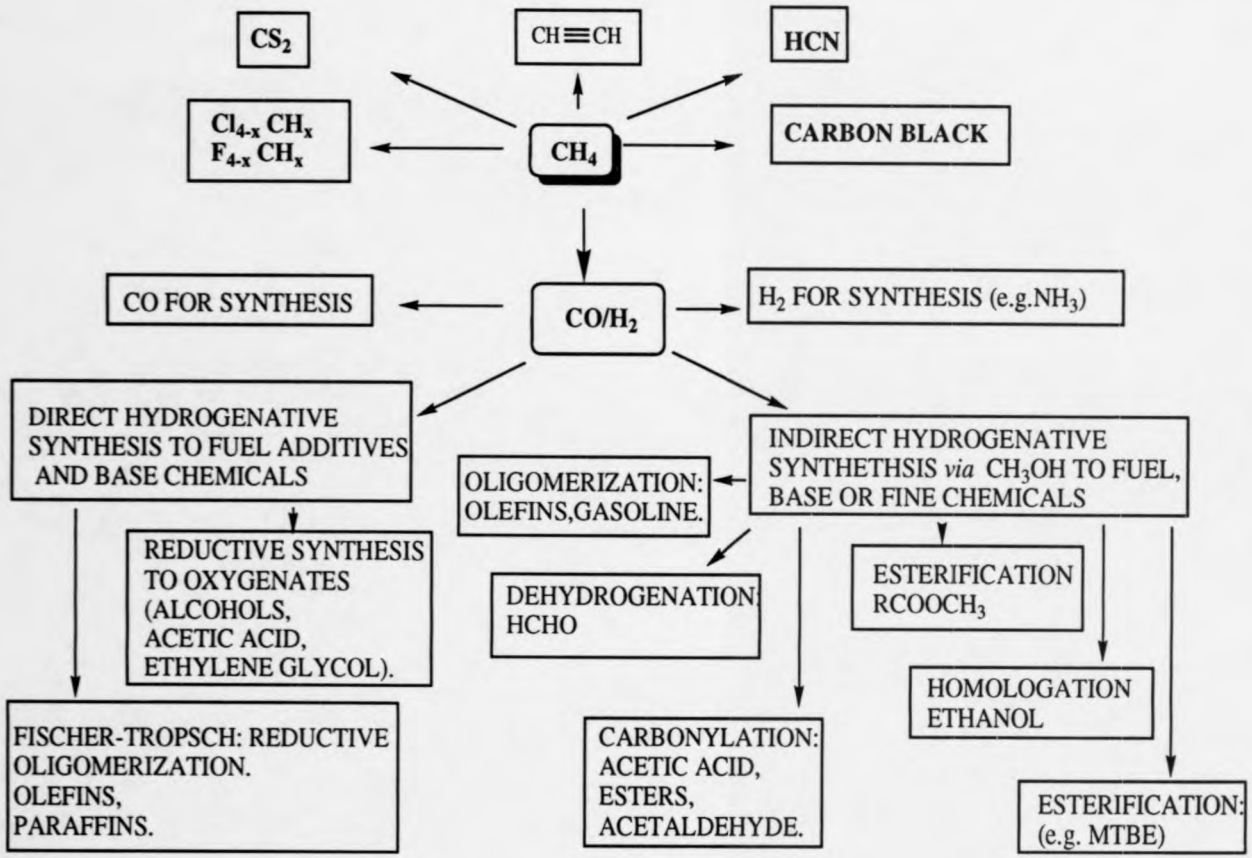


Figure 1-1 Conventional use of methane for base, intermediate and fine chemicals

$$CO_2 + H_2 \xrightarrow{\text{Ni catalyst}} CO + H_2O \qquad (1\text{-}2)$$

$$CO + 2H_2 \xrightarrow[250\text{-}280^oC,\ 70\text{-}110\text{bar}]{\text{Cu/Zn catalyst}} CH_3OH \qquad (1\text{-}3)$$

Methanol is a valuable product, either as a chemical feedstock or as a fuel. The one-step conversion of methane to methanol by chemical catalysts is a challenging research problem. A small proportion of research effort has been made to focus on the production of methanol (Foster, 1985; Pitchai and Klier, 1986; Burch *et al.*, 1989; Periana *et al.*, 1993) with limited success. For example, the research in Reading, in collaboration with British Gas, reported that 'catalysts' for this reaction probably have little effect other than to modify slightly the homogeneous gas phase reactions that occurred at moderate temperature (*ca* 400°C) and pressure (>10 bar) when methane and oxygen were brought into contact in a glass-lined tube (Burch *et al.*, 1989). The research also showed that the selectivity for methanol production could be high, but the maximum yield of methanol, defined as the percentage of methane converted in a single pass into methanol, was about 2% - much too low to form the basis of an economic process. Recently, a mercury ($Hg^{(II)}$)-catalysed the oxidation of methane to methanol showed that the reaction could occur at 180°C with reagents that could potentially be used on a large scale (Periana *et al.*, 1993). The oxidation of methane was through an electrophilic displacement reaction and the production of the monomethyl ester of sulfuric acid, methyl bisulfate, as a methanol precursor. The ~43% yield of methanol was achieved, but the major side product was carbon dioxide.

By far the majority of research has been carried out into the oxidative coupling of methane using oxide catalysts to form higher hydrocarbon products, mainly ethane and ethene. The oxidative coupling of methane to ethane and ethene is the first step in the direct synthesis of higher hydrocarbons, because ethane can react further and

oligomerisation catalysts are now being developed for converting ethane to liquid hydrocarbons. Oxidative coupling of methane to ethane, and preferably ethene, can occur on a wide range of different oxide catalysts, such as $BaCO_3/La_2O_3$, Li/MgO, N_2O, and a number of molybdate compounds and oxychloride catalysts (Ito and Lunsford, 1985; Helton *et al.*, 1988; France *et al.*, 1988; McCarty *et al.*, 1988; Kiwi *et al.*, 1990; Olsbye *et al.*, 1991; Swaan *et al.*, 1991; Burch *et al.*, 1991; Otsuka *et al.*, 1991), and selectivities of *ca* 60% at a methane conversion of 35% have been achieved. This process has attracted industrial interest in upgrading methane to higher hydrocarbons, but there are still many process engineering difficulties. The methodologies of activating methane for this purpose, so far reported, could be divided into three categories (Figure 1-2). First, methane is activated by the radicals which could be generated by a high temperature (the pyrolysis temperature of methane normally exceeds 1270°K) or by other radicals, such as halogen. The second type of methane activation occurs via oxidative addition of methane to a low valent metal cation. The third way of activating methane is the electrophilic attack by a metal atom. The first method is a high energy demanding process, apparent activation energies range from 100 to 250 KJ/mol, resulting in a high cost for the industrial production. Metal catalysts are involved in the second and the third methods, but selective reaction of methane in high yield is very difficult. Also, deactivation of the catalysts and the understanding of the nature of the active site of the catalysts are always a troublesome problem for the scientists.

As described above, a lot of hard work has been done to develop a convenient chemical process to convert methane to other general organic chemicals, unfortunately so far none of them showed the promising advantage in their technological and economic aspects. There is a strong need for alternative technologies which could complete this conversion using mild and hence economic conditions. It is well understood that most organic materials are ultermately degraded to methane (which is an end product of the anaerobic decay of plants, animals and other complicated

PYROLYSIS

$$2CH_4 \xrightarrow{>800^{\circ}K} HC{\equiv}CH + 3H_2$$

$$6CH_4 \xrightarrow{1500^{\circ}K} C_6H_6 + 9H_2$$

OXIDATIVE COUPLING

$$CH_4 + O_2 \xrightarrow[\substack{850\text{-}1150^{\circ}K \\ CH_4\text{: }0.001\text{-}0.09MPa \\ O_2\text{: }0.0003\text{-}0.03MPa}]{\text{Oxide catalysts}} CH_2{=}CH_2 + CH_3{\cdot}CH_3 \text{ etc.}$$

ALKYLATION

$$CH_4 + CH_2{=}CH_2 \xrightarrow[343K]{TaF_5} CH_3{-}CH_2{-}CH_3$$

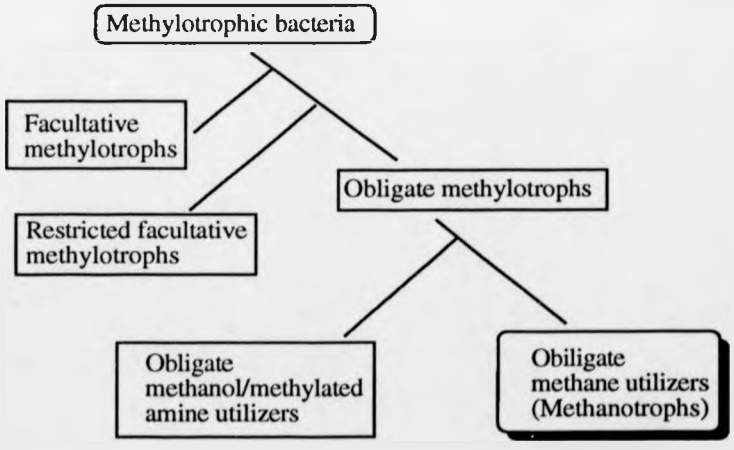
Figure 1- 2 Ccatalytic conditions for upgrading methane into higher n-alkanes or alkenes

molecules) and carbon dioxide, so why not use biological methods to convert methane to high grade organic materials particularly since bacteria can grow on methane? This stimulated scientists to look for a directly biological process to convert methane into methanol or higher hydrocarbons. Undoubtedly, this biological process could bring about an impotant breakthrough in the utilisation of natural gas and may be achieved catalytically via a number of different routes.

1.2 C_1-utilising organisms

1.2.1 Methylotrophic bacteria

Methylotrophic bacteria are a very diverse group of organisms united by a common physiological ability to obtain energy by utilising one-carbon (C_1) compounds for growth. These could be either methane, methanol, formic acid, methylamines, carbon monoxide, cyanide or methylated sulphur species. In 1892 Leow was the first to isolate and to identify a methylotrophic bacterium (Quayle, 1987; Hanson, 1992). Since then many methylotrophic bacteria have been isolated and characterised. The major genera of methylotrophic bacteria were subdivided as follows (Green, 1992):



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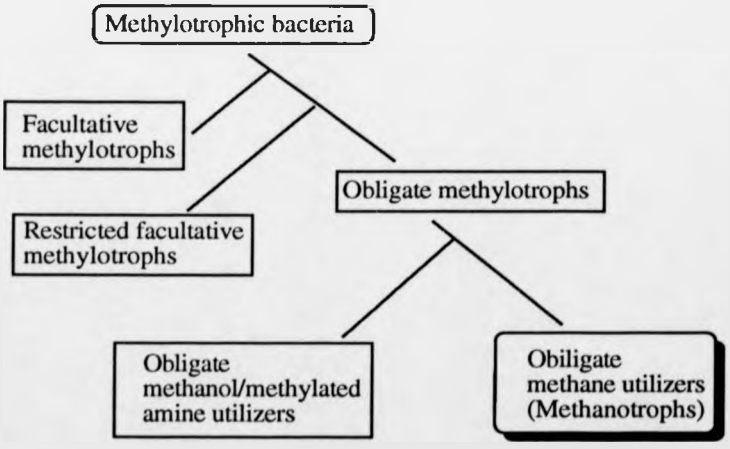


Of these the facultative methylotrophs not only utilise C_1 compounds as an energy source but also are able to grow on multicarbon compounds. However, the restricted facultative methylotrophs can only utilise a relatively narrow range of multicarbon compounds. The obligate methylotrophs, which are rod-shaped Gram-negative organisms, only have the ability to utilise C_1 compounds such as methane, methanol or methylamine as the sole carbon and energy source (Colby *et al.*, 1979). The obligate methylotrophs consist of two groups of microbes, the methane utilizers (methanotrophs), which can also grow on limited amounts of methanol; and the methanol/methylated amine utilizers. Interest in oxidising methane to CO_2 by methanotrophs and the role played by methane as a potent "greenhouse" gas have greatly stimulated scientists in recent years to study methane metabolism by these organisms as a possible solution to the removal of methane from the atmosphere (CH_4 is 17 times more potent than CO_2 as a greenhouse gas).

1.2.2 Methanotrophs

Methanotrophs occur in aerobic environments where methane is available and establish a vital part of the cycle of methane in nature. The characteristics of methanotrophs are that all such organisms possess the ability to utilise methane as sole carbon and energy source, and all strains have a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor (Green, 1992). The enrichment and isolation of methanotrophs from different freshwater and marine environments have been reported on numerous occasions. The first methane- oxidising bacterium, named *Bacillus methanicus*, was isolated by Sohngen in 1906. Later this bacterium was renamed *Methanomonas methanica* by Orla-Jenson in 1909 and subsequently *Pseudomonas methanica* by Dworkin and Foster in 1956 (Hanson, 1992). Since then, several strains of methane-utilising bacteria, for example, *Pseudomonas methanitrificans* (Davis *et al.*, 1964), *Methanomonas methano-oxidans* (Brown *et al.*, 1964) and *Methylococcus capsulatus* (Foster and Davis, 1966) were reported.

An outstanding contribution was made by Whittenbury and his co-workers in 1970, who isolated and compared over 100 strains of methanotrophs and classified them into five groups (or genera): *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylosinus* and *Methylocystis* (Whittenbury *et al.*, 1970). The assignment of the methanotrophs into five groups (or genera) was based on morphology, resting stages formed, intracytoplasmic membrane structures and some physiological characteristics (Whittenbury *et al.*, 1970; Whittenbury and Dalton, 1981). Furthermore, the methanotrophs were also divided into three types, I, II and X, based on the major carbon assimilation pathway, DNA base composition and resting stages formed etc. The type I and X assimilate formaldehyde via the ribulose monophosphate pathway, whereas the type II organisms utilized the serine pathway (Lawrence and Quayle, 1970). The type X, genera of *Methylococcus capsulatus*, is the only group of methanotrophs capable of autotrophic CO_2 fixation (Taylor *et al.*, 1980; Stanley and Dalton, 1982).

The difference between the methanotrophs and other C_1-utilising organisms is that the methanotrophs possess methane monooxygenase. The enzyme is capable of oxidising methane to methanol, which is then subsquently oxidized to formaldehyde. The route of methane metabolism due to the methanotrophs under aerobic conditions is described in Figure 1-3.

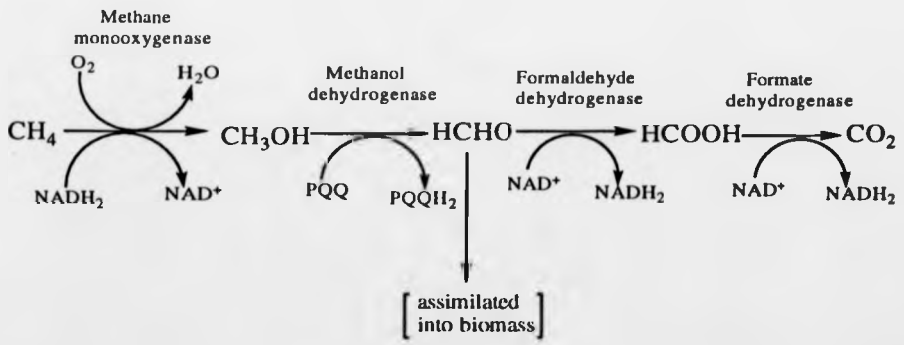


Figure 1-3. The pathway of methane oxidation.

1.3 General properties of methane monooxygenase

1.3.1 Determination and analysis of methane monooxygenase

Methane monooxygenase (MMO) (EC. 1. 14. 13. 25) is the enzyme that catalyses the initial step in the assimilation of methane in the methanotrophs.

The involvement of an oxygenase in the initial oxidative attack on methane by methanotrophs was suggested by Leadbetter and Foster (1959), based on the incorporation of ^{18}O from $^{18}O_2$ into the cellular constituents of *Pseudomonas methanica*. Further evidence for the involvement of this oxygenase in the oxidation of methane was provided by Higgins and Quayle (1970) who showed that $CH_3{}^{18}OH$ as the product of methane oxidation was isolated when suspensions of *Pseudomonas methanica* or *Methanomonas methanooxidans* were allowed to oxidise methane in ^{18}O-enriched atmospheres. The further observation of methane oxidation catalyzed by cell-free extracts of *Methylococcus capsulatus* (Ribbons and Michalover, 1970; Ribbons, 1975) or *Methylomonas methanica* (Ferenci, 1974) suggested that the oxygenase responsible for the methane oxygenation was a monooxygenase which catalysed the incorporation of 1 atom of oxygen into the substrate.

However, at that time only indirect methods, which measured methane-stimulated NADH disappearance spectrophotometrically or methane-stimulated O_2 disappearance polarographically (Ribbons and Michalover, 1970; Ferenci, 1974; Ribbons, 1975), were available as an assay for methane monooxygenase. In 1975, a convenient and rapid method for assaying methane monooxygenase was reported (Colby *et al.*, 1975) in which the disappearance of a soluble derivative of methane, bromomethane, was determined directly via gas chromatography. The extracts from *Methylomonas methanica* catalysed the O_2- and NAD(P)H-dependent disappearance of bromomethane. In these experiments the bromomethane monooxygenase was membrane bound (particulate) and was inhibited by metal-binding reagents, by some metal ions and by acetylene. These results with inhibitors resembled those obtained by Hubley *et al.* (1975) with methane-oxidizing bacterial suspensions of *Methylosinus*

trichosporium suggesting that the bromomethane monooxygenase activity measured in vitro was identical with the enzyme responsible for methane oxidation in whole bacteria. In 1976, analysis of the soluble extracts from *Methylococcus capsulatus* (Bath) confirmed that methane monooxygenase activity could be measured either by following bromomethane disappearance or by measuring methanol accumulation via gas chromatography (Colby and Dalton, 1976). In 1977, it was reported that soluble methane monooxygenase from *Methylococcus capsulatus* (Bath) catalysed a variety of hydrocarbon oxidations including methanol, carbon monoxide, alkanes, alkenes, ethers, cyclic alkanes and aromatic compounds (Colby *et al.*, 1977). It was found that propylene was a preferred substrate because the product of its oxidation, epoxypropane, was not susceptible to further oxidation by methane monooxygenase. Thus, the standard method for following MMO activity is direct gas chromatographic measurement of epoxypropane formation.

1.3.2 Particulate methane monooxygenase (pMMO)

During the 1970's studies of MMO obtained from several methanotrophs revealed that the enzyme existed in two forms differing in cellular location. Soluble methane monooxygenase (sMMO) was found in the cytosolic fraction of the bacteria and particulate methane monooxygenase (pMMO) was found in the cellular membranes. Ribbons and Michalover (1970) were the first to report a cell-free methane-oxidizing activity that was associated with the particulate fractions of extracts from *Methylococcus capsulatus*. Ferenci (1974) and Colby *et al.*, (1975) also observed the particulate activity in extracts from *Methylomonas methanica*. Tonge *et al.* (1975, 1977) and Patel *et al.* (1979) reported that the enzyme from *Methylosinus trichosporium* OB3b was associated with the particulate fraction of cell extracts. However, the enzyme system from *Methylococcus capsulatus* (Bath) (Colby and Dalton, 1976) and from *Methylosinus trichosporium* OB3b (Stirling and Dalton, 1979) could also be obtained in a soluble form. At around this time, Hubley *et al.*

(1975) and Takeda *et al.* (1976) indicated that copper ions might play a role in methane oxidation. In order to understand the factors which affected the expression of the particulate or soluble form of MMO, the nature of the growth-medium was studied (Stanley *et al.*, 1983). It bacame clear that the environmental growth conditions were responsible for dictating which type of enzyme was present in the cell. Stanley *et al.* reported that the intracellular location of methane monooxygenase activity depended on the copper concentration in the growth-medium. For instance, during cultivation of *Methylococcus capsulatus* (Bath), only sMMO was obtained when the concentration of copper was maintained below 1 μM, whereas when the bacterium was grown in copper-enriched media, increased pMMO activity was observed (Stanley *et al.*, 1983). In that paper the authors also reported that the enzyme from *Methylosinus trichosporium* OB3b could also be switched from a soluble to a particulate form by increasing the copper availability to the cell. High copper concentrations also stabilize and enhance pMMO activity in *vitro* (Burrows *et al.*, 1984; Prior and Dalton, 1985). Later more evidence (Cornish *et al.*, 1985; Davis *et al.*, 1987; Park *et al.*, 1991; 1992; Shah *et al.*, 1992) in support of the copper:biomass theory was reported. Recently some biochemical and biophysical characterization of pMMO from *Methylococcus capsulatus* (Bath) was reported. It was shown that the pMMO activity was directly proportional to the copper/total membrane protein ratio, the membrane-bound copper ions were EPR detectable, the copper ions appear to exist as trinuclear clusters associated primarily with the pMMO and there were many such clusters per protein molecule (Chan *et al.*, 1993).

The pMMO is different form the sMMO in several aspects. Particulate MMO will function with several electron donors, i.e., NADH, ethanol and succinate (Stanley *et al.*, 1983; Cornish *et al.*, 1985; Davis *et al.*, 1987). The substrates of pMMO are limited to a few of alkanes and alkenes (Burrows *et al.*, 1984; Smith and Dalton, 1989). There are only two reports on the purification of pMMO. The first was by Akent'eva and Gvozdev (1988) who purified two protein components from

membranes of *Methylococcus capsulatus* (strain M) on DEAE cellulose which were the hydroxylase (200-240 kDa; six subunits) and the reductase (180 kDa; four subunits). The enzyme was shown to contain 4 g atoms of non-heme iron and 1 g atom of copper. In its purified form the hydroxylase lost most of its activity at 4°C and 77°K; the reductase was completely inactivated after a few days at 77°K. The second was by Smith and Dalton (1989) who solubilized pMMO from *Methylococcus capsulatus* (Bath). Only a partial purification could be effected since activity was lost if attempts were made to purify the solubilized proteins. To date, too little is known about pMMO to draw any useful mechanistic conclusions.

1.3.3 Soluble methane monooxygenase (sMMO)

In 1976, Colby and Dalton first reported soluble MMO activity in *Methylococcus capsulatus* (Bath). They found that sMMO was not inhibited by metal ion chelating agents or by most electron-transport inhibitors and that it was a multicomponent enzyme. Subsequently, it was established that soluble MMO consisted of a reductase (previously named protein C) (Colby and Dalton, 1978), an hydroxylase (previously named protein A) (Woodland and Dalton, 1984) and a regulatory protein (protein B) (Green and Dalton, 1985). It was concluded that sMMO-catalyzed reactions required all three components, NADH and oxygen (Figure 1-4).

Soluble methane monooxygenase has now been purified and characterised from a number of methanotrophs, including *Methylococcus capsulatus* (Bath) (Colby and Dalton, 1978; Woodland and Dalton, 1984; Green and Dalton, 1985), *Methylobacterium* sp. strain CRL-26 (Patel and Savas, 1987), *Methylosinus trichosporium* OB3b (Fox et al, 1989), *Methylosinus sporium* 5 (Pilkington and Dalton, 1991), *Methylomonas* GYJ3 (Liu *et al.*, 1991) and *Methylocystis* sp. M (Nakajima *et al.*, 1992).

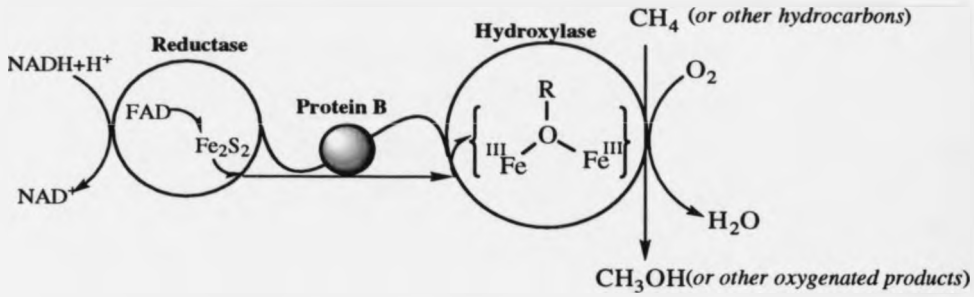


Figure 1-4. The functions of the three protein components of the soluble methane monooxygenase complex.

The well characterised soluble methane monooxygenase from *Methylosinus trichosporium* OB3b (Fox *et al.*, 1989; 1991) was shown to be similar to that from *Methylococcus capsulatus* (Bath). The enzyme from *Methylobacterium* CRL-26 may not contain a regulatory protein. The characteristics of purified soluble MMO's from several methanotrophs are summarised in Table 1-1.

The three components of sMMO from *Methylococcus capsulatus* (Bath) will be described separately in the following sections.

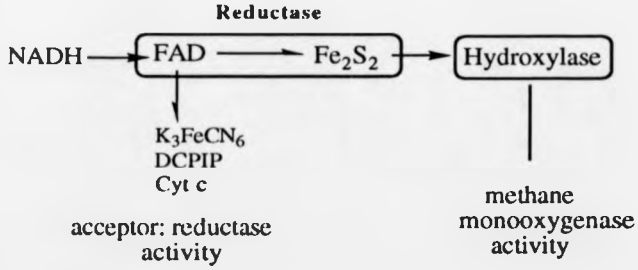
1.3.4 The reductase of soluble methane monooxygenase

The original separation of the sMMO from *Methylococcus capsulatus* (Bath) into three fractions, A, B and C, showed that fractions A and B were comparatively stable at 0°C, whereas fraction C was very unstable unless a reducing agent such as sodium thioglycollate or dithiothreitol was added (Colby and Dalton, 1978). At that time, it was thought that fraction C most likely contained the protein responsible for electron transfer from NADH to the hydroxylase. The purified active component from fraction C appeared yellow in colour and had absorption maxima at 270 and 465 nm with a shoulder at 395 nm. The 465 nm peak could be reduced in intensity by the addition of

Table 1-1 Characteristics of Purified Soluble Methane Monooxygenase

Organism	*Methylococcus capsulatus* (Bath)	*Methylobacterium* CRL-26	*Methylosinus trichosporium* OB3b	*Methylosinus spoium* strain 5	*Methylomonas* sp. GYJ3	*Methylocystis* sp. M
Classification	Type X	Type II	Type II	Type II	Type I	Type II
Number of component proteins	Three: Hydroxylase Regulator protein B Reductase	Two: Hydroxylase Reductase	Three: Hydroxylase Regulator protein B Reductase	Three: Hydroxylase Regulator protein B Reductase	Three: Hydroxylase Regulator protein B Reductase	Three: Hydroxylase Regulator protein B Reductase
Hydroxylase M_r	250,000	220,000	245,000	220,000	220,000	233,000
Number of subunits	Three	Three	Three	Three	Three	Three
Subunit M_r	60600,44700,19800	55000,40000,20000	54400,43000,22700	56000,40000,20000	54000,39000,20000	55000,44000,21000
Structure	$\alpha_2\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$
Metal content (mol/mol protein)	Fe 2.3	Fe 2.8	Fe 2-7.6	Fe 2.1	Fe 2.4	Fe 2-7.6
Protein B *Mr*	16,000	not identified	15,000-31,000	not purified	14,000	35,000-40,000
Number of subunits	One		One		One	
Metal content	none		none		none	
Reductase M_r	38,500	40,000	38,300	40,000	44,000	39,000
number of subunits	One	One	One	One	One	One
Prosthetic groups	1 FAD/1 Fe_2S_2	1 FAD/1 Fe_2S_2	1 FAD/1 Fe_2S_2	1 FAD/1 Fe_2S_2	1 FAD/1 Fe_2S_2	1 FAD/1 Fe_2S_2
Electron donor	NADH	NADH	NADH	NADH	NADH	NADH

NADH. Further studies revealed that the reductase contained 2 moles of iron, 2 moles of acid-labile sulfur and 1 mole of FAD per mole of protein (Colby and Dalton, 1978; 1979). The Fe_2S_2 redox cluster of the reductase was identified by electron paramagnetic resonance (EPR) spectroscopy (Colby and Dalton, 1979). Further characterisation of the FAD and Fe_2S_2 redox centres of the reductase showed that the Fe_2S_2 was similar to that of spinach ferredoxin (Lund and Dalton, 1985). If the Fe_2S_2 center of the protein was removed, this apo-Fe_2S_2 reductase possessed NADH acceptor:reductase activity but was inactive in the sMMO assay. If the FAD center was also removed, all activities were abolished. Addition of FAD to the apo-reductase restored the NADH:reductase activity. Reconstitution of the iron-sulfur and FAD centers restored full enzyme activity. These experiments (Lund and Dalton, 1985; Lund *et al.*, 1985) clearly indicated that the order of electron flow within the reductase followed the course :



This order is consistent with the reductase being a $2e^{-1}/1e^{-1}$ transformase. Electrons are first transferred to the FAD center, which is fully reduced by the addition of two electrons, whence it is able to effect intramolecular electron transfer of one electron to the Fe_2S_2 center (Figure 1-5) very rapidly with a second-order rate constant of 3.6×10^6 $M^{-1}sec^{-1}$ at 18°C (Green and Dalton, 1989a). The reduced Fe_2S_2 center then provides single constant-potential electrons to the acceptor-the hydroxylase.

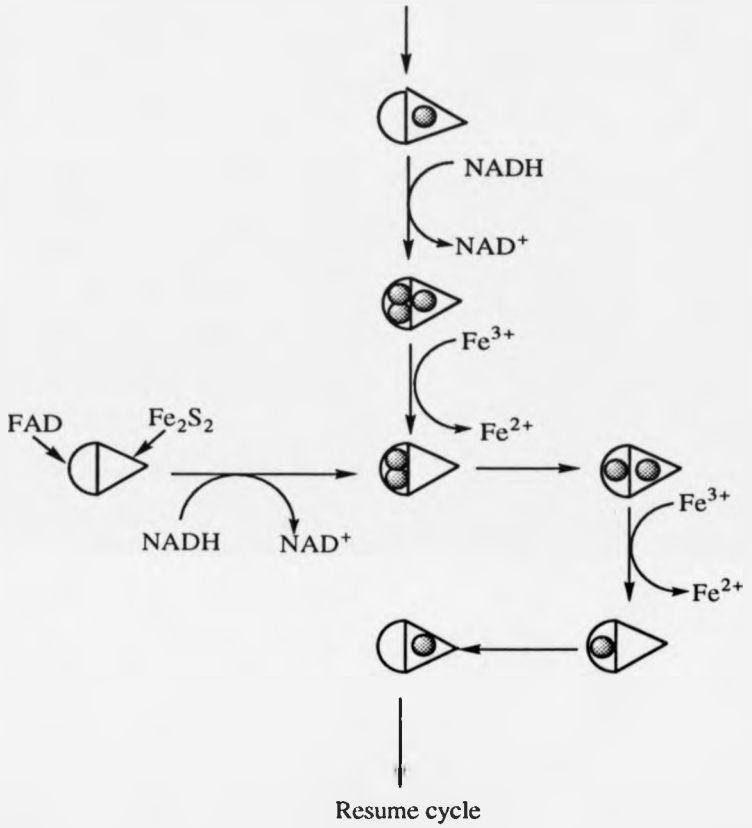


Figure 1-5. Catalytic cycle of the reductase and its interaction with the hydroxylase of sMMO. Fe represents the Fe centre of the hydroxylase.

The purified reductase components of sMMO from *Methylosinus trichosporium* OB3b (Fox et al, 1989), *Methylobacterium* sp. CRL26 (Patel and Savas, 1987), *Methylosinus sporium* 5 (Pilkington and Dalton, 1991), *Methylomonas* GYJ3 (Liu *et al.*, 1991) and *Methylocystis* sp. M (Nakajima *et al.*, 1992) all are similar to the

reductase from *Methylococcus capsulatus* (Bath). A common feature of these reductase is that all of them have 1 FAD and 1 Fe_2S_2 cluster within the protein with NADH acceptor:reductase activity.

1.3.5 The regulatory protein (protein B) of soluble methane monooxygenase

Very early in the enzyme studies it was observed that protein B of sMMO from *Methylococcus capsulatus* (Bath) was capable of stimulating methane monooxygenase activity (Colby and Dalton, 1978). It was found that protein B was a low molecular weight acidic protein (Colby and Dalton, 1978; Dalton *et al.*, 1984) and had the ability of modulating, in some way, the transfer of electrons between the reductase and the hydroxylase in the sMMO complex (Lund *et al.*, 1985; Dalton and Leak, 1985).

Subsequent studies using pure protein B established that it was a regulator of soluble methane monooxygenase activity (Green and Dalton, 1985). It was observed that in the presence of protein B, NADH oxidation was tightly coupled to substrate oxidation. In the absence of substrate both oxygen reduction and NADH consumption were prevented by protein B and when protein B was absent, the rate of oxygen (or NADH) consumed was similar whether or not substrate was present. These results suggested that protein B possessed the ability to regulate the flow of electrons between the reductase and the hydroxylase. There was very little reduction of the reductase by NADH alone, however the rates of oxidation of NADH and O_2 reduction were greatly enhanced by the addition of the hydroxylase. This implied that oxygen reduction occurred at the hydroxylase active site. If the hydroxylase was incubated with the apo-Fe_2S_2 reductase, enhanced rates of oxygen uptake were not observed regardless of presence or absence of protein B. In addition, the rate of electron transfer between the reductase and the hydroxylase was slowed in the absence of substrate with an increase in the concentration of protein B. The conclusion was made that protein B is a regulator of sMMO activity converting the enzyme from an oxidase to an oxygenase

(Green and Dalton, 1985). Figure 1-6 illustraties the function of protein B in the soluble methane monooxygenase complex.

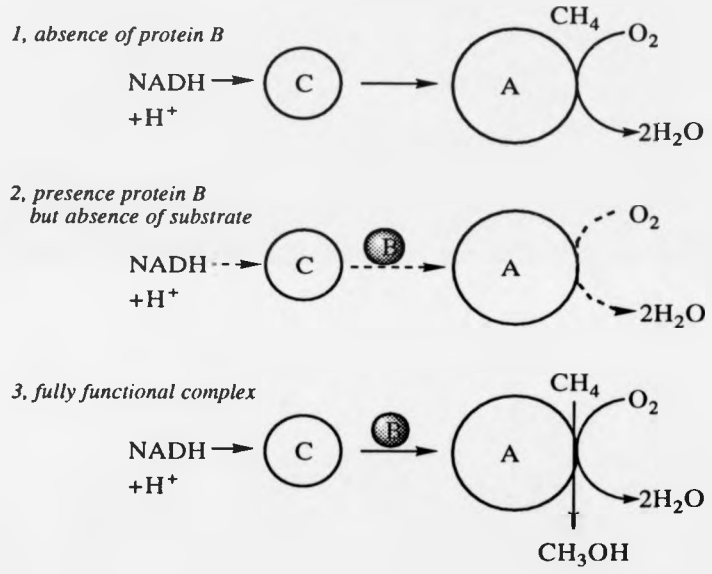


Figure 1-6. Role of protein B in regulation of electron transfer within the methane monooxygenase complex.
(1) In the absence of protein B, methane is not oxidized but dioxygen is reduced exclusively to water.
(2) In the presence of protein B but absence of substrate, there is very little electron transfer or oxygen reduction.
(3) The fully functional complex.

Protein B purified from *Methylosinus trichosporium* OB3b (Fox *et al.*, 1989) was physically similar to the protein B from *Methylococcus capsulatus* (Bath). Purification and characterisation of sMMO from *Methylosinus sporium* 5 indicated that a component which was lost during purification of the hydroxylase and the reductase was equivalent to protein B from *Methylococcus capsulatus* (Bath) and essential for enzyme activity (Pilkington and Dalton, 1991). Recently purification of the enzyme components from *Methylomonas* GYJ3 (Liu *et al.*, 1991) and *Methylocystis* sp. M (Nakajima *et al.*, 1992) also illustrated that protein B (named component I in *Methylocystis* sp. M) was capable of stimulating the sMMO activity. Only in *Methylobacterium* sp. CRL-26 has protein B not been identified (Patel and Savas, 1987).

The role of protein B in *Methylosinus trichosporium* OB3b in multiple turnover experiments appeared different from that observed in *Methylococcus capsulatus* (Bath). The reductase and the hydroxylase were still capable of NADH and O_2 utilisation in the absence of substrate with different concentrations of added protein B (Fox *et al.*, 1989). It was also reported that when the ratio of protein B to hydroxylase exceeded 5, a decrease of sMMO activity was observed (Fox *et al.*, 1989).

In addition, during purification of the component B of sMMO from *Methylococcus capsulatus* (Bath), two forms of this protein were found, one of which was a carboxy-terminal truncate, designated B' and was completely inactive (Pilkington *et al.*, 1990). The B' appeared to be a specific breakdown product of protein B with a molecular mass of approximately 1 kDa less than protein B. Limited proteolysis of protein B with trypsin resulted in the formation of B' and loss of activity. Recently, B and B' formation was also observed in the recombinant protein from *E. coli*. This indicated that the process was not under the specific control of the *Methylococcus capsulatus* chromosome, and any regulation must lie entirely within the conformation of protein B alone (West *et al.*, 1992).

1.3.6 The hydroxylase of soluble methane monooxygenase

The hydroxylase (previously named protein A), a non-heme iron containing protein, is believed to be the component of interaction with both hydrocarbon substrates and oxygen in sMMO catalyzed oxygenation reaction (Colby and Dalton, 1978; Green and Dalton, 1985). The purification and characterisation of the hydroxylase component of sMMO was first achieved by Woodland and Dalton (1984). They reported that the purified hydroxylase from *Methylococcus capsulatus* (Bath) had a molecular weight of approximately 210 kDa based on gel fitration chromatography and gel electrophoresis, and that the hydroxylase was comprised of three subunits of α = 54,000, β = 42,000, and γ = 17,000 which were present in stoichiometric amounts suggesting an $\alpha_2\beta_2\gamma_2$ arrangement in the native protein. Recent studies from DNA sequence and mass spectrosmetry showed that the protein molecular weight was 250 kDa, and the subunit molecular weights were α = 60,000, β = 45,000, and γ = 20,000. Analysis of the metal ion content of the purified hydroxylase from *Methylococcus capsulatus* (Bath) revealed the presence of two iron atoms (Woodland and Dalton, 1984).

The purification of sMMO hydroxylases from *Methylobacterium* sp. strain CRL-26 (Patel and Savas, 1987), *Methylosinus trichosporium* OB3b (Fox and Lipscomb, 1988), *Methylosinus sporium* 5 (Pilkington and Dalton, 1991), *Methylomonas* GYJ3 (Liu *et al.*, 1991) and *Methylocystis* sp. M (Nakajima *et al.*, 1992) have been reported. Each protein has a molecular mass of between 220 and 250 kDa, with three subunits in a $\alpha_2\beta_2\gamma_2$ configuration and is similar to that from *Methylococcus capsulatus* (Bath) (Table 1-1). The iron content of the hydroxylase from *Methylobacterium* sp. strain CRL-26, *Methylosinus sporium* 5 and *Methylomonas* GYJ3 is as same as that in *Methylococcus capsulatus* (Bath) (Patel and Savas, 1987; Pilkington and Dalton, 1991; Liu *et al.*, 1991). However, the hydroxylase from *Methylosinus trichosporium* OB3b and *Methylocystis* sp. M appeared to differ from the others in that they were reported to contain variable iron atoms from 2 to 7.6 moles

Fe/mole protein (Fox and Lipscomb, 1988; Nakajima *et al.*, 1992). It has been shown that there was a direct correlation between the amount of iron in the protein and its specific activity (Dalton, 1992a).

The first EPR spectra of the hydroxylase from *Methylococcus capsulatus* (Bath) revealed that the two iron atoms appeared to be coupled via a μ-oxo bridge (Woodland and Dalton, 1984; Woodland *et al.*, 1986). The native hydroxylase was EPR-silent with a minor signal at $g = 4.3$ suggesting the presence of high-spin ferric iron in a rhombically distorted site (Woodland *et al.*, 1986). Thus, the redox centre in the hydroxylase was believed to be a pair of μ-oxo bridged antiferromagnetically coupled high-spin ferric ions. The Mössbauer and EPR spectra of the hydroxylase from *Methylosinus trichosporium* OB3b also provided similar evidence for a μ-oxo-bridged binuclear iron cluster (Fox *et al.*, 1988). Further studies with extended X-ray absorption fine structure (EXAFS) spectroscopy indicated that the bridge in the hydroxylase from *Methylococcus capsulatus* (Bath) was more likely to be μ-hydroxo or -alkoxy rather than μ-oxo based on comparison with characterised model complexes (Ericson *et al.*, 1988). The first coordination shell was composed of approximately six nearest N/O neighbours at an average distance of ~2.05 Å and the Fe-Fe distance was 3.41 Å. EXAFS spectroscopy of the hydroxylase from *Methylobacterium* CRL-26 gave an Fe-Fe distance of 3.05 Å (Prince *et al.*, 1988).

The binuclear iron structure present in the hydroxylase of sMMO is similar to those found in other non-heme diiron oxoproteins including haemerythrin (Muhoberac *et al.*, 1980), R2 protein of ribonucleotide reductase (Petersson *et al.*, 1980), purple acid phosphatase (Davis and Averill, 1982) and rubrerythrin (LeGall *et al.*, 1988). Clearly, the function of these proteins is quite different although they all contain at least one binuclear iron centre (Wilkins, 1992). In haemerythrin, the diiron centre is involved in oxygen transport (Wilkins and Wilkins, 1987a). In the R2 protein of ribonucleotide reductase, the diiron centre and oxygen generate a stable tyrosyl radical

that is involved in the reduction of ribonucleoside diphosphate to deoxyribonucleoside diphosphate for DNA synthesis (Stubbe, 1990). In purple acid phosphatase, the iron centre is the catalytic site for the hydrolysis of phosphate esters (Doi *et al.*, 1988).

Further studies (Green and Dalton, 1988) have indicated that the binuclear iron centre of the hydroxylase of sMMO is directly involved in catalysing the oxidation of methane to methanol and the insertion of an O atom into a C-H bond in a variety of hydrocarbons. The role of the binuclear iron center was suggested by the removal and readdition of iron to the hydroxylase. It was observed that the iron-depleted hydroxylase [Fe content 0.26 mol/mol protein] showed loss of the catalytic activity. Addition of the iron to the iron-depleted hydroxylase gave the functionally active protein. This clearly implicated the iron atoms in the catalytic activity of the hydroxylase. A similar evidence was reported that the highest specific activity preparations (a value approximate to 1700 nmol/min/mg) contained ~2 iron clusters/mol protein of the hydroxylase from *Methylosinus trichosporium* OB3b, and the low specific activity preparations (below 500 nmol/min/mg) contained ~1 cluster/mol protein on average (Fox *et al.*, 1989). All these data demonstrated that the iron cluster of the hydroxylase of sMMO had an important role in catalysis.

It was also found that reconstitution of iron into the iron-depleted hydroxylase was inhibited by a number of metal ions including Ni^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}. None of of these metalloforms gave functionally active protein (Green and Dalton, 1988). This implied that the iron atoms were essential for the function of the hydroxylase of sMMO.

Furthermore, it was observed that, *in vitro* translation of total RNA isolated from *Methylococcus capsulatus* (Bath) produced hydroxylase subunits with protein molecular weights equal to the native but with no MMO activity. Addition of the iron to the translated hydroxylase could resulted a functionally active protein. The results suggested that the subunits of the hydroxylase were synthesised in their native, mature

form and self-assemble into an apoprotein, into which the iron was inserted (Green and Dalton, 1988).

The iron atoms of the hydroxylase can exist in three oxidation states: oxidised {$Fe^{(III)}$-$Fe^{(III)}$}, half-reduced {$Fe^{(III)}$-$Fe^{(II)}$} or fully reduced {$Fe^{(II)}$-$Fe^{(II)}$} (Woodland *et al.*, 1986). The half-reduced (or mixed valence) state exhibited a relatively axial EPR spectrum with g_z = 1.78, g_y = 1.88, and g_x = 1.95 with g_{ave} = 1.87 for the hydroxylase protein from *Methylococcus capsulatus* (Bath). The shape and amplitude of the g = 1.95 signal changed little when reacted with oxygen, suggesting that the half-reduced state did not interact with oxygen. The reductase-catalyzed NADH reduction of the hydroxylase from the mixed valent to the fully reduced state resulted in the disappearance of the g_{ave} = 1.87 signal (Woodland *et al.*, 1986).

Similarly, the EPR spectrum of the half-reduced state for the hydroxylase protein from *Methylosinus trichosporium* OB3b showed g_z = 1.94, g_y = 1.86, and g_x = 1.75 with g_{ave} = 1.85 (Fox *et al.*, 1988; 1989). Also, investigation of the mixed valence state with EPR spectroscopy showed it to be unreactive toward O_2 and no hydroxlated products were produced in a single turnover. It was observed that during reductive titration of the hydroxylase the g_{ave} = 1.85 signal increased on partial reduction and decreased on full reduction (Fox *et al.*, 1988), and the signal near g = 15 [later corrected to g = 16 by Hendrich *et al.*, (1990)] appeared with the disappearance of the g_{ave} = 1.85 signal. Addition of O_2 to the fully reduced hydroxylase resulted in the disappearance of the g = 15 signal (Fox *et al.*, 1989) with no g_{ave} = 1.85 signal appearing. This suggested that the fully reduced form could react with dioxygen. Also, single turnover experiments have shown that the fully reduced hydroxylase was capable of oxidising propane or propene (Fox *et al.*, 1989).

Analysis of the data suggests that g = 16 resonance represents the majority of iron in the diferrous hydroxylase (Hendrich *et al.*, 1990), so that this signal can now be used as measure of the Fe^{II}/Fe^{II} form of the protein. Similar observations were also

made by Bentsen *et al.* (1989) in which a broad $g = 12.3$ signal was observed on full reduction of the hydroxylase from *Methylococcus capsulatus* (Bath).

1.4 Characterisation of sMMO reaction

1.4.1 Catalysis of hydrocarbon oxidation

Early observation of methane monooxygenase showed that the enzyme was not only able to oxidize methane, but also was capable of oxidising other organic compounds. There were several reports that the enzyme from *Methylococcus capsulatus* (Bath) could oxidize bromomethane (Colby and Dalton, 1976), the enzyme from *Methylomonas methanica* could oxidise CO (Ferenci *et al.*, 1975), bromomethane and ammonium chloride (Colby *et al.*, 1975), and the enzyme from *Methylosinus trichosporium* could oxidize CO, ethane, propane and butane. Further research (Colby and Dalton, 1977) on soluble methane monooxygenase from *Methylococcus capsulatus* (Bath) confirmed that the enzyme has a very broad substrate specificity. The studies showed that the soluble extract from *Methylococcus capsulatus* (Bath) was able to oxidise various hydrocarbons including alkanes, haloalkanes, alkenes, ethers, alicyclic, aromatic and heterocyclic compounds, CO, methanol (Colby and Dalton, 1977), and ammonia as well (Dalton, 1977). The experiments demonstrated that all unsubstituted linear and most unsubstituted branched hydrocarbons including saturated and unsaturated up to approximately C_8 in length were readily oxidised to alcohols or epoxides. Similarly, the sMMO from *Methylosinus trichosporium* OB3b was observed to oxidize many hydrocarbons (Burrows *et al.*, 1984) including halogenated alkenes, such as trichloroethylene a widely distributed ground-water pollutant (Fox *et al.*, 1990). The sMMO's from other methanotrophs, such as *Methylobacterium* sp. strain CRL-26 (Patel and Savas, 1987), *Methylosinus sporium* 5 (Pilkington and Dalton, 1991), *Methylomonas* GYJ3 (Liu *et al.*, 1991) and *Methylocystis* sp. M (Nakajima *et al.*, 1992), also possess the ability to catalyse the oxidations of various hydrocarbons. Table 1-2 gives some substrates and

products of reactions catalysed by the soluble MMO from *Methylococcus capsulatus* (Bath).

The broad substrate specificity and possible economic significance of the products of MMO catalysed reactions has attracted industrialists' and environmentalists' attention. There is interest in the prospect of applying the enzyme to biotransformations and to bioremediation of contaminated water and soils. Possible applications which have been investigated are, for example, formation of epoxides (Large and Bamforth, 1988; Stanley and Dalton, 1992a; Leak, 1992; 1993) and biodegradation of trichloroethylene in contaminated ground water and soils (Tsien et al, 1989; Fox *et al.*, 1990).

Table 1-2 Some substrates and products of soluble MMO form *Methylococcus capsulatus* (Bath)

Substrate	Product(s)
Methane	Methanol
Ethane	Ethanol
Hexane	Hexan-1-ol, hexan-2-ol
Ethylene	Epoxyethane
Propylene	Epoxypropane
trans-2-Butene	*trans*-2,3-Epoxybutane, *trans*-2-Butene-1-ol
Chloromethane	formaldehyde
Dichloromethane	Carbon monoxide
Dimethyl ether	Methanol, formaldehyde
Diethyl ether	Ethanol, acetaldehyde
Cyclohexane	Cyclohexanol
Cyclohexene	cyclohexene oxide, 2-cyclohexenol, cyclohexanone
Benzene	Phenol
Toluene	Benzyl alcohol, *p*-hydroxy toluene
Styrene	Styrene epoxide
Pyridine	Pyridine-*N*-oxide
Methanol	Formaldehyde
Carbon monoxide	carbon dioxide

1.4.2 Kinetic analysis of sMMO reaction

The steady-state kinetic analysis of sMMO from *Methylococcus capsulatus* (Bath) illustrated that the enzyme complex follows a concerted-substitution mechanism (Green and Dalton, 1986) as shown in Figure 1-7 (Dalton, 1992a). First methane binds to the enzyme followed by NADH reduction. The reduced enzyme-methane complex then binds O_2 to give a second ternary complex, which breaks down to release the products water and methanol.

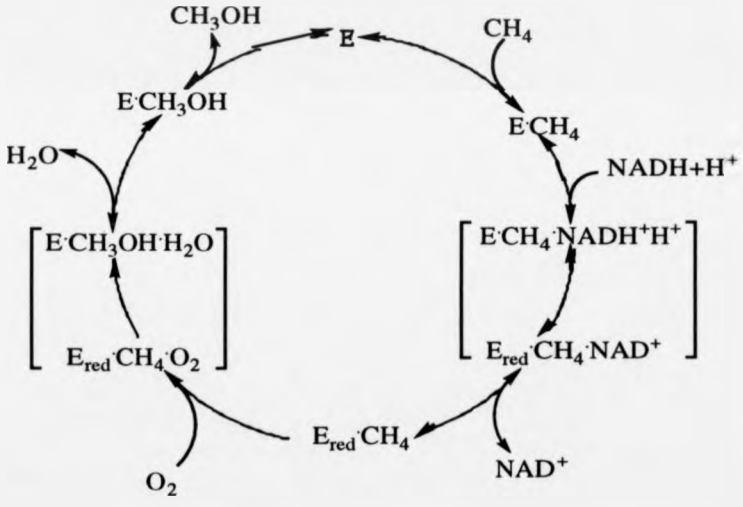


Figure 1-7. Catalytic cycle of methane monooxygenase. Dioxygen is bound to the reduced enzyme/methane complex utimately to release water, methanol, and reoxidized enzyme.

The sequence of events during the catalytic cycle was based on product-inhibition studies using propene as substrate and the determination of the values of K_m for

NADH, O_2 and substrate. The inhibition patterns observed were that (a) NAD^+ showed competitive inhibition with O_2, non-competitive inhibition with propene and mixed competitive/non-competitive inhibition with NADH; (b) propene oxide showed competitive inhibition with propene, uncompetitive inhibition with O_2 and non-competitive inhibition with NADH. The K_m value for NADH varied from 25 μM to 300 μM depending on the nature of the hydrocarbon substrate. For example, when methane is used as a substrate, the K_m values for methane, NADH and O_2 were 3 μM, 55.8 μM and 16.8 μM respectively; when propylene is used as a substrate, the K_m values for propylene, NADH and O_2 were 0.94 μM, 25.2 μM and 12.7-15.9 μM respectively (Green and Dalton, 1986).

The studies of electron transfer through the soluble methane monooxygenase complex (Lund et al, 1985) revealed that neither the reduction of the reductase by NADH nor electron transfer from the reductase to the hydroxylase was the rate-limiting step in the MMO reaction. Further observation showed that the reduction of the reductase and the intramolecular transfer of electrons through the reductase were much more rapid than the turnover rate of the enzyme. In a study with methane-d_4 as substrate (Green and Dalton, 1989a), it was demonstrated that C-H bond breakage was probably the rate-limiting step in the conversion of methane to methanol since the kinetic isotope effect was $K_H / K_D = 11.8$. This value was higher than that reported by Gvozdev *et al.* (1984) for the particulate enzyme from *Methylococcus capsulatus* (strain M) of 5, but comparable to that for *Methylomonas rubrum* 12.5 reported by Belova *et al.* (1976).

1.4.3. The three components complex of sMMO

The formation of specific complexes among the protein components of methane monooxygenase from *Methylosinus trichosporium* OB3b has been observed (Fox *et al.*, 1991). Protein B binds to the hydroxylase, perturbing the structure of the binuclear iron centre, as revealed by EPR spectroscopic studies. Complex formation

between the reductase and protein B and between the reductase and the hydroxylase was also demonstrated by fluorescence spectroscopy. Moreover, chemical crosslinking experiments showed that protein B bound to the α subunit of the hydroxylase, the reductase interacted with the β subunit of the hydroxylase and the α and β subunits were close to each other. Early studies using the radioactive acetylene (a suicide substrate for MMO)(Prior and Dalton, 1985) showed that the active site was quite likely to the site in the α subunit of the hydroxylase.

A study of the redox properties of the hydroxylase through potentiometric titrations (Liu and Lippard, 1991) showed that the potentials for the formation of the mixed valent $Fe^{(III)}$-$Fe^{(II)}$ and the fully reduced $Fe^{(II)}$-$Fe^{(II)}$ species were 48 mV and -135 mV, respectively. Addition of the substrate propene to the hydroxylase did not have a major effect on the reduction potentials. Introduction of protein B and the reductase components, however, completely inhibited reduction of the hydroxylase at potentials as far negative as -200 mV. Addition of propene to all three methane monooxygenase components greatly facilitated hydroxylase reduction. Under these conditions, the fully reduced form of the protein was obtained at potentials of >150 mV. These results indicated that the electron transfer from mediators to the hydroxylase in the presence of the reductase and protein B was kinetically controlled. The binding of substrate to the hydroxylase reversed this effect and rendered the hydroxylase more electron-deficient hence easier to reduce. This thermodynamic effect would occur only when all three components were present, and the greater driving force could explain the faster rates of NADH oxidation observed when protein B was available. These findings were consistent with the steady-state kinetic analysis of the methane monooxygenase complex described previously, where electron transfer was observed to occur only after substrate was bound (Green and Dalton, 1986). The obvious advantage is that, when no substrate is present, no NADH consumption takes place, therefore preventing the wasteful consumption of reducing equvalents (Green and Dalton, 1985).

Moreover, such an increase in reduction potential is similar to the behavior seen with cytochrome P-450, where small changes in the coordination sphere of heme iron due to the binding of the substrate can raise the reduction potential from -300 to -170 mV (Raag and Poulos, 1989).

The data presented so far clearly indicate that all three components of sMMO are necessary for enzyme activity. The hydroxylase of sMMO is responsible for the activation of O_2 and the oxidation of substrate, the reductase is responsible for electron transfer from NADH to the hydroxylase, and protein B is proposed to play a regulatory role.

1.5 The mechanism of action of soluble methane monooxygenase

It has been shown that in sMMO catalyzed reactions the reduction of O_2 and activation of the C-H bond occur in the hydroxylase. The binuclear iron centre of the hydroxylase is involved in the catalytic oxidations. The question is how activation of the C-H bond is achieved at this active site?

1.5.1 Oxygen activation

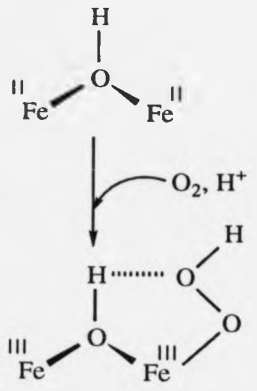
Several well-studied mechanisms for hydrocarbon functionalization all rely on oxygen activation to effect C-H bond activation. The Fenton reagent (essentially ferrous iron and hydrogen peroxide) leads to the production of an intermediate, which is believed to be $L_xFe^{II}OOH$ (Sawyer *et al.*, 1993) rather than hydroxyl radicals (Walling, 1975), and is able to oxidize many organic compounds. The well-studied cytochrome P-450 mechanism revealed that a high-valent iron-oxo species is involved in the oxidation of hydrocarbons and this high-valent iron-oxo species is believed to be created from splitting a reactive peroxide O-O bond formed from the reduction of O_2 by the reduced heme iron (Fe^{II}) at the active centre (Ortiz de Montellano, 1986; McMurray and Croves, 1986).

One of the key features to understanding the prerequisite for the reactivity of O_2 is its greater tendency to react as a two-electron oxidant rather than a one-electron oxidant (Drago, 1992). Oxygen in its common or ground state contains two unpaired electrons ("triplet state"). The triplet state is unreactive in part because the one-electron reduction has a large positive free energy change, and in part because, according to the law of spin conservation, the number of unpaired electrons in the products of oxygen reactions must be the same as in the starting reagents, whereas the products of the biological reactions of molecular oxygen contains no unpaired electrons. As a result, O_2 is a poor initiator of reactions with diamagnetic organic substrates that can only provide one electron or one hydrogen atom per molecule. This property makes O_2 kinetically unreactive with organic compounds. How does this come about and how is it possible to overcome this kinetic obstacle? Taube (1965) and especially Hamilton (1974) pointed out that there are two ways in which the reaction restrictions posed by the triplet state of O_2 can be circumvented: (1) by complexation with a transition metal ion at the active site of an enzyme, since it is a spin-allowed process for a system with delocalized spins to react with organic compounds, producing products (no unpaired electrons) if the number of unpaired electrons on the overall metal ion complex remains constant throughout the reaction. In this case, O_2 reactions can proceed by ionic (nonradical) mechanisms with low energy barriers. Alternatively, (2) if an intermediate free radical is part of a resonance-stabilized system (such as flavin semiquinone), radical reactions may take place readily at physiological temperatures. Clearly, the latter case is not present in sMMO reaction system because the reductase (containing a FAD and a Fe_2-S_2 cluster) of sMMO is responsible for electron transfer from NADH to the hydroxylase and is not able to function in activation of molecular oxygen and can't interact with the hydrocarbons. This raised the question as to what sort of complex is formed between molecular oxygen and the iron at the active centre of sMMO (O_2 binding) and how the O atom from O_2 is incorporated into the substrate?

Generally, the enzyme-catalysed reactions of molecular oxgen appear to take place in two distinguishable phases (Keevil and Mason, 1978): (1) the *oxygen-binding phase*, in which oxygen is bound to the active site, usually in the presence of the original or unoxidized substrate; (2) the *reaction phase*, in which the bound oxygen is reduced and/or transferred to the substrate.

The binding phase has been observed with representatives of every class of prosthetic group (Keevil and Mason, 1978), such as the cuprous proteins laccase (Andreasson *et al.*, 1973) and tyrosinase (Jolley *et al.*, 1974), the non-heme iron proteins haemerythrin (Bargett *et al.*, 1969) and protocatechuate 3,4-dioxygenase (Fujisawa *et al.*, 1971), the heme proteins cytochrome P-450 (Tyson *et al.*, 1972) and hemoglobin, and the flavoproteins *p*-hydroxybenzoate hydroxylase and phenol hydroxylase (Entsch *et al.*, 1976), in these proteins the oxygen is reduced to the peroxo level (haemerythrin and cytochrome P-450) and the superoxo level (hemoglobin). Here, "superoxo is *not* a superoxide ion (O_2^-), and peroxo is *not* a peroxide ion (HO_2^-)... thus, coordinated O_2 may not be expected to exhibit properties identical with those of dixoygen ions" (Vaska, 1976).

In its native state, the iron species within the hydroxylase of sMMO exists as a pair of antiferro-magnetically coupled high spin ferric ions (Woodland *et al.*, 1986) which upon one electron reduction reduces one of the iron atoms to high spin ferrous (the mixed valent species). A further one electron reduction produces the fully reduced form in which both iron atoms are high-spin ferrous. Addition of O_2 leads to a dioxygen complex with the iron atoms. The nature of the diiron-oxygen species has not been characterised, but the binding of dioxygen could occur with oxidation of the diiron centre leading to the formation of a iron bound peroxide intermediate by analogue with the well-characterised heme-iron systems in cytochrome p450 (Green and Dalton, 1989b; Dalton *et al.*, 1990) (Figure 1-8).



fully reduced form

iron bound peroxide

Figure 1-8. Dioxygen binding occurs with oxidation of the diiron centre (Fe^{II}/Fe^{II}) of sMMO and formation of a bound peroxide.

The next question concerns how the bound oxygen becomes the active state to react with C-H bond. The well-studied mechanism of cytochrome P450 (Ortiz de Montellano, 1986, McMurray and Groves, 1986) and of models of this enzyme (Tabushi, 1988; Dawson, 1988; Mansuy and Battioni, 1989; Shilov, 1989) have provided valuable information that high-valent iron-oxo species are formed via a peroxide (Fe^{III}-O-O-H). The proposed pathway is shown in Figure 1-9 (Mansuy, 1987).

The high valent iron-oxo species, which has been observed in model complexes (Lichtenberger *et al.*, 1976; Groves and McClusky, 1976; Battioni *et al.*, 1986; Mansuy, 1987; Gunter and Turner, 1991), is thought to be generated by heterolytic cleavage of the iron-bound peroxy O-O bond. Recently, the first *direct* observation of heterolytic O-O bond cleavage in acylperoxo-iron(III) porphyrin-imidazole complexes to yield oxo-ferryl porphyrin π-cation radicals was reported (Yamaguchi *et al.*, 1992). Determination of the activation parameters suggested that the coordination of a sixth

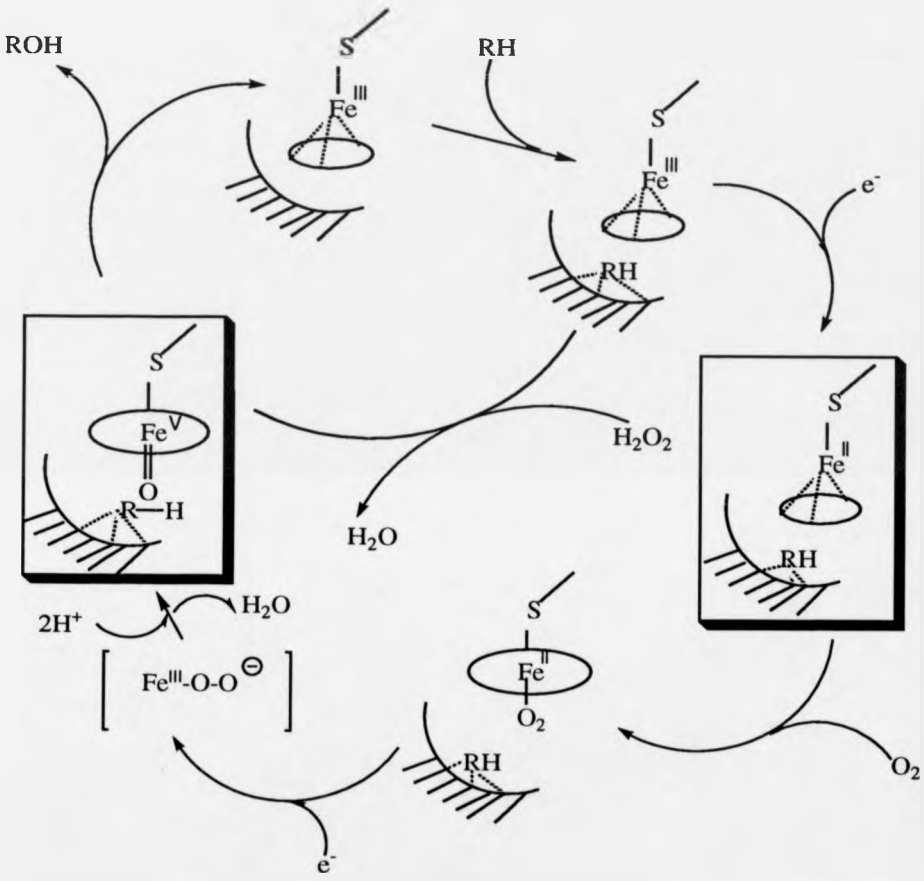


Figure 1-9. Proposed catalytic cycle of cytochrome P450.

Here, RH, a substrate; ROH, the corresponding product;
, means the substrate binding hydrophobic site close to the heme;
, means the heme-iron active site and with a thiol ligand (S).

ligand stabilised the transition state in the O-O bond-cleavage reaction. If H_2O_2 was used instead of O_2, the reaction involved conversion of Fe(III)(P)+ complex to $Fe(IV)O(P^{\cdot})^+$ (where (P)+ means porphyrin complex). This has been proved by the evidence that hydrogen peroxide and other surrogate oxygen donors such as iodosobenzene, chloroperbenzoic acid or cumyl hydroperoxide could replace the electron and O_2 donors for cytochrome P450 to effect substrate hydroxylation (White and Coon, 1980; Gugenerich and MacDonald, 1984; Ortiz de Montellano, 1986; Mansuy and Battioni, 1989). It was concluded that the mechanism of cytochrome P-450 was non-concerted and involved abstraction of a hydrogen atom from the substrate by the high-valent iron species to yield a carbon radical and an iron-bound hydroxyl species which recombine to form the alcohol.

Therefore, the mechanism by which molecular oxygen is brought into the substrate by sMMO has been a major focus of attention. To understand the nature of the active species involved and what mechanism could be operative in soluble methane monooxygenase-catalyzed oxidation of hydrocarbons, several investigations of the mechanistic aspects of the enzyme have been undertaken.

1.5.2 Mechanistic aspects-substrate oxidation

Some mechanistic aspects of sMMO in the oxidation of hydrocarbons were investigated by Green and Dalton (1989b) using purified enzyme from *Methylococcus capsulatus* (Bath) and taking advantage of its broad substrate specificity. The authors' approach was to choose key substrates which had been used to deduce the mechanism of oxidation reactions catalyzed by cytochrome P-450.

The oxidation of linear alkanes (C_4-C_7) by sMMO showed that the insertion of oxygen into secondary C-H bonds was favoured over attack at primary carbons (there being no tertiary positions available to attack). A substrate containing one tertiary and three primary carbon atoms, 2-methylpropane, was oxidised to yield 70% tertiary (2-

methyl-2-propanol) and 30% primary alcohol products. In the oxidations of 2-methylpentane and 2,4-dimethylpentane attack at the tertiary position was also preferred. The results seemed to indicate that a nonconcerted reaction mechanism was operative in the oxidations of these substrates because direct insertion of oxygen into a C-H bond (concerted mechanism) would show little preference for primary, secondary, or tertiary positions. In the case of the substrate of 2,3-dimethylpentane containing primary, secondary and tertiary positions, oxidation occurred at the secondary position to yield 3,4-dimethyl-2-pentanol and some oxidation at primary positions occurred as well. In the case of the substrate of 2,2-dimethylbutane, oxidation at primary positions was also observed. The oxidation of admantane gave the products 1-adamantanol (50%) and 2-adamantanol (50%) (Green and Dalton, 1989b). The selectivity (see p126) of C_2/C_3 was 0.334 which was close to the typical C_2/C_3 ratios of 0.05-0.15 for radical reactions of this substrate. Therefore, the evidence suggested that a substrate radical intermediate might be involved in the catalysis by sMMO.

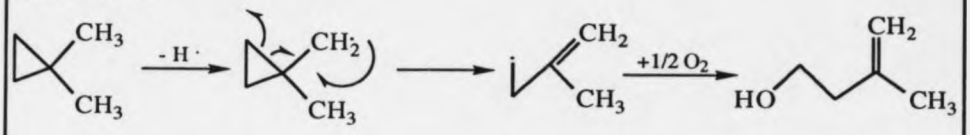
In oxidation of *cis* -1,4-dimethylcyclohexane and *cis* -1,3-dimethylcyclohexane, both the *cis* and the *trans* cyclohexanol products were detected showing non-stereoselectivity. Also, in oxidation of methylenecyclohexane and β-pinene, the allylic rearrangement of double bonds to 1-cyclohexene-1-methanol and 6,6-dimethylbicyclo [3.1.1] hept-2-ene-2-methanol was observed respectively (Green and Dalton, 1989b). This transposition of functionality implied the formation of an allylic free radical intermediate. Such an intermediate may arise in two ways: as a result of hydrogen abstraction or by sequential loss of a π-bond electron and a proton termed an aborted epoxidation (Ortiz de Montellano, 1986). Indeed, epoxidated products were generated by MMO from these substrates (Green and Dalton, 1989b). The oxidation of cyclopropylbenzene yielded a cyclopropyl ring-opened product indicating the presence of a charged or radical intermediate. The oxidation of norbornadiene and quadricyclane by MMO each gave an aldehyde product which had also been observed in the

cytochrome P450 catalyzed the oxidation of these substrates. It suggested that a common intermediate was generated which the enzymaticlly activated oxygen captured and the resulting cationic intermediate then rearranged to form the observed aldehyde products (Stearns and Ortiz de Montellano, 1985).

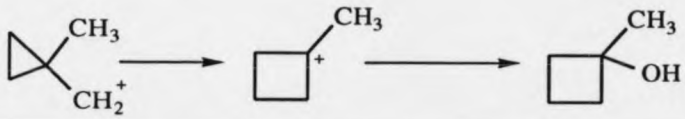
Both dichloroethylene (DCE) and trichloroethylene (TCE) were shown to rapidly inactivate the sMMO enzyme from *Methylococcus capsulatus* (Bath). It was observed that inactivation of MMO by DCE and TCE was associated with loss of iron from the hydroxylase component, and it was not possible to replace the iron suggesting that the radical substantially modified the enzyme in close to the active site (Green and Dalton, 1989b). Subsequent studies by Fox *et al.* (1990) showed that similar effects were observed with sMMO from *Methylosinus trichosporium* OB3b.

Evidence for a radical or carbocationic intermediate involved in hydroxylations catalyzed by sMMO was reported by Frey's group (Ruzicka *et al.*, 1990), who studied the oxygenation of 1,1-dimethylcyclopropane with purified enzyme from *Methylosinus trichosporium* OB3b. The products (1-methylcyclopropyl)methanol (81%), 3-methyl-3-butene-1-ol (6%) and 1-methyl-cyclobutanol (13%) were detected. It was rationalised that the rearrangement product 3-methyl-3-butene-1-ol was due to the oxygenation of 1,1-dimethylcyclopropane via the (1-methylcyclopropyl)carbinyl radical, which might undergo rearrangement with ring opening to the homoallylic 3-methyl-3-butene-1-yl radical in competition with conventional oxygenation giving 3-methyl-3-butene-1-ol (Scheme 1-1) (Ruzicka *et al.*, 1990). The formation of 1-methyl-cyclobutanol, which was a ring-expansion product, was explained on the basis that the 1-methylcyclobutyl tertiary carbocation was an intermediate. This cation would result from rearrangements of carbocations derived by one-electron oxidation of either radical intermediate (Scheme 1-2) (Ruzicka *et al.*, 1990). Therefore, these studies suggested that the oxygenation mechanism of sMMO could involve both radical and carbocationic intermediates.

Scheme 1-1.



Scheme 1-2.



In addition, the oxidation of a series of deuterated substrates by sMMO from *Methylosinus trichosporium* OB3b was shown to proceed by a mechanism analogous to that for similar reactions catalyzed by cytochrome P-450 (Rataj *et al.*, 1991). The only difference was that in propene epoxidation in D_2O and of d_6-propene in H_2O there was no exchange of substrate protons or deuterons with solvent, in contrast to what was observed with cytochrome P450 (Groves *et al.*, 1986). This suggested that the mechanism of epoxidation of olefins by sMMO differs at least in part from that of cytochrome P450 (Rataj *et al.*, 1991). Morever, stereospecificity in the epoxidation of *cis*- and *trans*-2-butene was observed with sMMO from *Methylococcus capsulatus* (Bath) (Green and Dalton, 1989b).

Recently, it was reported that in the oxidation of (*S*)- or (*R*)-[1-$^2H_1$,1-$^3H_1$]ethane to ethanol catalyzed by sMMO from *Methylosinus trichosporium* OB3b the products with retention of configuration were about 64-68% and the inversion products were about 32-36% (Priestley *et al.*, 1992). The relatively high amount of inversion products suggested that a substrate radical intermediate was involved.

The evidence overwhelmingly supports the presence of an intermediate in soluble methane monooxygenase catalysed oxidations of hydrocarbons. The intermediate (nonconcerted mechanism) is most likely a radical formed by H atom abstraction from substrate and direct addition of oxygen (concerted mechanism) seemed less probable. Thus, the reaction mechanism for soluble methane monooxygenase is very similar to that for cytochrome P450 despite the fact that the active sites of these two enzymes are very different, cytochrome P450 being a heme protein and methane monooxygenase possessing a μ-hydroxo-bridged binuclear iron centre.

1.5.3 Proposed mechanism

The nature of the products provides valuable mechanistic information on C-H bond activation and also gives insights into the topography of the active site pocket (Dalton, 1992b). Abstraction of a hydrogen atom from the substrate prior to insertion of oxygen implies that the generation of a strong electrophile similar to the ferryl species proposed for cytochrome P450 (Hamilton, 1974) may be formed in sMMO. In cytochrome P450 the strong electrophile is stabilised by the porphyrin ring system (Ortiz de Montellano, 1986). In the case of MMO, there is no such porphyrin ring system present, stabilisation could arise from the binuclear iron cluster (Shilov, 1984; Green and Dalton, 1989b) through delocalization of electron density (Fox *et al.*, 1990).

From the data accumulated for sMMO a scheme (Figure 1-10) for the oxidation of methane by soluble methane monooxygenase has been proposed (Green and Dalton, 1989b). In the native state, the iron species at the active site is present as a pair of high-spin ferric irons [Fe(III)/Fe(III)] (structure 1). Methane is believed to bind to this oxidised state of the enzyme at a site close to the binuclear centre (structure 2), presumably in some hydrophobic pocket in which displacement of water by methane would provide the entropy for binding, since there is no direct evidence that methane binds to the iron. Then, one of the iron atoms is reduced to give a mixed valence

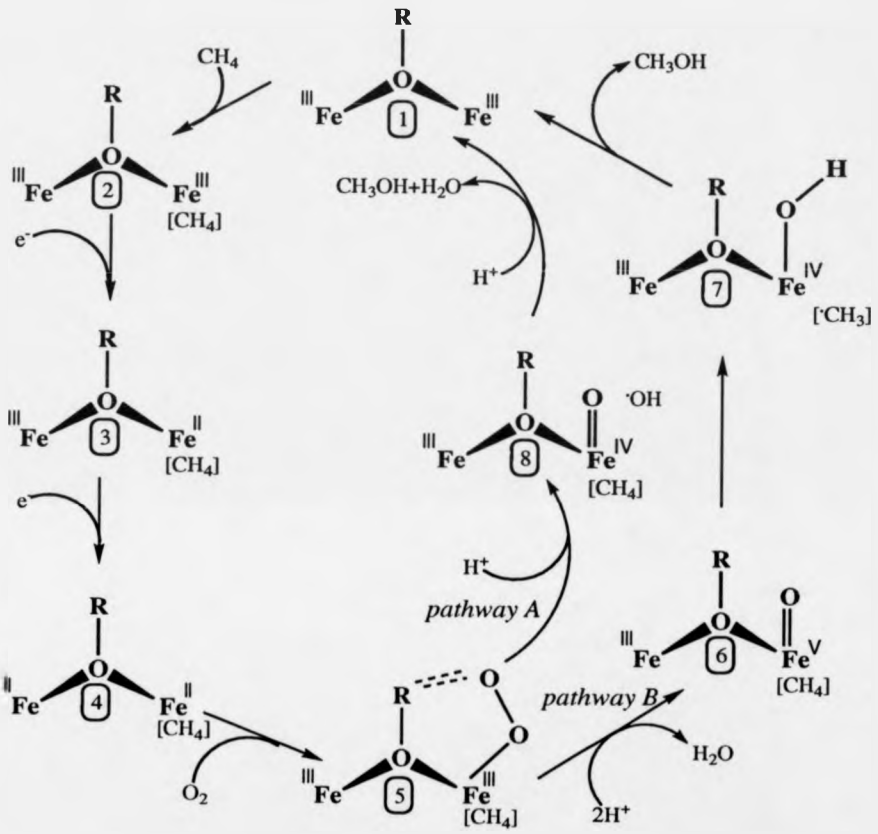


Figure 1-10. Proposed mechanism for the catalytic cycle of the soluble methane monooxygenase. *Pathway A represents homolytic cleavage of peroxide; pathway B represents heterolytic cleavage of peroxide. CH4, free methane, [CH_4], enzyme-bound methane. R means H or alkyl group.*

[Fe(II)/Fe(III)] form of the enzyme (structure 3) since electrons are delivered to the hydroxylase one at a time from the reductase. A second electron reduction of the diiron centre then occurs yielding the fully-reduced [Fe(II)/Fe(II)] enzyme species (structure 4). Dioxygen binding occurs with oxidation of the diiron centre and formation of a bound peroxide (structure 5). Two possible fates of the peroxide can then be envisaged. Homolysis of the peroxide would lead to the generation of a hydroxyl radical that would then abstract hydrogen from the methane in a Fenton-like reaction to form a methyl radical and water. The methyl radical would then collapse with the resultant $Fe^{4+}O^{-}$ species (structure 8) and a proton to form methanol. Heterolysis of the peroxide would generate water and a ferryl species (structure 6) (formally Fe^{IV}-Fe^{IV}-O^{2-} or Fe^{III}-Fe^{V}-O^{2-}). The ferryl species would then abstract a hydrogen atom from the methane to form a transient methyl radical in proximity to an iron-bound hydroxyl group (structure 7). The methyl radical through radical recombination with OH on the iron forms methanol with the concomitant regeneration of the oxidised form of the enzyme (structure 1).

1.5.4 Spin-trapping experiments

As described in Figure 1-10 two potential pathways were proposed for sMMO-catalyzed hydroxylations. The two possible pathways both invoke free-radical intermediates, i.e. hydroxyl radical from the homolysis of peroxo pathway or carbon-centred radical from the heterolysis of peroxide pathway. An effective method for dertermining which radical is involved is to employ the spin-trapping technique. Spin-trapping had proved to be very useful in identifying free radicals in a number of enzyme-catalyzed reactions, such as cytochrome P450 (Augusto *et al.*, 1982; Ortiz de Montellano, *et al.*, 1983; Kennedy and Mason, 1990; Nishida *et al.*, 1992), chloroperoxidase (Chamulitrat *et al.*, 1989), lipoxygenase (Chamulitrat *et al.*, 1991), peroxidase (Sinha, 1983; Mottley *et al.*, 1991), dopamine β-hydroxylase (Fitzpatrick

and Villafranca, 1986), flavoenzymes (Shi and Dalal, 1991), xanthine oxidase (Kohno *et al.*, 1991) and other complicated biological systems (Bynoe *et al.*, 1991; Hiramatsu *et al.*, 1991; Packer *et al.*, 1991).

Studies using spin-traps showed that only carbon-centred radicals were formed during catalysis with sMMO from *Methylococcus capsulatus* (Bath) and no hydroxyl radicals were detected (Deighton *et al.*, 1991; Wilkins *et al.*, 1992; Dalton *et al.*, 1992). In the spin-traping experiments the water soluble nitrones α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were employed (Deighton *et al.*, 1991; Wilkins *et al.*, 1992). The spin-trapping reagents and the reaction of formation of a spin-adduct are shown in Figure 1-11.

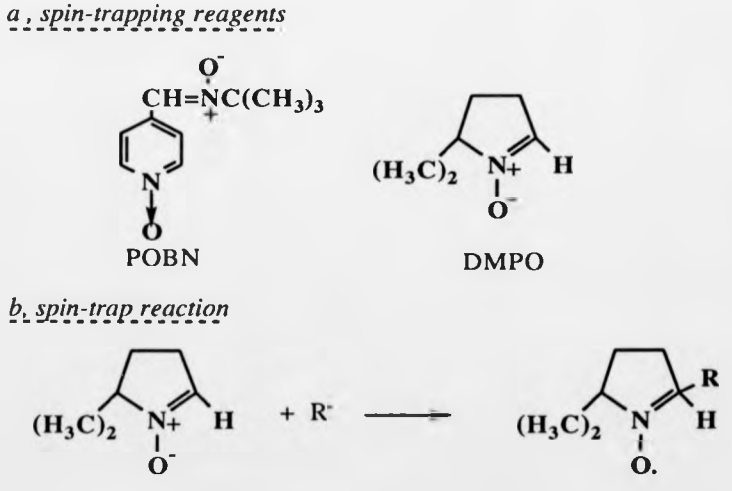


Figure 1-11. a, The spin-trapping reagents POBN and DMPO.
b, The reaction of formation of a spin-adduct.

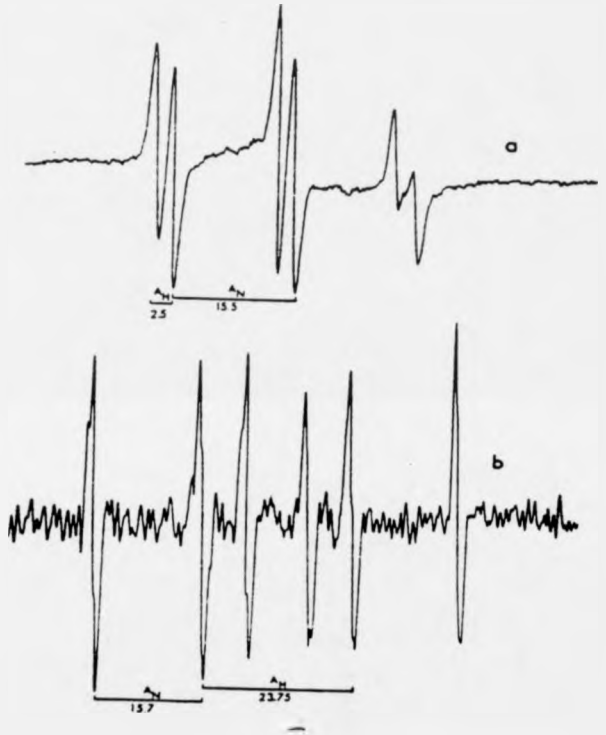


Figure 1-12. X-band EPR spectra of the spin-adduct.
(a), POBN CH_3 spin-adduct; (b), DMPO CH_3 spin-adduct.

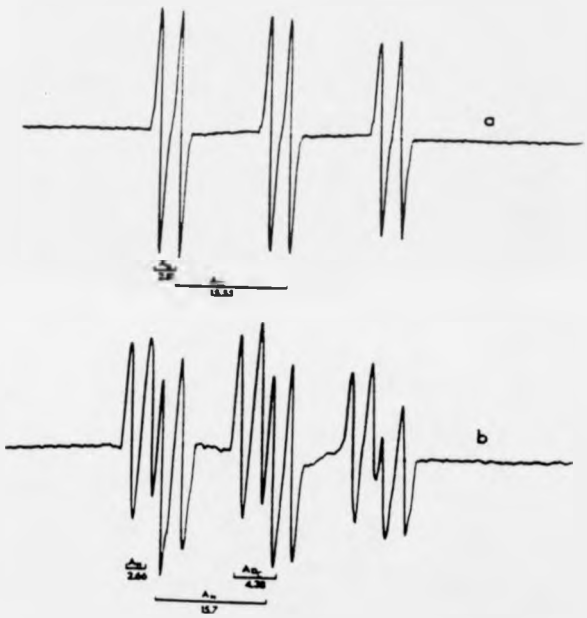


Figure 1-13. X-band EPR spectra of the (a) POBN·CH_2OH spin-adduct and (b) the POBN·$^{13}CH_2OH$ spin-adduct.

The X-band EPR spectra of the POBN·CH_3 and DMPO·CH_3 spin-adducts are shown in Figure 1-12. Isotope labelling experiments, as with $^{13}CH_3OH$, for unequivocal identification of the trapped radical were undertaken as shown in Figure 1-13. Splitting of the EPR signals due to ^{13}C was observed with both POBN and DMPO, showing that the trapped radicals were derived from substrate. A number of control experiments have confirmed that the observed carbon-centred radicals were produced by the enzyme during turnover. Many different substrate radicals were detected with spin traps, but no oxygen-based radicals of any kind were observed. These studies provided the first *direct* evidence that heterolytic cleavage of iron bound peroxide is operative in sMMO. The carbon-centred radicals are believed to result from hydrogen-atom abstraction by a ferryl species in a cytochrome-P450-like mechanism.

The heterolytic pathway requires formation a high electrophilic intermediate which may gain stability from the second iron in the binuclear centre. Thus an equilibrium between [-Fe^{IV}-Fe^{IV}=O] and [-Fe^{III}-Fe^{V}=O] probably exists (Dalton, 1992a). The results support the conclusion that hydrocarbon oxidation catalyzed by sMMO takes place in two distinct stages, C-H bond cleavage followed by -OH addition.

1.5.5 Alternative mechanisms for soluble methane monooxygenase

Several types of substrates were studied in the spin-trapping experiments. In the case of CO and pyridine oxidations, there were no detectable spin-adducts formed (Dalton *et al.*, 1992). Direct oxygen addition may occur in these reactions. The results seem to establish two independent mechanistic pathways for sMMO. The observation of weak spin-adduct EPR signals in the epoxidation of alkenes led to the suggestion that in these reactions both routes were possible (Dalton *et al.*, 1992).

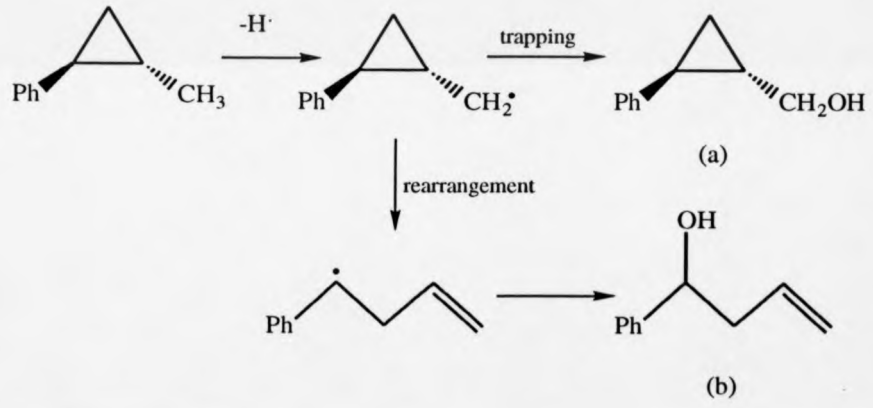
Intriguing results were recently reported by Lippard's group (Liu *et al.*, 1993) that when*trans*-2-phenylmethylcyclopropane, used as a mechanistic probe, was oxidized by sMMO from *Methylococcus capsulatus* (Bath), no ring-opened (a rearranged alcohol) product was observed suggesting that the hydroxylation reaction might not proceed through formation the cyclopropylcarbinyl radical or cation. If hydrogen atom abstraction from the probe *trans*-2-phenylmethylcyclopropane at the methyl position occurs, the resulting cyclopropycarbinyl radical either would be trapped before ring opening to *trans*-(2-phenylcyclopropyl)methanol or would ring open to the 1-phenylbut-3-enyl radical, subsequently forming 1-phenylbut-3-en-1-ol (Scheme 1-3). When using 2,2-diphenylmethylcyclopropane, *trans*-1,2-dimethylcyclopropane and bicyclo[2.1.0]pentane as substrates, similar results were also observed. It seemed like that hydroxylation reaction pathway did not proceed through a radical intermediate for these substrates. However, the hydroxylation reaction with sMMO from *Methylosinus trichosporium* OB3b gave a small amount (3-5%) of rearranged products with *trans*-2-phenylmethylcyclopropane.

Furthermore, when comparing the rate of conversion of the undeuterated and trideuterated *trans*-2-phenylmethylcyclopropane catalysed by sMMO from *Methylococcus capsulatus* (Bath), no intermolecular isotope effect was observed and the intramolecular isotope effects, k_H/k_D , were 5.15 and 5.03 from studies of the mono- and dideuterated *rans*-2-phenylmethylcyclopropane, respectively, suggesting that the C-H bond breakage was not the rate-limiting step of the enzymatic reaction and the hydroxylation reaction involved a substantial C-H bond stretching component.

Obviously, these results do not support many previous studies in which the proposed mechanism has resulted from the investigation a great number of substrates and the direct observation of carbon-centred radicals formed during catalysis. And these kinds of substrates oxidation by sMMO seemed to be favouring a non-radical mechanism and was similar to an observation made 12 years earlier, using

cyclopropane and methycyclopropane as MMO substrate (Dalton *et al.*, 1981). Certainly, no single unifying mechanism could account for all of the data (Liu *et al.*, 1993). It seems that more than one mechanistic pathway might be operative in soluble methane monooxygenase catalyzed reactions (Dalton *et al.*, 1992; Liu *et al.*, 1993). and the mechanism of action may well be substrate dependent (Dalton *et al.*, 1993b).

Scheme 1-3.



1.5.6 Chemical models and mimics for soluble methane monooxygenase

Substantial progress has been made in modelling diferric (Fe^{III}/Fe^{III}) or diferrous (Fe^{II}/Fe^{II}) protein centres using a variety of mutidentate ligands, and considerable insight has been gained into the magnetic and electronic properties of the active sites of these metalloproteins (Hagen and Lachicotte, 1992; Rardin *et al.*, 1991; Tolman *et al.*, 1991). There are a number of models which mimic μ-oxo bridged binuclear iron

centres. Some of them have been proposed as sMMO models and a few showed some catalysis of oxidation of C-H bonds (Kitajima *et al.*, 1988; Vincent *et al.*, 1988; Sheu and Sawyer, 1990; Stassinopoulos and Caradonna, 1990; Kurusu and Neckers, 1991), but none of them was capable of catalysing the oxidation of methane. Only one model of a binuclear iron peroxide complex was capable of olefin epoxidation (Murch *et al.*, 1986). Recently, a new model based on Gif chemistry was reported. The binuclear iron complex was supported on an imidazole-containing silica support and was prepared as a sMMO active site mimic (Dalton, 1992b and references within). This model was reported to be capable of oxidising methane to methanol and formaldehyde, ethane and hexane to alcohols, cyclohexane to alcohol and ketone and sulfoxides to sulfones in the presence of a proton donor (acetic acid), a reductant (zinc dust), a mediator (methyl viologen) and acetonitrile.

Gif chemistry was originally designed to mimic the non-heme enzymatic oxidations of alkanes. This group of enzymes includes soluble methane monooxygenase, prolyl 4-hydroxylase (Kivirikko *et al.*, 1989), isopenicillin N-synthase (Baldwin, 1990), γ-butyrobetaine hydroxylase (Ziering and Pascal, 1990), as well as biologically active systems like bleomycin (Suga *et al.*, 1991). There are a number of similarities between the sMMO catalytic reaction and Gif reactions for the functionalization of alkanes (Dalton, 1992a). Gif chemistry has developed into several systems, Gif^{III}, Gif^{IV}, GO, $GoAgg^{I}$, $GoAgg^{II}$, $GoAgg^{III}$, GoChAgg and Cu^0/O_2. In the first four systems the catalysts are Fe(II) with O_2; in GoAgg II and III the catalysts are Fe(III) with H_2O_2 as the electron source and the oxidant; in GoChAgg the catalyst is Cu(II) with H_2O_2 and in the last system the catalyst is Cu(I) with O_2 (Barton and Doller, 1992). The activation of a C-H bond in Gif systems gave alcohol and ketone as products. The proposed mechanism of C-H bond activation involved two-intermediates as shown in Figure 1-14 (Barton *et al.*, 1992a). The intermediate B has been shown to be an alkyl hydroperoxide (Barton *et al.*, 1992b). The intermediate A

was not thought to be a carbon radical based on the mechanistic studies showing that there was not any evidence supporting a carbon radical formation in Gif reactions. It was proposed that the active iron species (Fe^{V}) reacts with the C-H bond to form an iron bound carbon intermediate (intermediate A) in the initial step (Figure 1-15) (Barton and Doller, 1992; Barton *et al.*, 1992b).

So far there is no direct evidence which supports iron-carbon bond formation in either sMMO or Gif catalysed reactions. Although the Gif system is indeed capable of mimicking some of the alkane oxidations catalyzed by sMMO, it is still unclear whether the mechanism proposed for Gif chemistry could be operative in soluble methane monooxygenase. A drawback of Gif systems as sMMO models is that they are unable to epoxidize alkenes and are unable to oxidise methane.

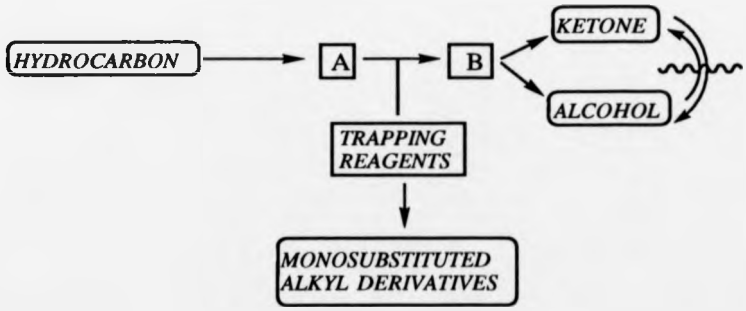


Figure 1-14. The hypothesis of two-intermediates in Gif chemistry.

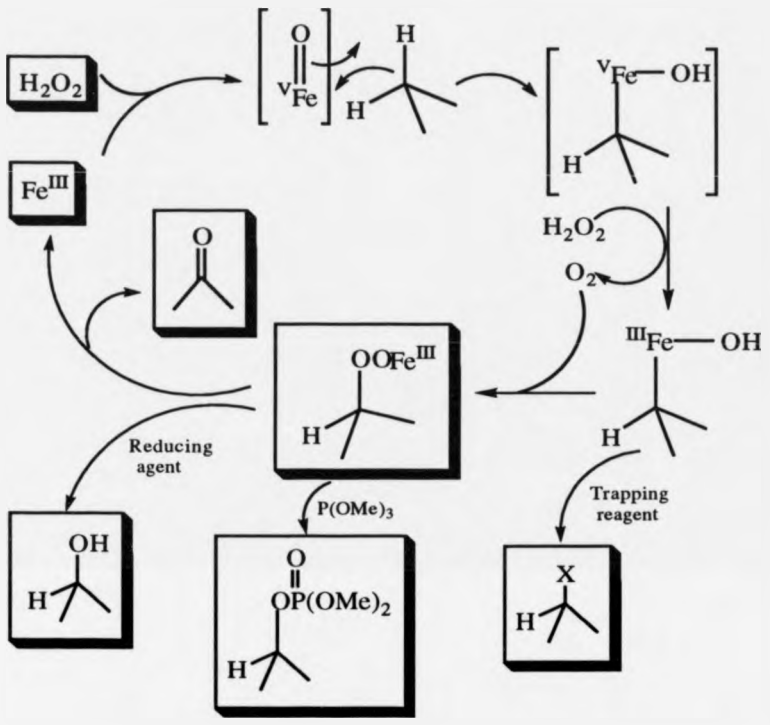


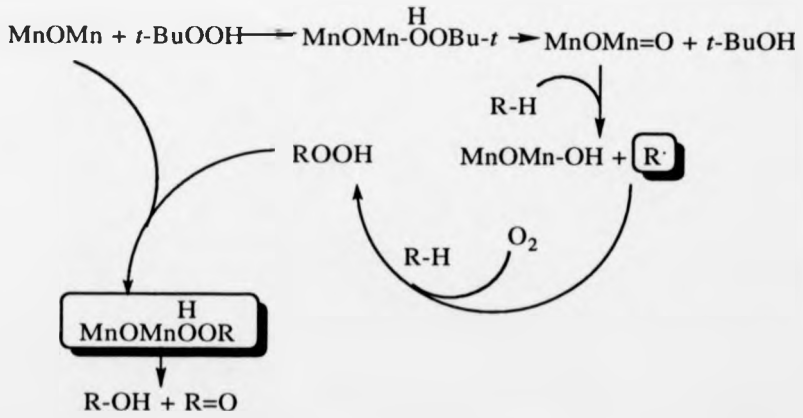
Figure 1-15. Proposed catalytic cycle for $GoAgg^{III}$ reactions.

Recently, a model complex containing a μ-oxo-bridged-dimanganese cluster was established (Fish *et al.*, 1992). This compound in the presence of O_2 was reported to catalyse conversion of ethane, propane, cyclohexane, adamantane and toluene to their respective alcohols, aldehydes or ketones with a oxygen atom transfer reagent, such as *t*-butyl hydroperoxide or iodosobenzene. The results implied that a putative Mn=O species was formed and it homolytically abstracted hydrogen from C-H bonds

(substrate, R-H) to give a carbon radical (R·) and Mn-OH. The carbon radical was proposed to be trapped by O_2 to form a alkyl hydroperoxide (ROOH), which decomposed via a alkyl peroxy-Mn complex intermediate to alcohol, ketone, or aldehyde and Mn=O complex (Scheme 1-3). This mechanism also proposed that two intermediates might be present in the reaction cycle. The first intermediate is thought to be a carbon-centred radical and the second an alkylperoxy-Mn complex which is similiar to the alkylperoxy-Fe and alkylperoxy-Cu complexes found in Gif chemistry (Barton *et al.*, 1992a; Barton and Doller, 1992).

Information from chemical models reaction systems could be helpful in understanding the mechanism of action of soluble methane monooxygenase.

Scheme 1-4.



1.6 Possible structure of the active site

1.6.1 Gene sequence of sMMO

The cloning of the structural genes of sMMO from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b has given complete amino acid sequences for the hydroxylase, reductase and protein B (Mullens and Dalton, 1987; Stainthorpe *et al.*, 1989, 1990; Pilkington *et al.*, 1990; Cardy *et al.*, 1991a). DNA sequencing of the entire sMMO gene cluster of *Methylococcus capsulatus* (Bath) (Figure 1-16), contained on plasmid pCH4, has shown that the genes encoding the α, β and γ subunits of the hydroxylase (*mmoX, Y*, and *Z*), protein B (*mmoB*) and the reductase (*mmoC*) were all linked on the chromosome of this methanotroph (Murrell, 1992).

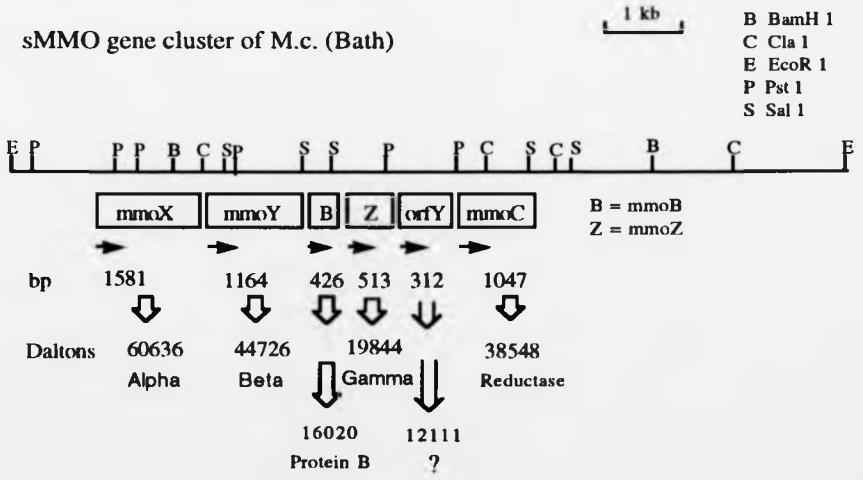


Figure 1-16 . Recombinant plasmid pCH4 containing the soluble MMO gene cluster of *Methylococcus capsulatus* (Bath).

It was found that the reductase exhibited significantly homology at the N-terminal amino acid sequence with ferredoxins of plant and bacterial origin (Cardy *et al.*, 1991b; Murrell, 1992). The function of *orfY* is unknown and the protein product has not knowingly been isolated from *Methylococcus capsulatus* (Bath) (Murrell, 1992). The genes encoding the α, β and γ subunits of the hydroxylase were interrupted by the regulatory protein B gene *mmoB* (Pilkington *et al.*, 1990). Expression of sMMO genes has been achieved in an in vitro transcription/translation assay system (Cardy *et al.*, 1991a). The *mmoB* and *mmoC* genes from *Methylococcus* have been expressed in *E. coli* and the proteins produced were functionally active (West *et al.*, 1992).

1.5.2 The physical nature of the active site

Protein homology studies revealed that the α subunit of the hydroxylase possesses conserved regions which show homology to the R2 protein of ribonucleotide reductase (Stainthorpe *et al.*, 1990). It was noted that the Glu-x-x-His sequence motif which has been identified to coordinate to the irons in the R2 protein of ribonucleotide reductase appeared twice in the α-subunit (Cardy *et al.*, 1991) (Figure 1-17). The X-ray crystal structure of the R2 protein of ribonucleotide reductase from *E. coli* has given the ligands associated with the binuclear iron centre of this protein (Nordlund *et al.*, 1990) (Figure 1-18). Comparison of the alignment of the sequences of the α subunit of the hydroxylase with the sequences for the iron binding site of the R2 protein showed that they were very complementary (Dalton, 1992a). The α-subunit sequences of the *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b hydroxylases have been aligned with the four helix iron coordination bundle of R2 protein (Nordlund *et al.*, 1992). The corresponding iron ligands for the sMMO hydroxylase are His 147 and bidentate Glu 114 on Fe1, His 246 and monodentate Glu 209 and Glu 243 on Fe2 and bridging Glu 144 (Dalton et al, 1993a) (Figure 1-18). The iron coordination in sMMO is similar to the R2 protein with two histidine ligands and four carboxyl ligands. The residues lining the

proposed oxygen binding site in MMO are smaller than those in R2 protein. An electron nuclear double resonance (ENDOR) spectroscopy study (Hendrich *et al.*, 1992) and the chemical modification of the hydroxylase with diethylpyrocarbonate (DEPC) (Smith and Dalton, 1992) indicated that two histidine ligands were present at the diiron site of the hydroxylase of sMMO. Also, circular dichroism (CD) spectroscopy has shown that the hydroxylase component of sMMO contains a high degree of helical structure and is similar to that in the R2 protein of ribonucleotide reductase (Smith and Dalton, 1992). Therefore, the active site structure of sMMO may be closely related to the binuclear iron centre of ribonucleotide reductase.

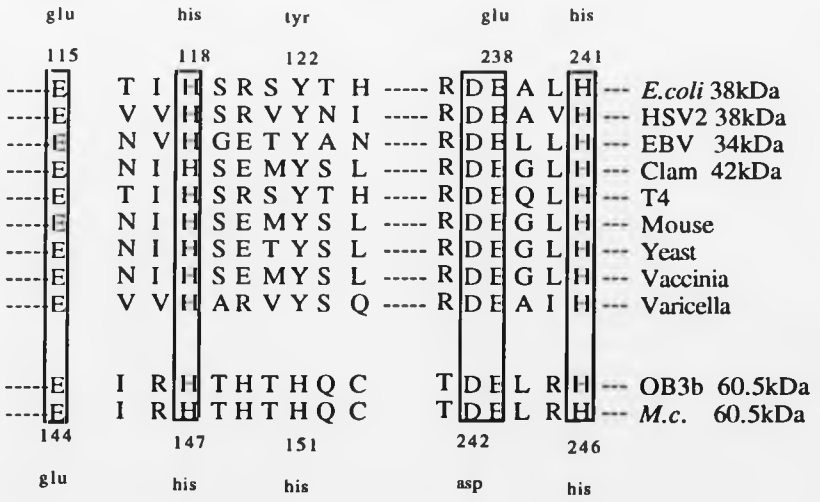


Figure 1-17. Sequence homology between the Fe-binding sites in the R2 protein of ribonucleotide reductase from nine different sources and the α–subunit of the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b.

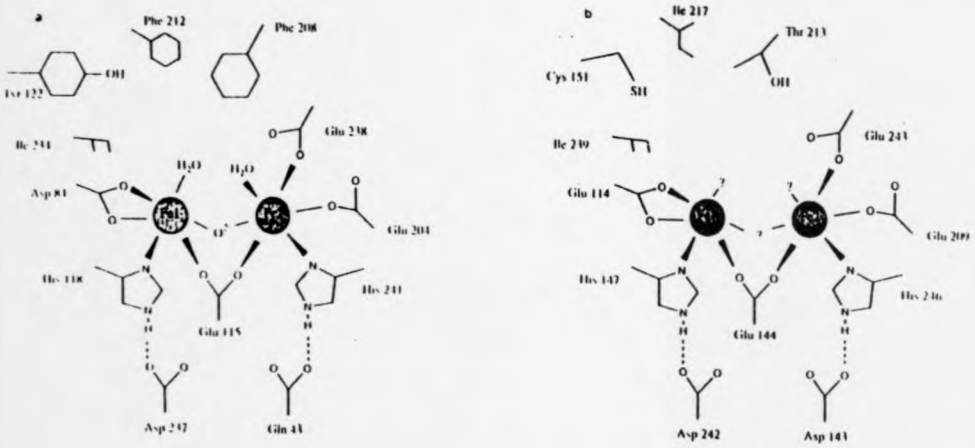


Figure 1-18. Schematic view of the iron sites in the R2 protein of ribonucleotide reductase and the hydroxylase subunit of sMMO. (a) The diiron site in the R2 protein of ribonucleotide reductase. (b) The active site model for sMMO hydroxylase.

It appears that a number of amino acids near the active site of the sMMO hydroxylase are effectively neutral in character (Thr, Ile) (Nordlund *et al.*, 1992). The presence of these neutral amino acids might minimise any damaging effects of the radicals formed in sMMO reactions (Dalton *et al.*, 1993a). A cysteine residue in sMMO was suggested to be at the position equivalent to that of the tyrosyl radical in the R2 protein of ribonucleotide reductase. This residue might be involved in reactivation of sMMO apoenzyme (Smith and Dalton, 1992). A recent study using UV-Vis, CD and resonance Raman spectra demonstrated that phenol bound to Fe(III) when added to native hydroxylase indicating that relatively large molecules have access to the diiron cluster in sMMO (Andersson *et al.*, 1992).

Recently, the hydroxylase proteins from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b have been crystallized from aqueous solutions containing polyethyleneglycol, lithium sulfate and ammonium acetate (Rosenzweig *et al.*, 1992). A preliminary X-ray analysis of the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath) has been made and indicates that the proposed model for the sMMO active site (Nordlund *et al.*, 1992) fits quite well with the unrefined X-ray data (to 3 Å) (Lippard, 1993; Rosenzweig et al., 1993).

1.7 Project objectives

The functionalization of C-H bonds, especially the activation of methane, by soluble methane monooxygenase at ambient temperature and pressure is difficult to achieve. C-H bond breakage and insertion of an O atom into a C-H bond, i.e., H_3C-OH, under mild conditions has not been possible by chemical means. Also, most biochemical catalysts, such as monooxygenases including cytochrome P450 and many one or two non-heme iron proteins, can not oxidise methane to methanol. Significantly, only the methane monooxygenases possesses this unique characteristic among biochemical catalysts able to functionalize C-H bonds. Stimulating the study of

soluble methane monooxygenase are the catalytic features of the enzyme from an industrial point of view. Understanding gained on how this is achieved biologically may help in the preparation of direct methane oxygenation catalysts that are more robust than the enzyme and which can withstand the rigours imposed on it in industrial use (Dalton, 1992a).

Therefore, the objectives of this project are as follows:

(1) The idea that is initially addressed in this project is to mimic chemical oxidative-coupling reactions. Since our mechanism studies have indicated that radicals are involved in the sMMO reaction, it might be possible that their lifetime in the active site is such that they could recombine to produce higher alkanes homologue. Such a precedent is observed in the oxidative coupling over basic oxides (Ito and Lunsford, 1985; Tong et al., 1989; 1990; Lunsford, 1990; 1991). One might expect that if the oxygen concentration was controlled or replaced by alternative donors, then the carbon-centred radicals generated in the sMMO reaction might be released and react with each other to form a C-C bond.

(2) In cytochrome P450-catalysed reactions it has been demonstrated that a number of peroxides, such as hydrogen peroxide or iodosobenzene, can replace both the reductant and oxygen to effect hydrocarbon oxidation. Recent studies by the Lipscomb group (Andersson *et al.*, 1991) has shown that hydrogen peroxide could activate the hydroxylase of sMMO from *Methylosinus trichosporium* OB3b. Thus, the effect of peroxide compounds on the hydroxylase component of sMMO from *Methylococcus capsulatus* (Bath) will be adressed. If hydrogen peroxide could activate the hydroxylase of *Methylococcus capsulatus* (Bath), then it would be possible to simplify the reaction and study its properties in the absence of other proteins.

(3) If the hydroxylase alone plus hydrogen peroxide will catalyse the oxidation of methane and other hydrocarbons, then we can ask "how much of the hydroxylase is necessary to effect such an oxidation"? Since there is no longer the requirement to bind O_2, NADH or the reductase and protein B, it is possible that much of the hydroxylase is superfluous to requirements when H_2O_2 is used. proteolytic digestion of the hydroxylase could lead to a truncated, but functionally active form of the protein that only has to bind substrate and H_2O_2 and this trimmed down version of the hydroxylase may be helpful in defining the smallest catalytic unit necessary to oxidise hydrocarbons.

(4) Modification of the hydroxylase with specific chemical or cross-linking reagents and determination of the physical and catalytic properties of the altered protein is the next stage. The modified hydroxylase could be used in extreme conditions, such as non-aqueous solvents. There have been many reports on the use of whole cells of methane oxidising bacteria to effect the production of oxy-chemicals from simple hydrocarbons with industrial application in mind (Lidstrom and Stirling, 1993). For methane monooxgenase that catalyses the mono-oxygenation of a wide variety of hydrophobic substrates it is believed that reaction rates may be limited by the solubility of the substrates in aqueous medium. Furthermore, the inherent instability of the enzyme precludes its use as a general oxidising catalyst. Any improvements that could be made to such a utilitarian catalyst could have important implications in its industrial use.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation of soluble methane monooxygenase

2.1.1 Growth of *Methylococcus capsulatus* (Bath)

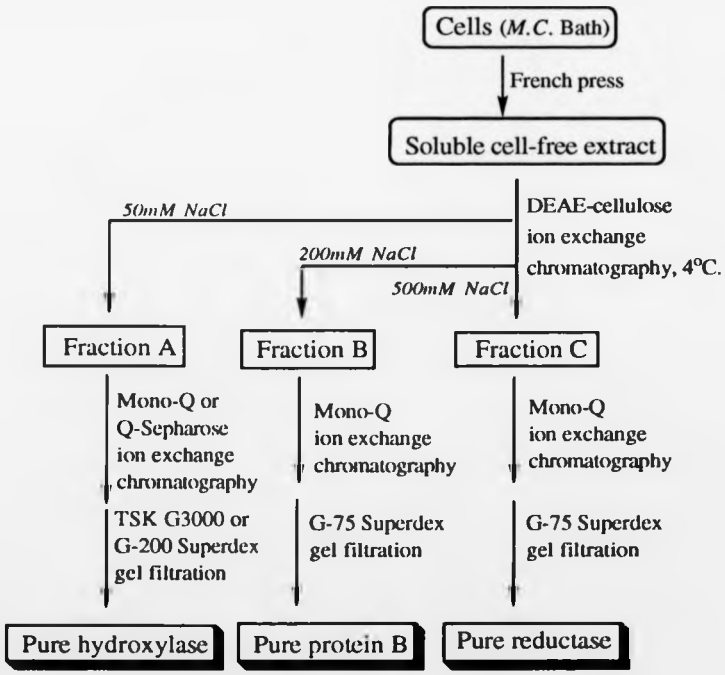
Methylococcus capsulatus (Bath) was grown in a low-copper nitrate mineral salts medium with methane as carbon source in continuous culture (Whittenbury *et al*, 1970; Stanley *et al*, 1983).

The fermentation was performed in a 2-L working volume LH1000 fermenter (LH Engineering Ltd, Stoke Poges, Bucks, U.K.) in continuous culture at 45°C. The medium (per litre) contained 1 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$, 200 mg $CaCl_2$, 4 mg FeEDTA, 0.26 mg $NaMnO_4$, 15 ml pH 6.8 buffer of 6.5% Na_2HPO_4/KH_2PO_4, and 1 ml of stock trace element solution which consisted of 100 mg $CuSO_4 \cdot 5H_2O$, 500 mg $FeSO_4 \cdot 7H_2O$, 400 mg $ZnSO_4 \cdot 7H_2O$, 15 mg H_3BO_4, 50 mg $CoCl_2 \cdot 6H_2O$, 250 mg EDTA-Na_2, 20 mg $MnCl_2 \cdot 4H_2O$ and 10 mg $NiCl_2 \cdot 6H_2O$ in 1 litre of water. The 2-L working volume fermentation was grown for 72-96 hours until the optical density (A_{550nm}) of the culture was 7 - 9, at which point continuous medium addition was initiated. During continuous culture fermentation, the medium flow rate was maintained at 90 ml/h; the volume was 20 L; the pH value of the culture was maintained at 6.8 by automatic titration with 6 M of HCl; air and methane were sparged into the fermenter with gas flow rates of 40 ml/min of methane and 200 ml/min of air. The 20 L of cells from the overflow reservoir were harvested using a Westfalia continuous centrifuge (Westfalia Separator Ltd, Wolverton, Bucks, U.K.).

The resultant cell paste was washed once with ice-cooled buffer {25 mM MOPS (3-[N-morpholino]propane sulfonic acid, *sodium salt*) pH 7.0} and was resuspended in the same buffer containing DTT (D,L-dithiothreitol, $C_4H_{10}O_2S_2$, Cleland's reagent) and benzamindine ($C_7H_8N_2$·HCl) in the concentration of 5 mM and 1 mM, respectively. The suspension was frozen in liquid nitrogen and stored at -80°C until required.

2.1.2 Purification of sMMO-components

2.1.2.1 Flowchart of the purification of sMMO-components



2.1.2.2 Preparation of soluble cell-free extract

The cell paste was thawed (120 ml) and diluted with 25 mM MOPS buffer (pH 7.0) to a final volume of 150 ml, followed by the addition of 5 mM sodium thioglycollate (thioglycolic acid, *sodium salt,* grade V) and 2-4 mg of DNase (Deoxyribonuclease I, from bovine pancreas, E.C. 3.1.21.1). The resulting mixture was loaded into a pre-cooled French Pressure Cell (American Instrument Company, Silver Spring, MD) and the cells were broken by two-passages at 137 MPa. Unbroken cells and debris were removed by centrifugation at 18,000 g, 4°C for 60 minutes with a J2-21M/E Centrifuge (Beckman, U.K.) to yield a soluble cell-free extract.

The resulting soluble crude methane monooxygenase could be either directly loaded onto DEAE-cellulose column to complete a further purification, or was frozen using liquid nitrogen and stored at -80°C until required.

2.1.2.3 DEAE-cellulose ion exchange chromatography

Resolution of the sMMO into three components was effected by ion-exchange chromatography (Colby and Dalton, 1978; Pilkington and Dalton, 1990) and the whole process was carried out at 4°C.

The soluble extract was thawed and placed onto a column of DEAE-cellulose (4.5 x 8.0 cm) equilibrated with buffer of 25 mM MOPS(pH 7.0) containing 5 mM sodium thioglycollate and 50 mM NaCl. Material not binding to DEAE-cellulose was eluted with MOPS buffer to give *fraction A* containing the hydroxylase. The column was then eluted with MOPS buffer containing 200 mM NaCl, 1 mM benzamindine and 1 mM EDTA (ethylenediaminetetraacetic acid, disodium salt) to yield *fraction B* containing protein B. Finally the column was eluted with MOPS buffer containing 500 mM NaCl and 5 mM DTT to produce *fraction C* containing the reductase.

The further purification of the separated three components of soluble methane monooxygenase was undertaken separately.

2.1.2.4 Purification of the hydroxylase

Purification of the hydroxylase of soluble methane monooxygenase was described by Woodland & Dalton (1984) and Pilkington & Dalton (1990). In this thesis the purification procedures of the hydroxylase were basically adopted from the literature using more efficient purification columns.

Step 1: Mono Q or Q-Sepharose Ion-Exchange Chromatography.

Centrifugation of the *fraction A* from the DEAE-cellulose was performed by a J2-21M/E Centrifuge (Beckman, U.K.) at 18,000 g, at 4°C for 20 minutes, then the supernatant was filtered through a 0.2 μm syringe filter. The resulting material could be loaded (10 ml in volume and the maximum loading amount < 0.3 g protein) onto a prepacked Mono Q ion-exchange column (HR 10/10) (Pharmacia, LKB Biotechnology, Uppsala, Sweden), followed by elution with a 0-300 mM linear gradient of NaCl in 25 mM MOPS buffer (pH 7.0) using a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, LKB Biotechnology) at room temperature. The hydroxylase fractions elute from the column at approximately 150 mM NaCl.

A second method was also used in this work. The filtered fraction A was loaded (40 ml in volume with a maximum loading of < 1.5 g protein) onto a prepacked Q-Sepharose ion-exchange column (26 x 100 mm) (Pharmacia, LKB Biotechnology) maintained at 0-4°C by a cooler (Grant Instruments Ltd, Barrington, Cambridge, England). The hydroxylase fractions elute from the Q-Sepharose column at approximately 220 mM NaCl.

Active hydroxylase fractions were then concentrated by ultrafiltration over a PM30 membrane (Amicon, Corporation, Danvers, MA.). Enzyme assays showed that both columns of Mono Q and Q-Sepharose gave similar results as shown in Table 2-1.

Table 2-1 Purification of the hydroxylase

Step	Vol. (ml)	Protein content (mg/ml)	Total protein (mg)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Purification factor	Yield (%)
Soluble extract	90	55	4,950	40.8	201,960	1.00	100
DEAE-cellulose	90	25	2,250	85.6	147,600	2.10	73.1
Mono-Q	6	95	570	138.5	78,945	3.39	39.1
TSK G3000	20	18	360	178.7	64,332	4.38	32
Soluble extract	120	35	4,200	41	172,200	1.00	100
DEAE-cellulose	120	12	1,440	88	126,720	2.15	73.6
Q-sepharose	5	88	440	142.7	62,788	3.48	36.5
G200 superdex	21	16	336	176.5	59,304	4.29	34.4

Step 2: TSK-G 3000 SWG or G-200 Superdex Gel Filtration.

The concentrated material (40-80 mg/ml) was then applied in 2 ml aliquots to a TSK-G 3000 SWG (21.5 x 600 mm) gel permeation column preceded by a guard column of Ultra-Pak TSK-G SWPG (21.5 x 75 mm) (Tosoh Corporation, Tokyo, Japan) using a High Performance Liquid Chromatography (HPLC) system (Pharmacia, LKB Biotechnology) at room temperature. Degassed and filtered buffer (25 mM MOPS, pH 7.0) was supplied to the column at a flow rate of 3 ml/min. The eluate was collected by means of a Multirac-2m fraction collector (Bromma, Sweden) and monitored for protein by UV absorbance at 280 nm with UV-M Optical Unit (Pharmacia, LKB).

Also, the material could be applied in 4 ml aliquots to a prepacked G-200 Superdex gel column (26 x 600 mm) (Pharmacia, LKB Biotechnology) using an FPLC system which gave almost the same purity as that using TSK-G 3000 SWG column with HPLC system. Degassed and filtered buffer (25 mM MOPS, pH 7.0) was supplied to the column at a flow rate of 3 ml/min and the process was carried out at 4°C. The eluate was collected by means of a FRAC-100 fraction collector (Pharmacia, LKB) and monitored for protein by UV absorbance at 280 nm with UV-1 Optical Unit (Pharmacia, LKB).

The combined active fractions were concentrated by ultrafiltration over a PM30 membrane and then frozen in liquid nitrogen and stored at -80°C until required. This method of storage was applied to all intermediary steps of purification without loss in activity. Purity of hydroxylase was assessed by SDS-polyacrylamide gel electrophoresis.

2.1.2.5 Purification of protein B

The process of purification of protein B of soluble methane monooxygenase was described as previously (Green and Dalton, 1985; Pilkington and Dalton, 1990).

Step 1: Mono-Q Ion-Exchange Chromatography.

Firstly, the *fraction B* from DEAE-cellulose was treated with 0.3 mM of phenylmethylsulphonyl fluoride (PMSF, 0.4 M ethanol solution), a serine protease inhibitor. Secondly, the ionic strength of the fraction B was reduced either by ultrafiltration through an Amicon PM10 ultrafiltration membrane and then made up to the original volume with buffer, or by dilution to two-fold volume with 25 mM MOPS buffer (pH 7.0). The material was then applied onto a Mono-Q (HR 10/10) ion-exchange column and eluted with a 0-350 mM (non-linear) gradient of NaCl in 25 mM MOPS buffer (pH 7.0) containing 1 mM benzamindine and 1 mM EDTA on the FPLC system. The fractions containing protein B were eluted off the column at approximately 220 mM NaCl concentration. The combined active protein B fractions were concentrated over a PM10 ultrafiltration membrane.

Step 2: G-75 Superdex Gel Filtration Chromatography.

The resulting concentrated material (10-25 mg/ml) was then applied in 4 ml aliquots to a G-75 Superdex column (26 x 600 mm, prep grade, Pharmacia) on the FPLC system. Degassed and filtered 25 mM MOPS (pH 7.0) buffer containing 1 mM of each benzamindine and EDTA was supplied to the column at a flow rate of 4 ml/min. Gel filtration was carried out at 4°C. The combined active protein B fractions were concentrated and frozen in liquid nitrogen and stored at -80°C without loss in activity. Purity of protein B was assessed by SDS-polyacrylamide gel electrophoresis.

2.1.2.6 Purification of the reductase

The purification of the reductase of sMMO was described by Colby & Dalton (1978, 1979), and Pilkington & Dalton (1990).

Step 1: Mono-Q Ion-Exchange Chromatography.

The *fraction C* from DEAE-cellulose was diluted two-fold with 25 mM MOPS buffer (pH 7.0) to reduce its ionic strength. The sample was then applied to a Mono-Q (HR 10/10) ion exchange column and was eluted with a 0-600 mM linear gradient of NaCl in 25 mM MOPS buffer (pH 7.0) containing 5 mM DTT on the FPLC system. The fractions containing reductase eluted from the column at approximately 300 mM NaCl. The combined reductase fractions were concentrated over a PM10 ultrafiltration membrane.

Step 2: G-75 Superdex Gel Filtration Chromatography.

The concentrated material (10-15 mg/ml) was applied in 4 ml aliquots onto a G-75 Superdex column (26 x 600 mm, prep grade, Pharmacia) on the FPLC system. Degassed and filtered buffer of 25 mM MOPS (pH 7.0) containing 5 mM DTT was supplied to the column at a flow rate of 3 ml/min. The gel filtration was carried out at 4°C. The combined reductase fractions were concentrated then frozen in liquid nitrogen and stored at -80°C without loss in activity. The purity of the reductase was assessed by SDS-polyacrylamide gel electrophoresis.

2.2 Analytical determinations of protein and enzyme

2.2.1 Estimation of protein

Protein concentration was estimated by the method of Bradford (1976) using commercially available Bio-Rad reagent (Bio-Rad Ltd. Watford, Herts, U.K.) and compared with a standard curve using bovine serum albumin as a protein standard.

2.2.2 Estimation of iron content of the hydroxylase

Iron content of the hydroxylase was determined by a method adopted from Woodland and Dalton (1984) and Lynch *et al* (1989).

The protein was wet ashed in concentrated trichloroacetic acid(TCA), i.e., 500 µl of protein solution (2-5 mg protein) plus 50 µl of 50% of TCA for 2 minutes. After centrifugation the acid digest (500 µl) was transferred to a 1 ml cuvette which contained 400 µl saturated sodium acetate, 90 µl 20% ascorbic acid (fresh solution) and 10 µl 50 mM bathophenanthroline sulphonate (4,7-dipheny-1,10-phenanthroline di-sulfonic acid) and absorbance was measured at 535 nm. The data was either compared with a standard curve prepared from solutions of ferrous ammonium sulphate, or calculated by the extinction coefficient (ε = 22,000 M^{-1}) (Woodland and Dalton, 1984) to estimate the iron content of the hydroxylase.

2.2.3 Enzyme activity assays

Enzyme specific activity in all purification steps was assayed by the observation of propene oxidation to epoxypropane by sMMO complex. All figures for the activity of enzymes are the average of at least three assays.

Soluble extract activity.

The soluble extract consists of the three components of sMMO in which the specific activity was demonstrated to show a non-linear relationship with the concentration of the protein. When the concentration of the protein was 5 mg/ml in the assay, the maximum specific activity was observed (Colby and Dalton, 1976). Therefore, the amount of protein used to assay the soluble extract was 5 mg/ml throughout this thesis.

Hydroxylase activity.

Assay of the hydroxylase could use either crude or purified preparations of Protein B and reductase. The assay was performed in 25 mM MOPS (pH 7.0) buffer in a Suba-sealed (W.H. Freeman, Barnsley, U.K.) 5-ml conical flask, at 45°C as described by Colby and Dalton (1978) and Woodland and Dalton (1984).

In each reaction mixture containing (final concentration) 8 μM hydroxylase, 1.5 mg each of crude protein B and crude reductase, or 8 μM each of purified protein B and reductase was added in a 500 μl working volume and sealed with a Suba-seal. 3 ml of propene was added to replace the same volume of gas phase above the reaction solution. The flask was incubated in a Gyratory water bath shaker (G76 model, New Brunswick Scientific Co Ltd., Edison, New Jersey, U.S.A.) shaking at 60-70 oscillations/min. After 1 minute preincubation, the reaction was initiated by the addition of 5 mM NADH (ethanol-free). After 3 minutes incubation, a sample (5 μl) of reaction mixture was injected into the gas chromatograph for analysis of epoxypropane formed.

Protein B activity

8 μM of pure protein B was added in a 500 μl reaction solution containing 8 μM of the hydroxylase and 1.5 mg of crude reductase (or 8 μM of purified reductase) and sealed with a Suba-seal. 3 ml of propene was added to replace the same volume of gas phase above the reaction solution. The flask was incubated in the water bath shaking at 60-70 oscillations/min. After 1 minute preincubation, the reaction was initiated by the addition of 5 mM NADH (ethanol-free). After 3 minutes incubation, a sample (5 μl) of reaction mixture was injected into the gas chromatograph for analysis of epoxypropane formed.

Reductase activity

8 μM of pure reductase was added to the reaction solution (500 μl) containing 8 μM of the hydroxylase and 1.5 mg of crude protein B (or 8 μM of purified protein B) and sealed with a Suba-seal. 3 ml of propene was added to replace the same volume of gas phase above the reaction solution. The flask was incubated in the water bath shaking at 60-70 oscillations/min. After 1 minute preincubation, the reaction was initiated by the addition of 5 mM NADH (ethanol-free).After 3 minutes incubation, a

sample (5 µl) of reaction mixture was injected into the gas chromatograph for analysis of epoxypropane formed.

NADH: acceptor reductase assay.

NADH:acceptor reductase activity assay was performed as described in the literature (Colby and Dalton, 1979; Lund and Dalton, 1985; Lund *et al*, 1985).

Analysis of NADH:acceptor reductase was carried out in a 1-ml quartz cuvette in the presence of 0.1 mM potassium ferricyanide (anhydrous), 0.12 µM of the reductase and 1 mM NADH in buffer of 25 mM MOPS (pH 7.0). The reaction was monitored at 420 nm.

2.3 Enzymatic reaction assays

2.3.1 Catalytic system and reaction conditions

All the enzymatic reactions were performed at 45°C in a Gyratory water bath shaker shaking at 60-70 oscillations/min.

sMMO complex system

The reaction solution (500 µl) contained 8 µM of the hydroxylase, 1.5 mg of each crude protein B and crude reductase (or 8 µM of each purified protein B and reductase) in 25 mM MOPS buffer, pH 7.0. Liquid substrate was added to 10 mM final concentration. For gaseous substrates, 3 ml of the gas phase was removed from the flask and replaced the same volume of test substrate. After 1 minute preincubation, the reaction was initiated by the addition of 5 mM NADH (ethanol-free). After 3 minutes (except indicated otherwise) incubation, analysis of the reaction mixture was undertaken. The rate of oxidation were calculated from the total amount of products formed.

The hydroxylase/H_2O_2 system

The reaction solution (500 µl) contained 24-120 µM of hydroxylase in 25 mM MOPS buffer, pH 7.0. The substrates were introduced to the reaction solution as described above. Hydrogen peroxide (100 mM) was added as an initiator after 1 minute preincubation. The rate of oxidation were calculated from the total amount of products formed after 30 minutes for most substrates and 15 minutes when propene or methanol was used as substrate.

When studying the effect of hydrogen peroxide in sMMO-catalysed hydrocarbon oxidations, a variety of combinations of the enzyme complex and H_2O_2 were used (Table 2-2) and these reactions were carried out under either aerobic or anaerobic conditions with propene, methane or methanol as substrates.

In studying the effect of protein B in H_2O_2-driven system, the reactions were undertaken in the selected combinations of the enzyme components and reagents (Table 2-3), and in these experiments propene, methanol or 2-methylbutane were used as substrate.

Table 2-2 The incubations of sMMO complex with H_2O_2 (reaction vol. 500 µl)

Incubation	Hydroxylase	Protein B (crude)	Reductase (crude)	NADH (ethanol-free)	H_2O_2
1	8 µM	1.5 mg	1.5 mg	5 mM	0-40 mM
2	8 µM	1.5 mg	1.5 mg	-	0-40 mM
3	8-24 µM	-	-	-	0-100 mM
4	8-16 µM	1.5-3 mg	-	-	0-45 mM
5	8-16 µM	-	1.5-3 mg	-	0-45 mM
6	-	-	-	-	0-300 mM

Table 2-3 Combination of the hydroxylase/H_2O_2 system with sMMO components (reaction vol. 500 µl)

Incubation	µM Hydroxylase / 100 mM H_2O_2	Protein B(pure) (µM)	Reductase(pure) (µM)	NADH (ethanol-free)
1	24	0-72	-	-
2	80	-	-	-
3	80	40	-	-
4	80	-	40	-
5	80	-	-	5 mM
6	80	40	40	5 mM

Other peroxide compounds with the hydroxylase

Activation of the hydroxylase with potassium superoxide (KO_2) was performed in 2.5 M MOPS buffer, pH 7.0. The reaction solution (500 µl) contained 24 µM of the hydroxylase. The substrates propene, methanol, acetonitrile, or cyclohexane were introduced into the reaction solution as described above. Freshly made KO_2 solution (50-100 mM) was added as an initiator after 1 minute preincubation. The reaction was carried out for 15 minutes.

When sodium periodate, sodium chlorite, *t*-butylperoxide or *t*-butylhydroperoxide were used to activate the hydroxylase, they were added to 0.1 M final concentration. When iodosobenzene (PhIO) was used, it was added either in a liquid form (dissolved in methanol) to 20 mM final concentration or in a solid state to 0.1 M. The reaction solution (500 µl) contained 24-120 µM of the hydroxylase and

the reactions were performed in 25 mM MOPS buffer, pH 7.0, with propene, methanol, acetonitrile, or cyclohexene as substrates for 30 minutes.

2.3.2 Reactions under controlled conditions

Anaerobic conditions

Where required, the solutions were made anaerobic by repeated evacuating and gassing with argon five times. Transfer of the reaction solutions were accomplished via a gas-tight syringe (Scientific Glass Engineering PTY Ltd., Victoria, Australia). If necessary, glucose oxidase (10 units/ml) and glucose (10 mM) were mixed in the argon-saturated solutions to remove any trace of dioxygen.

Oxygen-controlled conditions

Before addition of O_2-saturated buffer the reaction flask and the reaction solution were purged repeatedly under high vacuum and refilled with argon or nitrogen five times prior to the addition of other reagents. The different volumes of O_2-saturated buffer were added by gas-tight syringe. Under O_2-saturated conditions all the reaction solutions were gassed with dioxygen.

The experiments for assay of toxic oxygen species, which could be generated under high oxygen concentration, were performed in the presence of superoxide dismutase (5-12 units/ml) or catalase (5-12 units/ml). When studying the effect of superoxide anion on the sMMO activity, xanthine (0.05 mM) and xanthine oxidase (0-0.5 units/ml) were added in the reaction solution.

Reactions under nitric oxide (NO) or nitrous oxide (N_2O) atmosphere

The reactions under nitric oxide or nitrous oxide atmosphere were performed under anaerobic conditions.

The preparation of NO-solutions was carried out under an argon atmosphere with scrupulous exclusion of oxygen as described by Wilkins and Wilkins (1987).

Nitric oxide gas was passed through a solution of 4 M NaOH and degassed the argon-saturated buffer (25 mM MOPS, pH 7.0) at ambient pressure and room temperature. If the nitric oxide gas was not scrubbed with base, it was found that the NO-saturated buffer solutions absorbed at 355 nm which was caused by NO_2^- (Springborg *et al.*, 1989). The concentration of nitric oxide was determined prior to use by colorimetric measurements at 434 nm using the reaction of $Fe^{(II)}(EDTA)^{2-}$ with nitric oxide as described by Springborg *et al.* (1989).

The preparation of N_2O-solutions was performed by gassing the argon-saturated buffer with N_2O.

The NO- or N_2O-saturated buffer solutions were carefully added to the reaction system under anaerobic conditions by gas-tight syringe. The protein solution was degassed and placed under an argon atmosphere before to mixture with the NO-, or N_2O-saturated buffer. The reaction solution (500 µl) contained 8-24 µM each of hydroxylase, protein B and reductase with methane or propene as substrate. 5 mM of anaerobic NADH (ethanol-free) solution was added as initiator after 1 minute preincubation. The reaction was carried out for 0-15 minutes.

Reactions under an atmosphere of $^{18}O_2$

The reaction solutions (500 µl) contained (a) 8 µM each of hydroxylase, protein B and reductase, and 5 mM NADH for the sMMO system, (b) 24 µM, or 120 µM of the hydroxylase and 100 mM H_2O_2 for the hydroxylase/H_2O_2 system. The flasks were sealed with Suba-seals and the stock solutions were degassed and placed under an argon atmosphere prior to use. Several different concentrations of oxygen ($^{18}O_2$) (1 atm) were introduced in both the sMMO and the hydroxylase/H_2O_2 reaction systems with propene or ethane as substrate. The reactions were performed in 25 mM MOPS buffer, pH 7.0, and carried out for 3 minutes with sMMO complex system and 15 minutes for H_2O_2-driven system. The product was identified and estimated by GC/MS spectrometry.

Reactions with mixtures of substrates

The reactions with mixtures of substrates were carried out in 25 mM MOPS buffer, pH 7.0. The mixtures of substrates were methane with (a) methanol, (b) acetonitrile, (c) acrylonitrile, (d) ethene, (e) propene, (f) ethane, (g) styrene, (h) pyridine or (i) 1,4- cyclohexadiene. For mixtures of two gaseous substrates, 3 ml of the gas phase from the reaction system was replaced with 1.5 ml of each substrate or in a variety of ratios between the two substrates used. For mixtures of gas and liquid substrates, the liquid substrate was added at a concentration between 1 mM to 250 mM with 3 ml of gaseous substrate as before.

The reactions were performed using both the sMMO complex and the H_2O_2-driven reaction systems. When the sMMO complex system was used to catalyse the reactions, the reaction solution contained 8-40 µM of the hydroxylase, 1.5-8 mg of each crude protein B and crude reductase, and 5 mM of NADH as an initiator. In the case of H_2O_2-driven system, 24-120 µM of the hydroxylase and 100 mM of H_2O_2 were added. The reactions were performed at 45°C for 3-15 minutes with sMMO system and for 15-30 minutes with the hydroxylase/H_2O_2 system.

2.4 Identification and estimation of the reaction products

2.4.1 Gas chromatography

Measurement of the reaction products was carried out by gas chromatography using a Pye Unicam Series 104 gas chromatograph (Pye Unicam, Cambridge, U.K.) fitted with a flame ionisation detector and linked to a Hewlett-packard 3390A integrator (Hewlett-packard, Avondale, Pennsylvania, U.S.A.). All analyses were performed with N_2 as a carrier gas at a flow rate of 25 or 30 ml/min.

The products propene oxide, methanol, and lower molecular weight alkane alcohol were assayed on a Porapak Q (Waters Associates, Milford Massachusetts, U.S.A.) column (1 m x 4 mm) with an oven temperature of 140-180°C. 5 µl of

liquid phase of reaction mixture was injected for assay. The amount of product was estimated by comparison with a 2 mM freshly made standard solution.

The products phenol, styrene oxide, and oxidation of alicyclic, heterocyclic, high molecular weight alkane and alkene compounds were assayed on BP-1 (25 m x 0.1 mm), or BP-5 (50 m x 0.1 mm) capillary columns operating either at a fixed temperature (isothermally) or using a temperature gradient 60-250°C depended the products assay. The products were extracted with ethyl acetate (HPLC grade), or chloroform (HPLC grade) from the aqueous reaction solution and the organic layer was assayed by GC. In some cases, the extracted sample need to be concentrated by removing the organic solvent under reduced pressure (1 mmHg) at 30-35°C, then 50 µl organic solvent was added to dissolve the residue for assay. 1 µl extracted solution (or concentrated sample) was injected to GC and the amount of product was calibrated with a standard curve prepared from a 0.2-10 mM standard.

When analysing methane oxidation using the H_2O_2-driven system the product methanol was extracted with the equal volume of ethyl acetate and assayed by using a BP-5 column or by GC/MS.

When analysing of the reactions with mixtures of substrates, the products were assayed with a number of columns of Porapak Q, R, N, and BP-1 or BP-5. The oven temperatures were operated with a temperature gradient of 130-185°C for the Porapak R column and 50-90°C for the Porapak N column. The products were identified and quantitied by comparing their retention times with standard samples and by GC/MS.

2.4.2 GC/MS spectrometry

GC/MS spectrometry (Kratos Analytical MS25 RFA instrument, Kratos Ltd, Manchester, U.K.) was used for the analysis of the products from the reactions catalyzed by sMMO complex and the hydroxylase/H_2O_2 system. All samples for GC/MS assay were prepared in the same way as those for GC.

The products of the oxidation of propene and ethane under an atmosphere of $^{18}O_2$ were analysed by GC/MS spectrometry. The results showed that the molecular ion intensity of propene oxide was ca. 98% (*m/z* 58), but ethanol had a molecular ion of medium intensity (*m/z* 46; ca. 45%). So, the ion $[M-H]^+$ (*m/z*=45) was therefore used for ethanol mass spectrometric analysis since it showed peak intensities of ca. 98% of base peak. Isotopic compositions were calculated by averaging the peak intensities throughout the entire GC peak.

The reaction mixtures from the hydroxylase/H_2O_2 system, after GC analysis, were also analysed by GC/MS spectrometry for confirmation. The reaction mixtures were assayed with its ethyl acetate extract except propene oxide which was directly estimated in buffer solution.

2.4.3 Thin layer chromatography (t.l.c.)

Pyridine *N*-oxide was identified by t.l.c. on silica gel as described by Colby *et al* (1977). After incubation, reaction mixtures containing 90 µmol of pyridine as test substrate were extracted with an equal volume of dichloromethane. The dichloromethane layer was taken off and evaporated to dryness. The residue was then redissolved in 0.1 ml of dichloromethane and spotted on to the plate together with a dichloromethane solution of authentic pyridine *N*-oxide. The chromatograms were developed in either methanol or acetone and the R_f values for the reaction product in each solvent compared with those for authentic pyridine *N*-oxide.

2.4.4 Estimation of formaldehyde

Formaldehyde was assayed by the method of Nash (1953).

Nash reagent contained 2 ml of acetyl acetone, 3 ml of glacial acetic acid and 150 g of ammonium acetate per litre of deionised water.

Analysis of formaldehyde was performed by mixing 0.5 ml of the reaction mixtures with 2.5 ml Nash reagent and incubating at either 37°C for 45 minutes, or at 60°C for 10 minutes. After incubation, the sample was diluted to 10 ml with deionised water and measured at 420 nm. The data were compared with a standard curve of formaldehyde.

2.5 Preparation of apohydroxylase and reconstitution of the active enzyme

Hydroxylase was depleted of iron atoms by incubation with 3,4-dihydroxybenzaldehyde followed by ligand exchange using 8-hydroxyquinoline sulphonate (Smith and Dalton, 1992).

Preparation of the apohydroxylase was performed in a 3-ml quartz cuvette which was closed with a white ceramic plug. 3 ml of the hydroxylase (120 μM, [Fe] = 2.15 mol/mol protein) in 25 mM MOPS buffer (pH 7.0) was mixed with 30 mM of 3,4-dihydroxybenzaldehyde in the cuvette. Chelation of the iron atoms of the hydroxylase was completed in 3 hours at room temperature and observed at 450 nm ($\varepsilon = 8370\ M^{-1}$). 0.4 ml of 8-hydroxyquinoline-5-sulfonate (400 mM) was then added and gently mixed. The cuvette was then immediately placed on ice. The apohydroxylase could be obtained either by ultrafiltration through a PM30 membrane (Amicon, Corporation, Danvers, MA.) with 25 mM MOPS buffer, pH 7.0, as the diluting solution, or by applying the reaction mixture to a desalting column (G25 Sephadex, Pharmacia) on the FPLC system to separate the apohydroxylase, ligands and Fe[(8-hydroxyquinoline-5-sulfonate)$_3$$^{3-}$] complex. Analysis of the activity of the apohydroxylase was performed in both the sMMO-complex and the H_2O_2-driven systems. Analysis of the iron content of the apohydroxylase was as described in Section 2.2. The iron content of apohydroxylase prepared in this thesis was 0.105 mol/mol protein.

Reconstitution of the active enzyme was effected by the addition of ferrous ions in the presence of dithiothreitol (Woodland and Dalton, 1984).

A solution of 10 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$/DTT was prepared under anaerobic conditions. The apohydroxylase (1 ml, 60 μM) was placed under anaerobic conditions with evacuation and gassing with argon (not bubbling). 0.12 ml of the ferrous iron solution was added to the apohydroxylase by a gas-tight syringe and the mixture was placed either at room temperature for 5 minutes, or at 0-4°C for 15 minutes under anaerobic conditions. The mixture was then placed under an air atmosphere for a further 10 minutes. Any extra iron atoms could be removed by using gel filtration on a G25 Sephadex column, but their presence had no effect on enzyme activity. Analysis of the activity of the iron-reconstituted hydroxylase was performed on both sMMO-complex system and H_2O_2-driven system.

2.6 Preparation of protein B'

Protein B has previously been shown to be sensitive to the action of proteases (Green and Dalton, 1985). Limited proteolysis of protein B resulted in the appearance of a lower molecular weight form, named protein B', which was a carboxyl terminal truncated version of protein B which had lost all activity. Protein B' was prepared as described by Pilkington *et al* (1990).

Purified protein B (4 mg/ml) was treated with trypsin (10 μg/ml) on ice for 3 minutes. The reaction was stopped by the addition of soybean trypsin inhibitor (15 μg/ml). Protein B' was analysed by SDS-polyacrylamide gel electrophoresis (12%) and stored at -80°C until required.

2.7 Preparation ethanol-free NADH

Ethanol-free NADH was prepared by the extraction of NADH buffer solution with diethyl ether by the following procedure. A solution (20 ml, 25 mM MOPS, pH

7.0) of 5 g of NADH (grade II, Boehringer Mannheim GmbH, Germany) was placed into a separating funnel to which was added 150 ml of diethyl ether (A.R. grade) and vigorously shaken for five minutes. The ether layer was removed and the same amount of fresh ether was added again. After repeating the extraction five times, the NADH solution (20 ml) was placed under vacuum to remove the remaining ether until no more ether or ethanol were detected. The levels of ethanol and ether in the NADH solution were monitored by gas chromatography. The NADH solution was then adjusted to 100 mM concentration by spectroscopy at 340 nm (ε = 622,000 M^{-1}) and stored at -20°C in vials until required.

2.8 Lyophilization of the hydroxylase component of sMMO

The hydroxylase was produced either in 25 mM MOPS buffer, pH 7.0, or in deionised water before lyophilization. If in buffer, the hydroxylase was passed twice through a desalting column (G-25 Sephadex) and eluted with deionised water to remove any salt in the protein solution. The protein solutions were then frozen using liquid nitrogen and dried under reducing pressure (1 mmHg).

2.9 Proteolysis of the Hydroxylase component of sMMO

2.9.1 Proteases and inhibitors

The proteases of thermolysin (protease type X, from *Bacillus*), subtilisin (protease type XXVII, Nagarse), α-chymotrypsin (E.C. 3.4.21.1, type II, from bovine pancreas) and trypsin (E.C. 3.4.21.4, from pancreas) were used for proteolysis of the hydroxylase component of sMMO. Phenylmethylsulfonyl fluoride (PMSF) (334 mM stock solution in ethanol) was used for inhibition of subtilisin and phosphoramidon (60 mM stock solution in H_2O) was used for inhibition of thermolysin. The soybean trypsin inhibitor (attached to 4% beaded agarose) was used for inhibition of chymotrypsin and trypsin.

2.9.2 The procedures of proteolysis

Proteolysis of the hydroxylase was performed in 25 mM Tris-HCl buffer at different pH values (pH 7.0 or pH 7.5) and temperatures. The incubations were (per mg hydroxylase) (1) 5-35 μg of thermolysin at 0-4°C or 37°C for 5-30 minutes; (2) 10-40 μg of subtilisin at 0-4°C or 37°C for 5-30 minutes; (3) 5-133 μg of α-chymotrypsin at 0-4°C for 30-240 minutes, or at room temperature for 10-90 minutes; (4) 2-150 μg of trypsin at 0-4°C for 30-120 minutes, or at room temperature for 2-60 minutes. The proteolytic reactions were terminated by addition of proteinase inhibitor (PMSF) or phosphoramidon at a concentration of 1 mM/mg protease. Soybean trypsin-inhibitor-agarose was added in a concentration of 0.4 ml gel/mg protease.

The protease-inhibitor complexes and the hydroxylase were separated by chromatography. When using subtilisin or thermolysin, the separation was performed by loading the reaction solution onto a Mono-Q (HR 10/10) column via the FPLC system. When using soybean trypsin inhibitor-attached on agarose to inhibit chymotrypsin or trypsin, the separation was effected by passing the mixture through a glass-wool column (5 x 20 mm) and eluted with 25 mM MOPS buffer, pH 7.0.

For further study the samples of the hydroxylase proteolysed by trypsin or chymotrypsin were loaded onto a G-75 Superdex column and eluted with 25 mM MOPS buffer, pH 7.0 to separate the active species and the smaller inactive fragments. The combined active fractions of the hydroxylase were concentrated by ultrafiltration over a PM30 membrane.

Analysis of the activity of the proteolysed hydroxylase was carried out on both the sMMO complex and the H_2O_2-driven systems using propene, methane, ethane or propane as substrate.

Reconstitution of the iron atoms to the hydroxylase proteolysed by chymotrypsin was by the addition of ferrous ions in the presence of dithiothreitol as described previously in Section 2.5.

2.10 Chemical modification of the hydroxylase component

2.10.1 Modification of the hydroxylase by crosslinking

Partially proteolysed hydroxylase using low amount of trypsin (2 μg /mg hydroxylase) was chosen for the crosslinking experiments. The crosslinking reagents diamines and polyoxyethylene bis(imidazolyl carbonyl) were used.

Diamines

The carboxyl groups of the hydroxylase were first treated with carbodiimide using a slight modification of published procedures (Caraway *et al.*, 1969; Perfetti *et al.*, 1976; Torchilin *et al.*, 1978), followed by treament of the activated protein with diamines.

To 20 ml of the hydroxylase (40 μM) was added 2 ml of a 10 mM aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The solution was incubated in a bufferless medium at pH 4.5 for 1 hour at 20°C. At this stage, the activity of the hydroxylase was assayed and a 3-fold decrease of the activity in both the sMMO complex and the H_2O_2-driven systems was observed.

To 10 ml of the carbodiimide-activated hydroxylase solution was added 4 ml of 20 mM phosphate buffer, pH 8.2, and 1 ml of the following solutions respectively: 10 mM hexamethylenediamine, 10 mM ethylenediamine, 10 mM tetramethylene-diamine, 10 mM pentamethylenediamine and 50 mM hydrazine. The reaction was carried out at 20°C, pH 8.2 for 1-2 hours. The unreacted crosslinking reagents were removed by ultrafiltration over a PM30 membrane (30 KDa cut off) at 4°C with 25 mM MOPS, pH 7.0, as the diluting solution.

Polyoxyethylene bis (imidazolyl carbonyl)

The bifunctional reagent-polyoxyethylene bis(imidazolyl carbonyl) can react with protein amine groups under appropriate conditions (Beauchamp *et al.*, 1983).

The hydroxylase (20 ml, 10 mg/ml) and the commercial polyoxyethylene bis(imidazolyl carbonyl) (MW 3350)(12 mM) were incubated in 0.1 M borate buffer, pH 8.5, at 4°C for 24 hours. After which, the reaction mixture was diluted to 120 ml with 15 mM MOPS, pH 7.0. Excess polymer was removed by ultrafiltration over a PM30 membrane at 4°C with 15 mM MOPS, pH 7.0, as the diluting solution. After several dilutions, the buffer was changed to 25 mM MOPS, pH 7.0, and the crosslinked protein was used to assay its thermostability.

2.10.2 Modification of the hydroxylase using activated methoxypolyethylene glycols

Methoxypolyethylene glycol-activated with cyanuric chloride (PEG-cyanuric chloride) (MW 5000) is commercially available and ethanal ω-methoxypolyethylene glycol (PEG-aldehyde) is synthesised from the oxidation of methoxypolyethylene glycol (MW 5000). The both chemicals were used for modification of the native hydroxylase component of sMMO.

Synthesis of ethanal ω-methoxypolyethylene glycol (PEG-aldehyde)

The oxidation of methoxypolyethylene glycol to the corresponding aldehyde is described by Wirth *et al.* (1991) and the principle of the synthesis was based on the Moffatt-Swern method (Mancuso *et al.*, 1978; 1979).

Commercial methoxypolyethylene glycol (Av. mol.wt. 5000) was dried under vacuum for 12 hours. A solution of dimethyl sulfoxide (48 mmol) in methylene chloride (5 ml) was added dropwise to a cold (-78°C) solution of oxalyl chloride (22 mmol) in methylene chloride (25 ml). The rate of addition was very slow to prevent the temperature increasing above -55°C. After 10 minutes, a solution of methoxypolyethylene glycol (10 mmol) in methylene chloride (20 ml) was added. The temperature was kept at -20°C for 1 hour. After addition of 1,4-diazabicyclo[2.2.2]octane (60 mmol), the reaction mixture was warmed to room temperature. Water (50 ml) was then added, and the reaction mixture was extracted

with methylene chloride. The organic layer was separated, dried over magnesium sulfate, and evaporated. The ethanal ω-methoxypolyethylene glycol (PEG-aldehyde) was precipitated from the solution in acetone with ether. The precipitate was filtered and dried under vacuum for 24 hours.

The aldehyde content was determined with the oxime formation test. The hydroxylamine solution was prepared by adding ethanol (80 ml) and 0.5 M potassium hydroxide solution in methanol (90 ml) to hydroxylamine hydrochloride (4 g) dissovled in water (8 ml). A solution of PEG-aldehyde (200-300 mg) dissolved in benzene (20 ml) was added to the hydroxylamine solution (10 ml). After 1 hour at 70°C, the solution was cooled to 20°C, and methanol (30 ml) was added. Excess of base was titrated with a 0.2 M hydrochloric acid solution. The blank value was determined in the absence of PEG-aldehyde.

Modification with PEG-aldehyde

The aldehyde groups of the activated PEG will react with accessible amino groups on the protein by reductive amination. PEG-aldehyde (MW 5000) (20 mM) was added to 10 ml solution of the hydroxylase (30 μM) in 0.1 M borate buffer, pH 8.2. After incubation 3 hours at room temperature, sodium cyanoborohydride (20 mM) was then added and incubated for various time intervals (up to 24 hours). After the incubations were completed, the buffer was changed to 15 mM MOPS buffer, pH 7.0, and the excess PEG-aldehyde was removed by ultrafiltration using an Amicon PM30 membrane. The concentrated PEG/hydroxylase solution was applied to a G-200 Superdex column to separate the modified and unmodified protein using 25 mM MOPS, pH 7.0, as eluant. The modified protein was then lyophilised.

Modification with PEG-cyanuric chloride

Cyanuric chloride can react with amino, imino and hydroxyl groups (Abuchowsk *et al.*, 1977). The chlorine atom reacts readily with amino groups at

approximately 4°C, with imino groups at 25°C, and with hydroxyl groups at 80°C in aqueous solution at pH 9.2. The conditions for modification protein using PEG-cyanuric chloride had been reported by Abuchowsk *et al.* (1977), Jackson *et al.* (1987) and Ljunger *et al.* (1993). In this thesis, modification of the hydroxylase by PEG-cyanuric chloride was carried out in 0.1 M borate buffer, pH 9.2, at 4°C and then at room temperature.

400 mg of PEG-cyanuric chloride (MW 5000) was added to a 24 μM solution of the hydroxylase (10 ml) at 4°C at various time intervals (up to 5 hours). After the incubations were completed, the reaction mixture was allowed to warm up to room temperature over a 30 minutes continue. Then, unreacted PEG-cyanuric chloride was removed by ultrafiltration using a PM30 membrane and 15 mM MOPS, pH 7.0, as the diluting solution. The modified protein was obtained by elution from a G-200 Superdex column with 25 mM MOPS, pH 7.0, followed by lyophilisation.

Gel filtration on G-200 Superdex column with different samples showed when the hydroxylase was modified with PEG-cyanuric chloride, no unreacted protein appeared.

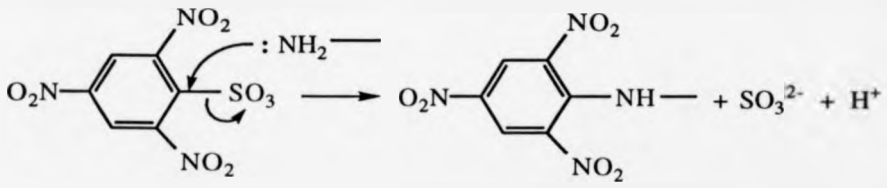
2.10.3 Determination of the concentration of chemically modified protein

The concentrations of native and modified hydroxylase were determined by using Bio-Rad reagent as described previously, since the Bradford method is known to be unaffected by the presence of PEG (Wirth *et al.*, 1991; Ljunger *et al.*, 1993). For the hydroxylase dissolved in organic solvent, the solution was filtered through a 0.4 μm Acrodisc filter and a known volume of the clear solution was evaporated to dryness under vacuum. The protein was then dissolved in a known volume of buffer and the concentration was determined by the Bradford method.

2.10.4 Titration of amino groups

The number of accessible amino groups of native and modified hydroxylase could be determined with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Habeeb, 1966; Goldfarb, 1966; Fields, 1972; Wirth *et al.*, 1991).

TNBS was originally proposed by Okuyama and Satake (1960) for determining amino acids and peptides in protein modification studies, as the conditions required for its reaction were mild compared with those with ninhydrin (Moore and Stein, 1954; Rosen, 1957), and the reaction was more specific than that of aryl halides: no reaction with tyrosine or with histidine side chains was detected (Fields, 1972). Sulfite is displaced from TNBS by an attacking nucleophile:



Its generation has presented a serious nuisance, since it associated reversibly with TNP-amino groups or TNP-thiol groups (where TNP- = 2,4,6-trinitrophenyl-) to form complexes whose absorption spectrum is altered, thus making quantitation of the reaction diffcult (Fields, 1972). Many efforts had been made such as acidification of reaction mixtures to dissociate the sulfite complexes before reading (Habeeb, 1966), empirical correction was applied to the absorption data (Goldfarb, 1966), or readings were made at the isosbestic point for the TNP-amino group and its sulfite complex (Plapp *et al.*, 1971). However, an improvement in the technique was made by Fields (1971, 1972) who measured the orange color of the sulfite complex at 420

nm. This wavelength was removed from the region of absorption of TNBS, thus permitting a high concentration of reagent to be used, to shorten the time required for reaction without increasing the blank readings (Fields, 1972). Titration of amino groups of the hydroxylase protein before and after modification used the Fields methods.

The protein was added to 0.5 ml solution of 0.1 M $Na_2B_4O_7$ in 0.1 M NaOH, and the volume made up to 1.0 ml in which the protein concentration for assay was 2 μM. Then, 220 μl of 5% TNBS solution was added and the reaction solution was rapidly mixed. After 15 minutes in the dark at 40°C, the reaction was stopped by adding 2.0 ml of 0.1 M NaH_2PO_4 solution containing 1.5 mM sulfite and absorbance of the sample was measured at 420 nm. The concentration of amino groups was determined from the extinction coefficient with the TNBS ($\varepsilon = 19200\ M^{-1}$). A blank was also prepared with buffer replacing protein solution.

2.10.5 Thermoinactivation experiments

The native, proteolysed and the polyoxyethylene bis(imidazolyl carbonyl)-crosslinked hydroxylase in 25 mM MOPS, pH 7.0, were incubated at 45°, 55°, 65°, 75°, and 95°C. Aliquots were taken at certain time intervals and immediately placed on ice. The residual catalytic activity in the sMMO complex and the H_2O_2-driven systems were determined as described previously.

2.10.6 Determination of the catalytic activity in organic solvents

The activity of the hydroxylase modified by PEG derivatives in organic solvents was determined by the following procedure. Propene was introduced to a protein-saturated organic solution (containing approximately 3 mg protein) in a 50-ml conical flask sealed with a Suba-seal and 100 mM H_2O_2 was added to initiate the reaction. The reaction was allowed to proceed at 45°C for 15 minutes. The product was

analysed by gas chromatography on a BP-5 column. All analyses were performed with a temperature ramp program from 50°C to 220°C at 8°C/min with N_2 as the carrier gas.

2.11 Polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis (PAGE) was carried out using a vertical 5-20% linear gradient gel with a 4% stacking gel. The discontinuous buffer system consisted of (1) 0.375 M Tris/HCl (pH 8.8) resolving gel buffer; (2) 0.125 M Tris/HCl (pH 6.8) stacking gel buffer and (3) 0.025 M Tris/glycine (pH 8.3) reservoir buffer. The gel was run at 150 mV constant voltage for at least 2000 Vh.

In the purity assessment, the samples were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) as described by Laemmli (1970). Furthermore in order to run SDS-PAGE (7.5% or 12.5% gel) all buffers were supplemented with 0.1% sodium dodecyl sulphate (SDS) and protein samples were treated with β-mercaptoethanol and 0.2% SDS for 3 minutes at 100°C prior to application to the gel. 10 µl sample, containing about 1 mg protein/ml, was placed on the gel and then run under 40 mA constant current. The protein standard was from the low molecular weight electrophoresis calibration kit (Pharmacia AB, Laboratory Separation Division, Uppsala, Sweden) which contained phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100) and α-lactalbumin (M_r 14,400). After electrophoresis the gel was stained Coomassie brilliant blue R-250 solution (0.1% w/v) in the mixed solvent of methanol:acetic acid:water (4:1:5) and then destained with the same solvent.

In the assessment of the proteolysed hydroxylase, the PAGE and SDS-PAGE electrophoresis were carried out by using a Pharmacia PhastSystem (Pharmacia, LKB Biotechnology, Uppsala, Sweden) with PhastGel of homogeneous-gel (12.5% or

20%), and gradient-gel (8-25% or 10-15%) (Pharmacia, LKB Biotechnology). Each sample of proteolysed protein was subject to electrophoresis using the PhastGel native buffer strips (Pharmacia, LKB Biotechnology) for the native-gel and PhastGel SDS buffer strips (Pharmacia, LKB Biotechnology) for the SDS-gel. The lower molecular weight electrophoresis calibration kit was used for SDS-gel as marker protein and for the native-gel the higher molecular weight electrophoresis calibration kit (Pharmacia AB, Laboratory Separation Division, Uppsala, Sweden) was used. This contained thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000), lactate dehydrogenase (M_r 140,000) and albumin (M_r 67,000). After electrophoresis the gel was stained in Coomassie brilliant blue R-250 solution (0.1% w/v) as before, and then preserved with a solution of glycerol:acetic acid:water (at a ratio of 1:1:8 for SDS-PAGE, and 0.25:1:8.75 for PAGE).

2.12 Spectrophotometry

Absorption spectra were recorded on an ultraviolet/visible HP8452A Diode Array Spectrophotometer (Hewlett-Packard GmbH, Germany) using HP89531A UV/VIS operating software.

2.13 Chemicals and biochemicals

Gases, methane, propene, ethane, dioxygen and isotopic oxygen ($^{18}O_2$), argon, O_2-free nitrogen, nitric oxide, nitrogen monoxide and air etc., were of technical grade and were obtained from British Oxygen Co., London.

DNase (Deoxyribonuclease I, from bovine pancreas, E.C. 3.1.21.1), sodium thioglycollate (thioglycolic acid, sodium salt; grade V), catalase (E.C. 1.11.1.6., from bovine liver), superoxide dismutase (E.C. 1.15.1.1.), glucose oxidase (E.C. 1.1.3.4., type VII), thermolysin (protease type X, from bacillus), subtilisin (protease type XXVII, Nagarse), α-chymotrypsin (E.C. 3.4.21.1, type II, from bovine pancreas), trypsin (E.C. 3.4.21.4, from pancreas), soybean trypsin inhibitor

(attached to agrose), polyoxyethylene bis(imidazolyl carbonyl) (Av. Mol.Wt.: 3350), methoxypolyethylene glycol (PEG) (Av. Mol.Wt.: 5000), methoxypolyethylene glycol-activated with cyanuric chloride (PEG-cyanuric chloride) (Av. Mol.Wt.: 5000), 2,4,6-trinitrobenzenesulfonic acid (TNBS), DTT (D,L-dithiothreitol, $C_4H_{10}O_2S_2$, FW. 154.2), benzamindine ($C_7H_8N_2$·HCl, FW. 156.6) and MOPS (3-[N-morpholino]propane-sulfonic acid, sodium salt; $C_7H_{14}NO_4SNa$, FW. 231.3) were obtained from Sigma (Poole, Dorset) Chemical Co. Phenylmethylsulfonyl fluoride (PMSF), phosphoramidon and NADH (grad II, $C_{21}H_{27}N_7O_{14}P_2Na_2$, *Mr.* 7094) were purchased from Boehringer Mannheim GmbH (Germany).

All chemicals, inorganic and organic, and other biochemicals were obtained from a number of commercial sources and were of the best grade available. In analysis of the PEG-modified hydroxylase, organic solvents of highest purity from commercial sources were used without further purification.

CHAPTER 3

CHARACTERISATION OF OXYGEN DONOR AND ALTERNATIVE DONORS FOR SOLUBLE METHANE MONOOXYGENASE

RADICAL-COUPLING REACTION?

3.1 Introduction

Soluble methane monooxygenase is a three component enzyme that catalyses the $NAD(P)H_2$- and O_2-dependent oxidation of methane to methanol. This direct oxidation by the enzyme occurs at ambient temperature and pressure, and the selectivity of the biological system is 50% since methanol and water are normally produced from methane. Exceptionally, formaldehyde may be produced, but only when the methane concentration is vanishingly small and the methanol concentration is sufficiently high to be converted to formaldehyde (Dalton, 1992a). Clearly, the direct oxidation of methane to methanol, which is chemically the most difficult step, by the enzyme system is a remarkably efficient reaction and the process is exergonic. Hence, application of the biological system to direct utilisation of natural gas might be economic for industry.

Methane upgrading could be achieved by using very reactive species. Radicals are among the most reactive species, and radical chemistry has been traditionally used for reaction with methane to form higher hydrocarbons. Significantly, studies of the mechanism of action of sMMO in catalysing methane oxidation has indicated that radicals are involved in the catalytic alcohol synthesis cycle, and the spin-trapping

experiments have shown that the alkyl radicals are involved and can be captured (Deighton *et al.*, 1991; Wilkins *et al.*, 1992). This poses a interesting possibility. If we could trap or release these alkyl radicals we might be able to create conditions in which they could react with each other to form higher hydrocarbons through radical recombination. Obviously, we would like to know whether those alkyl radicals generated by the sMMO system would interact with each other. If the alkyl radicals can be captured by spin-traps, why could they not capture each other to form a C-C bond? We assume therefore that if the alkyl radicals could be released to form "free" radicals, the capture of one alkyl radical by another may not be impossible in the sMMO-catalysed reactions. In principle, it would be possible to devise conditions that could lead to radical recombination between alkyl radicals rather than with an iron-bound hydroxyl group (see Section 1.5.3) using the enzyme system.

Therefore, further investigation of the chemical and physical properties of the sMMO system was undertaken to "persuade" it to effect carbon radical recombination. On the basis of the biological system characteristics and the chemical radical-reaction mechanism, the following investigations were undertaken: (1) studies of the effect of oxygen concentration on sMMO-catalysed oxygenation, (2) use of alternative donors to replace oxygen for the sMMO system, (3) use of mixtures of substrates to mimic chemical oxidative coupling reaction.

3.2 Oxygen donor

Soluble methane monooxygenase is an oxygen dependent enzyme, in its catalytic process O_2 is cleaved and one oxygen atom is inserted into a C-H bond of methane to yield methanol (eq 3-1).

$$CH_4 + O_2 + NADH + H^+ \xrightarrow{MMO} CH_3OH + NAD^+ + H_2O \qquad (3\text{-}1)$$

To find conditions which might allow methyl radicals to combine with each other, the effect of oxygen concentration present in the reaction system was investigated. It is argued that at low oxygen concentration the methyl radicals, which would be generated, might react with each other rather than with ·OH which could be made limiting at low oxygen regimes.

3.2.1 The effect of oxygen concentration on the sMMO activity

Studies of the effect of oxygen concentration on the sMMO activity were performed using oxygen-free to oxygen-saturated conditions with methane or propene as substrate. To test any available products, analysis of the reaction samples was performed by gas chromatography using porapak Q, R and N columns, respectively. The reaction solution (500 µl) contained 8 µM of the hydroxylase, 1.5 mg of each crude protein B and reductase, and 5 mM NADH (ethanol-free). Every solution was made under an argon atomsphere and transferred by using gas-tight syringe. After the gas substrate was introduced into the reaction system, the oxygen-saturated buffer (under room temperature and ambient pressure the oxygen concentration in water is 0.25 -0.30 mM) was added to give a known concentration of oxygen.

Observation of the reactions showed that there was no sMMO activity under oxygen-free conditions (argon-saturated and the presence of glucose and glucose oxidase to remove any oxygen). The enzyme activity increased as the oxygen concentration was increased before the oxygen reached its ambient concentration (0.25-0.30 mM in water) (Figure 3-1). However, the enzyme activity decreased when the oxygen concentration exceeded the ambient concentration and under oxygen-saturated conditions the enzyme activity was at minimal.

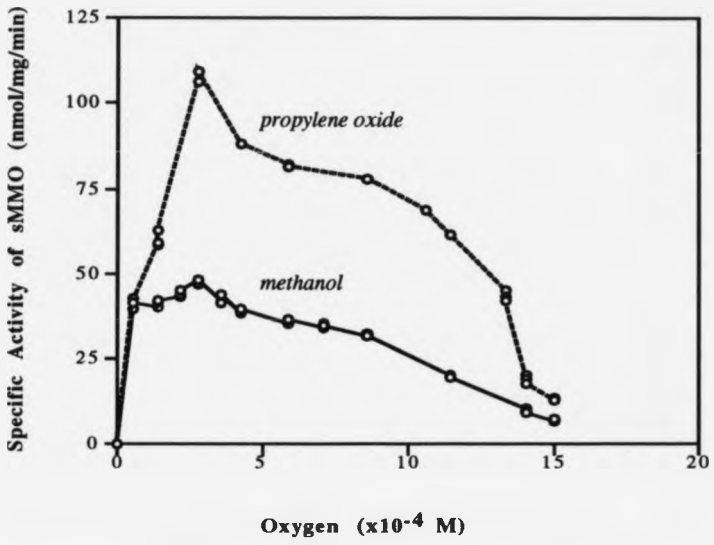


Figure 3-1 The effect of the concentration of oxygen on sMMO-catalyzed oxygenation of methane and propene.

----o---- *propene oxide produced from propene*

—··o—· *methanol produced from methane*

Note: The reaction solution contained 8 μM hydroxylase, 1.5 mg each of crude protein B and reductase, and 5 mM NADH. The reactions were performed at 45°C, for 3 minutes, and analysis was performed by GC using a Porapak Q column.

The results demonstrated that oxygen is essential for the sMMO-catalysed oxygenation of substrate. Under oxygen-free condition there was not any product observed. In the presence of oxygen the sMMO-catalysed reactions result in the production of oxgenated products. It is obvious that the presence of oxygen leads to a rapid recombination of the methyl radical with any oxygen present to form methanol, for example. Unfortunately, at low concentrations of oxygen, presumably, alkyl radicals were generated, but no C-C bond was formed since on the GC assay no

ethane or ethene, for example, was observed. This poses the question that "could an alternative donor be used to replace oxygen in the search for forming alkyl radical recombination products?" Another important question is "why did high oxygen concentrations inhibit an oxygen-dependent enzyme?"

3.2.2 Generation of toxic oxygen species at high oxygen concentrations

The $1/v \sim 1/[O_2]$ plot (Figure 3-2) clearly indicated that oxygen is a complex donor for sMMO in the oxidation of substrates. When oxygen concentration was less than ambient the rate of the enzymatic reaction increased with increase of oxygen concentration, however when oxygen concentration was greater than ambient in the reaction system, it appeared to be an inhibitor. One possible reason for inhibition observed at high oxygen concentrations may be that toxic oxygen species (e.g., H_2O_2 or O_2^-) may have been generated through the reactions with organic material.

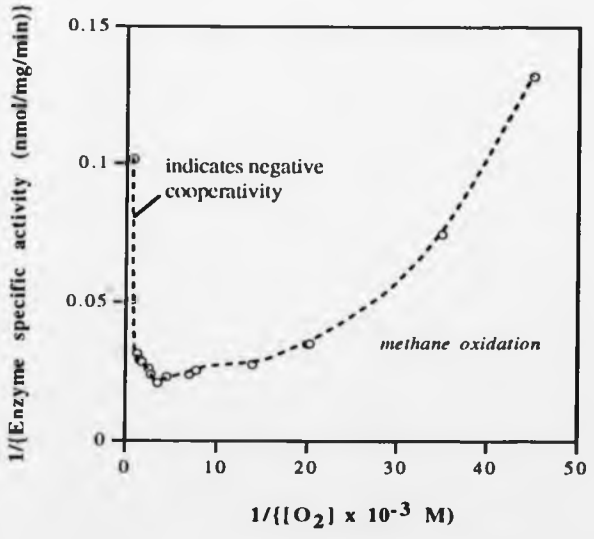


Figure 3-2 The $1/v \sim 1/[O_2]$ plot for sMMO-catalyzed methane oxidation

In order to determine which of these, if any, was responsible for inhibition, the reaction mixtures with high oxygen concentrations were incubated with catalase or superoxide dismutase which would remove any H_2O_2 or O_2^- generated. It is well known that superoxide dismutase (SOD) is an oxidoreductase enzyme (EC 1.15.1.1) catalysing the disproportionation of superoxide anion (eq 3-2) and catalase (EC 1.11.1.6) catalyses the decomposition of hydrogen peroxide (eq 3-3).

$$2O_2^- + 2H^+ \xrightarrow[\text{pH 7.8, 25°C}]{\text{SOD}} O_2 + H_2O_2 \qquad (3\text{-}2)$$

$$H_2O_2 \xrightarrow[\text{pH 7.0, 25°C}]{\text{Catalase}} H_2O + 1/2\ O_2 \qquad (3\text{-}3)$$

Analysis was carried out under oxygen-saturated conditions with methane as a substrate at pH 7.0 and at 45°C. Under oxygen-saturated conditions sMMO activity was at a minimum with a specific activity of 12 nmol/mg/min. The sMMO activity increased in the presence of superoxide dismutase and catalase. The results of addition of these enzymes to the sMMO reactions showed that sMMO activity was increased to 65 nmol/mg/min (the normal reaction ~76 nmol/mg/min) when both catalase and superoxide dismutase were added to the reaction system (Figure 3-3). Interestingly, the sMMO activity could be increased to 64 nmol/mg/min in the presence of catalase alone, whereas superoxide dismutase increased the sMMO activity very slightly (~14 nmol/mg/min). These results seemed to suggest that under oxygen-saturated conditions the peroxide anion was produced and it was harmful to the sMMO enzyme catalytic activity. The fact that superoxide dismutase showed little effect on sMMO activity raised another question: either there was no superoxide anion generated under oxygen-saturated conditions or the superoxide anion dismutated to peroxide anion by

superoxide dismutase resulting in the production of the peroxide anion. The measurement of the absorption spectrum of oxygen-saturated buffer (25 mM MOPS, pH 7.0) showed that at room temperature in the UV range from 200 nm to 300 nm, an absorption at 220-230 nm (O_2^- ion spectrum, Bradiç and Wilkins, 1984) increased with increasing time of buffer bubbling with O_2 (Figure 3-4). This absorption disappeared when superoxide dismutase was added.

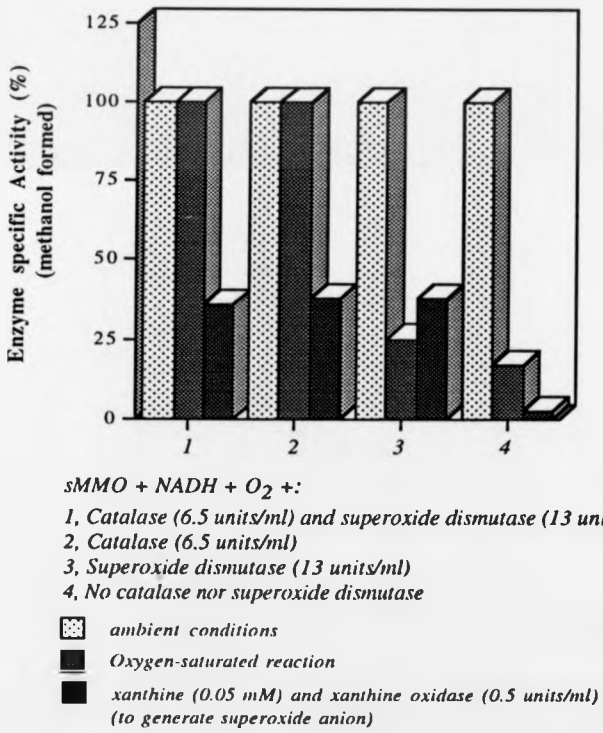


Figure 3-3 The effect of catalase and superoxide dismutase on sMMO-catalyzed methane oxidation under different conditions.
Note: The sMMO reaction solution contained 8 μM of each hydroxylase, protein B and reductase, and 5 mM NADH (ethanol free). The reactions were performed at 45°C for 3 minutes. Analysis was performed by GC using a Porapak Q column.

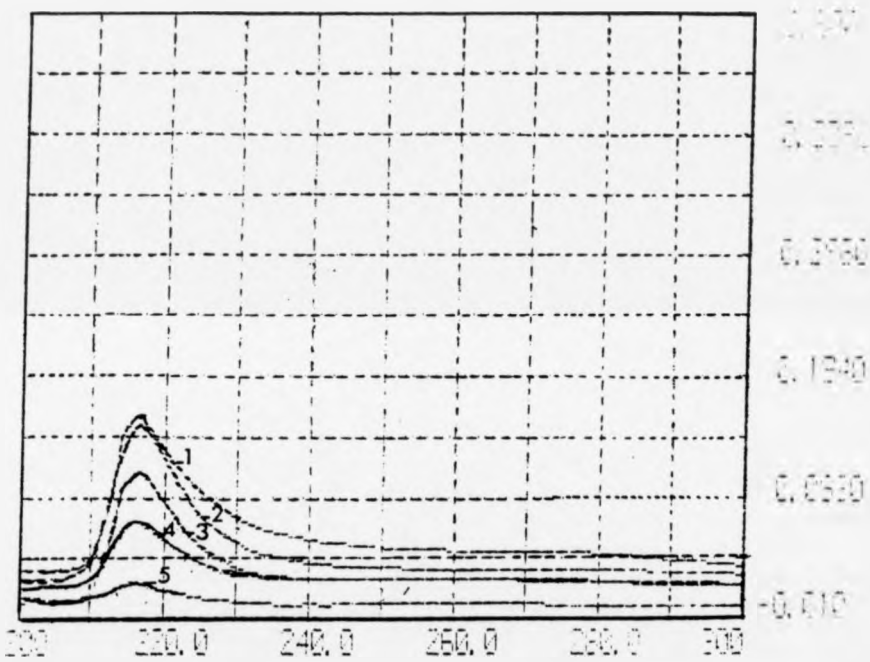


Figure 3-4 UV-spectra of the buffer (25 mM MOPS, pH 7.0) at different time with dioxygen bubbling.
1, 120 minutes; 2, 60 minutes; 3, 30 minutes; 4, 10 minutes; 5, 0 minute.

The following experiment clearly showed that O_2^- was indeed harmful to the sMMO reactions. Superoxide anions, generated by xanthine (0.05 mM) and xanthine oxidase (0.05-0.5 units/ml), decreased sMMO activity (Figure 3-5) with increasing xanthine oxidase (i.e., O_2^-) concentration in the reaction system when using methane as a substrate.

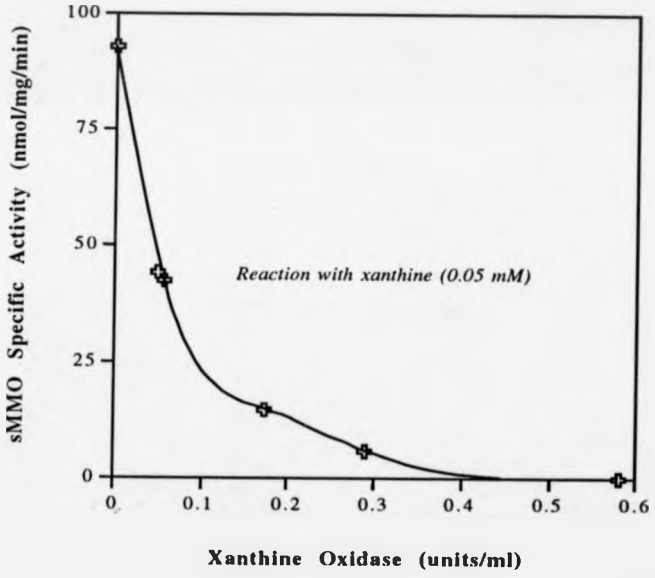


Figure 3-5 The effect of generated O_2^- on sMMO-catalyzed methane oxygenation under ambient conditions.
Note: The reaction solution contained 8 μM of each hydroxylase, protein B and reductase, 5 mM NADH (ethanol free). The reaction was performed at 45°C for 3 mintues. Analysis was performed by GC using a Porapak Q column.

Thus, the results from these experiments on sMMO activity in the presence of superoxide dismutase or catalase under high oxygen conditions suggested that both O_2^- and HO_2^- could be present in the oxygen-saturated buffer and the combined action of superoxide dismutase and catalase abate the formation of the toxic oxygen species.

As shown already (Figure 3-3), in the presence of xanthine and xanthine oxidase when superoxide dismutase (13 or 60 units/ml) or catalase (6.5 or 75 units/ml) were added, increase of the sMMO activity was indeed observed but it did not reach its high activity observed in the ambient reaction. Possibly, the reason for the impaired function of superoxide dismutase could be due to the reaction temperature (45°C) which was not optimum for its function. Also, the product hydrogen peroxide of superoxide dismutation by superoxide dimutase may be harmful to the sMMO reaction.

What caused lower catalase function in the presence of xanthine and xanthine oxidase (Figure 3-3)? Further experiments (Figure 3-6) showed that it was possibly due to the xanthine present. Under ambient reaction conditions the sMMO activity was ~80 nmol/mg/min with methane as substrate and was uneffected by the presence of xanthine (0.05 mM). When hydrogen peroxide (5 mM) was added, the sMMO activity was reduced dramatically. On addition of catalase (5 units/ml) the sMMO activity was raised to 75 nmol/mg/min, but when xanthine (0.05 mM) was present, the catalase-catalysed decomposition of hydrogen peroxide was impeded and the sMMO activity was not increased. Xanthine has been reported to inhibit cAMP phosphodiesterase (Beavo *et al.*, 1970), however, there has not been any report that xanthine inhibits catalase. The experiments shown here strongly suggest that xanthine *per se* inhibits catalase such that the hydrogen peroxide could not be removed from the reaction system, and the sMMO was therefore inhibited at high O_2 + catalase + xanthine, whereas high O_2 + catalase gave no inhibition.

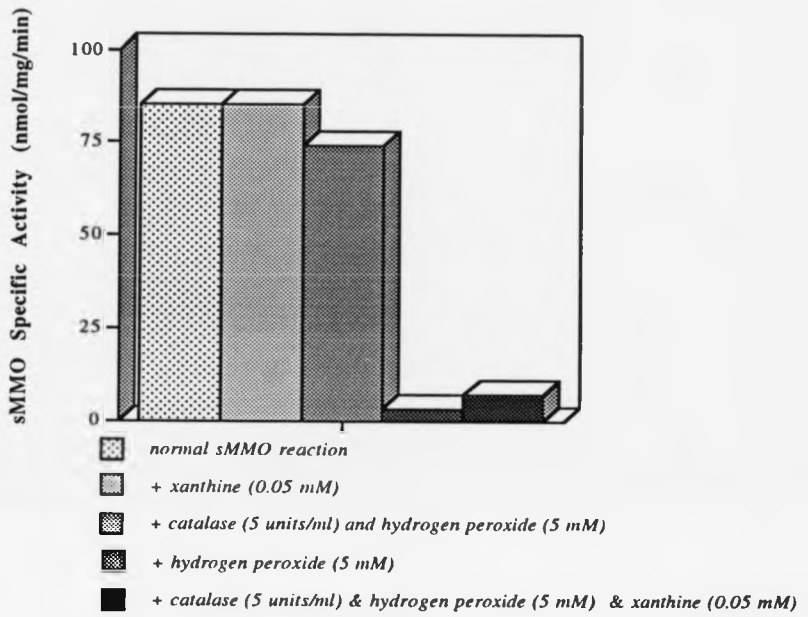


Figure 3-6 The influence of xanthine on catalase-catalyzed H_2O_2 decomposion in the sMMO activity assay.

Note: The normal reaction was carried out under ambient conditions and the reaction solution contained 8 μM each of hydroxylase, protein B and reductase and 5 mM NADH (ethanol free). The reactions were performed with methane as substrate and at 45°C for 3 minutes. Analysis was performed by GC using a Porapak Q column.

The conclusions from above results are: (1) oxygen is necessary for sMMO-catalysed oxygenation, (2) the high oxygen concentrations result in a decrease of sMMO activity, (3) toxic oxygen species, superoxide ion or peroxide ion, appeared in the reaction system under high oxygen concentration conditions, (4) both the superoxide ion and the peroxide ion inhibits sMMO activity, (5) the sMMO activity can be relieved by the addition of catalase at high oxygen concentration.

3.3 NO and N_2O as donors?

3.3.1 Why we chose an alternative donor to replace O_2

The presence of oxygen results in the production of oxygenated hydrocarbons by the sMMO system. We attempted to minimise this reaction and promote alkyl radical recombination by replacement of oxygen from the reaction system. Some form of oxidant is clearly necessary for any sMMO reaction to proceed since no reaction products at all were observed in the complete absence of O_2. One might argue that if an alternative donor could be used to replace oxygen in sMMO-catalysed reaction then alkyl radicals may be generated, such that newly-coupled carbon species would then be produced. If so, this would imply that the alkyl radicals could be captured or released from the active centre of the enzyme and the conditions for minimising attack of any iron bound oxy-species leading to alkyl radical recombination would be satisfied. In this thesis, we chose nitric oxide (NO) and nitrous oxide (N_2O) as alternative donors to replace O_2 in sMMO-catalysed reactions.

3.3.2 Alternative donors characteristics

Nitric oxide (NO)

NO is an unstable free radical species which is highly reactive towards atoms or molecules containing unpaired electrons such as molecular oxygen, superoxide anions or protein-bound metals. The NO molecule has the electron configuration $(\sigma)^2(\sigma^*)^2(\sigma)^2(\pi)^4(\pi^*)^1$. The unpaired π^* electron renders the molecule paramagnetic and partly cancels the effect of the π-bonding electrons. Thus, nitric oxide is at an intermediate oxidation state and can act as both an oxidant and a reductant (Cotton and Wilkinson, 1988).

NO could be presented as a very peculiar ligand for heme and non-heme iron proteins especially with regard to its unusual binding affinity to various iron(II) porphyrins (Traylor *et al.*, 1992). For example, previous work (Springborg *et al.*, 1989) showed that nitric oxide could bind on a non-heme iron site on Fe(II) in

deoxyhemerythrin, which enzyme iron centre was similar to the iron centre of the hydroxylase of sMMO, as does oxygen. Furthermore, NO binds iron of guanylate cyclase, a heme containing enzyme which catalyses the reaction GTP --> cGMP (Ignarro *et al.*, 1981; Murad, 1986), suggesting a scenario involving imidazole release and participation of NO in the mechanism of guanylate cyclase activation (Ignarro, 1989).

Recent studies of NO- or some nitric oxide carriers have shown that NO could play a major role in at least three biological processes: smooth muscle relaxation, neurotransmission modulation and macrophage cytotoxicity (Furchgott and Zawadzki, 1980; Palmer *et al.*, 1987; Moncada *et al.*, 1991; Bredt and Snyder, 1992; Girard and Potier, 1993). For example, NO is a biatomic radical-molecule bearing an unpaired electron, which could be suitable for electron transport. In appropriate conditions, NO can react with thiol groups and disulfides to form *S*-nitroso compounds in a more or less reversible manner (Williams, 1985).

Obviously, NO can play an activation or electron transfer role in enzymatic reactions and some biological systems. Why could we not try to use it as an alternative donor to replace oxygen in sMMO reaction? Also, we already have evidence that nitric oxide could bind to and cause a change in the EPR spectrum of the hydroxylase of sMMO (Wilkins, personal communication). This presumably occurs at the iron centre of the hydroxylase where dioxygen also binds and the evidence suggested that nitric oxide seemed to be like an active donor to sMMO. We hoped that nitric oxide may have a similar function to oxygen, i.e., to form a high valent electrophilic iron species and abstract a hydrogen atom from substrate to create a "free" alkyl radical followed by radical recombination to form C-C bond.

Nitrous oxide (N_2O)

Nitrous oxide, laughing gas, is a hyponitrous acid anhydride. N_2O is relatively unreactive, being inert to the halogens, alkali mtals, and ozone at room temperature. It

will oxidise some low-valent transition metal complexes to oxo species. At elevated temperatures it decomposes to nitrogen and oxygen, reacts with alkai metals and many organic compounds, and supports combustion (Cotton and Wilkinson, 1988).

A number of studies in chemistry have specifically utilised N_2O as oxidant for methane conversion and the specific effect has been achieved. For example, methane could be oxidised to methanol with N_2O as oxidant by using Mo and V as catalysts (Zhen *et al.*, 1985). Solymosi *et al.* (1985) demonstrated that use of N_2O with catalyst of Bi_2O_3 resulted in the formation of high selectivities for formaldehyde. Furthermore, a number of other studies have shown that use of N_2O with catalysts of Sm_2O_3, MgO and Li/MgO could increase C_2 selectivity, particularly to ethane, under comparable reaction conditions although a lower methane conversion was observed (Otsuka and Nakajima, 1987; Hutchings *et al.*, 1987; 1989a; Roguleva *et al.*, 1991). There was evidence that at comparable conversions N_2O was always more selective for C_2 formation than O_2 as oxidant (Hutchings *et al.*, 1989b; Roguleva *et al.*, 1991).

The characteristics of N_2O on chemical catalytic conversion of methane led us to consider utilisation of N_2O as donor to place O_2 in sMMO-catalysed reaction.

3.3.3 The effect of NO as a donor in sMMO-catalysed reactions

It is well known that nitric oxide reacts instantly with O_2:

$$2NO + O_2 \longrightarrow 2NO_2 \qquad (3\text{-}4)$$

Therefore the experiments using NO as donor were performed under anaerobic conditions. Methane and propene were used as hydrocarbon substrates. Analysis of products was performed by gas chromatography with several columns of Porapak Q, R and N, respectively. The reaction solution (500 µl) contained 8 µM each of hydroxylase, protein B and reductase, and 5 mM of NADH (ethanol free). The reaction was performed at 45°C for 0-15 minutes. Anaerobic conditions were made

initially by gassing the reaction solution with argon and then the NO-saturated solution was carefully transferred to the reaction system by a gas-tight syringe. The results (Figure 3-7) showed that the products epoxypropane and methanol were produced from the oxidation of propene and methane catalysed by sMMO complex, but production decreased with increasing nitric oxide concentration in the reaction system. Also, analysis of the reaction mixture on the GC showed that no other products such as ethane or ethene were produced by sMMO in the presence of nitric oxide.

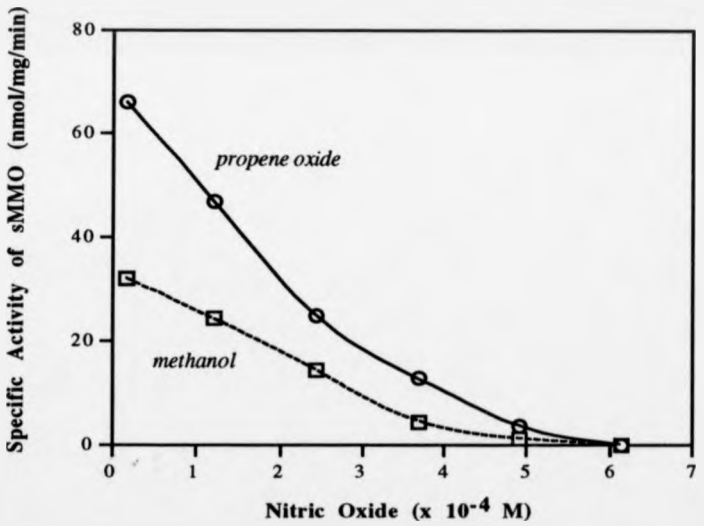


Figure 3-7 The effect of nitric oxide as a donor on the sMMO-catalyzed reactions.

○ *propylene as substrate*

□ *methane as substrate*

Note : sMMO reaction (500 µl) contained 8 µM of each hydroxylase, protein B and reductase, and 5 mM NADH (ethanol free). The reactions were performed at 45°C for 15 minutes and analysed by GC. These reactions were undertaken under anaerobic conditions in the absence of glucose/glucose oxidase system.

The observation of activity in the presence of NO suggested that the NO might have been contaminated by traces of oxygen. To remove any trace amounts of oxygen from the reaction system, the same experiments were undertaken in the presence of glucose/glucose oxidase. It is well known that glucose oxidase (EC 1.1.3.4, b-D-glucose: oxygen 1-oxidoreductase) catalyses the reaction:

$$\beta\text{-D-glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \qquad (3\text{-}5)$$

and has been made to remove remaining oxygen from anaerobic systems. When the glucose/glucose oxidase system was used in the sMMO reaction system with NO as an donor to replace oxygen, then no products were detected at all. We assumed that trace amounts of hydrogen peroxide would be produced by incubation with glucose/glucose oxidase, which could then react with NO to generate a potentially singlet oxygen (Noronha-Dutra *et al.*, 1993) that had been reported to be a highly cytotoxic species-mediated killing and this species, if formed, might be harmful to the enzyme-sMMO complex system catalysed reactions. Further experiments were therefore carried out in the presence of glucose/glucose oxidase and catalase (5 units/ml) which would inhibit the production of singlet oxygen. No products were formed. At this stage, if O_2 was introduced into the reaction system (gassing with air) to replace nitric oxide, unexpectedly, there was still no products produced from the reaction system.

These results would suggest that NO might bind on the iron atoms to produce an inactive complex with the Fe species at the active site. There was report that the NO binding rate on the iron for activation of guanylate cyclase was $K^{NO} = \sim 10^{15}$ (Traylor and Sharma, 1992). In our case NO binding rate to the hydroxylase active site was unknown, but the results showed that using NO as alternative donor was unsuccessful to activate the hydroxylase to catalyse the hydrocarbon oxidations. Similar evidence

has been reported that NO could inactivate ribonucleotide reductase in microoganisms (Marletta *et al.*, 1988; Hibbs *et al.*, 1988; Stuehr *et al.*, 1989; Moncada *et al.*, 1991).

Another possibility may be that nitric oxide has an effect on the reductase component of sMMO. Recent studies on inactivation of *Azotobacter vinelandii* nitrogenase by nitric oxide (Hyman *et al.*, 1992) has shown that the effect of NO on the Av2 protein of nitrogenase involved oxidation of the [4Fe-4S] centre and caused inactivation of the Av1 protein of nitrogenase, an Mo-Fe protein. However, the inactivating effects of nitric oxide on the individual components of sMMO from *Methylococcus capsulatus* (Bath) were not examined, so the complete inactivation of sMMO by NO is unclear. Since no products were detected, then it was not considered important to carry on studies with NO.

3.3.4 The effect of N_2O as donor in sMMO-catalysed reactions

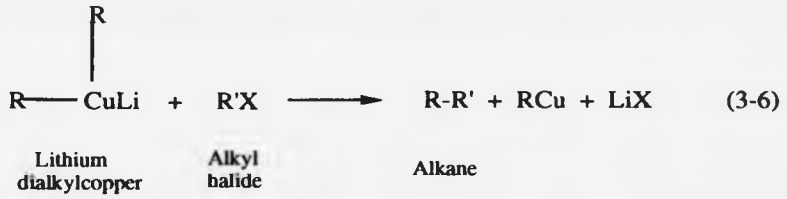
Using N_2O as donor to replace O_2, the sMMO reaction was carried out under anaerobic conditions in the presence of glucose/glucose oxidase system. The reaction solution (500 µl) contained 8 µM of each hydroxylase, protein and reductase, and 5 mM NADH (ethanol free). The reaction was performed at 45°C for 0-15 minutes. Methane or propene were used as hydrocarbon substrates. Analysis of the reactions showed that no products were produced under the N_2O atmosphere. At this stage, introduction of O_2 into the reaction system (gassing with air) to replace nitrous oxide, a slight oxidation of substrate could be observed. It seemed that the use of nitrous oxide as a donor in place of molecular oxygen had no effect on the sMMO complex. On a molar basis N_2O can be considered to have only half the potential for stoichiometric reaction compared to O_2, but the experiment here has shown that it was zero. It is possible that the nitrous oxide-iron complexes (oxo metal species) may not be formed in the reaction system, thus no products were observed under N_2O atomsphere and once O_2 was present the enzyme utilised it to oxidise hydrocarbons.

3.4 Radical recombination using mixtures of substrates?

3.4.1 Coupling and oxidative coupling

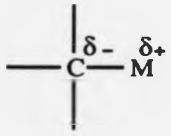
Coupling

To make an alkane of higher carbon number than the starting material requires formation of carbon-carbon bonds, most directly by the coupling together of two alkyl groups. The most versatile method of doing this is through a synthesis developed during the late 1960s by E.J. Corey and Herbert House (Morrison and Boyd, 1987). Coupling takes place in the reaction between a lithium dialkylcopper, R_2CuLi, and an alkyl halide, R'X (R' stands for an alkyl group that may be the same as, or different from, R.), for example, the reaction is:



Carbon-carbon bonds are most commonly formed heterolytically. This means that one of the carbons furnishes a pair of electrons, and the other carbon accepts them: that is, reaction occurs between a nucleophilic carbon and an electrophilic carbon (Morrison and Boyd, 1987). Except for hydrogen or another carbon, the elements that are generally found attached to carbon are more electronegative than carbon, and pull electrons away from it: halogen in alkyl halides, for example, or oxygen in aldehydes and ketones (Morrison and Boyd, 1987). The carbon in such compounds is electron-deficient and hence *electrophilic*; it tends to react with nucleophiles. If such reactions are to result in the formation of a C-C bond, we must use reagents in which the nucleophilic element is carbon (Morrison and Boyd, 1987). The Corey-House contributions giving the answer is the use of *organometallic compounds* (Morrrison

and Boyd, 1987). Just as electronegative elements make carbon electrophilic, so electropositive elements-metals make carbon nucleophilic (Morrison and Boyd, 1987). Therefore the reaction between the nucleophilic carbon of an organometallic reagent and the electrophilic carbon of a substrate that gives rise to a new carbon-carbon bond (Morrison and Boyd, 1987).



Oxidative coupling

The first step in the upgrading of methane into higher hydrocarbons (C_2+) is the formation of the first C-C bond. As mentioned before, the condensation of methane into acetylene or benzene requires thermodynamically a very high temperature reaction (Figure 1-1). A suitable oxidant can provide the energy necessary for this unfavourable reaction (Hutchings *et al.*, 1989b). For economic reasons the most obvious oxidant is dioxygen from the air. The thermodynamic feasibility of the oxidative coupling reaction can be written as follows:

$$2\,CH_4 + 1/2\,O_2 \longrightarrow CH_3\text{-}CH_3 + H_2O \qquad (3\text{-}7)$$

$$2\,CH_4 + O_2 \longrightarrow CH_2{=}CH_2 + H_2O \qquad (3\text{-}8)$$

However, the deep oxidation of methane into carbon oxides is even more favoured (Lunsford, 1985; Justin *et al.*, 1990), such as:

$$CH_4 + 3/2\ O_2 \longrightarrow CO + 2\ H_2O \qquad (3\text{-}9)$$

$$CH_4 + 2\ O_2 \longrightarrow CO_2 + 2H_2O \qquad (3\text{-}10)$$

This implies that the reaction system which approaches its thermodynamic equilibrium will produce exclusively carbon dioxide. The great challenge is to find a catalytic system that produces higher hydrocarbons and suppresses the deep oxidation.

Since the pioneering work of Keller and Bhasin (1982), a large number of articles and patents have been published on this subject. The oxidative coupling of methane over metal oxide catalysts is an example of a heterogeneous-homogeneous reaction network. Many investigations have shown that transition metal oxides which form anion deficient non-stoichiometric phases act as catalysts in the activation of methane to form higher hydrocarbons (Helton *et al.*, 1988; Judd *et al.*, 1991; Campbell, 1991; Ross *et al.*, 1991).

Essentially, two methods have been developed for the oxidative coupling of methane with dioxygen. The first is denoted as the *cyclic redox mode*, as methane and dioxygen are alternatively contacted with the catalyst. This method was first used by Keller and Bhasin (1982) and further developed by Jones *et al.* (1984). Methane is passed over a metal oxide in a high oxidation state at reaction temperatures between 950 and 1200 K. C_{2+} hydrocarbons and water are produced and, consequently, the metal oxide is reduced. The methane conversion progressively decreases and finally the reduced metal oxide has to be reoxidised in a second step with air. The second method, introduced in 1983 (Hinsen and Baerns, 1983; Shepelev and Ione, 1983), is denoted as the *cofeed mode*. Methane and dioxygen are simultaneously contacted with a heterogeneous catalyst, at temperatures between 950 K and 1150 K and atmospheric

pressure. Suitable catalysts for the cofeed mode are metal oxides supported and/or promoted by additives.

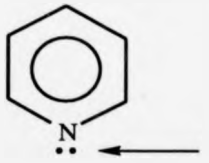
With respect to the reaction mechanism of oxidative coupling, there is not much coherence in the available literature data. The main reason is that the reaction is performed at such a high temperature that physicochemical characterisation of the working catalyst is very difficult (Vermeiren, 1991). There is still no agreement on the nature of the active oxygen species, on the hydrogen abstraction mode, which is either homo- or heterolytic and on the contribution of homogeneous gas phase reactions to the overall reaction (Vermeiren, 1991). So far, it is assumed that methyl radicals are produced from methane (Lunsford, 1991; Swaan *et al.*, 1991). These methyl radicals recombination to ethane, and ethene is formed out of ethane via C_2 radicals.

3.4.2 Mimicing the oxidative coupling reaction with mixtures of substrates in the sMMO system

In the study of the sMMO reaction mechanism the spin-traps have been used to successfully capture the alkyl radicals. We designed similar experiments to allow the radicals generated by sMMO to react with each other. We used a mixtures of substrates to perform the sMMO catalysed reaction under an O_2 atmosphere (at ambient pressure). If different substrates were used in sMMO reaction system, different alkyl radicals should be generated, such that there might be a possibility of producing coupled "adducts" in the sMMO reaction system.

Several combinations of substrates have been investigated. The mixtures of substrates were methane with ethene, propene, benzene, acetonitrile, acrylonitrile, methanol, pyridine, styrene, 1,4-cyclohexadiene or carbon monoxide. We have known that ethene, propene, benzene, acetonitrile, acrylonitrile, methanol and styrene would be generated to their corresponding alkyl radicals by sMMO (Deighton *et al.*, 1991; Wilkins *et al.*, 1992; Dalton *et al.*, 1993). If these various alkyl radicals and

methyl radicals from methane were present in the reaction system at the same time then they might be expected to react with each other. It has been suggested that the oxidation of pyridine and CO may not occur via radical reaction cycle in sMMO-catalysed reactions (Dalton *et al.*, 1992). Why then should we chose them to combine with methane in sMMO reactions? The nitrogen atom, like each of the carbon atoms in pyridine, is bonded to other members of the ring via sp^2 orbitals and provides one electron for the π cloud. The third sp^2 orbital of nitrogen simply contains a pair of electrons which makes pyridine a much stronger base and affects the reactivity of the ring in quite a different way-the ring can undergo the substitution, both electrophilic and nucleophilic (Morrison and Boyd, 1987).

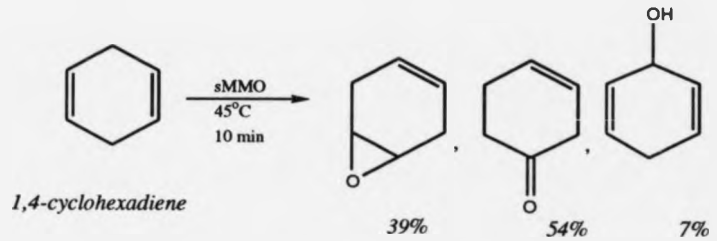


Unshared pair in sp^2 orbital

Our interest would lie chiefly in the way the nitrogen atom affects the reaction of mixtures of pyridine and methane oxidation catalysed by sMMO.

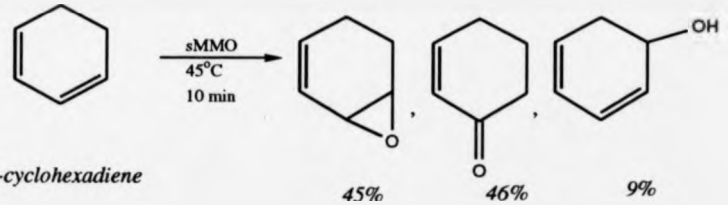
The most important properties of carbon monoxide is its ability to act as a donor ligand toward transition metals. Direct reaction of iron with CO is feasible only at high temperatures and pressures (Cotton and Wilkinson, 1988). However, oxidation of CO to CO_2 by sMMO occurred at ambient temperature and pressure even though this reaction mechanism has been unclear. We assumed that if CO, during the oxidation, could ligate to the iron atoms of the active site of the enzyme the oxidation of mixtures of carbon monoxide and methane by sMMO would lead to the formation of a new product.

1,4-cyclohexadiene, 1,3-cyclohexadiene and 1,3,5-hexatriene have also been chosen for study. Analysis of their oxidation products was performed by GC/MS as shown in Figures 3-8, 3-9, 3-10. The reaction products of these substrates by sMMO are:



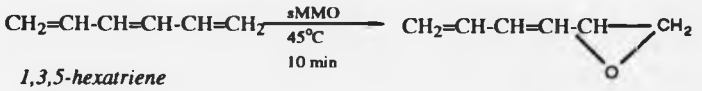
1,4-cyclohexadiene

39% *54%* *7%*



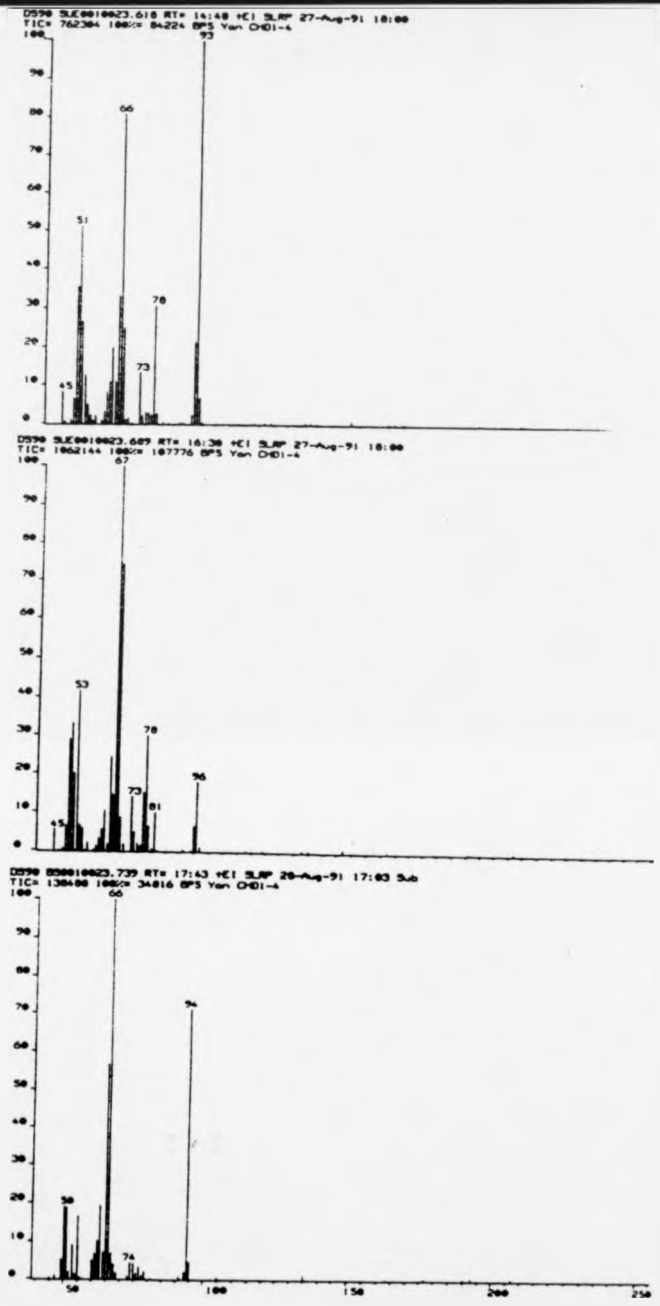
1,3-cyclohexadiene

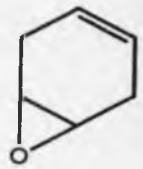
45% *46%* *9%*



1,3,5-hexatriene

These results indicated that these substrates were similar to benzene or propene undergoing a radical reaction with sMMO. In the mixture substrates experiments, 1,4-cyclohexadiene was chosen to mix with methane in which it was expected that the different radicals present in the reaction system would attack each other to produce a coupling product.





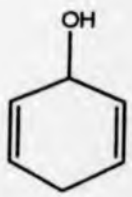
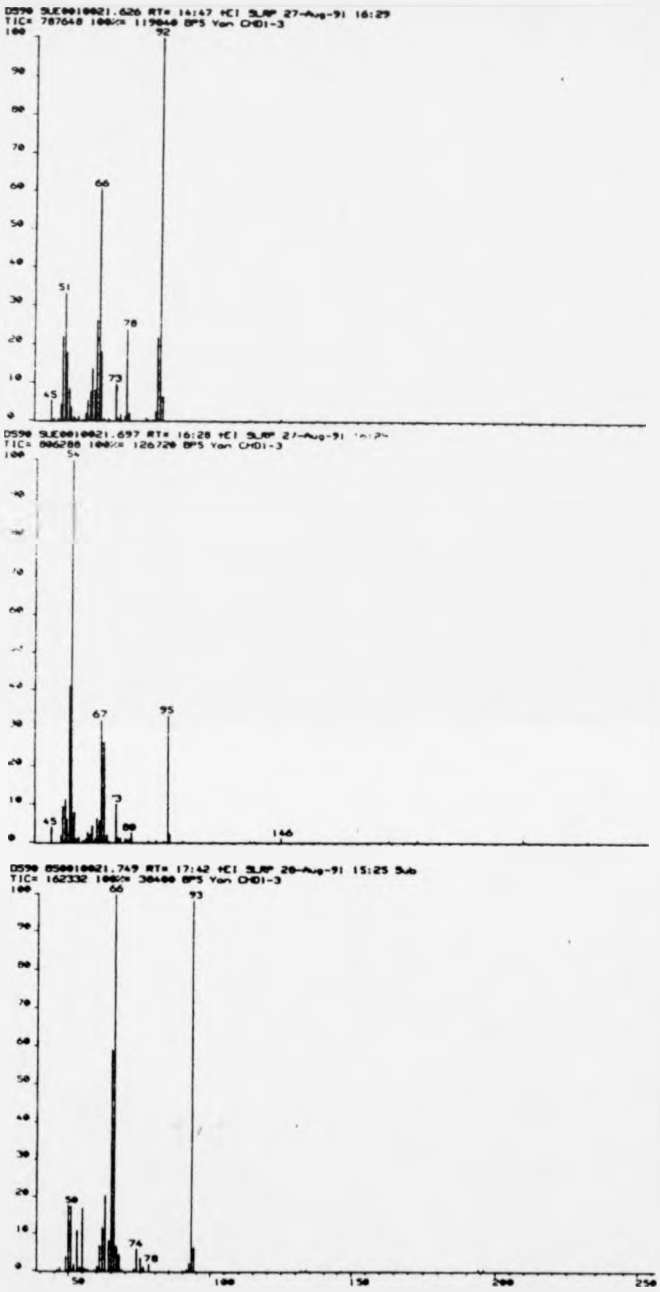
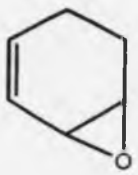


Figure 3-8 The mass spectra of the products of 1,4-cyclohexadiene oxidation-catalysed by sMMO.





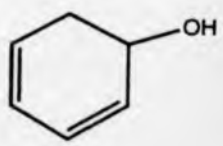
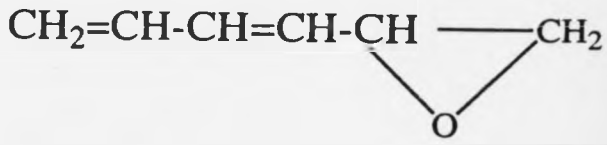


Figure 3-9 The mass spectra of the products of 1,3-cyclohexadiene oxidation-catalysed by sMMO.



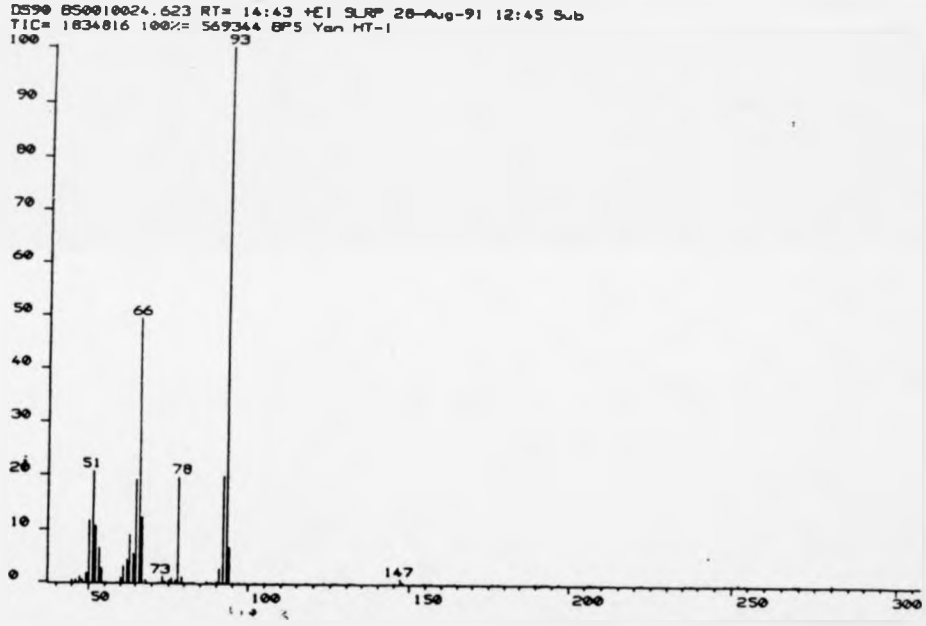


Figure 3-10 Mass spectrum of the product of 1,3,5-hexatriene oxidation-catalysed by sMMO.

The experiments using mixtures of substrates were carried out under aerobic conditions using sMMO complex system containing 8-40 μM hydroxylase, 1.5-8 mg each of crude protein B and crude reductase, and 5 mM NADH (ethanol free). The reactions were performed at 45°C for 3-15 minutes. Analysis of any possible radical coupling products was performed by GC, t.l.c., and GC/MS.

However, the reactions with mixtures of substrates for radical coupling were not successful. The product analysis of the reactions showed that no higher hydrocarbons were observed. Only a mixture of the expected products of oxygenative reaction with substrates was observed.

We can ask the question "why could the spin-tapping reagents react with alkyl radicals generated by sMMO but the radicals were not able to react with each other?" Presumably, the spin-trapping reagents are *free* from the enzymatic system and because they were present at a high concentration (0.1 M), when they were added to the sMMO-catalysed reaction they could react readily with any radicals present in the reaction system. The characteristic EPR spectrum could be observed and the reaction with spin-traps also gave a 17% inhibition of sMMO activity.

Comparison of experiments using mixtures of substrates with the studies of chemical oxidative coupling of methane indicated that the high energy of the methyl radical may be crucial for the radical interaction, since a high temperature was an important condition for generation of methyl radicals. For example, if using molybdenum-based catalysts, C_2+ products began to form at 600°C (Kiui *et al.*, 1990); if using Li/MgO catalyst, the high yield for C_2+ compounds required temperature around 720°C (Ito and Lunsford, 1985). Another crucial condition is the concentration of the methyl radicals on the catalytic site in the chemical process. It has been observed that the competition between coupling and oxidation occurred both in the gas phase and at the catalyst surface when magnesium oxide and lithium-promoted magnesium oxide catalysts were used, the rate of production of ethane depended on the square of the concentration of methyl radicals (Hargreaves *et al.*, 1990). However,

the sMMO-catalysed reaction is much lower in temperature than the chemical oxidative coupling system, and furthermore the alkyl radicals generated in the enzyme active site could be caged, such that they were not present in a sufficiently high concentration to react with each other.

3.5 Preface for next stage

As shown above, we designed experiments to encourage sMMO to catalyse *radical recombination* reactions but were not successful using non-natural donors, and we were unable to mimic the conditions used for chemical oxidative coupling. These studies may indicate that upgrading of methane by the native sMMO complex would be impossible.

O_2 has been shown to be a very important donor for sMMO. Is it possible to obviate the need for the molecular oxygen from the sMMO reaction but still allowing the enzyme to function? When we heard from the Lipscomb group that hydrogen peroxide could be used to activate the hydroxylase of sMMO from *Methylosinus trichosporium* OB3b (Andersson, *et al.*, 1991), this stimulated us to search for peroxide-driven sMMO from *Methylococcus capsulatus* (Bath). If hydrogen peroxide could activate the hydroxylase and provide a source of O_2 and electrons, the sMMO catalysed reactions would not require the other components (the reductase and protein B) and cofactors (O_2 and NADH), thereby simplifying it for study. The peroxide-driven reaction was well known for cytochrome P450 (Ortiz de Montellano, 1986) and because there appeared to be a characteristic similarity between MMO and cytochrome P450, it seemed quite reasonable to expect that peroxide could activate MMO reaction. Therefore, the investigation of the sMMO-catalysed reactions driven by hydrogen peroxide could be helpful to study the mechanism in both systems. One hope was that alkyl radicals could be

encouraged to interact with each other in the absence of dioxygen if an alternative oxygen donor like H_2O_2 could be made to work.

CHAPTER 4

THE EFFECT OF HYDROGEN PEROXIDE ON THE HYDROXYLASE COMPONENT OF SOLUBLE METHANE MONOOXYGENASE

4.1 Introduction

In many respects the mechanism of action of sMMO closely resembles that of cytochrome P450 in which a high valent iron-oxo species effects hydroxylation of C-H bonds through abstraction of hydrogen and incorporation of one atom of oxygen into substrate via radical recombination (Ortiz de Montellano, 1986). Normally, the cytochrome P450 hydroxylase requires a reductase, NAD(P)H and O_2 to effect substrate hydroxylation, but these can be replaced by hydrogen peroxide (the peroxide shunt) or other surrogate oxygen donors. How does oxygen activation and transfer occur in cytochrome P450 and how is the mechanism realised?

4.1.1 Cytochrome P450 reactions and catalytic mechanisms

The cytochrome P450 enzymes were discovered in the 1950s and occur widely in nature in most animal tissues and organelles and in plants and microorganisms. The P450 cytochromes are ubiquitous membrane bound monooxygenase enzymes that catalyse oxygen atom transfer to a number of substrates such as steroids, eicosanoids, fatty acids, lipid hydroperoxides, retinoids and other lipid metabolites and xenobiotics such as drugs, alcohols, procarcinogens, antioxidants, organic solvents, anesthetics, dyes, pesticides, odourants, and flavourants. Since the initial report of the

solubilization, separation and reconstitution of the components of the cytochrome P450 monooxgenase system by Coon in 1968 rapid progress in estabilishing the mechanisms of catalysis by the enzyme has been made (Guengerich, 1992). The term cytochrome P450 is commonly used to refer to a group of heme proteins that are apparently unique in having a sulfur atom ligated to the iron and which form carbon monoxide complexes with an absorption band at an unusually long wavelength (about 450 nm) (White and Coon, 1980).

The cytochrome P450-catalysed insertion of oxygen into a substrate culminates a process that reduces molecular oxygen to a species equivalent, in terms of formal electron count and reactivity, to an oxygen atom. The catalytic sequence for cytochrome P450 enzymes involves the following steps: (1) binding of substrate, (2) reduction of the two flavin prosthetic groups of cytochrome P450 reductase by NAD(P)H, (3) transfer of one of the two electrons to the iron reducing it to the ferrous state, (4) binding of molecular oxygen to give a ferrous-dioxygen complex, (5) transfer of a second electron from the reductase to the Fe(II)-O_2 complex to yield an iron peroxo species, (6) cleavage of the O-O bond with concurrent incorporation of the distal oxygen atom into a molecule of water, (7) transfer of the second oxygen atom to the substrate and (8) dissociation of the product. The binding of oxygen to ferrous P450, the event that commits the enzyme to the production of some form of activated oxygen is the starting point for the ensuing discussion.

Oxygen binding and formation of the iron-peroxide

The binding of oxygen to ferrous P450 yields a dioxygen complex [Fe^{2+}][O_2] characterised by absorption maxima at approximately 420-440 and 558-590 nm (Estabrook *et al.*, 1971; Peterson *et al.*, 1972; Bonfils *et al.*, 1979). The ferrous dioxygen complex can oxidize by directly transferring an electron (Ullrich *et al.*, 1981) to form an iron-coordinated peroxide ([Fe^{3+}-O-O^-]) complex. The direct involvement of the iron-oxygen species in normal catalytic turnover was estabilished by single

turnover experiments in which the ferrous dioxygen complex decays in parallel with the *O*-deethylation of 7-ethoxycoumarin (Anderson *et al.*, 1979). Mössbauer studies of the $P450_{cam}$ dioxygen complex indicated that the iron was diamagnetic and had properties similar to those of the iron in oxyhemoglobin (Sharrock *et al.*, 1973; 1976) and accumulated information on model systems supported a formal $[Fe^{3+}][O_2^-]$ structure for the complex in which the iron is low-spin and hexacoordinate. Earlier studies found that the $P450_{LM2}$ ferrous dioxygen complex, which has a half-life of approximately 11 hours at -30°C in water-glycerol (Bonfils *et al.*, 1979), autooxidizes cleanly to the ferric enzyme under stopped-flow conditions with a rate constant $k = 12$ min^{-1} or 90 min^{-1} in the presence of reduced cytochrome b_5 (Guengerich *et al.*, 1976). Also, the observation that the autoxidation of rabbit liver $P450_{LM4}$ yielded hydrogen peroxide without the detectable formation of superoxide indicated that an autoxidation mechanism was operative.

O-O bond cleavage and the active intermediate

O-O bond-splitting must occur since a ferric peroxide complex *per se* is not expected to be reactive toward alkanes without further activation (Hamilton, 1974). In fact, O-O bond cleavage is required for normal catalytic turnover of P450 and the second electron initiates the O-O bond cleavage to result in a catalytically active species. The O-O bond cleavage could be either via a heterolytic or a homolytic pathway, but the two pathways are fundamentally different. Heterolysis of the peroxide O-O bond would generate a high valent iron-oxo (ferryl) species which is capable of abstraction of a hydrogen atom to produce a substrate radical. Homolysis of the O-O bond would require participation of both hydroxyl radical and the iron-bound oxygen in the catalytic reaction.

In 1976, Dawson *et al.* suggested that the relatively polarizable thiolate ligand might push electron density toward the position *trans* to it, thereby weakening the O-O bond and facilitating its heterolytic cleavage and formation of a reactive iron-oxo

intermediate (Dawson *et al.*, 1976). However, White and Coon (1980) suggested that the axial ligand could also enhance homolytic cleavage. Evidence in support of a shift of electron density through the iron to the *trans* ligand was discussed by Mansuy *et al.* (1984) and Dawson and Sono (1987), who suggested that a key role of the thiolate ligand in P450 is to facilitate the O-O bond cleavage in a lipophilic, electrostatically neutral environment. The influence of the *trans*-axial ligand on O-O bond cleavage is evident in the shift from homolytic to heterolytc cleavage in metallotetraphenylporphyrin alkyl hydroperoxide complexes when imidazole rather than thiolate is the *trans* substituent. It appears that heterolytic cleavage of the O-O bond is favoured in P450.

The mechanism of P450-catalysed reactions has been studied by using molecular probes such as stereoselectivity, molecular rearrangements and deuterium isotope effects (White and Coon, 1980). The hydroxylation of saturated methylene units is accompanied by epimerization and a large kinetic isotope effect, k_H/k_d = 10-12. The results of the molecular probe studies strongly suggested that oxidation proceeds with preferential hydrogen abstraction by an iron-oxo intermediate (ferryl species) followed by scrambling or epimerization of the caged alkyl radical and subsequent radical recombination. Furthermore, the observation of substrate radicals involved in P450 reactions using the spin-trapping technique also supported this suggestion.

Peroxide shunt and chemical models

Studies of iron-porphyrins model systems (McMurry and Groves, 1986; Mansuy, 1987) provided information valuable to understanding the detailed molecular mechanism of the reactions catalysed by cytochrome P450. An important observation with regard to oxygen activation by P450 was that the binding and reduction of dioxygen could be circumvented by the addition of surrogate oxygen sources. This is referred to as the peroxide shunt pathway. This observation gave considerable impetus

to the development of functional model systems for cytochrome P450 and was exploited in mechanistic studies.

Hrycay and O'Brien (Hrycay and O'Brien, 1971; 1972) found that the cytochrome P450 of liver microsomes could function as a peroxidase utilising a lipid peroxide substrate. However, the reaction that cytochrome P450 was able to catalyse, or at least promote, the oxidation of an organic substrate at the expense of an alkyl hydroperoxide was first discovered by Kadlubar *et al.* (1973). Since then, a number of oxidants such as hydrogen peroxide, peroxyacids, sodium periodate, sodium chlorite, iodosobenzene, iodosobenzene diacetate and pyridine N-oxide (Rahimtula and O'Brien, 1974; Hrycay *et al*, 1975a; 1975b; Nordblom *et al.*, 1976; Lichtenberger *et al.*, 1976; Gustafsson *et al.*, 1979; Berg *et al.*, 1979; Sligar *et al.*, 1980) have been found to support catalytic turnover of cytochrome P450 in the absence of electron transfer proteins, NAD(P)H and O_2.

There is a clear similarity of P450 to typical peroxidatic reactions, but no peroxidase is known to catalyse aliphatic hydroxylation. It may be assumed in the peroxide shunt and the normal NAD(P)H-and O_2-dependent mechanisms that the ferric peroxide species generated are the same and that the mechanisms are essentially identical from that point on. It is generally accepted that a ferryl or oxenoid iron(IV) porphyrin π cation radical is ultimately responsible for the oxygen atom transfer. Stable intermediates formed during peroxidase and catalase turnover have been extensively studied (Groves and McMurray, 1986). Horseradish peroxidase (HRP) reacts with hydrogen peroxide to form a 2-electron oxidised complex which is designated as compound I. Compound I has been characterised as an oxoiron(IV)porphyrin π radical cation (Penner-Hahn *et al.*, 1983; 1986) and the one-electron reduced species, compound II, is a neutral oxoiron(IV) complex. The discovery that P450 exhibits "peroxidase activity" has encouraged the belief that the two enzymes have analogous reaction cycles that utilise similar intermediates. Iron

porphyrin model compounds that are capable of substrate hydroxylation or epoxidation have supported this analogy.

The axial coordination site in HRP analogous to the thiolate site in P450 is occupied by a histidine (Yonetani *et al.*, 1972). This difference has been suggested to account for the fact that HRP does not catalyse the oxygenation of hydrocarbons as does P450. Therefore, the thiolate ligand may be essential to the physical and functional character of the P450 cytochromes. Removal of the cysteinate ligand results in both the loss of the unique spectral properties and of the monooxygenase activity (Ullrich *et al.*, 1977; 1980). Recent studies on the mechanisms of cytochrome P450 and peroxidase-catalysed xenobiotic metabolism (Hollenberg, 1992) indicated that the axial heme-iron thiolate moiety in P450 plays a critical role in directing the α-carbon-hydrogen elimination involved in N-demethylations. It has been suggested that the differences in the catalytic mechanisms of the two enzymes is due to the environments provided by the separate proteins for the heme active sites. Replacement of the proximal histidine ligand of human myoglobin with cysteine and tyrosine by site-directed mutagenesis has shown that the thiolate ligand enhances heterolytic O-O bond cleavage, but the phenolate ligand enhanced neither heterolytic nor homolytic O-O bond cleavage (Adachi *et al.*, 1993).

Accumulated evidence has suggested that the oxidant derived via the peroxide shunt pathway is similar to that formed by the reduction of dioxygen (Groves and Van Der Puy, 1974; Groves and McClusky, 1976; Groves and Subramanian, 1984; McMurry and Groves, 1986). Since the first report by Groves *et al.* (1979) showing that iodosobenzene in the presence of catalytic amounts of iron-*meso*-tetraarylporphyrins was able to epoxidize alkenes and to hydroxylate alkanes, a great deal of work has been devoted to iodosoarene-iron-porphyrin systems. It is clear that these systems are able to perform all the reactions catalysed by cytochrome P450 with very similar characteristics. For example, alkane hydroxylation occurs with high kinetic isotope effects (k_H/k_d of about 13 for cyclohexane) (Groves and Nemo, 1983),

with retention of configuration of the C-H bond (for hydroxylation of *cis*-decaline to 9-decalol) (Groves and Nemo, 1983; Smith and Sleath, 1983) and with preferential reaction of tertiary C-H bonds (Groves and Nemo, 1983). A high-valent porphyrin-iron-oxo complex corresponding formally to a Fe(V)=O structure has been prepared by reaction of metachloroperbenzoic acid with Fe(tetramesity-porphyrin)Cl (Groves *et al.*, 1981) and studied by ^{1}H NMR, EPR, Mössbauer and EXAFS spectroscopies (Boso *et al.*, 1983; Balch *et al.*, 1983). All its characteristics are compatible with a (porphyrin-radical cation)Fe(IV)=O structure and similar to those of horseradish peroxidase compound I. Also, this complex transfers its oxygen to alkenes almost quantitatively, as does the analogous complex of cytochrome P450.

It is evident, however, that inferences drawn from work with peroxides must be carefully evaluated to determine their relevance to the normal function of the enzyme (Ortiz de Montellano, 1986). The finding that hydroperoxides did not support the turnover of all P450 isozymes with equal facility (Coon *et al.*, 1979) and differences in the regiochemistry of warfarin metabolism observed when cumene hydroperoxide was substituted for NADH and oxygen (Fasco *et al.*, 1979) indicated that the two processes differ in some essential manner (Ortiz de Montellano, 1986). The reaction of the P450-dependent conversion of cyclohexane carboxylaldehyde to cyclohexene with loss of the aldehyde carbon as formate (Vaz *et al.*, 1991) can be catalysed by hydrogen peroxide replacement NADH and the reductase, however, iodosobenzene, *m*-chloroperbenzoic acid and cumyl hydroperoxide are ineffective, indicating that an oxene is not the oxidant (Coon *et al.*, 1992). The suggestion has been made that deformylation by H_2O_2 driven P450 is mechanistically distinct from the hydroxylation reactions catalysed by these oxidants.

Thus, many studies on cytochrome P450 reactions including peroxide activated and iron porphyrin based model systems have largely indicated that the peroxide shunt is similar to the NAD(P)H- and O_2-dependent native enzyme in that a high valent iron-oxo intermediate is formed. Also, several catalytic mechanisms could be operative in

cytochrome P450's depending on which oxygen donor is used and which substrate is being oxidised.

4.1.2 Hydrogen peroxide-driven substrate oxidation by sMMO

As already indicated, the hydroxylation reactions catalysed by sMMO involve hydrophobic binding of substrate to the hydroxylase near the binuclear iron active site, followed by transfer of two electrons from NADH via the reductase to form fully reduced enzyme (Fe^{II}/Fe^{II}) and O_2 binding with formation of an iron bound peroxide. After O-O bond cleavage one atom of O_2 leaves in a water molecule and the other remains bound to iron in the ferryl (Fe^{III}-Fe^{V}=O or Fe^{IV}-Fe^{IV}=O) species. Substrate activation in many cases occurs by hydrogen atom abstraction with formation of a transient substrate carbon radical prior to recombination with the iron-bound oxygen. This heterolytic cleavage of the O-O bond of the iron-bound peroxide has been established by spin-trapping experiments.

The report (Andersson *et al.*, 1991) on the ability of hydrogen peroxide to act as O_2 and electron donor in sMMO-catalysed hydroxylation reactions raised the intriguing question of whether a similar "activated oxygen species" to that already described is formed. Whether a common intermediate is generated from peroxy compounds and from O_2 in the non-heme iron protein and whether the peroxide route has biological significance remain to be established. Also, the peroxide shunt in sMMO from *Methylococcus capsulatus* (Bath) had to be addressed.

Clearly, study of the peroxide shunt in sMMO reactions would be mechanistically interesting because (1) there is a similarity to cytochrome P450 which can be activated by peroxide compounds including hydrogen peroxide, (2) the sMMO-catalysed reactions can be studied in the absence of NADH, O_2 and the other components.

Only a few reports that peroxide shunt might be operative in binuclear iron enzymes, such as the R2 subunit of ribonucleotide reductase (Fontecave *et al.*, 1990;

Sahlin *et al.*, 1990) have appeared. A peroxide shunt in ribonucleotide reductase was expected to be very similar to that studied in detail in cytochrome P450 monooxygenases. The suggestion has been made that the process of peroxide-dependent production of tyrosyl radical in the R2 subunit involved a high-valent iron-oxo intermediate which was similar to those peroxidase and cytochrome P450. A similar suggestion has also been made by Andersson *et al.* (1991) for the activation of the hydroxylase of sMMO from *Methylosinus trichosporium* OB3b by hydrogen peroxide. Based on the above work, we studied the ability of various oxygen atom donors to support oxidations catalysed by the binuclear iron containing hydroxylase of sMMO from *Methylococcus capsulatus* (Bath).

4.2 Effect of oxygen atom donors

-Activation of the hydroxylase by hydrogen peroxide

A number of peroxide compounds have been tested for their ability to activate the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath).

The reaction mixtures contained hydroxylase with H_2O_2, potassium superoxide (KO_2), iodosobenzene (PhIO), sodium periodate ($NaIO_4$), sodium chlorite ($NaClO_2$), *t*-butylperoxide or *t*-butylhydroxyperoxide as potential oxygen and electron donors. The experiments were performed at 45°C under aerobic conditions using propene, methanol, cyclohexene or acetonitrile as substrate. The investigations showed that both H_2O_2 and KO_2 were able to activate the hydroxylase catalysing the oxygenation of propene to propene oxide and methanol to formaldehyde. PhIO, $NaIO_4$, $NaClO_2$, *t*-butylperoxide and *t*-butylhydroxyperoxide were unable to activate the hydroxylase.

To test which species, either O_2^- or H_2O_2, was able to activate the hydroxylase the following experiments were undertaken. In either the KO_2, or H_2O_2-driven systems, the reaction mixtures contained superoxide dismutase (SOD) or catalase

(CAT). The results showed that in the presence of SOD the oxygenation of propene and methanol were still observed in the presence of KO_2- or H_2O_2 and complete inhibition of the reactions was observed when catalase replaced SOD (Table 1). This indicates that it is hydrogen peroxide, rather than the superoxide anion, that is acting as electron and oxygen donor for the hydroxylase. In a previous study the superoxide anion was implicated as a reductant in a Fenton reaction and may be involved in the hydroxylation of aromatic compounds (Fee 1980). If O_2^- was required for the oxygenation of substrate by the hydroxylase/H_2O_2 system, SOD would be expected to inhibit the reaction. However, the reactions with SOD present illustrated that the H_2O_2- and KO_2-driven reactions did not involve superoxide anion, whereas complete inhibition by catalase clearly indicated that H_2O_2 itself is essential for the observed reactions. The effects of catalase and SOD in the KO_2-driven oxidation of substrates by the hydroxylase indicated that KO_2 disimutates to hydrogen peroxide.

Table 4-1 Oxygenation of propene and methanol by the hydroxylase coupled with H_2O_2 or KO_2

Conditions	Propene ⟶ Propene oxide (nmol/min.)	Methanol ⟶ Formaldehyde (nmol/min.)
Hydroxylase (24 μM)		
+ H_2O_2 (100 mM)	64	35
+ KO_2 (140 mM)	47	21
+ KO_2 (50 mM)	26	13
+ SOD (5 units/ml)/H_2O_2 (100 mM)	59	32
+ SOD (30 units/ml)/KO_2 (50 mM)	26	13
+ CAT (5 units/ml)/H_2O_2 (100 mM)	0	0
+ CAT (30 units/ml)/KO_2 (50 mM)	0	0

More difficult to explain is the inability of other oxygen donors used to convert the sMMO hydroxylase to the active intermediate required for catalysis. There is a distinct difference between P450 and sMMO in that other surrogate oxygen donors, such as *t*-butylhydroperoxide and iodosobenzene, are effective in the former in the catalysis of hydrocarbon oxygenation. Apparently, hydrogen peroxide behaves somewhat differently from the other oxygen donors. The ineffectiveness of oxidants such as iodosobenzene may imply that an iron oxene is not the oxidant (Coon *et al.*, 1992) in the diiron active site of the hydroxylase of sMMO (Jiang *et al.*, 1993) when H_2O_2 acts as O and electron donor.

4.3 Catalysis via the activated hydroxylase

The generation of hydroxyl radicals from ferrous ions and hydrogen peroxide (Fenton's reagent) has been known for many years (Fenton, 1894; Walling, 1975). The generation of hydroxyl radicals from iron(III) and hydrogen peroxide has also been proposed (Walling, 1975). It must therefore be considered that the free iron ions might initiate the Fenton reaction (Fenton, 1894) in which powerful oxidizing species are generated which can hydroxylate a wide variety of organic compounds. The species responsible for the hydroxylation reactions might be the nucleophilic adduct $[L_xFe^{II}OOH(BH^+)]$ ("bound ·OH") (Yamazaki and Piette, 1991; Sawyer *et al.*, 1993) or the *free* hydroxyl radical (Walling, 1975).

To eliminate the possibility of such purely chemical oxidations in hydrogen peroxide activation of the hydroxylase, the following investigations were undertaken. There was no oxidation of propene and methanol observed by hydrogen peroxide alone in the absence of the hydroxylase. The heat-inactivated protein (100°C/5min) could not catalyse oxidation of substrates (Table 4-2) and the protein thus treated still retained its two atoms of iron. Therefore, purely chemical oxidation can be eliminated

by the demonstration that no free iron is present in the reaction system, and that none is released from even the heat-inactivated hydroxylase.

Furthermore, in the presence of up to 500 mM mannitol (a hydroxyl radical scavenger) no inhibition of propene epoxidation was observed indicating that no *free* hydroxyl radical was produced during the activation of the hydroxylase by hydrogen peroxide. In contrast to the mannitol incubated reaction, in the presence of 2,4,6-tri-*tert*-butylphenol (TBPH) (4-40 mM) little or no propene oxide production was observed in both the H_2O_2-driven and the sMMO systems. If there were *free* hydroxyl radical present, it would react rapidly with TBPH to give the highly coloured 2,4,6-tri-*tert*-butylphenoxy radical (Nam *et al.*, 1991), and propene epoxidation would be inhibited by incubation with TBPH. However, no colour appeared in either the H_2O_2-driven or the sMMO system in the presence of TBPH. The inhibition observed in both systems by TBPH is perhaps due to trapping of the reactive Fe intermediate by TBPH (Balasubramanian and Bruice, 1986; Stassinopoulos and Caradonna, 1990). Currently, the exact nature of the inhibition is unclear.

Acetylene, a potent inhibitor of sMMO, lowered the yield of product in the hydroxylase/H_2O_2 system, rather than completely inhibiting product formation as in the native sMMO system. This will be discussed later.

Finally, apohydroxylase (0.105 mol Fe/mol hydroxylase) exhibited no activity in the H_2O_2-driven system and reconstitution with Fe restored nearly 90% activity, thus implicating the active site irons in the catalytic process. Presumably, the activation of the hydroxylase by hydrogen peroxide in catalysing hydrocarbon oxidation is similar to cytochrome P450 in that an Fe-OOH species is formed initially followed by O atom transfer either directly or after O-O bond breakage.

Table 4-2 Effect of various additions on substrate oxidation by the hydroxylase/H_2O_2 system

Conditions	Propylene oxide[a] (nmol/min)	Formaldehyde[b] (nmol/min)
Hydroxylase/H_2O_2	64	35
-hydroxylase	0	0
Heated hydroxylase		
60°C, 5 mins	9.6	nd.*
100°C, 5 mins	0	0
+acetylene (10%(v/v) in air)	20	16
+mannitol (20-500mM)	64	nd.*
+2,4,6-tri-*tert*-butylphenol (4-40mM)	36-0	nd.*
Apohydroxylase (-Fe)	0	0
Apohydroxylase (+Fe)	55	nd.*

a, 24μM hydroxylase, 100mM H_2O_2; GC assay.
b, 24μM hydroxylase, 100mM H_2O_2; assayed by Nash method.
**"nd" means not done.*

4.4 Hydroxylase/H_2O_2 system

The oxidations of propene and methanol by the H_2O_2-driven system were performed with 0-300 mM peroxide and 24 μM hydroxylase. The results (Figure 4-1) showed that the oxidations of propene to propene oxide and methanol to formaldehyde were dependent on the concentration of hydrogen peroxide. The $Km_{(app)}$ for H_2O_2 was similar for both substrates at approximately 66 mM, which is orders of magnitude higher than the Km ~16 μM for O_2, and Km ~25 μM for NADH (with propene as substrate) in the native sMMO-catalysed reactions (Green and Dalton, 1986). This may be a reflection of the fact that H_2O_2 is acting both as an electron and oxygen donor in replacing NADH, O_2, reductase and protein B and that one of these functions is a rate limiting step in the reaction.

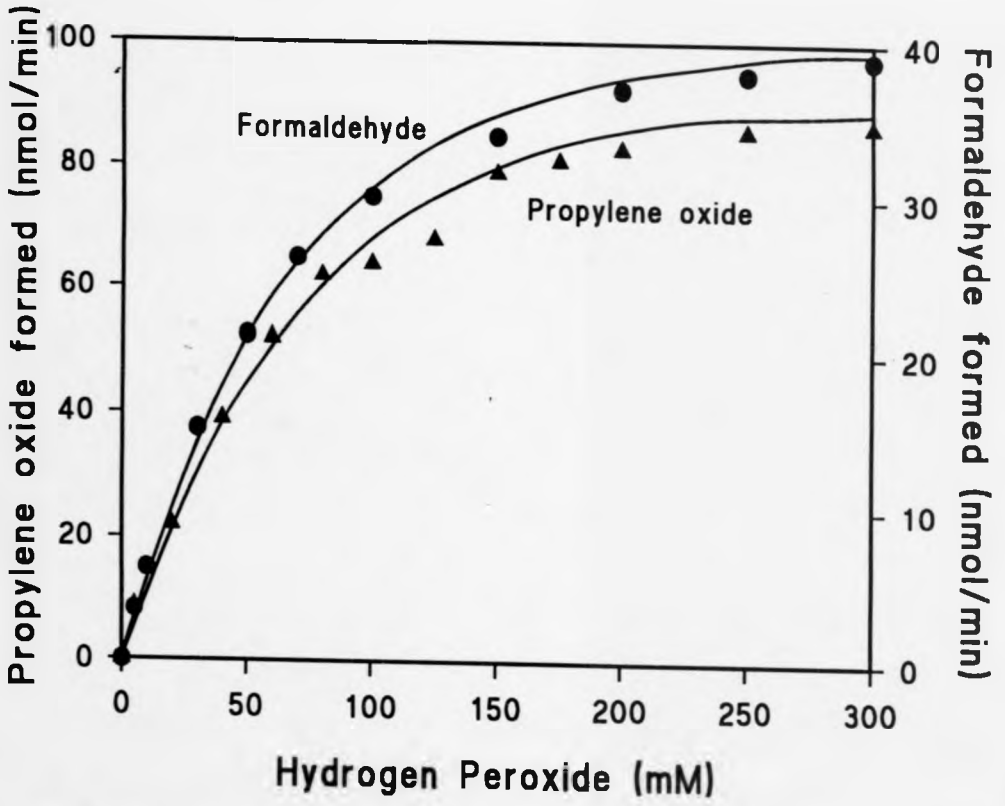


Figure 4-1 The oxidation of propene and methanol by 24 μM hydroxylase and various concentrations of H_2O_2.

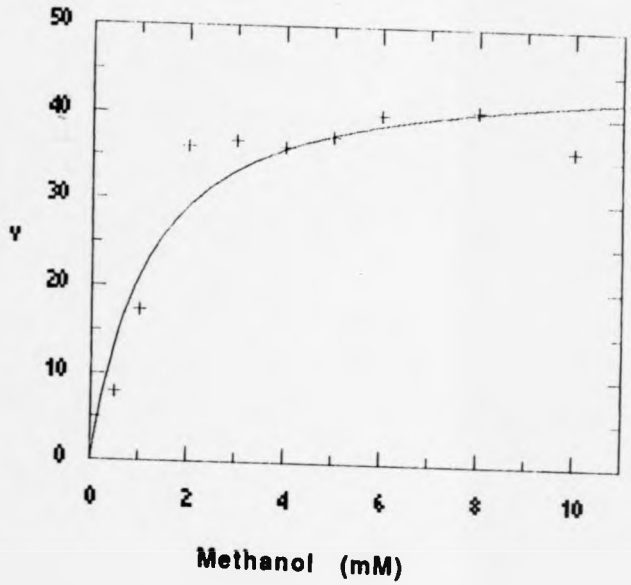


Figure 4-2 The velocity of methanol oxidation with 100 mM hydrogen peroxide and 24 μM hydroxylase

The Km for methanol in the H_2O_2-driven system was also determined. Using 100 mM H_2O_2 and 24 μM hydroxylase and various concentrations of methanol the hyperbolic saturation curve expected for an enzyme catalysed reaction was observed (Figure 4-2), from which the $Km_{(app)}$ for methanol was calculated to be 1.2 mM which is close to the value of ~ 0.95 mM in the complete sMMO system (Colby *et al.*, 1977). This result suggests that the binding of methanol to the hydroxylase was no different in the sMMO and the H_2O_2-driven systems.

Investigation of the most effective ratio of the hydroxylase to H_2O_2 for the oxidation of substrates was carried out. This study was performed by using 8-40 μM of the hydroxylase and 40 mM or 100 mM H_2O_2 with propene as substrate. The results (Figure 4-3) showed that high concentrations of hydroxylase (40 μM) and H_2O_2 (100 mM) gave good catalytic activity, otherwise, the yield of product was low. This indicates that the oxidation of hydrocarbons in H_2O_2-driven system is dependent on an optimal ratio of the hydroxylase and hydrogen peroxide. On the other hand, in the H_2O_2-driven system the activation of the hydroxylase is a peroxide-dependent process similar to the NADH- and O_2-dependent sMMO-catalysed oxygenation.

The oxidation of propene to the epoxide was also carried out with several concentrations of hydrogen peroxide over various times (Figure 4-4). When catalase (30 units/ml) was added after 40 minutes to these samples to remove H_2O_2 and the reaction mixture tested for specific activity in a conventional assay mixture ($NADH_2/O_2$/protein B and reductase), only 8% of the activity remained in the 100 mM H_2O_2 sample, whereas 70% remained in the 20 mM H_2O_2 sample indicating that destruction of the protein at high concentrations of peroxide occurs.

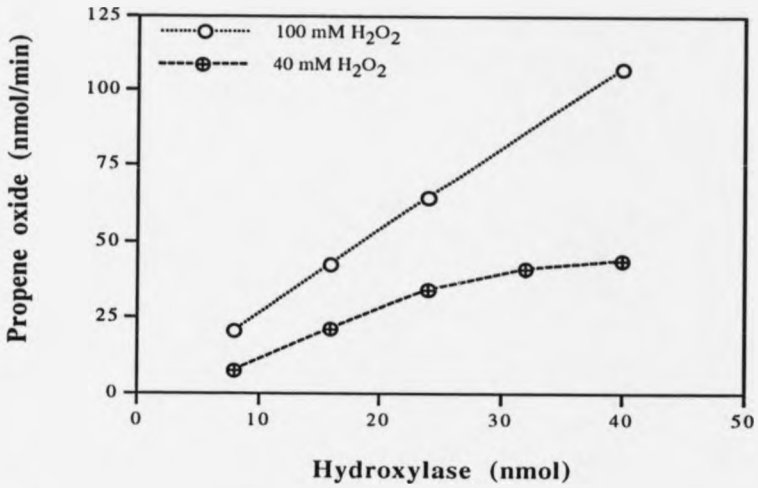


Figure 4-3 Effect the hydrogen peroxide concentration on propene oxide formation at different concentrations of the hydroxylase.

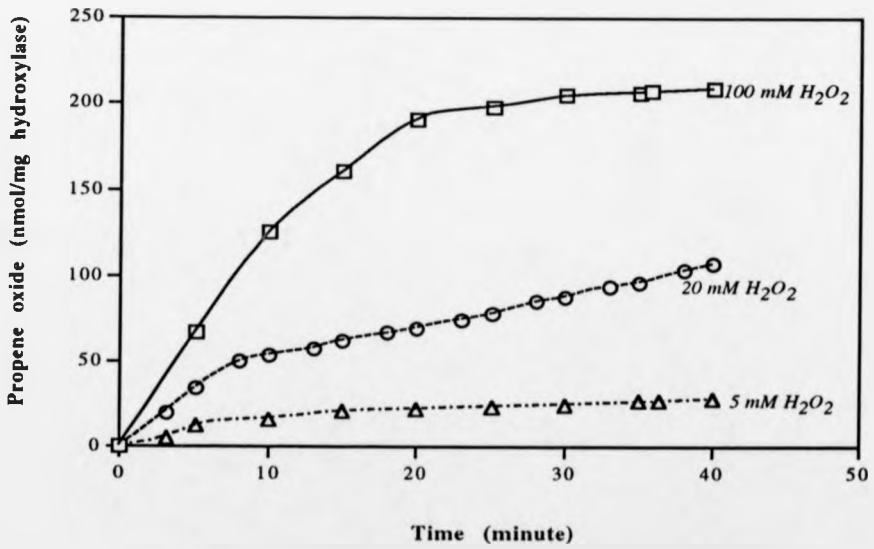


Figure 4-4 The effect of peroxide concentration in epoxidation of propene

Further experiments showed that the hydroxylase/H_2O_2 system was capable of catalysing the oxidation of other known sMMO substrates, e.g., methane, when using 100 mM H_2O_2 as the donor (Table 4-3). Relatively high concentrations of the hydroxylase were required to effect the oxidation of methane, ethane, benzene and styrene when compared to that required for propene oxidation. Also, the oxidation of propene and ethane occurred under anaerobic conditions at almost the same rate as the aerobic reaction (Table 4-3). The activity of the hydroxylase/H_2O_2 system towards these substrates was generally lower than that observed for the native sMMO system with the rate for methane being about 10% and that for propene about 35%, of the complete system.

Table 4-3 Oxidation of substrates by the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath) using 100mM H_2O_2

Substrate	Product (nmol/min)
Methane[a]	Methanol (19)
Ethane[b]	Ethanol (56)
Ethane[b] (anaerobic)	Ethanol (50)
Propene[c]	Propene oxide (64)
Propene[c] (anaerobic)	Propene oxide (56)
Cyclohexane[d]	Cyclohexanol (11)
Cyclohexene[d]	Cyclohexene oxide (20)
Benzene[e]	Phenol (24)
Styrene[e]	Styrene oxide (17)
Methanol[f]	Formaldehyde (35)

a, 120 μM hydroxylase; Ethyl acetate extracted; GC/BP-5 and GC/MS assay.
b, 120 μM hydroxylase; GC and GC/MS assay.
c, 24 μM hydroxylase; GC and GC/MS assay.
d, 80 μM hydroxylase; Ethyl acetate extracted; GC/BP-5 assay.
e, 120 μM hydroxylase; Ethyl acetate extracted; GC/BP-1 assay.
f, 24 μM hydroxylase; assayed by Nash method.

The fact that high concentrations of the hydroxylase was required to effect the oxidation of methane, ethane, benzene and styrene whereas relatively low concentration of the hydroxylase could carry out epoxidation of propene indicated that substrate oxidation will be much slower for these substrates at low concentrations of the hydroxylase. This may indicate that two different mechanisms of catalysis by sMMO could be operative which are dependent upon the nature of the substrate. Such a notion is supported by earlier observations (Dalton *et al.*, 1993) in our laboratory that carbon monoxide and pyridine are oxidised by sMMO to products (carbon dioxide and pyridine-N-oxide) which gave no detectable carbon-centred radical intermediates. The suggestion made for these compounds is that oxidation is via direct oxygen insertion (addition). Furthermore, the oxidation of alkenes may also proceed this way since the EPR signals observed for the alkene radical adducts (produced by H-atom abstraction) were extremely weak even though they are good substrates. Thus different mechanisms appear to operate for the complete sMMO complex and these mechanisms may also be manifest in the H_2O_2/hydroxylase system. It is possible that direct insertion (addition) of an oxygen atom from Fe^{III}-Fe^{III}-O-O^- occurs, especially into alkene substrates, followed by O-O bond breakage to form H_2O and epoxide (Nam *et al.*, 1991).

4.5 Hydrogen peroxide decomposition?

The oxidation of propene and ethane occured under anaerobic conditions, which is similar to the observation that in the *Methylosinus trichosporium* OB3b study, a slightly enhanced rate of oxidation under extremely anaerobic conditions was detected when a second oxygenase was used to scavenge oxygen (Andersson *et al.*, 1991). These observations suggest that H_2O_2 is the source of the O atom inserted into the substrates.

It is necessary to consider whether the O atom which inserts (adds) into the substrates arises directly from H_2O_2 or is derived from the "O_2" produced by the enzyme oxidising H_2O_2 via a "Gif"-type reaction. Peroxide decomposition by Fe^{III} to O_2 which was then incorporated into an alkyl hydroperoxide and finally into the alcohol product (Barton *et al.*, 1992a) was demonstrated in the "Gif" oxidation system of cycloalkanes (Knight and Perkins, 1991; Barton *et al.*, 1992b; 1992c).

To determine whether the O atom was derived from H_2O_2 or from "O_2" via a "Gif"-type reaction, isotopically labelled oxygen was used. If decomposition of hydrogen peroxide in the H_2O_2-driven system occurred when the reaction was run in the presence of hydrogen peroxide and dioxygen-$^{18}O_2$, any ^{18}O-incorporated into the oxidised substrate could be detected by mass spectrometry. The experiments were performed in the presence of labelled oxygen ($^{18}O_2$ and [^{18}O]-H_2O) in both the sMMO and the H_2O_2/hydroxylase systems with propene and ethane as substrates.

The results (Table 4-4) showed that the isotopic abundance pattern observed for the $[M-H]^+$ ion of ethanol and the molecular ion region of propene oxide show that both products have incorporated oxygen from $^{18}O_2$ with the sMMO system under an atmosphere of labelled oxygen, but in the case of the H_2O_2-driven system no $^{18}O_2$-labelled products were isolated. That is, no $^{18}O_2$ was incorporated into propene oxide or ethanol when the H_2O_2-driven assays were run in the presence of $^{18}O_2$.

Parallel experiments were performed in [^{18}O]-H_2O with both substrates using both the native sMMO and the H_2O_2-driven systems. There were no ^{18}O-products isolated from either of the reaction systems in ^{18}O-labelled water, again indicating that in the H_2O_2-driven oxidation hydrogen peroxide decomposition does not occur.

These experiments (Figure 4-5) illustrated that in H_2O_2-driven oxidations, regardless of whether the substrate is a saturated- or unsaturated-hydrocarbon, the oxygen atom in the product is derived directly from H_2O_2 rather than from "O_2"

formed from decomposition of hydrogen peroxide. A "Gif"-type reaction is ruled out in the H_2O_2-driven hydrocarbon oxidations. It is likely that insertion of oxygen atom into substrate is via the iron-bound peroxide (Fe^{III}-Fe^{III}-OOH) or by an iron-oxo species after O-O bond breakage. Whether it is the same active intermediate in the native sMMO and hydroxylase/H_2O_2 systems is a question which must be addressed.

Table 4-4 Oxidation of substrate in the presence of labelled oxygen in the sMMO and hydroxylase/H_2O_2 systems (Experiments with $^{18}O_2$)

I. Isotopic abundance for the ion $[M-H]^+$ (*m/z*=45) in ethanol

Sample	ion (*m/z*)			
	45	46	47	48
Hydroxylase/H_2O_2 (anaerobic)	100	45	2	
sMMO/NADH+$^{18}O_2$	100	36	104	36
Hydroxylase/H_2O_2+$^{18}O_2$	100	45	2	

II. Isotopic abundance for the molecular ion (*m/z*=58) in epoxypropane

Sample	ion (*m/z*)			
	58	59	60	61
Hydroxylase/H_2O_2 (anaerobic)	100	4	2	
sMMO/NADH+$^{18}O_2$	100	20	102	5
Hydroxylase/H_2O_2+$^{18}O_2$	100	3	1	

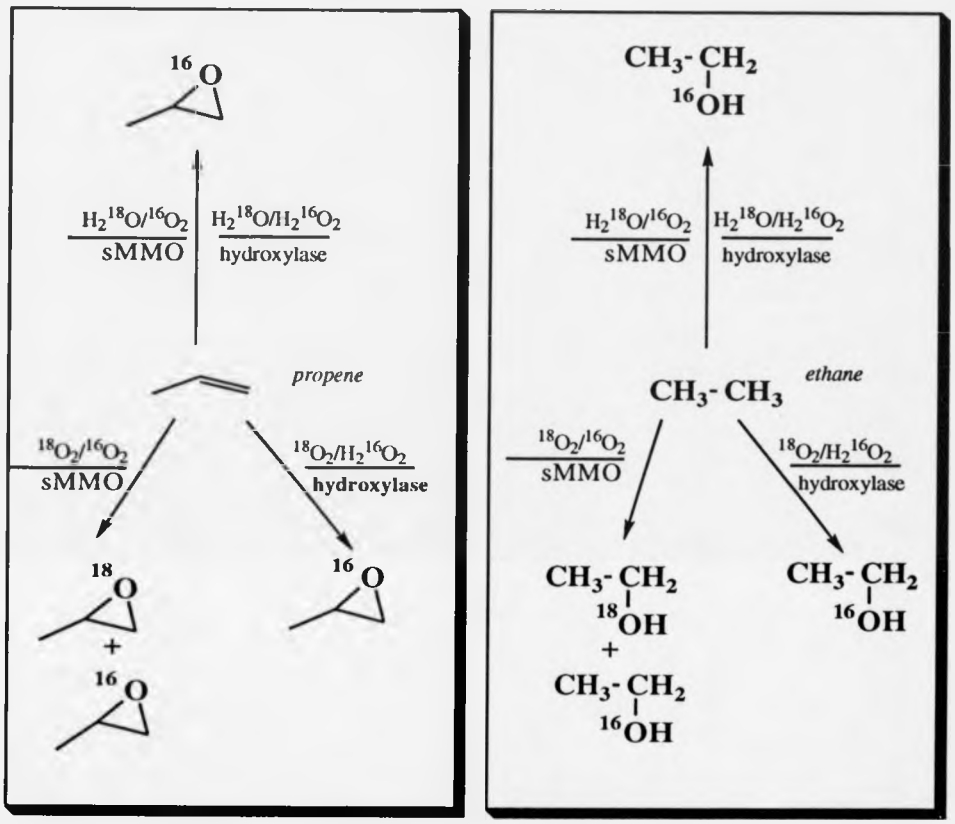


Figure 4-5 The labelled-oxygen experiments using both the sMMO and the H_2O_2-driven systems with propene and ethane as substrates.

4.6 Mechanistic implications

The demonstration of catalysis by the hydroxylase/H_2O_2 system is further evidence of the similarities between the mechanisms of sMMO and cytochrome P450 where a "peroxide shunt" has been well-documented. In both cases, generation of a high valent ferryl species is proposed which, in the case of P450, appears to be stabilised by the porphyrin ring (Ortiz de Montellano, 1986). Structurally, however,

the two enzymes are quite different. In the case of sMMO no such porphyrin ring system is present, so it is likely that stabilisation could arise from the binuclear iron cluster through delocalization of electron density (Fox *et al.*, 1990). Whether the same high valent species is formed when hydrogen peroxide is used in place of the reductase, protein B, NADH and O_2 is a point in question.

The current mechanism proposed for sMMO catalysis has been derived, in part, from the nature of products produced from substrate oxidation. Product distribution as a probe to investigate the mechanism operative in the H_2O_2-driven system was therefore studied. The experiments were performed with several alkanes containing primary-, secondary- or tertiary-carbons and with 2-butene as substrates. If the same mechanism were operative for the hydroxylase/H_2O_2 system, the oxidation of alkanes and 2-butene would be expected to give a similar distribution of products as that observed when using the complete sMMO system. Also, this investigation could be helpful in distinguishing between a radical or non-radical mechanism.

The oxidations of several alkanes and 2-butene by both the sMMO complex and the hydroxylase/H_2O_2 system were observed (Table 4-5). The results showed that in the case of 2-methylpropane the ratio of products was almost identical for the two systems, but the ratio of products from propane and 2-methylbutane were quite different. The selectivity showed that the oxidation of secondary- or tertiary-carbons is more favourable in the H_2O_2-driven reactions than in the sMMO complex. Selectivity was expressed as a percentage of the total amount of product divided by the number of available hydrogen atoms at that position. In the case of 2-methylbutane, for example, there are 9 available primary hydrogens, 2 secondary hydrogens and 1 tertiary hydrogen so that the selectivity should be divided by these numbers and expressed as a percentage of the total amount of product formed.

The radical attack reaction seemed to occur in H_2O_2-driven oxidation based on the investigation of the oxidation of substrates described above. There are major differences between the two systems with propane and 2-methylbutane as substrates.

Table 4-5 Oxidation of alkanes and 2-butene by the hydroxylase/H_2O_2 system and sMMO complex

Substrates	Products	sMMO+NADH[a]		Hydroxylase/H_2O_2[b]	
		Ratio (%)	Selectivity*(%)	Ratio (%)	Selectivity*(%)
Propane	1-propanol	33	1^o-, 14	5.5	1^o-, 2
	2-propanol	67	2^o-, 86	94.5	2^o-, 98
2-methylpropane	2-methyl-1-propanol	51	1^o-, 15	50	1^o-, 14
	2-methyl-2-propanol	49	3^o-, 85	50	3^o-, 86
2-methylbutane	2-methyl-1-butanol	26	1^o-, 16	0	1^o-, 6
	3-methyl-1-butanol	26	2^o-, 50	27	2^o-, 42
	3-methyl-2-butanol	36	3^o-, 34	45	3^o-, 52
	tert-amylalcohol	12		28	
trans-2-butene	*trans*-2-epoxybutane	32[c] {33.73 nmol/min}		97[d] {60.27 nmol/min}	
	trans-2-butene-1-ol	68[c] {71.67 nmol/min}		3[d] {1.86 nmol/min}	

a, Hydroxylase 8μM; protein B(crude), 1.2mg; protein C(crude), 1.5mg; NADH, 5mM. Reaction 10min.GC assay.
b, Hydroxylase 80μM; H_2O_2, 100mM Reaction 30min. GC and GC/MS assay.
c, Reaction 5min.
d, Hydroxylase 24μM, H_2O_2 100mM. Reaction 15min. GC assay.
** Selectivity was calculated as a percentage of the total ammount of product divided by the number of availuble hydrogen atoms at that position.*
The data shown were average values from a number of experiments.

The primary positions were quite susceptible to attack by native sMMO, but not by the hydroxylase/H_2O_2 system. The complete sMMO system involves the generation of a high valent ferryl species which acts as a strong electrophile and abstracts hydrogen atom from the substrate. Thus in the hydroxylation of branched n-alkanes such as 2-methylbutane all positions (primary, secondary and tertiary) were attacked by the complete sMMO system with little selectivity for any particular position (Table 4-5). This lack of selectivity is characteristic of the involvement of a highly reactive electrophilic species that would produce radicals with little discrimination for the ease of H atom abstraction. A similar observation has been reported by Froland *et al* in study of the *Methylosinus trichosporium* OB3b (Froland *et al.*, 1991). In the case of the H_2O_2/hydroxylase system, however, tertiary and secondary positions were clearly favoured with only slight activation of the primary position. Energetically, the tertiary and secondary positions are easier to attack than the primary positions and this indicates that a weaker electrophilic species may be involved in hydrogen abstraction from the substrate when the H_2O_2-driven system is used. This selectivity implied that the active species is lower in free energy and thus less reactive than a non-selective species (Hill, 1989). The ability of H_2O_2 to drive the hydroxylation reaction raises the possibility that hydroxyl radicals may be involved in the catalytic reaction. Such radicals react readily with alkanes to give the observed selectivities. If iron-peroxy bound O-O bond cleavage was homolytic, hydroxyl radicals would be produced (Green and Dalton, 1989) and the remaining iron-oxo species would be less electrophilic than the ferryl species produced from heterolytic cleavage.

The oxidation of *trans*-2-butene showed the significantly different ratios of production of epoxide and alcohol in the two systems (Table 4-5). The amounts of *trans*-2-epoxybutane and *trans*-2-butene-1-ol were 32% and 68% in the sMMO system, but 97% and 3% in the hydroxylase/H_2O_2 system. Obviously, the weaker electrophile (·OH), which may be involved in H_2O_2-driven oxidation, would have difficulty in abstracting H from RCH_2-H (104 kcal/$mol_{(R=H)}$ or ~90

kcal/mol$_{(R=alkene)}$) (Morrison and Boyd, 1987) in forming the alcohol and would more easily attack (break) the -HC=CH- bond in 2-butene (π bond, 70 kcal/mol) (Morrison and Boyd, 1987) in forming the epoxide. This again indicates that different reactive intermediates may be involved in the hydroxylase/H_2O_2 system and sMMO complex. On the other hand, epoxide formation in the H_2O_2-driven system could be almost entirely via *direct* oxygen insertion (vide supra).

4.7 Proposal-the mechanism of action in H_2O_2-driven system

As discussed above attempts have been made to determine whether hydroxyl radicals were directly involved in the H_2O_2-driven oxidations. In their study of the peroxide/hydroxylase reaction Froland *et al.* (1991) dismissed the involvement of hydroxyl radicals on the basis that the scavenger mannitol did not affect the reaction. The concentration of mannitol used was only 50 mM and as such may have been too low to trap radicals associated with the active site of the enzyme. Thus, up to 500 mM mannitol was used here, but no inhibition of H_2O_2-driven oxidations was observed (Table 4-2). Also, the experiments using 2,4,6-tri-*tert*-butylphenol (4-40 mM) were unsuccessful in an attempt to trap hydroxyl radicals as inhibition of propene oxide formation was observed in both the H_2O_2/hydroxylase and the complete sMMO systems. However, the participation of $^{\cdot}OH$ can not be completely ruled out as the hydroxyl radical in the active site may not be accessible to mannitol.

Inhibition by acetylene

Acetylene, a potent inhibitor of native sMMO, only caused a lower yield of product in H_2O_2-driven oxidations. This partial inhibition of activity is a most intriguing result. The complete sMMO system is extremely susceptible to inhibition by acetylene (Prior and Dalton, 1985) in which a concentration as low as 0.5% causes total inhibition of activity by acting as a suicide substrate. In the hydroxylase/H_2O_2

system 30% of the activity with propene and 45% of the activity with methanol remained in the presence of 10% acetylene. When the amount of acetylene was increased to 40% (v/v air) complete inhibition was observed. It is possible that hydrogen peroxide itself serves to quench the suicide reaction of the ketene intermediate (Prior and Dalton, 1985) formed from acetylene and this is why its inhibition is so weak. To test this hypothesis studies on the effect of peroxide on the epoxidation of propene in the complete sMMO system in the presence of acetylene were undertaken. When propene oxidation was inhibited by acetylene, the same level of inhibition was observed when concentrations of H_2O_2 up to 5 mM were added, indicating no direct reaction of H_2O_2 with ketene. These results with acetylene are further evidence for the suggestion of the possibility of hydroxyl radical participation in the H_2O_2-driven system. The ~50% inhibition by acetylene in the H_2O_2-driven oxidation could be explained by assuming that it is acting as a competitive inhibitor of propene epoxidation. Reaction of acetylene with ·OH would produce vinyl radicals which in the presence of Fe^{2+} and H^+ would yield acetaldehyde (Figure 4-6) in a manner similar to that observed in Fenton chemistry (Walling, 1975). Hydroxyl radicals and acetylene can also react to form ·CH=CHOH radicals which rapidly combine with oxygen to produce glyoxal (Figure 4-6) (Anderson and Schulte-Frohlinde, 1978). So far we have been unable to detect the production of acetaldehyde or glyoxal, and in the presence of up to 2 mM acetaldehyde in propene epoxidation in the H_2O_2-driven system no inhibition was observed. Our understanding of these results is far from being complete.

Analysis of pyridine oxidation

Another interesting aspect of the possible involvement of hydroxyl radicals is the effect of some organic substrates on the iron-catalysed decomposition of hydrogen peroxide. Substances such as acetone, acetic acid, *tert*-butanol or catechol (Hamilton *et al.*, 1966a; 1966b; Flicstein and Kremer, 1967; Walling and Goosen, 1973) and

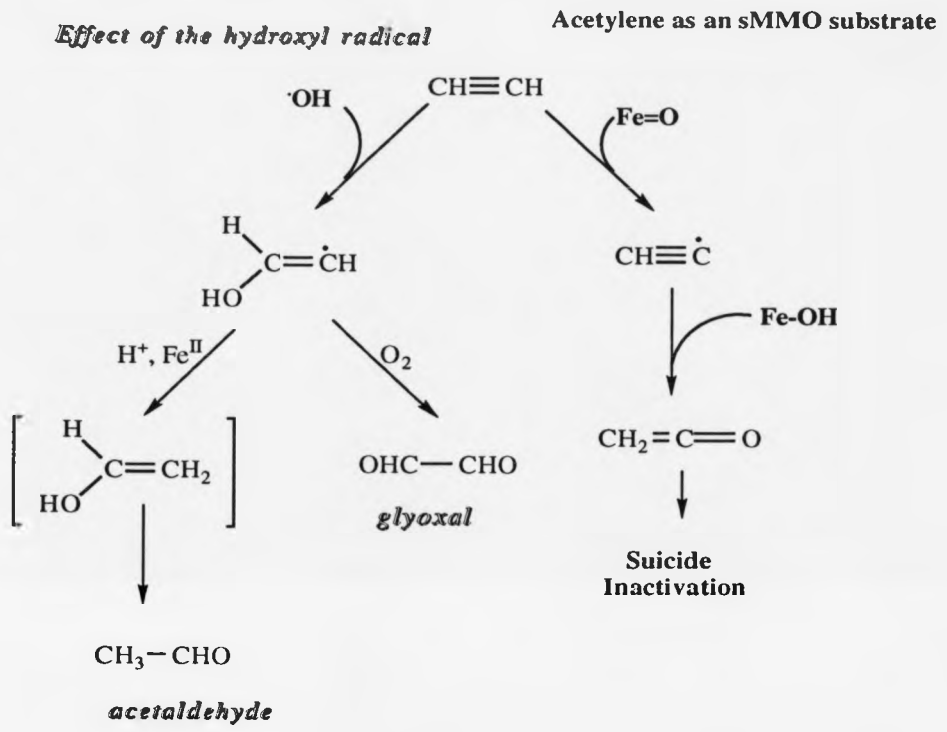


Figure 4-6 Proposal of a diffenert effect of acetylene in the sMMO complex and the H_2O_2-driven system.

pyridine recently reported in Gif chemistry (Barton *et al.*, 1992c) are known to alter the normal hydrogen peroxide dismutation pathway, probably by intercepting a reaction intermediate. Pyridine is supposed to be an excellent trap for hydroxyl radicals (Fenton, 1894; Roberts *et al.*, 1978; Citterio *et al.*, 1980; Barton *et al.*, 1992c). Measurement of the selectivity of hydroxyl radicals for the *ortho*-, *meta*-, or *para*-positions of pyridine was used to identify the role of hydroxyl radicals in Gif chemistry (Barton *et al.*, 1992c). It was assumed that if hydroxyl radicals were

involved in the H_2O_2-driven reaction, the pyridol might be detected. Thus, we attempted to use pyridine oxidation to detect hydroxy radicals in the hydroxylase/H_2O_2 system.

In the complete sMMO system, the reaction was carried out with 8 μM of the hydroxylase and 1.5 mg of each crude protein B and reductase, and 5 mM NADH in the presence of 10 mM of pyridine. In the hydroxylase/H_2O_2 system, the reaction was carried out with 40 μM of the hydroxylase and 100 mM H_2O_2 in the presence of 10 mM pyridine in buffer solution. Pyridine-N-oxide was assayed using a t.l.c. method and hydroxypyridine using GC and GC/MS with authentic standards. In the sMMO-catalysed pyridine oxidation the product is pyridine-N-oxide and no production of hydroxypyridine (any position) was observed. In the H_2O_2/hydroxylase system pyridine-N-oxide was detected, but hydroxypyridine (any position) was not. The results (Figure 4-7), in contrast to the Gif reaction, imply that the oxidation of pyridine in the hydroxylase/H_2O_2 system is by the same mechanism as that in the native sMMO system. Direct insertion of an O atom into pyridine could be via an Fe^{III}-O-O^- species followed O-O bond breakage.

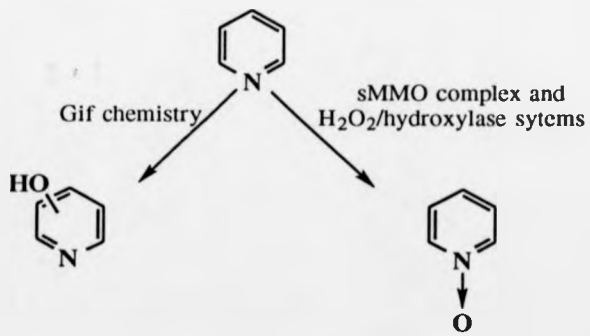


Figure 4-7 The difference between the chemical and the biochemical pathways in the oxidation of Pyridine.

However, the oxidation of propane and 2-methylbutane in the hydroxylase/H_2O_2 system suggested that a weaker electrophile-hydroxyl radical might be involved in the H_2O_2-driven oxidations. Probably, the generation of hydroxyl radicals by the two processes (Gif and enzyme) differ in some essential manner. Currently, *direct* evidence for hydroxyl radical generation in biologically relevant Fenton systems is lacking (Halliwell and Gutteridge, 1992). The recent report by Sawyer *et al.* (1993) has provided evidence that Fenton reactions may not produce a "free ·OH" from Fenton reagents, the species involved might be a nucleophilic adduct [$(EDTA)Fe^{II}OOH(H_3O^+)$, "bound ·OH"] which reacts directly with substrates. If a similar process occurs at binuclear iron active centre of the enzyme when activated by hydrogen peroxide, the generation of hydroxyl radicals would not be detected.

From above observations some conclusions (Jiang *et al.*, 1993) can be drawn (1) Hydrogen peroxide can replace protein B, the reductase, oxygen and NADH in activation of the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath) during catalysis of the oxidation of hydrocarbons. (2) Hydrogen peroxide activation of the hydroxylase of sMMO from *Methylococcus capsulatus* (Bath) occurs at the active site irons in the catalysis of the oxygenation of hydrocarbons. (3) The O atom derived from H_2O_2 is transferred to the substrates and decomposition of peroxide to O_2 does not occur in the reaction. (4) The homolytic cleavage of Fe bound O-O- pathway is favoured in the H_2O_2-driven system, and hydroxyl radicals may be involved in the reaction cycle. This is different from the sMMO system in which heterolytic breakage of the O-O bond occurs leading to the formation of a highly reactive electrophile which abstracts H atoms from substrates. Also, direct insertion of an O atom from Fe^{III}-O-O^- as proposed for ferrous cyclam complexes (Nam *et al.*, 1991) may occur in both the sMMO complex and the hydroxylase/H_2O_2 system, especially for CO, pyridine and alkenes (Dalton *et al.*, 1993b).

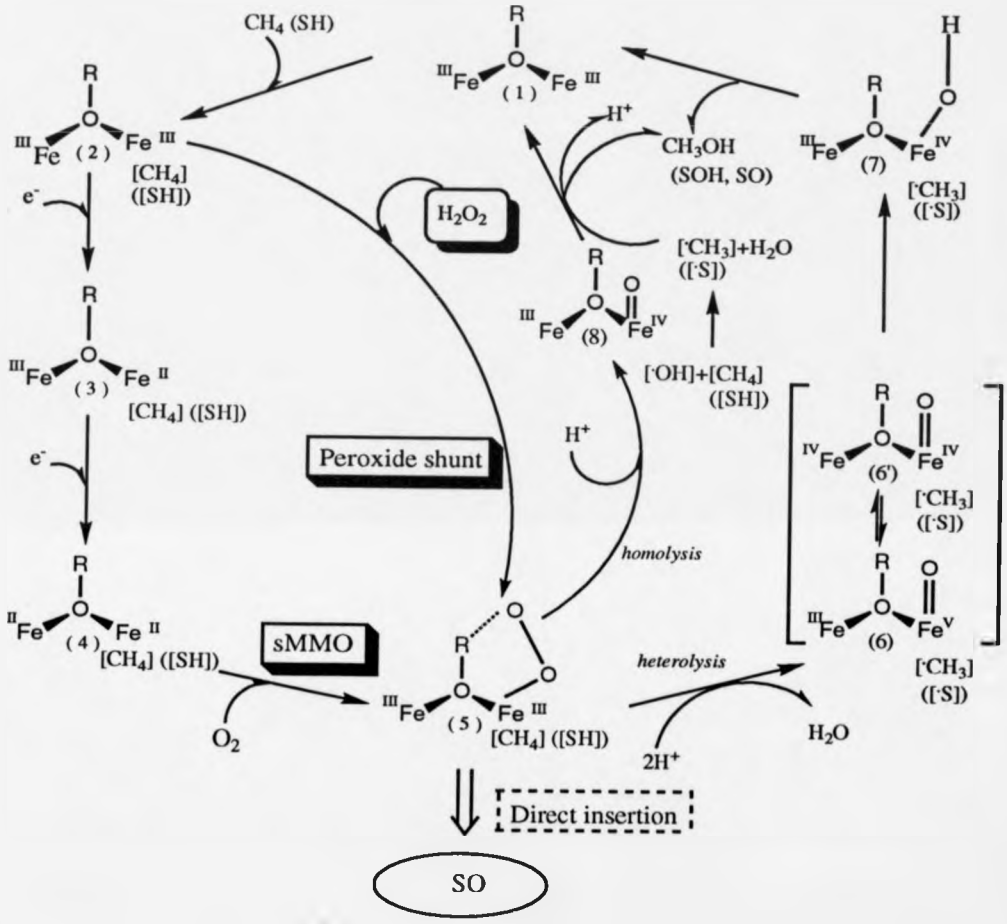


Figure 4-8. Proposed mechanism for the catalytic cycle of native sMMO and the H_2O_2-driven system

Note: CH_4 (SH), free methane (free other substrate);
[CH_4] ([SH]), enzyme-bound methane (or orther substrate);
SO, epoxide product; SOH, hydroxylated product.
R=H, alkyl.

This is summarised in Figure 4-8 illustrating the complete sMMO and H_2O_2-driven systems in catalysing hydrocarbon oxidations. The H_2O_2-driven mechanism is similar to that for native sMMO. The initial step is binding of substrate and hydrogen peroxide in unspecified order to give an iron-bound peroxide complex and the unactivated substrate. Step 2, however, involves *homolytic* rather than *heterolytic* cleavage of the peroxide bond. This step analogous to Fenton cleavage of hydrogen peroxide by ferrous ion, which produces a Fe^{III}-Fe^{IV}=O species and hydroxyl radical (Green and Dalton, 1989b). The following step is abstraction of an accessible, reactive hydrogen atom from the bound substrate by the hydroxyl radical. Finally, the nascent alkyl radical collapses with the oxygen atom on the nearby Fe^{III}-Fe^{IV}=O to form oxygenated product.

4.8 Effect of protein B in H_2O_2-driven oxidation

At an early stage in the study of the effect of hydrogen peroxide on the native sMMO complex it was found that added H_2O_2 inhibited substrate oxidation (Figure 4-9). Why, in the presence of the other components of soluble methane monooxygenase, was hydrogen peroxide such a strong inhibitor when it had been shown to act as an oxygen and electron donor to the hydroxylase?

Further observations with different combination of sMMO components (Table 4-6) indicated that the effect could be due to inhibition of protein B (or the reductase) by H_2O_2. The experiments were performed by incubation of hydrogen peroxide (0-45 mM) with individual and combined components of soluble methane monooxygenase. In the presence of protein B a minimum enzyme activity was observed in activation of the hydroxylase by hydrogen peroxide. The reductase also lowered the activity in H_2O_2-driven system.

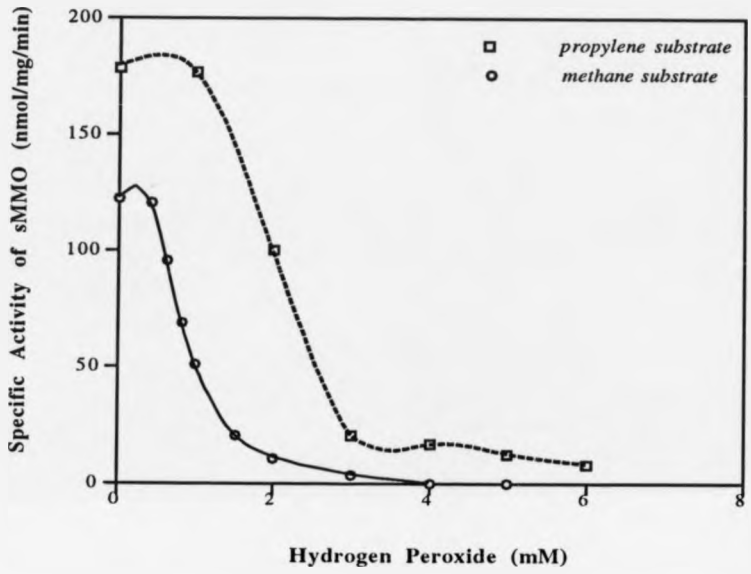


Figure 4-9 The effect of hydrogen peroxide on the sMMO complex.
The incubation contained 8 μM of each hydroxylase, reductase and protein B, and 5 mM NADH. Aerobic reaction at 45°C, for 3min

In the fully functional complex, protein B, which is absolutely required for enzyme activity, acts as a regulator by shutting down electron flow to the hydroxylase from the reductase when substrate is absent. In the presence of substrate normal electron flow is resumed (Green and Dalton, 1985). In contrast, protein B appeared to inhibit the H_2O_2-driven oxidation. The experiments were performed by using different ratios of the hydroxylase and protein B in H_2O_2-driven oxidations with propene or methanol as substrate. The results (Figure 4-10) showed that the enzyme activity decreased with increasing protein B concentration in the reaction system. When the ratio of protein B to hydroxylase was 2:1, almost complete inhibition of substrate

oxidation was observed, in complete contrast to its effect in the sMMO complex where it is absolutely required for activity and inhibition only occurs at a > 5:1 ratio (Fox *et al.*, 1991). It is possible that the protein-protein interaction between the hydroxylase and protein B obstructs hydrogen peroxide in activation of the hydroxylase.

Table 4-6 Effect of hydrogen peroxide on the individual and combined components of sMMO

sMMO components*	H_2O_2 (mM)	Enzyme activity** (nmol/mg/min)
sMMO+NADH	0	183
sMMO+NADH	45	0
sMMO-NADH	45	0
Hydroxylase	45	12
Hydroxylase+protein B	45	< 2
Hydroxylase+reductase	45	5
Hydroxylase+reductase+NADH	45	5

*These incubation contained 8 μM of each hydroxylase, protein B and reductase, and 5 mM of NADH.

**The reactions were performed at 45°C, when H_2O_2 was absent incubation was for 3 minutes; when H_2O_2 was present assay was for 15 minutes using propene as substrate.

We then investigated the effect of protein B' (Figure 4-11, Pilkington *et al.*, 1990), the truncated version of protein B, on the hydroxylase/H_2O_2 system. It is known that B' is completely inactive in the complete sMMO system. We supposed that if B' is unable to bind to the hydroxylase causing inactivation of the native sMMO system, it would then be possibly less effective in inhibiting the activation of the hydroxylase by hydrogen peroxide. A slight inhibition of activity by B' was observed in the propene epoxidation assay (Figure 4-12) which is consistent with its effect on the complete sMMO system. Indeed at equimolar concentrations of B' and the hydroxylase only 10% inhibition was observed.

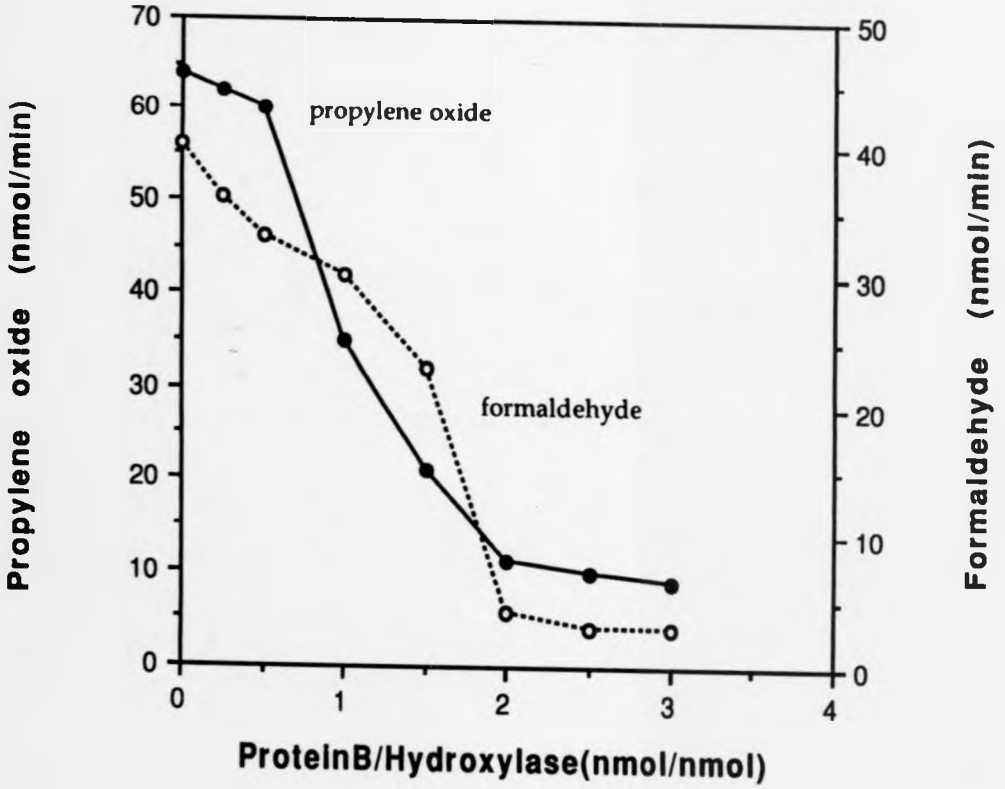


Figure 4-10 The effect of protein B in the oxidation of propene and methanol in the hydroxylase/H_2O_2 system. The hydroxylase was 24 μM and H_2O_2 was 100 mM, protein B was a range of 0-72 μM.

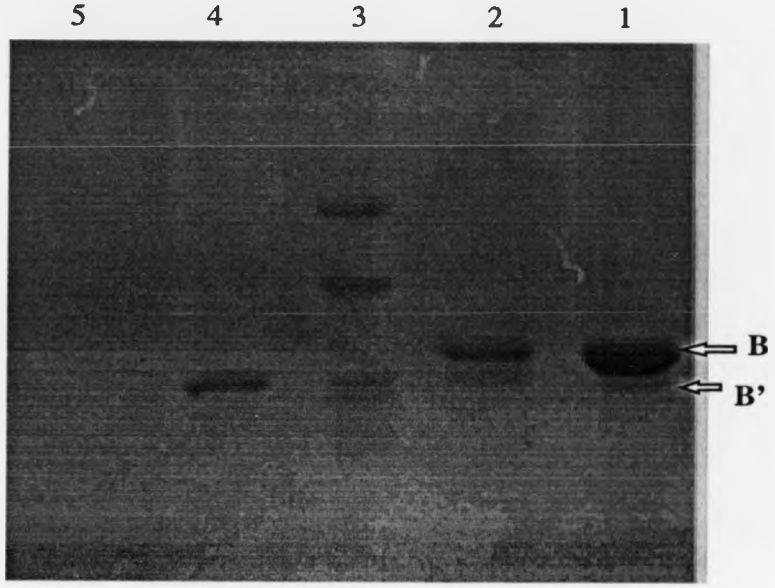


Figure 4-11 Electrophoretic analysis of protein B and B' on a SDS-polyacrylamide gel (8%). Lane 1, protein B; lane 2, protein B (95% purity); lane 3, incubation of protein B(3 mg/ml) with trypsin (10 mg/ml) on ice for 3 min, then stopped reaction by addition of soybean trypsin inhibitor; lane 4, protein B'; lane 5, trypsin plus soybean trypsin inhibitor.

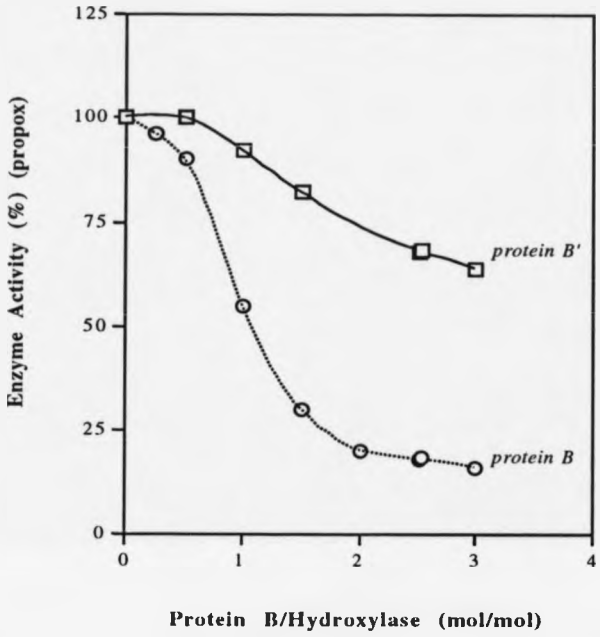


Figure 4-12 Effect of protein B and B' in the H_2O_2-driven system.

The results imply that the function of protein B may not only be in controlling electron flow in the sMMO system, but may also be connected with O_2 binding to the active site. This could be the reason that protein B hinders hydrogen peroxide in activation of the hydroxylase.

Of particular interest was the effect of protein B on the oxidation of 2-methylbutane. At a protein B: hydroxylase ratio of 0.5 a slight decrease of oxidation at primary carbon (Table 4-7) was observed either in the presence or absence of the reductase, and the overall amount of products decreased. This effect was also observed in the sMMO from *Methylosinus trichosporium* OB3b (Froland *et al.*, 1991). Clearly protein B is able to interact with the hydroxylase to significantly affect the hydroxylase/H_2O_2 system.

Table 4-7 Some characterization of the products in the hydroxylation of 2-methylbutane

System	[c]Product ratio (%)		
	Primary	Secondary	Tertiary
[a]sMMO+NADH	52	36	12
[b]Hydroxylase/H_2O_2	27	45	28
[b]Hydroxylase/protein B/H_2O_2	16	56	28
[b]Hydroxylase/reductase/H_2O_2	25	51	24
[b]Hydroxylase/protein B/reductase/H_2O_2	18	54	28
[b]Hydroxylase/NADH/H_2O_2	29	43	28
[b]sMMO+NADH/H_2O_2	38	46	16

a. Hydroxylase 8μM, protein B 8μM, reductase 8μM, NADH 5mM. Reaction 10 mins.
b. Hydroxylase 80μM, H_2O_2 100mM, protein B 40μM, reductase 40μM, NADH 5mM. Reaction 30 min.
c. Ethyl acetate extracted, GC/BP-5 or BP-1 and GC/MS assay. The data shown were average values from a number of experiments.

4.9 Radical recombination using mixtures of substrates?

Carbon radical recombination leading to higher n-alkanes in the sMMO system does not appear to be possible at this stage. It is thought that in activating the hydroxylase hydrogen peroxide replaced the electron donation system (NADH & reductase) and O_2 and in the simpler reaction system it might be possible for carbon radical recombination to occur. Experiments with mixtures of substrates using the H_2O_2-driven system were undertaken. The mixed substrates were methane with propane, 2-methylbutane, benzene and styrene. The incubation contained 100 mM of hydrogen peroxide and 120 μM of the hydroxylase. The reactions were performed under aerobic conditions at 45°C, for 30 minutes. Analysis of the reaction showed that using the hydroxylase/H_2O_2 system no C-C bond products were observed although the reactive intermediate could be different from that in the sMMO complex. On the other hand, this study suggested evidence for explaining the mechanism of recombination of carbon radicals with OH bound to the enzyme active site in both systems. In the future this study may contribute to the construction of catalysts for direct conversion of methane which are more robust than the enzyme and can withstand the rigors imposed on it as an industrial catalyst.

4.10 Discussion

Homolytic pathway for cleavage of O-O bond

The present results have shown that hydrogen peroxide is capable of substituting for O_2 and the electron donating system in sMMO-catalysed oxygenation reactions (eq.4-1).

$$CH_4 + H_2O_2 \xrightarrow{\text{Hydroxylase}} CH_3OH + H_2O \qquad (4\text{-}1)$$

This will contribute to investigation of oxygen activation by the binuclear iron active centre. Both the hydroxylases of sMMO from *Methylosinus trichosporium* OB3b (Andersson *et al.*, 1991) and from *Methylococcus capsulatus* (Bath) (Jiang *et al.*, 1993) can be activated by hydrogen peroxide providing further evidence that oxygen activation and insertion into substrate by the enzyme with a non-heme binuclear iron active site is analogous to the heme-protein cytochrome P450.

The ability of hydrogen peroxide to activate the hydroxylase of sMMO has led researchers to consider the possibility of a Fenton-like reaction being involved in the hydroxylation reactions (Jiang *et al.*, 1993). The homolytic cleavage of the O-O bond of Fe bound peroxide would generate hydroxyl radicals which could oxidize organic compounds (Walling, 1975). A Fenton-reaction mechanism has been used to explain several phenomena associated with hydroperoxide-dependent hydroxylations in chemical and biochemical reactions (Padbury and Sligar, 1988; Labeque and Marnett, 1989; Barton *et al.*, 1992b; Jiang *et al.*, 1993). As already described, selectivity for hydroxylation of alkanes at the 1^o-, 2^o- or 3^o-carbon by the H_2O_2-driven system has suggested that the active intermediate generated is different from that in the sMMO complex and a Fenton reaction mechanism seemed able to explain the available data. However, there is no direct evidence for the involvement of hydroxyl radicals in the H_2O_2-driven reactions. We were unable to detect acetyldehyde and glyoxal when acetylene was present in H_2O_2-driven reactions, or hydroxypyridine from pyridine oxidation. The chemistry of related processes which are mediated by non-heme iron protens, especially μ-hydroxo bridged binuclear iron proteins, is still to be developed. Several proteins are known to contain oxy-bridged diiron centres, such as haemerythrin, the R2 subunit of ribonucleotide reductase (RNR) and purple acid phosphatases. Only sMMO and R2 are known to generate radical species. In the case of R2 it is a protein-based radical whereas in the sMMO reaction it is a substrate-based radical. The fundamental difference may lie in the active-site topography (Dalton *et al.*, 1993b) even though there is a close structural similarity between the R2 and sMMO

active sites. It is possible that the ligands around the enzyme active site play an important role in caging the hydroxyl radicals generated which can carry out the hydroxylation reactions in the hydroxylase/H_2O_2 system and thus were not accessable to scavenging reagents. Indeed, the hydroxyl radical involvement has been reported in many biological systems playing a positive or a negative role (Percival *et al.*, 1992; Halliwell and Gutteridge, 1992). Whether a Fenton-like reaction (ferrous/ferric iron and hydrogen peroxide) is involved in the hydrogen peroxide activation of the hydroxylase of sMMO must be investigated further.

Radical reaction

In the H_2O_2-driven system the oxidation of alkanes may be via a radical reaction based on the observation that the order of selectivity of oxidation of substrates is $3^o > 2^o > 1^o$. It is different from the complete sMMO system where high valent iron-oxo species (Fe^{IV}-Fe^{IV}=O or Fe^{III}-Fe^{V}=O) is generated from the heterolytic pathway (Deighton *et al.*, 1991; Wilkins *et al.*, 1992). Both pathways involve generation of substrate radicals, but homolysis would produce hydroxyl radicals. Spin traps would be expected to inhibit the reactions. In our experiments, the oxidation of ethane and 2-methybutane by the H_2O_2-driven system in the presence of DMPO or POBN spin traps was undertaken. However, product analysis using gas chromatography (GC) was unsuccessful due to a large GC-peak which appeared earlier and obscured the product peak. The generation of hydroxyl radicals via a homolytic pathway is still unsure at this stage.

The effect of protein B

In the H_2O_2-driven system protein B plays a role different from that in the complete sMMO system. This might indicate that protein B as a regulator in the sMMO complex may function in both electron transfer from the reductase to the hydroxylase and oxygen addition to the iron (Fe^{II}/Fe^{II}) active centre. Understanding the regulatory

function of protein B is still unclear, especially in the H_2O_2-driven system. The activation of the hydroxylase by H_2O_2 may be connected with protein-protein interactions. Therefore, further study of the effect of protein B in the hydrogen peroxide-driven system is necessary and the added information will be helpful in understanding the function of protein B as a regulator in the native sMMO system.

The hydroxylase of sMMO can be activated by hydrogen peroxide to effect catalysis of hydrocarbon oxidation raising the interesting question that "how much of the hydroxylase (protein size) is necessary to effect such an oxidation?" Since there is no requirement for other proteins (the reductase and protein B) to associate with the hydroxylase in catalysing the O_2- and NADH-dependent oxidation, it is possible that much of the hydroxylase is superfluous to requirements when H_2O_2 is used. It is hoped that proteolytic digestion of the hydroxylase may give the answer. The next stage will therefore investigate the proteolysis of the hydroxylase component of sMMO.

CHAPTER 5

PROTEOLYSIS OF THE HYDROXYLASE COMPONENT OF SOLUBLE METHANE MONOOXYGENASE

5.1 Introduction

Proteolytic cleavage of peptide bonds is one of the most frequent and important enzymatic modifications of proteins. Historically, enzymatic proteolysis has generally been associated with protein digestion. More recently, proteolytic enzymes have been recognised as major tools in the sequence analysis of proteins and in the identification and isolation of domains of more complex multi-functional proteins (Walsh, 1986). For example, limited proteolysis of tyrosine hydroxylase (TH) by the Ca^{+}-activated neutral proteinase (calpain) has revealed that the regulatory function was in the N-terminal region of the TH molecule (Kiuchi et al., 1991). The eludation of the amino acid sequence of the α fragment of flavocytochrome b_2 by using proteinase is another instance (Ghrir et al., 1984). Also in the study of sMMO incubation of the regulatory protein (protein B) with trypsin (Pilkington et al., 1991) revealed that B' is a truncated version of protein B. This study implied that the C-terminal region of protein B could be involved in protein-protein interactions between the hydroxylase and protein B or the reductase and protein B in the native sMMO complex.

Therefore the study of the proteolysed hydroxylase component by the use of proteinases might be helpful in obtaining the information about the structure, conformation and function of the hydroxylase of sMMO. As already described the hydroxylase can be activated by hydrogen peroxide. We proposed an interesting

question: "how much of the hydroxylase protein is necessary for the H_2O_2-driven oxidation?" The entire native hydroxylase molecule may not be required when hydrogen peroxide is used since some part of protein which for connect with reductase or protein B could be unnecessary in the H_2O_2-driven reaction. Proteolytic digestion of the hydroxylase could lead to a truncated, but functionally active form of the protein that only has to bind methane and hydrogen peroxide, and may be substantially more stable than its progenitor. This trimmed down version of the hydroxylase may be helpful in defining the smallest catalytic unit neccessary to oxidise methane to methanol. So far, it is impossible to sparate subunits of the hydroxylase protein and remain its activity, therefore, the protolysis of the hydroxylase was performed with whole native protein.

The hydroxylase of sMMO from *Methylococcus capsulatus* (Bath) has a relative molecular mass 250 kDa with three subunits of 60 kDa, 45 kDa and 20 kDa in an $\alpha_2\beta_2\gamma_2$ arrangement (Woodland and Dalton, 1984). Previous studies of the cloning and sequenceing of the sMMO proteins from two methanotrophs (Stainthorpe *et al.*, 1990; Cardy *et al.*, 1991) revealed a high degree (94%) of conservation in the α-subunit of the hydroxylase, which was known to interact with both O_2 and substrates (Green and Dalton, 1988; Fox *et al.*, 1989). Also in the sequence of the sMMO α-subunit two Glu-X-X-His sequence motifs were identified, which are known to coordinate the iron atoms in the binuclear iron-binding site of the R2 protein of ribonucleotide reductase (RNR) (Nordlund *et al.*, 1990). Alignment of the sequences of the two proteins, R2 and the hydroxylase, indicated that the iron coordination may be similar (Nordlund *et al.*, 1992). Recently, the X-ray crystal structure of the hydroxylase (Rosenzweig *et al.*, 1993) indicated that the proposed structure for the active site (Nordlund *et al.*, 1992) was a very good prediction.

Based on our understanding of the hydroxylase of sMMO, if a proteolytic enzyme could trim away the unneccessary residues of the hydroxylase, the remaining active fragment might provide information for defining the smallest functional unit and the

interrelationship of the subunits when compared with the native protein. Unlike mutagenesis studies where specific amino acid residues may be changed and the protein remains intact, proteinase treatment will cause cleavage at any site that is accessible and that has the correct sequence specificity. Cleavage of the binuclear iron containing protein at specific sites potentially facilitates the study of metal clusters contained in smaller protein fragments and thereby can locate these clusters in the native protein. Hopefully, this study might be helpful in exploiting a catalytic model that can convert methane to methanol using a highly truncated and consequently more stable form of the enzyme.

5.2 Proteinases and their inactivation

Proteinases have been classified based on a comparision of active sites, mechanism of action and three-dimensional structure. Four mechanistic classes are recognized by the International Union of Biochemistry and within these classes six families of proteinases are recognized (Table 5-1) (Beynon and Bond, 1989). Each family has a characteristic set of functional amino acid residues in a particular configuration to form the active site. The serine proteinases include two distinct families: the mammmalian serine proteinases, such as chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), elastase (EC 3.4.21.11) and kallikrein (EC 3.4.21.8), and the bacterial serine proteinases, such as subtilisin (EC 3.4.21.14). They differ from each other in amino acid sequence and three-dimensional structure, despite a common active site geometry and catalytic mechanism. Analogously, the metallo-proteinases include two families: the mammalian pancreatic carboxypeptidase (EC 3.4.17.1) which differs from bacterial thermolysin (EC 3.4.24.4) in chemical structure even though both are zinc metallo-enzymes and have similar active site configurations.

The best characterised and physiologically most versatile proteinase family is that of the mammalian serine proteinases. The hallmark of their active sites is the catalytic

triad of Asp^{102}, His^{57}, Ser^{159} (in chymotrypsin numbering). Catalysis proceeds via a tetrahedral transition state intermediate during both the acylation and deacylation steps of catalysis (Kraut, 1977). The cysteine proteinases include several mammalian lysosomal cathepsins, the cytosolic calcium activated proteinases (calpains) and the plant proteinases papain and actinidin. Papain (EC 3.4.22.2) is the best-studied member of the family of cysteine proteinases (Drenth *et al.*, 1971). Catalysis proceeds via a thiol ester intermediate and is facilitated by the side chains of amino acids. The aspartic proteinases are enzymatically active in the pH range of 2-3. In this pH range the hydroxylase of sMMO denatures with loss catalytic activity so this family proteinases is unsuitable for the proteolysis of the protein. The metalloproteinases carboxypeptidase A and thermolysin prefer C-terminal aromatic and aliphatic side chains hydrophobic in nature or arginine and lysine residues (Vallee *et al.*, 1983; Matthew, 1988).

Table 5-1 Families of proteolytic enzymes

Families[a]	Protease(s)	characteristic active site residues
Serine protease I	Chymotrypsin Trypsin Elastase Pancreatic kallikrein	*Asp, Ser, His*
Serine protease II	Subtilisin	*Asp, Ser, His*
Cysteine proteases	Papain	*Cys, His, Asp*
Aspartic proteases	Penicillopepsin	*Asp*
Metallo-protease I	Bovine carboxypeptidase A	*Zn, Clu, Try*
Metallo-protease II	Thermolysin	*Zn, Glu, His*

[a]This table includes only enzymes of known amino acid sequence and three dimensional structure.

Proteolysis by these proteinases is limited to the cleavage of specific peptide bonds in the target protein. The key to this selectivity is the accessibility of the scissile peptide bond to the processing proteinase and its specificity.

Many proteinases can be inhibited (inactivated) by specific or general inhibitors. Proteinase inhibitors can be divided into two general classes: (i) active site-specific low-molecular-weight inhibitors that irreversibly modify an amino acid residue of the proteinase active site, such as the serine proteinase inhibitors DFP (diisopropyl phosphofluoridate) and PMSF (phenylmethanesulphonyl fluoride) which react with the active site serine; (ii) naturally occurring proteinase inhibitors, many of which behave as pseudosubstrates, such as soybean trypsin inhibitor. In most cases, commercially available inhibitors can be used to inactivate proteinases.

One potential general problem with some inhibitors is in the careful choice of a suitable method for easy separation of the target protein and the proteinase-inhibitor complex. A suitable method is one which obtains the best quality and quantity of the proteolysed protein (target protein) for further study. In this thesis, the serine proteinases chymotrypsin, trypsin and subtilisin and one of the metallo-proteinases thermolysin were chosen to attempt the proteolysis of the hydroxylase component. The serine proteinase inhibitor PMSF (334 mM stock solution in ethanol) was used (1 mM) for inhibition of subtilisin. Phosphoramidon (60 mM stock solution in water) was used (1 mM) for inhibitior of thermolysin. The proteinase-inhibitor complex and the hydroxylase were separated via ion-exchange chromatography (mono Q 10/10). The soybean-trypsin-inhibitor (attached to 4% beaded agarose, commercially) was used to inhibit the proteinases trypsin and chymotrysin. The separation of the hydroxylase and the proteinase-inhibitor complexes was through a glass wool column which was prepared dependent on the volume of reaction solution (2 ml reaction solution required a

0.5 x 1 cm glass wool column). PMSF also inhibits chymotrypsin and trypsin, but the instability of PMSF in aqueous solution and its toxicity made this an undesirable method for obtaining the active fragment of the hydroxylase and soybean-trypsin-inhibitor (attached to agarose) was more simple and effective .

5.3 The effect of proteinases on native hydroxylase

In considering the action of proteinases on unfolded proteins, the only factor that has to be taken into account is the bond specificity exhibited by the proteinase. Any given proteinase would then follow a characteristic digestion pattern to give a defined set of peptide fragments. In the case of the hydroxylase (M_r = 250 kDa) of sMMO from *Methylococcus capsulatus* (Bath), the accessibility of susceptible bonds, in the native folded state, to specific proteinases was found to be limited. Initially, it was necessary to screen the effects of a number of proteinases before more focused studies could be undertaken.

The proteinases α-chymotrypsin, trypsin, subtilisin and thermolysin were screened under various conditions and at a range of proteinase concentrations (Table 5-2). The proteinases used were all commercially available in a suitably pure form; this was important as contamination could give rise to spurious cleavage. The results showed that chymotrypsin and trypsin reacted effectively with the hydroxylase at pH 7.5 and at room temperature. Neither thermolysin nor subtilisin cleaved the hydroxylase as judged by gel-electrophoresis. The proteinases investigated and some of their properties are listed in Table 5-3. The resistance of the hydroxylase to substilisin and thermolysin indicated that it adopts a tight, compact or domain structure like that of most globular proteins. The parts of the hydroxylase polypeptide chain accessible to chymotrypsin and trypsin were thought to be exposed loops within domains or the

linking regions of polypeptide chains between domains. These accessible regions present in the native protein might arise from either protein folding or conformational changes with a pH change.

Table 5-2 The effect of proteases on the hydroxylase component of sMMO

Protease	$\frac{\mu g \text{ protease}}{mg \text{ hydroxylase}}$	Conditions	Gel-electrophoresis
Thermolysin	5-35	37°C, pH 7.0 (25mM MOPS)	-
		pH 7.5 (25mM Tris)	-
Subtilisin	10-40	37°C, pH 7.0 (25mM MOPS)	-
		pH 7.5 (25mM Tris)	-
Chymotrypsin	5-25	4°C, pH 7.0 (25mM MOPS)	-
		RT, pH 7.0 (25 mM MOPS)	+
		RT, pH 7.5 (25mM Tris)	++
Trypsin	2-25	RT, pH 7.5 (25mM Tris)	++

Note: -, means no truncated version of the hydroxylase on the gel;
+, means appearance of truncated version of the hydroxylase on the gel.

Table 5-3 Proteases used to investigate the hydroxylase of sMMO

Proteases	M_r (Da)	pH optimum	Specificity	Inhibitor
Chymotrypsin	25000	7.8	Aromatic amino acids	Soybean trypsin inhibitor
Trypsin	23500	7.8	Arg, Lys	
Substilisin	27600	7.5	Asp, Glu, Ala, Gly	PMSF
Thermolysin	35000	7.5	Basic amino acids	Phosphoramidon

5.4 Proteinases screening

To date, it has not been possible to map the positions of all the amino acid residues of the hydroxylase of sMMO from crystallographic data. However, the sequence of the hydroxylase is available and a structure for the active site has been proposed (Stainthorpe *et al.*, 1989; 1990; Cardy *et al.*, 1991a; Nordlund *et al.*, 1992).

Accordingly, in the α-subunit of the hydroxylase with a total of 527 amino acid residues, there are 55 (10.4%) possible cleavage sites for trypsin and 67 (12.7%) possible cleavage sites for chymotrypsin (Figure 5-1). The proposed structure of the active site indicates that the iron ligands are His^{147} and bidentate Glu^{144} on Fe1, and His^{246} and monodentate Glu^{209} and Glu^{243} on Fe2 and Glu^{144} bridging the two irons. The possible cleavage sites near the irons ligand for chymotrypsin are more numerous than those for trypsin. It was important to investigate the remaining activity and iron content of the hydroxylase after incubation with chymotrypsin and trypsin.

In the β-subunit there are 388 amino acid residues, with 47 (12%) possible cleavage sites for trypsin and 49 (12.6%) possible cleavage sites for chymotrypsin (Figure 5-2). In the γ-subunit there are 170 amino acid residues, with 27 (~16%) possible cleavage sites for trypsin and 16 (9.4%) for chymotrypsin (Figure 5-3).

It is clear that chymotrypsin might possibly be more effective in the α- or β-subunits and less so in the γ-subunit. Trypsin might be more effective in the γ-subunit than in the α- or β-subunits.

5.5 The effect of chymotrypsin on the hydroxylase component

The effect of different chymotrypsin incubation times was first investigated. The experiments were performed with chymotrypsin at 25 μg/mg hydroxylase at room temperature, the incubation times were 10-90 minutes. Non-denaturing gel-

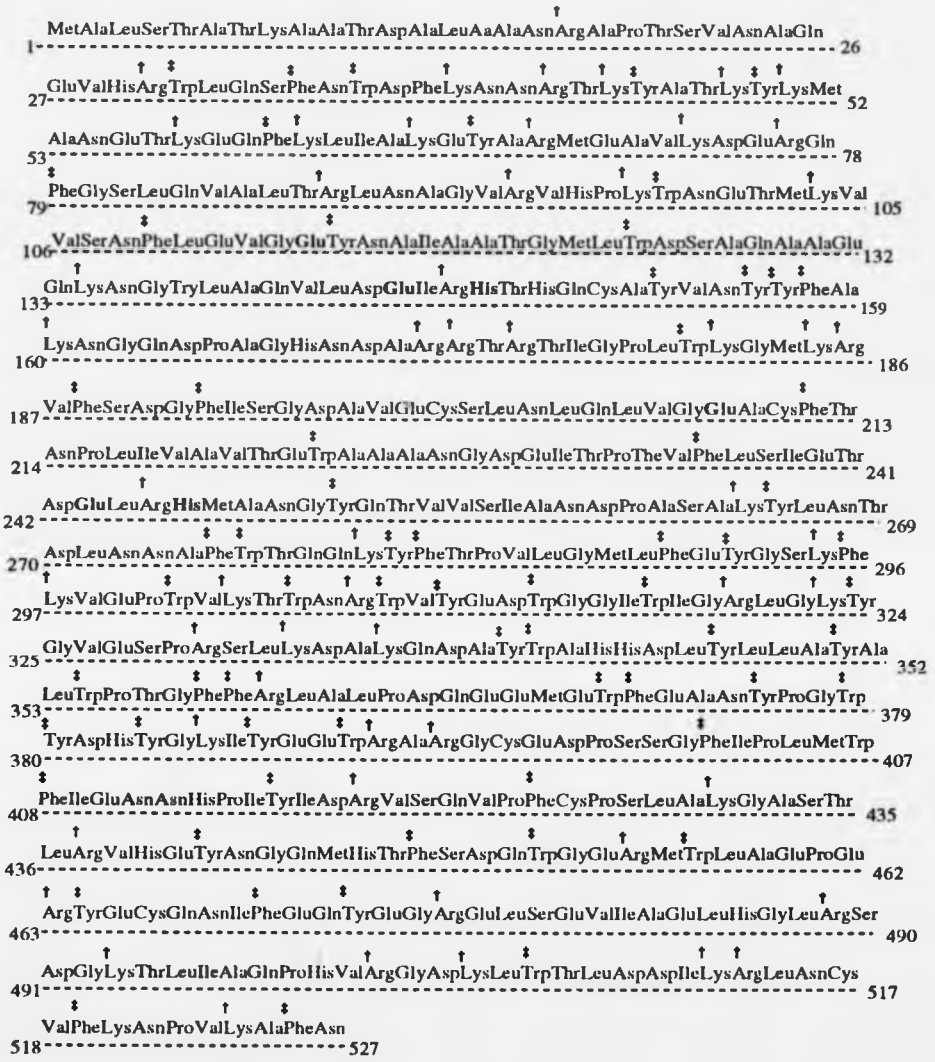


Figure 5-1 α-Subunit amino acid sequence of the hydroxylase of sMMO and the potential sites of chymotrypsin (↕) and trypsin (↑) cleavage.

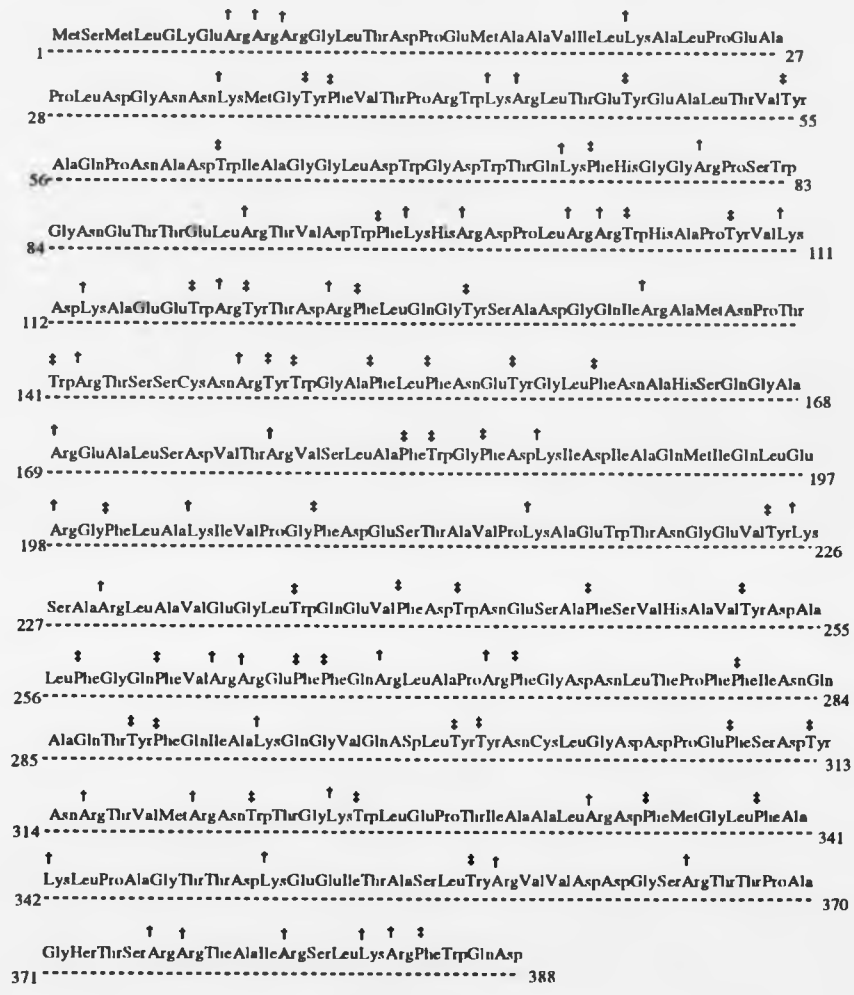


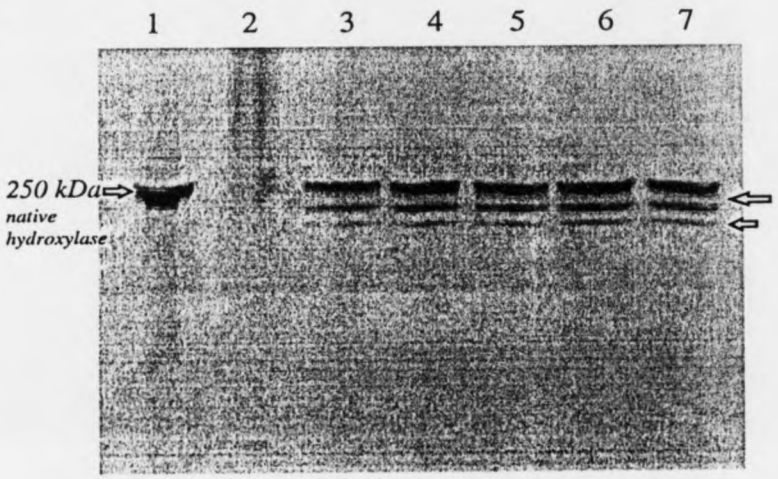
Figure 5-2 β-Subunit amino acid sequence of the hydroxylase of sMMO and the potential sites of chymotrypsin (↕) and trypsin (↑) cleavage.

Figure 5-3 γ-Subunit amino acid sequence of the hydroxylase of sMMO and the potential sites of chymotrypsin (↕) and trypsin (↑) cleavage.

electrophoresis showed that with increasing incubation time the hydroxylase was digested to several fagments by chymotrypsin and on SDS-gels (denaturing-gel) the α-, β and γ-subunits appeared to be all cleaved into smaller fragments (Figure 5-4). Analysis of the iron content of the hydroxylase showed that about 10-15% of the iron was lost during incubation with chymotrypsin (Figure 5-5). Determination of the remaining activity showed that in the H_2O_2-driven assay 35-40% of enzyme activity was lost with incubation times of 10-60 minutes and more than 60% activity was lost in the sMMO complex assay (Figure 5-5). The above analysis showed there was little effect on enzyme activity and iron content with increasing incubation time. Therefore, the effect of increasing chymotrypsin concentration was studied to achieve a greater degree of proteolysis.

The chymotrypsin was used in a range of 25-133 μg/mg of hydroxylase, the incubation was performed at room temperature for 10 minutes. Non-denaturing gel-electrophoresis showed that with increasing chymotrypsin the hydroxylase was digested to several fragments and on SDS-gels the α- and β-subunits had been almost trimmed down (Figure 5-6). This was as predicted that chymotrypsin would be more effective in cleavage of the α-subunit in which the active centre of irons was loaded. Analysis of the iron content of the hydroxylase after proteolysis showed that about 50% of the iron was lost when the chymotrypsin concentration was increased to 133 μg/mg of hydroxylase (Figure 5-7). Determination of the remaining activity showed that in the H_2O_2-driven assay 70-75% enzyme activity was lost and almost 100% activity was lost in the sMMO complex assay under these conditions (Figure 5-7). It is clear that the higher the conentration of chymotrypsin used, the greater the extent of cleavage.

(a)



(b)

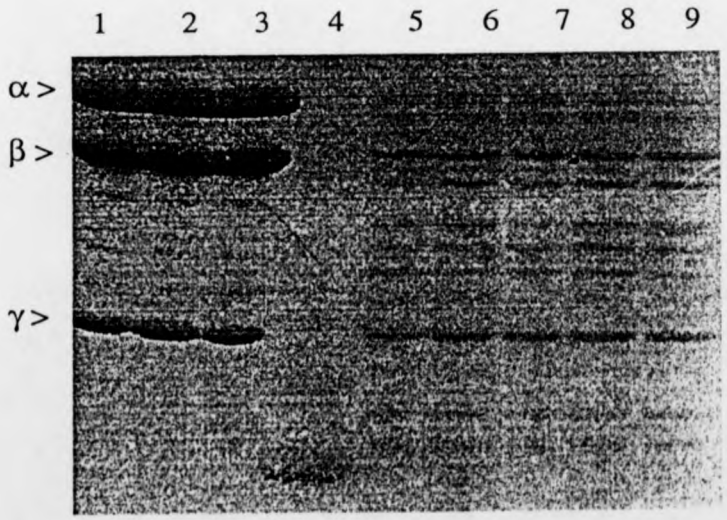


Figure 5-4 Gel-electrophoresis of the hydroxylase proteolysed by incubation with chymotrypsin at different times.

Note: The ratio is 25 μg chymotrypsin/mg hydroxylase.

The proteolysis was performed at room temperature and pH 7.5.

(a) non-denaturing-polyacrylamide gel (8-25%) electrophoresis:

lane 1, native hydroxylase; lane 2, chymotrypsin;

lanes 3-7, digested hydroxylase with chymotrypsin incubated for 10 minutes, 20 minutes, 40 minutes, 60 minutes and 90 minutes, respectively.

(b) SDS-polyacrylamide gel (8-25%) electrophoresis:

lanes 1 and 2, native hydroxylase;

lane 3, hydroxylase with chymotrypsin at zero time;

lane 4, chymotrypsin;

lanes 5-9, digested hydroxylase incubated with chymotrypsin for 10 minutes, 20 minutes, 40 minutes, 60 minutes and 90 minutes, respectively.

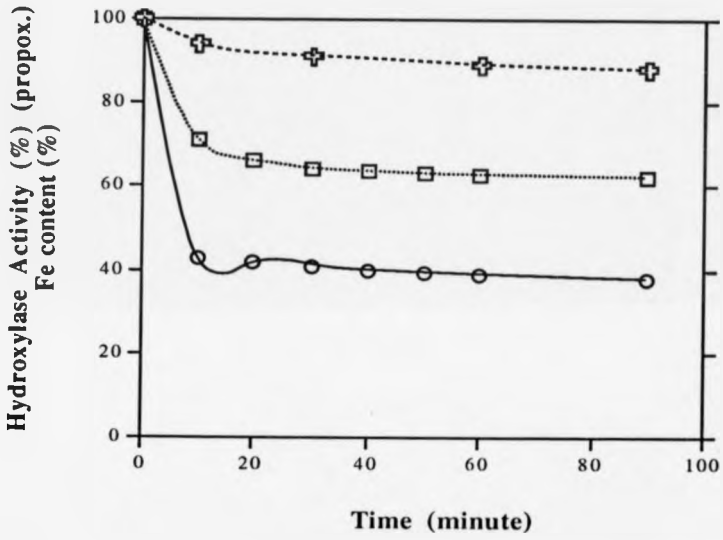


Figure 5-5 Change of the hydroxylase activity (%) and iron content (%) during the time course of proteolysis of the hydroxylase by chymotrypsin.

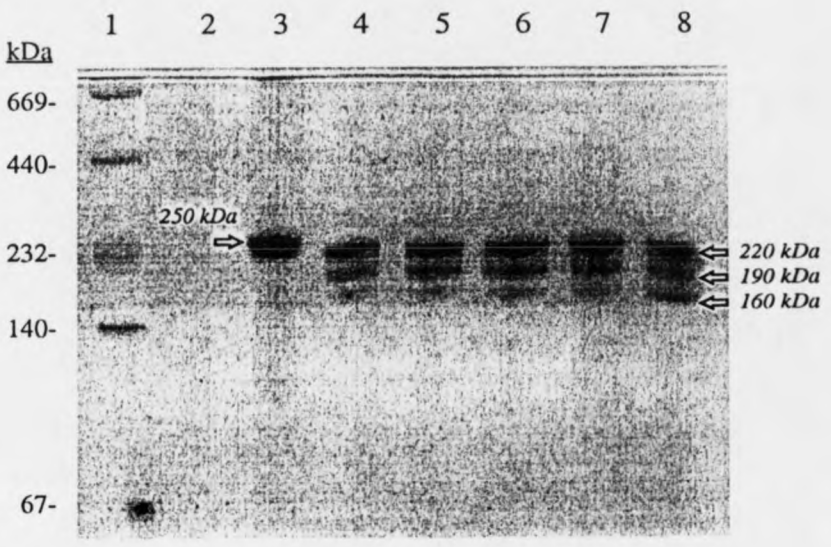
✣ [Fe] (%),100% [Fe] = 2.3 mol/mol protein

□ Hydroxylase/H_2O_2 system,100% activity = 75 nmol/min

○ sMMO complex, 100% activity = 178 nmol/mg/min

Note: The activity of the proteolysed hydroxylase was assayed using propene as substrate. The reaction was performed at 45°C, 15 min for the H_2O_2-driven system and 3 min for the sMMO system. In the H_2O_2-driven system, the hydroxylase was 24 μM and H_2O_2 was 100 mM. In the sMMO complex, the reaction solution contained 8 μM each of the hydroxylase, protein B and the reductase and 5 mM NADH.

(a)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kDa
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440-
250 kDa
232-
140-
67-
220 kDa
190 kDa
160 kDa

(b)

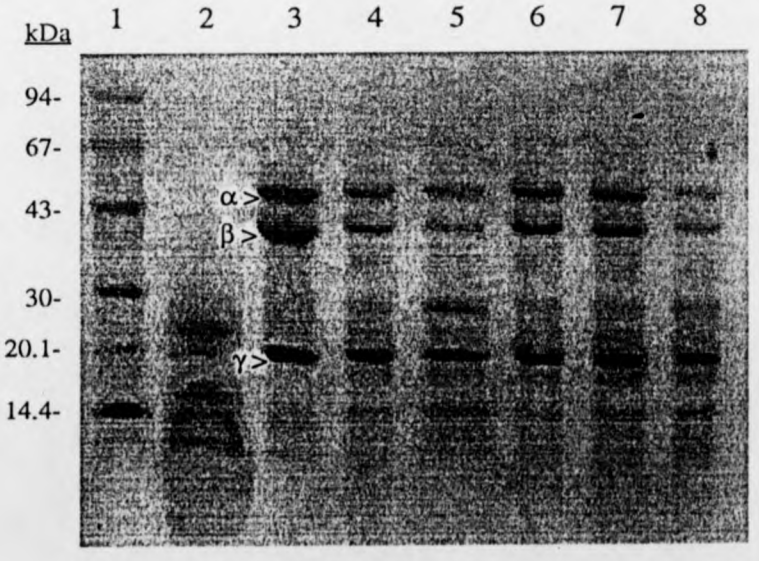

1 2 3 4 5 6 7 8
kDa
94-
67-
43-
30-
20.1-
14.4-
α >
β >
γ >

Figure 5-6 Gel-electrophoresis of the hydroxylase proteolysed by incubation with different ratios of chymotrypsin.

Note: The proteolysis was performed at room temperature and pH 7.5 for 10 minutes.

(a) non-denaturing-gel (8-25%):

lane 1, marker proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa);

lane 2, native hydroxylase;

lane 3, chymotrypsin;

lanes 4-8, hydroxylase digested with chymotrypsin at ratios of 25, 53, 80, 107, 133 μg /mg hydroxylase, respectively.

(b) SDS-gel (8-25%):

lane 1, marker proteins: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 Kda), trypsin inhibitor 20.1 kDa, α-lactalbumin 14.4 kDa;

lane 2, native hydroxylase;

lane 3, chymotrypsin;

lanes 4-8, hydroxylase digested with chymotrypsin at ratios of 25, 53, 80, 107, 133 μg /mg hydroxylase, respectively.

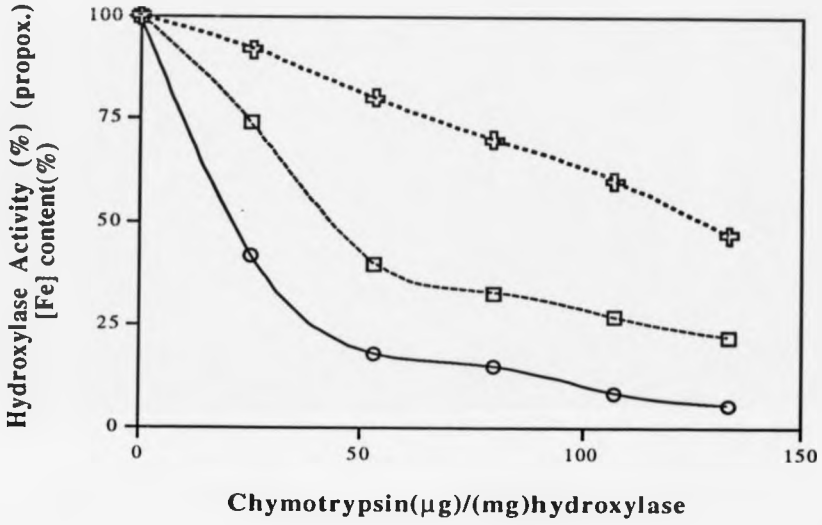


Figure 5-7 The effect of incubation with chymotrypsin (RT, 10 min) on the hydroxylase activity and iron content

- ✣ [Fe] (%), 100% [Fe] = 2.3 mol/mol protein
- □ hydroxylase/H_2O_2 system, 100% activity= 75 nmol/min
- ○ sMMO system,100% activity= 178 nmol/mg/min

Note: The proteolysed hydroxylase activity assay used propene as substrate. The reaction was performed at 45°C, 15 min for the H_2O_2-driven system and 3 min for the sMMO system. In the H_2O_2-driven assay, the hydroxylase was 24 μM and H_2O_2 was 100 mM. In the sMMO complex assay, the reaction solution contained 8 μM each of the hydroxylase, protein B and the reductase and 5 mM NADH.

As described above, chymotrypsin proteolysed the hydroxylase component into several fragments, but with much loss of the enzyme activity in both the complete sMMO and the H_2O_2-driven systems. Reconstitution of the truncated hydroxylase with iron was attempted but no enzyme activity was recovered in either assay system. Either ligands around the active site or the substrate binding site had been removed so that enzyme activity was not restored even though iron was incorporated in the truncated protein. These results suggest that chymotrypsin may not be the best proteinase to use in the search for the smallest active unit of the hydroxylase.

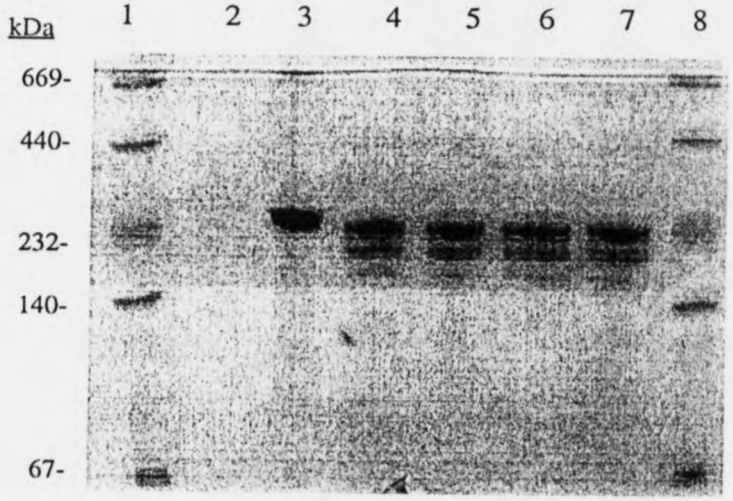
5.6 The effect of trypsin on the hydroxylase component

Proteolysis of the hydroxylase at various times using trypsin (50 μg trypsin/mg hydroxylase) at room temperature and at pH 7.5 was undertaken. The incubation was over the range of 10-60 minutes. One sample was incubated for 60 minutes, followed by continued incubation on ice overnight then the reaction was stopped. Non-denaturing gel-electrophoresis showed that with increase in the incubation time the hydroxylase was substantially reduced in size and on the SDS-gels the α- and β-subunits appeared to be little digested (Figure 5-8). The sample which was incubated overnight was most digested but not as much as with chymotrypsin which almost completely proteolysed the α- and β-subunits. Analysis of the iron content of the hydroxylase showed that about 10% of the iron was lost during incubation with trypsin and with increasing incubation time the iron content decreased no further (Figure 5-9). Determination of the remaining activity showed that in the H_2O_2-driven assay 30-40% enzyme activity was lost with increasing incubation time and about 50-60% activity was lost in the sMMO complex assay (Figure 5-9). The sample incubated overnight lost more activity in both assay systems. It seemed that with an increase in incubation time (from 10 minutes to 60 minutes) the digestion was increased, but there was almost no difference in the enzyme

activity and the iron content. Thus the limited proteolysis must have occurred after just 10 minutes.

Further investigation used different concentrations of trypsin to see the effect on the proteolysis of the hydroxylase. The trypsin was used in a range of 25-150 μg/mg of hydroxylase, the incubation was performed at room temperature, pH 7.5 for 10 minutes. Non-denaturing gel-electrophoresis showed that there was no significant difference in digestion of the hydroxylase with increasing concentrations of trypsin. The SDS-gels showed that the α- and β-subunits were only slightly trimmed down when a high concentration of trypsin (up to 150 μg/mg hydroxylase) was used and the γ-subunit was digested more than the other subunits (Figure 5-10). These observations indicated that the trimming of the hydroxylase into smaller fragments seemed to take place on all the three subunits and cleavage sites are mainly on the surface of the protein. Analysis of the iron content of the hydroxylase showed that only 10% of the iron was lost when incubation with trypsin was at a ratio of 150 μg/mg of hydroxylase (Figure 5-11). Determination of the remaining activity showed that in the H_2O_2-driven assay more than 50% enzyme activity could be detected and with increased ratio of trypsin, whereas 75% activity was lost in the sMMO complex assay (Figure 5-11). These results suggested that the catalytically active site and substrate binding site of the hydroxylase were affected very little by trypsin. Hydroxylase proteolysed with trypsin, either over a long time or with a high concentration, still retained most of its catalytic activity in the H_2O_2-driven system.

(a)


kDa
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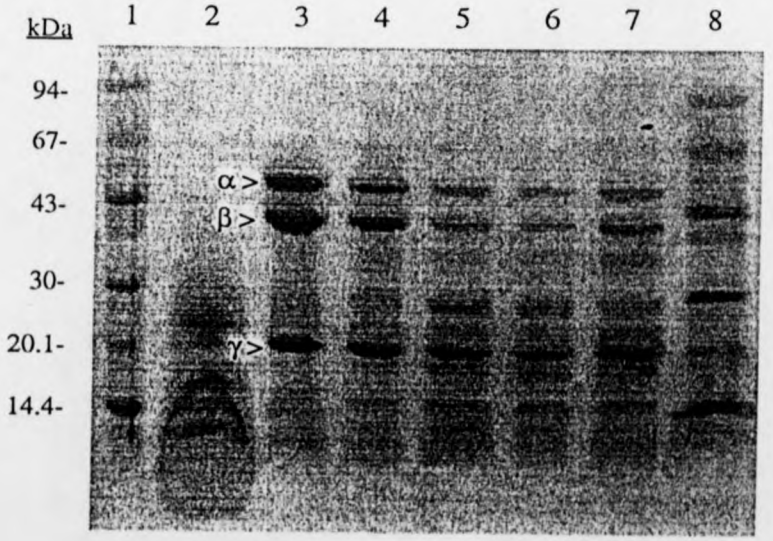
8
669-
440-
232-
140-
67-

(b)


kDa
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6
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8
94-
67-
43-
30-
20.1-
14.4-
α>
β>
γ>

Figure 5-8 Gel-electrophoresis of the hydroxylase proteolysed by trypsin at different times.

Note: The ratio is 50 μg trypsin/mg hydroxylase.
The proteolysis was performed at room temperature and pH 7.5 (25 mM Tris/HCl buffer).

(a) non-denaturing-gel (10-15%):
lane 1, marker proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa);
lane 2, trypsin; lane 3, native hydroxylase;
lanes 4-7, hydroxylase digested with trypsin for 10 minutes, 20 minutes, 40 minutes and 60 minutes, respectively.
lane 8, hydroxylase digested with trypsin after incubation for 60 minutes at room temperature followed by placing on ice overnight.

(b) SDS-gel (8-25%):
lane 1, marker proteins: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 Kda), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa);
lane 2, trypsin; lane 3, native hydroxylase;
lanes 4-7, hydroxylase digested with trypsin for 10 minutes, 20 minutes, 40 minutes and 60 minutes, respectively.
lane 8, hydroxylase digested with trypsin after incubation for 60 minutes at room temperature followed by placing on ice overnight.

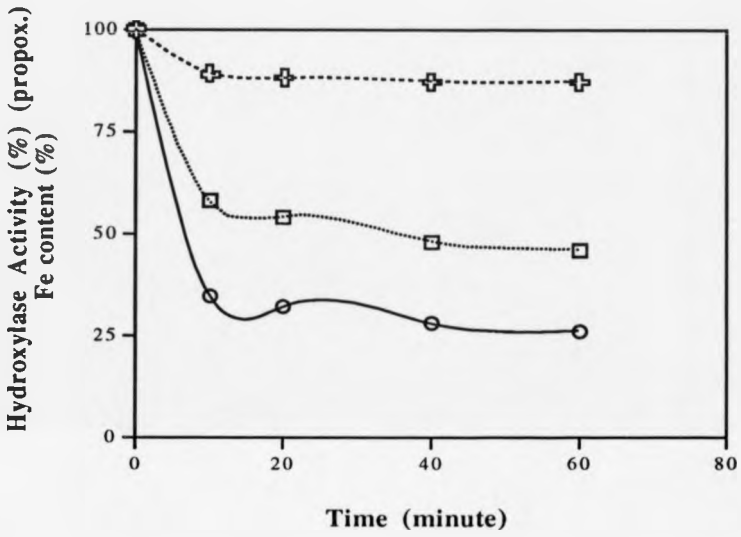
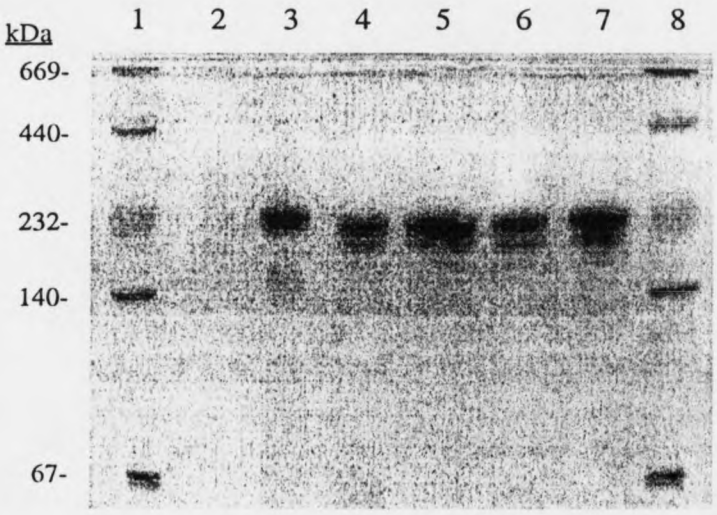


Figure 5-9 Change of the hydroxylase activity (%) and the iron content (%) during incubation with trypsin at various times.

- ✣ [Fe] (%), 100% [Fe]= 2.1 mol/mol protein.
- □ Hydroxylase/H_2O_2 system,100% activity = 65 nmol/min.
- O sMMO system,100% activity= 158 nmol/mg/min.

Note: The activity assay for proteolysed hydroxylase used propene as substrate. The reaction was performed at 45°C, 15 min. for the H_2O_2-driven system, 3 min for the sMMO system. In the H_2O_2-driven system the reaction solution contained 24 μM hydroxylase and 100 mM H_2O_2. The sMMO system contained 8 μM each of the hydroxylase, protein B and the reductase and 5 mM NADH.

(a)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669-
440-
232-
140-
67-

(b)

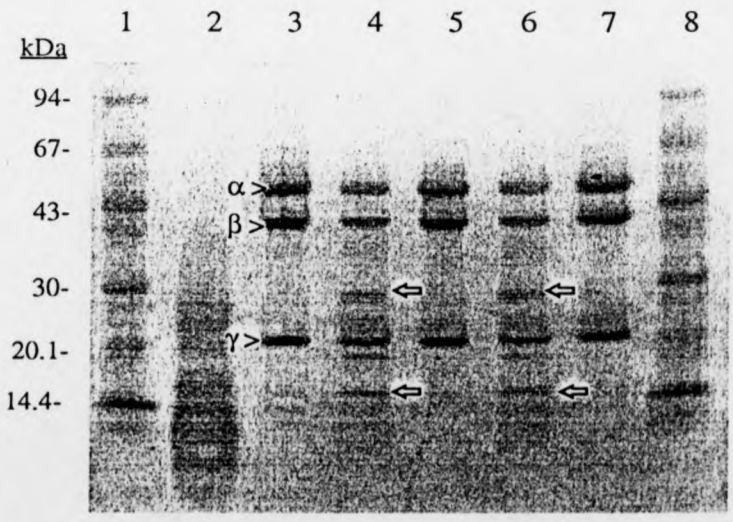

kDa
1 2 3 4 5 6 7 8
94-
67-
43-
30-
20.1-
14.4-
α >
β >
γ >

Figure 5-10 Gel-electrophoresis of the hydroxylase proteolysed by different concentrations of trypsin.

Note: The proteolysis was performed at room temperature and pH 7.5 for 10 minutes.

(a) non-denaturing-gel (10-15%):

lane 1 and 8, marker proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa);

lane 2, trypsin; lane 3, native hydroxylase;

lanes 4-7, digested hydroxylase with trypsin at ratios of 25, 50, 100 and 150 μg /mg hydroxylase, respectively.

(b) SDS-gel (8-25%):

lane 1 and 8, marker proteins: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 Kda), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa);

lane 2, trypsin; lane 3, native hydroxylase;

lanes 4-7, digested hydroxylase with trypsin at ratios of 25, 50, 100 and 150 μg /mg hydroxylase, respectively.

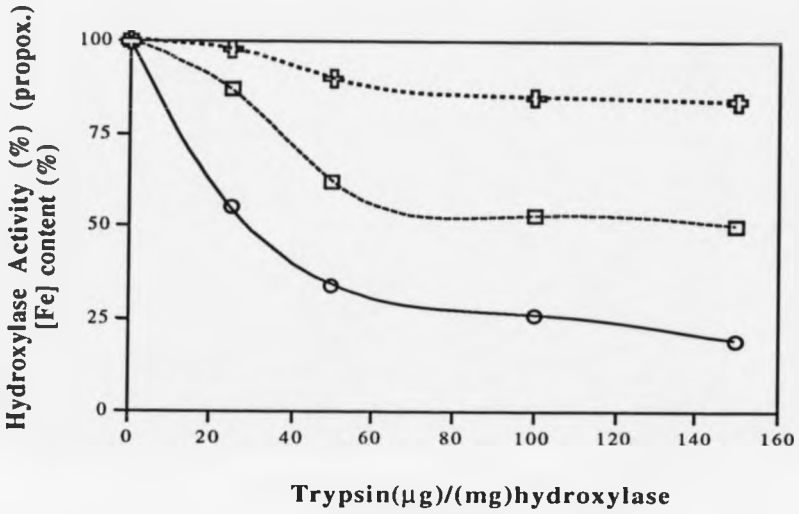


Figure 5-11 The effect of trypsin (RT, 10 min) on the hydroxylase activity and iron content.

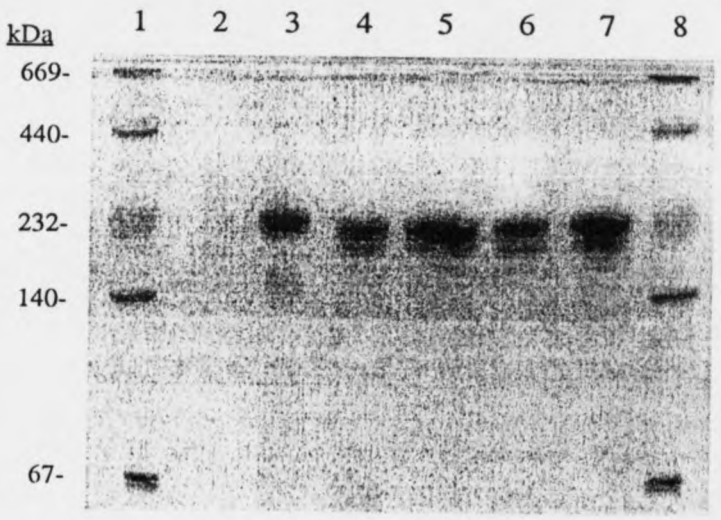
- ✤ [Fe](%),100% [Fe]= 2.1mol/mol protein
- □ Hydroxylase/H_2O_2 system, 100% activity= 65nmol/min
- ○ sMMO system,100% activity= 158 nmol/mg/min)

Note: Pproteolysed hydroxylase actvity assay used propene as substrate. The reaction was performed at 45°C, 15 min for the H_2O_2-driven system and 3 min for the sMMO system. In the H_2O_2-driven system the hydroxylase was 24 μM and H_2O_2 was 100 mM. In the sMMO system, the reaction solution contained 8 μM each of the hydroxylase, protein B and the reductase and 5 mM NADH.

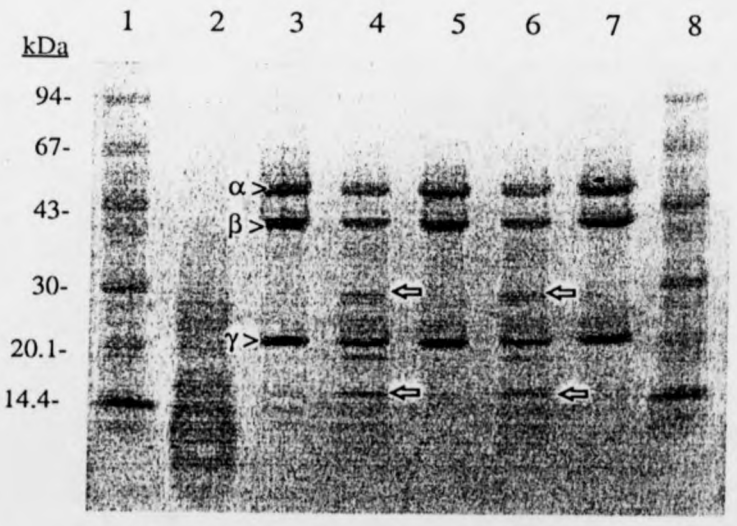
Indeed, trypsin was different from chymotrypsin in giving a degraded protein (approximately 35-40% degradation when using 150 μg trypsin/mg hydroxylase) of at least three fragments (Figures 5-8 and 5-10, on non-denaturing-gel). Attempts were made to separate the three fragments using gel-filtration chromatography (Superose 12, Superdex G200 or Superdex G75) in order to check each fragment for activity, however, the separation was not perfect. The 220 kDa fragment after gel-filtration (Superdex G75) retained more activity than the 170 kDa fragment in the sMMO activity assay, but in the H_2O_2-driven system the activities were similar. This indicated that 35-40% of the structure of the protein could be removed with retention of approximately 50% of the activity in the H_2O_2-driven system. The truncated protein retained approximately 90% of its iron, but the activity in the native sMMO assay was almost completely undetectable whereas in the H_2O_2-driven system 50% of the activity remained. It is possible that cleavage of the hydroxylase protein by trypsin destroyed its ability to interact with the other protein components of the native system (sMMO assay), but had little effect on its interaction with H_2O_2 *in vitro*. Comparsion of the results obtained using two different proteolytic enzymes, trypsin and chymotrypsin, revealed that the iron content was important for the enzyme activity and that the most structure of the protein may also be necessary.

Low concentration of trypsin (2-10 μg/mg hydroxylase) for limited proteolysis of the hydroxylase were also investigated (Figure 5-12). When using 2 μg trypsin/mg hydroxylase for 10 minutes at room temperature and pH 7.5, the protein was degraded by approximately 5-10%. Gel-filtration (Superdex G75) chromatography gave almost complete separation of the active fragments (Figure 5-13). We used this limited proteolysed hydroxylase (2 μg trypsin/mg hydroxylase) and the truncated protein purified by gel-filtration to investigate the catalysis of oxidation of substrates in the two reaction systems, the sMMO complex and the H_2O_2-driven system.

(a)


kDa
1
2
3
4
5
6
7
8
669-
440-
232-
140-
67-

(b)


kDa
1
2
3
4
5
6
7
8
94-
67-
43-
30-
20.1-
14.4-
α
β
γ

(a)

Figure 5-12 Non-denaturing gel (10-15%) electrophoresis of the hydroxylase from limited proteolysis by incubation with a low concentration of trypsin.

Note: The proteolysis was performed at room temperature and pH 7.5 for 10 minutes.

lane 1 and 8, marker proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa);
lane 2, trypsin; lane 3, native hydroxylase;
lanes 4-7, hydroxylase digested with trypsin at ratios of 2, 5, 10 and 25 μg /mg hydroxylase, respectively.

(b)

Figure 5-13 SDS-gel (8-25%) electrophoresis of the limited proteolysed and the purified limited proteolysed protein.

Note: The purified form was obtained from gel-filtration (Superdex G75).

lane 1 and 8, marker proteins: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 Kda), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa);
lane 2, trypsin; lane 3, native hydroxylase;
lane 4, hydroxylase digested with 2 μg trypsin/mg hydroxylase;
lane 5, purified digested hydroxylase (2 μg trypsin/mg hydroxylase);
lane 6, hydroxylase digested with 5 μg trypsin/mg hydroxylase;
lane 7, purified digested hydroxylase (5 μg trypsin/mg hydroxylase).

It was observed that the rate of oxidation of different substrates was decreased upon proteolysis when the complete sMMO system was used to assay the truncated hydroxylase (Table 5-4), but when the H_2O_2-driven system was used there was little or no change in the catalytic activity for all the substrates used except methane which showed a two to three fold increase in activity. This was repeated three times on different enzyme preparations with the same results.

Table 5-4. Comparation of native and limited proteolysed** hydroxylase activity in the sMMO complex and the H_2O_2-driven systems.

Substrate	*sMMO assay (nmol/mg/min)		^H_2O_2-driven assay (nmol/min)	
	native	proteolysed	native	proteolysed
Propene	163	111	64 [(a)]	65 [(a)]
Methane	79	46	8-13 [(b)]	21-26 [(b)]
Ethane	52	38	33 [(b)]	30 [(b)]
Propane	30 [(c)] 50 [(d)]	13 [(c)] 25 [(d)]	2 [(b,c)] 16 [(b,d)]	1.5 [(b,c)] 15 [(b,d)]

** The hydroxylase was proteolysed by 2 μg trypsin/mg hydroxylase at room temperature for 10 minutes.

* In sMMO assay the reaction solution contained 8 μM each of hydroxylase, protein B and reductase, and 5 mM NADH. The reaction was performed at 45°C for 3 minutes.

^ In H_2O_2-driven system, 100 mM H_2O_2 was used.

(a) 24 μM hydroxylase, the reaction was performed at 45°C for 15 minutes;

(b) 80 μM hydroxylase, the reaction was performed at 45°C for 30 minutes.

(c) product = 1-propanol, (d) = 2-propanol.

It is apparent from these experiments that the surface regions of the protein are those most readily attacked by the proteolytic enzyme and that these areas are resposible for interactions with the other components of the sMMO system. When H_2O_2 was used to drive the reaction there was no difference in activity between native and proteolysed proteins. This is presumably because that part of the hydroxylase responsible for its interaction with H_2O_2 is unaffected by proteolysis.

5.7 Discussion

Proteolysis of the hydroxylase

The hydroxylase was greatly digested by a high concentration of chymotrypsin resulting in a loss of enzyme activity. However, even when using a high concentration of trypsin to cleave the hydroxylase, a high percentage of the catalytic activity was still observed. When comparing the possible cleavage sites acted upon by trypsin and chymotrypsin there were almost the same number for each of the enzymes on the hydroxylase, especially on the α-subunit. Previously, we assumed that the aromatic residues might be buried within the protein and would therefore be inaccessable to chymorypsin, but this proved not to be so. It could be that the hydrophobic pocket for substrate binding was surrounded by aromatic residues such that they were accessible to attack by chymotrypsin. Trypsin proteolysis gave a degraded protein retaining half its activity. This would imply that some Lys and Arg residues are either buried in the molecule or in some way not accessible to trypsin. Also, it is possible that there are no Lys and Arg residues near the catalytic site and are therefore not very important for enzyme activity.

The iron atoms in the hydroxylase active site are known to play an impotant role in the oxidation of hydrocarbons. Proteolysis of the hydroxylase by chymotrypsin again demonstrated this fact. Proteolysis of the hydroxylase by trypsin indicated that not only

the iron atoms but also somehow structure of the hydroxylase protein were all necessary for enzyme activity. The unique characteristics of catalysis of methane oxidation to methanol could be due not only to the diiron site but to a unique protein structure. The hydrophobic substrate binding pocket could play a very important role in methane oxidation. This may be the reason why other non-heme binuclear iron proteins, such as the R2 protein of ribonucleotide reductase, do not possess this catalytic capacity and why the chemical models which try to mimic sMMO never achieve this purpose.

Catalytic capacity after limited proteolysis

The hydroxylase which had undergone limited proteolysis showed almost no reduction of catalytic capacity for oxidation of propene, ethane and propane and increased activity for methane oxidation in the H_2O_2-driven aassay. The overall increase (200-300%) of catalytic capacity in the methane/H_2O_2 driven assay can not be accounted for solely by the reduction of the hydroxylase, so some other explanation must be sought. It is possible that the truncated form of the enzyme has a more "accessible" active site to methane than the unproteolysed form-if this was the case we might expect other substrates to be affected in the same way, but they are not. Alternatively, since it is known that sMMO will also oxidise methanol to formaldehyde {albeit with a high Km value (0.95 mM)}, it is possible that the truncated form is unable to oxidise this product of the methane oxidation and therefore permits its higher accumulation during assay.

The thermostability of the hydroxylase, either native protein or proteolysed by trypsin is nearly the same for both. If the proteins were stored at 4°C, almost no activity was lost over 24 hours; if at room temperature, their catalytic capacity was reduced by about 5-10%. This led us to further studies on the stabilization of the hydroxylase component using specific chemicals or cross-linking reagents. There have been many reports on the use of whole cells of methane oxidising bacteria to effect the production

of oxy-chemicals from simple hydrocarbons with industrial applications in mind (Lidstrom and Stirling, 1993). If the hydroxylase of soluble methane monooxygenase, which catalyses the mono-oxygenation of a wide variety of hydrocarbons, could be modified to produce more stable protein, the enzyme could be used as a general oxidising catalyst in industry. Therefore modification of the hydroxylase and determination of the physical and catalytic properties of the altered protein was examined next.

CHAPTER 6

STUDIES OF THE CHEMICAL MODIFICATION OF THE HYDROXYLASE COMPONENT OF SOLUBLE METHANE MONOOXYGENASE

6.1 Introduction

The stability of proteins and enzymes is a major concern in their industrial applications. Biocatalysts with high thermostability will have prolonged viability (Mozhaev *et al.*, 1983). A lengthened operational stability will reduce the need for frequent enzyme replacement and these thermostable enzymes will enable the acceleration of chemical reaction rates at a higher temperature. In cases where several reaction steps are required, a thermostable biocatalyst will enable the process to continue at elevated temperatures without lowering the heat (Tatsumoto *et al.*, 1990). In addition, stability in organic solvents, at extreme pH values and pressures, and mechanical disturbances is also desirable for organic syntheses, chemical analysis, isolation and purification of chemicals in therapeutics and diagnostics, and in the study of protein structures and functions (Wong and Wong, 1992). Therefore enzyme stabilisation has received considerable attention and numerous searches for methods to prepare stable proteins and enzymes.

6.1.1 Chemical basis of protein stability

Interest in structure-stability relationships in proteins has led to calculations of the free energy contributed by various structural elements. A number of articles have been published on the subject (Jaenicke, 1991; Doig and Williams, 1991; Alonso and Dill, 1991; Serrano *et al.*, 1991; Sharp *et al.*, 1991). A brief account of structure-stability relationships in proteins is given here.

It has been generally accepted that the principal driving force in protein folding is the hydrophobic effect. The hydrophobic effect has been defined as the process by which non-polar groups are removed from contact with water (Doig and Williams, 1991). Hydrophobicity has always been considered one of the major factors of protein integrity (Kellis *et al.*, 1988; Zhou *et al.*, 1992). Hydrophobic side chains are usually buried inside protein molecules to induce tight packing. For proteins that associate with others, hydrophobic clusters are found localised on the surface of the protein molecules to induce solitary contacts with other proteins, or with other hydrophobic entities (Chothia and Janin, 1975). This shielding of nonpolar groups from interaction with water in the medium reduces the unfavourable entropy of the system and thus increases protein stability (Tanford, 1980).

In addition to hydrophobic forces, hydrogen bonding, salt bridges, dipole-dipole and other electrostatic interactions also contribute to protein stability (Stigter and Dill, 1990). Hydrogen bonds play an important role in the maintenance of the secondary structures of proteins, i.e. α-helixes, β-sheets, etc. They are also the basis of interactions of polar groups on the protein and water at the protein-water interface to form a hydration sphere around the molecule (Chothia and Janin, 1975). The formation of S-S disulfide "bridges" between two cysteine residues (forming cystine) is another important feature responsible for establishing the secondary structure of proteins. The disulfide bonds are very specific, they contribute to the stability of the folded structure since a molecule containing -S-S- bridges exhibits a smaller conformational entropy change (Matthews and van Holde, 1990). While the energy contribution of dipole-

dipole interactions is small, the large number of such interactions in proteins makes them significant (Schulz and Schirmer, 1979). On the other hand, the number of salt bridges is small, but their energy contribution is large (Barlow and Thornton, 1983; Gilson *et al*, 1985).

It is the sum of these various forces, together with the binding of metal ions, substrates, cofactors, and other low-molecular-weight ligands that maintains the structural integrity of a functional protein or enzyme (Mozhaev and Martinek, 1984).

A critical feature of a protein is its ability to fold into a three dimensional conformation. Each protein exists in a unique conformation (or sometimes a series of alternate conformations) (Mathews and van Holde, 1990; Lewin, 1990). Therefore, the molecular mechanism of protein inactivation may involve several conformational changes leading to the unfolding of the molecule, however the overall physical process can be represented simply by two steps (Zale and Klibanov, 1983):

where N, I, and D are native, intermediate, and denatured forms of the protein, respectively. The first step involves reversible conformational changes, followed by a second step of irreversible unfolding by which an enzyme loses its activity. The approach to stabilising proteins and enzymes, either for thermostability or for stability in organic solvents or extreme pH's and pressures, is to prevent irreversible unfolding form occurring and this can be achieved by several means. Enzyme immobilisation has long been recognised as a way to confer stability (Martinek *et al.*, 1977; Klibanov, 1979; Schmid, 1979) and this technique has been used most frequently to solve the problem of enzyme stabilisation. However, other methods have been suggested to achieve enzyme stabilisation: (1) addition of low molecular weight compounds to enzymes free in solution, (2) chemical modification of enzymes by reaction with low

molecular weight compounds and (3) use of bifunctional reagents to produce enzymes containing artificial intramolecular crosslinks (Martinek and Torchilin, 1988). Here, is briefly described the successful modification of proteins and enzymes using crosslinking or other selected chemical alterations.

6.1.2 Stability effected by chemical crosslinking

Many practical methods have been developed to preserve the integrity and activity of native proteins. Based on thermodynamic reasoning, proteins were invariably kept at low temperatures to prolong their useful lives. Many enzymes and proteins can be stored in this manner for extended periods of time, but low temperatures are not preferable in many industrial applications. In an attempt to increase the thermostability of proteins, a technique used to increase the various contributions to the free energy of stabilisation is protein engineering involving site-specific mutagenesis and cloning of thermostable molecules whose structures mimic those of proteins isolated from thermophilic organisms (Mozhaev *et al.*, 1988). This method is appealing, but tremendous difficulties associated with protein characterisation including three-dimensional structural analysis will have to be resolved before site-specific mutagenesis becomes widely applicable to the problem (Wong and Wong, 1992). Another approach to stabilising proteins and enzymes is to strengthen the compact structure of the molecule so that denaturation will not occur. This can be accomplished by chemical crosslinking.

Chemical crosslinking is based on diminishing the entropy of the polypeptide which is the principal thermodynamic quantity stabilising the denatured form (Flory, 1956). In 1967 Hartman and Wold (1967) first introduced the use of bifunctional reagents in protein chemistry. Later Husain and Lowe (1968) used protein crosslinking with a bifunctional reagent as a means to study the tertiary structure of an enzyme molecule consisting of a single polypeptide chain and Davies and Stark (1970) used

this technique to explore the quaternary structure of oligomeric enzymes. Since the publication of these pioneering works crosslinking of proteins has become a widely used technique for immunoanalysis, fundamental studies and protein and enzyme stabilisation.

Chemical crosslinking reagents contain bifunctional groups which can most easily react with proteins and enzymes. There are many bifunctional compounds available for the crossinking of proteins such as dialdehydes, diimido esters, diisocyanates, bisdiazonium salts, diamines and diacids, etc. The bifunctional reagents may be classified into zero-length, homobifunctional and heterobifunctional crosslinkers (Wong and Wong, 1992). The zero-length crosslinkers induce direct joining of two chemical components without the introduction of any extrinsic materials. This is in contrast to homo- and heterobifunctional reagents where a spacer is incorporated between the two crosslinked groups. When a crosslinker contains two identical reactive groups, it is referred to as a homobifunctional reagent. Heterobifunctional crosslinkers contain two different functionalities which offer the possibility of different functional groups of the protein to be modified. These crosslinkers, homo- and heterobifunctional reagents, will react with amino acid side chains of proteins, thus bridging two components (or subunits) together. These chemicals can react with nucleophilic side chains of amino acids such as the sulfhydryl group of cysteine, the amine groups of lysine and N-terminal amino acids, the carboxyl groups of aspartic, glutamic acids and C-terminal amino acids, the imidazolyl group of histidine and the thioether group of methionine. The specificity of these chemicals for a specific amino acid side chain depends on the relative reactivity of the nucleophile. Since nucleophicity is a function of the electronic structure, the pK_a and the microenvironment, the reactivity of an amino acid side chain is generally not specific and several side chains may react with the same bifunctional reagent (Wong, 1991).

Chemical crosslinks can reticulate the protein molecule to diminish the polypeptide entropy, which will decrease the rate of denaturation. The braces can be achieved by both intra- and intermolecular crosslinking. Many studies have shown that intramolecular crosslinking is effective in protein stabilisation. The principles of intramolecular crosslinking are shown schematically in Figure 6-1 (Martinek and Torchilin, 1988; Wong and Wong, 1992). The crossinking of catalase using dimethyl suberimidate and dimethyl adipimidate showed impressive stability and temperature (Shaked and Wolfe, 1988). Olsen *et al* (White and Olsen, 1987; Yang and Olsen, 1988; 1991) succeeded in crosslinking hemoglobin with bis(3,5-dibromosalicyl)fumarate to give a more stable protein with an increase of the denaturation transition temperature compared to that of native protein. Also, crosslinking of superoxide-generating respiratory burst oxidase in neutrophil plasma membranes with water-soluble carbodiimide or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide has significantly enhanced the stability of the enzyme towards heat, high salt and detergent (Tamura *et al.*, 1989). Recently, the preparation of crosslinked enzyme crystals (CLECs) with the bifunctional compound glutaraldehyde, $HCO(CH_2)_3CHO$, has been reported (St. Clair and Navia, 1992; Borman, 1992). Enzymes which are treated this way retain the same high activity as the natural form and are more resistant to extremes of temperature and pH.

Since the amino acid residues modifiable by crosslinking reagents are distributed randomly in a protein, their positions in the three-dimensional structure differ in space and microenvironment. Different crossslinking reagents may vary significantly in their ability to confer stability. Torchilin *et al.* (1978) have reported that the degree of increased thermostability of α-chymotrypsin depended on the length of the carbon chain in the intramolecular crosslinks. Intramolecularly crosslinked glyceraldehyde-3-phosphate dehydrogenase was more stable at 60°C than native enzyme and the degree of stabilisation was shown to depend on the chain length of the bifunctional reagent used (Torchilin *et al.*, 1983). Similarly, Tatsumoto *et al.* (1989) used a series of

bifunctional chemical modification reagents of different chain lengths to crosslink amyloglucosidase and came to the same conclusion, that the longer chain length the longer the half-life of the modified protein.

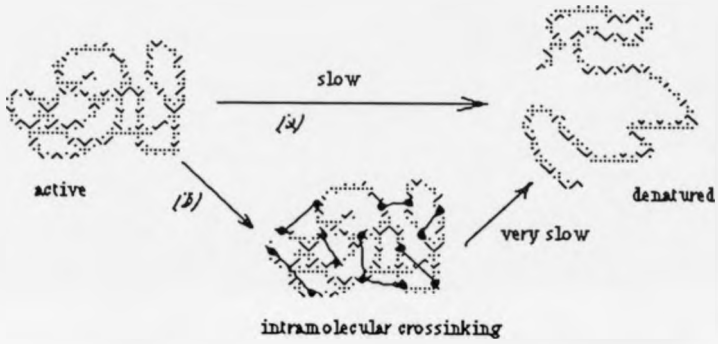


Figure 6-1 General scheme of enzyme stabilization effected by intramolecular crosslinking. (a) native enzyme irreversibly denatured, (b) crosslinked enzyme denaturation.

Intermolecular crosslinking of enzymes to themselves or to other soluble proteins is also beneficial. This was first demonstrated for ribonuclease when it was crosslinked to itself with dimethyl suberimidate to form a dimer or to polyspermine to yield a heteroconjugate (Wang *et al.*, 1976; Wang and Moore, 1977). The polyspermine-ribonuclease conjugate was 115 times more active towards poly(A)·poly(U) and 176 times more active towards poly(I)·poly(C) than the parent enzyme (Wang and Moore, 1977). Another study showed that when horseradish peroxidase is crosslinked to immunoglobulin G or Jacalin, the energy of inactivation of the enzyme increased from 35 to 51 and 43 kcal/mol, respectively (Wong *et al.*, 1992).

Other studies such as the crosslinking of the C-terminal domain of *E. coli* ribosomal protein L7/L12 by N-[4(p-azidosalicylamido)butyl]-3-(2'-pyridyldithio) propionamide (Zecherle *et al.*, 1992) and of egg white riboflavin-binding protein by calcium phosphate (Aoki *et al.*, 1993) have also been reported. Some new crosslinking reagents such as carbohydrate-based materials (Wang *et al.*, 1992a) have also been investigated. Thus, there are ample examples to illustrate that chemical crosslinking, either inter- or intramolecular, can provide a means of preparing stable enzymes and proteins.

6.1.3 Solubility in organic solvents effected by specific chemicals

Despite the phenomenal efficiency of enzymes in biological systems, they are not widely used in industrial processing because of their instability and their strong tendency to denature in aqueous-organic solvent mixtures. Many industrial substrates and products are unstable or insoluble in aqueous media where enzymes are most comfortable. The incompatibility of enzymes with many organic solvents also has precluded their use in processes in which solubility differences between aqueous and organic solvents are exploited to isolate products and improve synthetic yields. The technology of modification of proteins or enzymes with special chemicals can address such stability and solvent compatibility concerns head-on.

An important finding in Klibanov's laboratory was that many enzymes could function in organic solvents with little or no addition of water (Zaks and Klibanov, 1988). The main interest in organic-phase enzymology is for synthetic organic work and the field of enzymology may benefit greatly from this important work. Such organic-phase enzymology could offer a number of potential advantages compared to the common use of enzymes in aqueous solutions. These include increased solubility of hydrophobic substrates, enhanced thermostability, alteration of substrate specificity, shifts of thermodynamic equilibria or reduction of side reactions (Wang *et al.*, 1991).

Enzyme-based assays could also benefit from the use of nonaqueous solutions (Wang *et al.*, 1991; 1992b). Improved detection of phenolic compounds, cholesterol or hydrogen peroxide was obtained by operating amperometric or thermal transducers in chloroform, toluene or diethyl ether. The use of enzymes in nonaqueous environments is not restricted to man-made situations. Many enzymes or multienzyme complexes including lipases, esterases, dehydrogenases and those responsible for xenobiotic metabolism (e.g., cytochrome P450) function in natural hydrophobic environments, usually in or immobilised to a membrane (Gunsalus *et al.*, 1974; Borgstrom and Brockman, 1984). The concentration of water in the vicinity of these enzymes is significantly less than the bulk water concentration of 55 M in aqueous solutions, so it should not be surprising that enzymes are catalytically active in organic solvents (Dordick, 1989).

The more successful and expansive approach with possible industrial relevance to drug design for clinical uses and synthetic organic work is the covalent modification of enzymes with polyethylene glycol (PEG) derivatives, especially with the higher molecular weight polymers, to make enzymes soluble and retain their activity in organic solvents. The use of other chemicals such as dithioesters (Souppe *et al.*, 1988) and labelled PEG (Ladd and Snow, 1993) to increase protein stability has also been reported. The chemically modified enzymes, such as lipase (Yoshimoto *et al.*, 1984; Inada *et al.*, 1984; Takahashi *et al.*, 1985), α-chymotrypsin (Matsushima *et al.*, 1984; Gaertner and Puigserver, 1988; 1989; Babonneau *et al.*, 1989; Pina *et al.*, 1989; Ljunger *et al.*, 1993), trypsin (Abuchowski and Davis, 1979; Gaertner and Puigserve, 1989), thermolysin (Ferjancic *et al.*, 1988), papain (Lee *et al.*, 1988; Souppe *et al.*, 1988; 1989), catalase (Takahashi *et al.*, 1984a), peroxidase (Takahashi *et al.*, 1984b; 1984c; Souppe *et al.*, 1989; Urrutigoity and Souppe, 1989; Wirth *et al.*, 1991) and cholesterol oxidase (Yoshimoto *et al.*, 1987), have been rendered soluble in organic solvents and it has been shown that these modified enzymes could be used as catalysts of chemical reactions for a number of biotechnological applications.

6.1.4 Possiblity of modification of the hydroxylase of sMMO

It is well known that the significant and unique characteristic of methane monooxygenase is its ability to catalyse the oxidation of methane to methanol at ambient temperature and pressure. Soluble methane monooxygenase is also known to catalyse a wide variety of hydrocarbon oxidations. There have been many reports on the use of whole cells of methane oxidising bacteria to effect the production of oxy-chemicals from simple hydrocarbons with industrial applications in mind (Lidstrom and Stirling, 1990). However if methane monooxygenase were to be considered for use as an industrial catalyst, the inherent instability of the enzyme would be a troublesome problem precluding its use as a general oxidation catalyst. It is believed that for sMMO catalysed reactions the rates may be limited by the solubility of the substrate in aqueous medium. If the thermostability of the enzyme could be increased via chemical crosslinking, the enzyme might withstand the demands of temperature and nonaqueous solvents in industrial applications.

Previous work on the chemical modification of proteins lead us to consider the possibility of modification of the hydroxylase of sMMO with the aim of increasing its thermal stability and solubility in nonaqueous solvents. Any such improvements to this useful catalyst could have important implications for its industrial use.

6.2 Effect of chemical crosslinking

In Chapter 5 it it was reported that when the hydroxylase was degraded by approximately 10% in size using a low amount of trypsin (2 mg/mg hydroxylase) this had no effect on its activity in the H_2O_2 assay.

In the present work, this truncated but fully active version of the hydroxylase (which was purified on a Superdex G75 gel-filtration column to remove any inactive small fragments after proteolysis) was chosen for study with the chemical crosslinkers.

6.2.1 Diamines

Diamines are bifunctional reagents and are used as one sort of intramolecular crosslinker. The thermostability of α-chymotrypsin has been successfully increased by diamine crosslinking (Torchilin *et al.* 1978). Dimines can be used for crosslinking carboxyl groups in proteins if they are first activated with carbodiimide. As shown in Scheme 6-1, the carbodiimide reacts with the carboxyl groups of the protein at acid pH to form the active intermediate which then react with the diamine to form a bridge between the carboxyl groups.

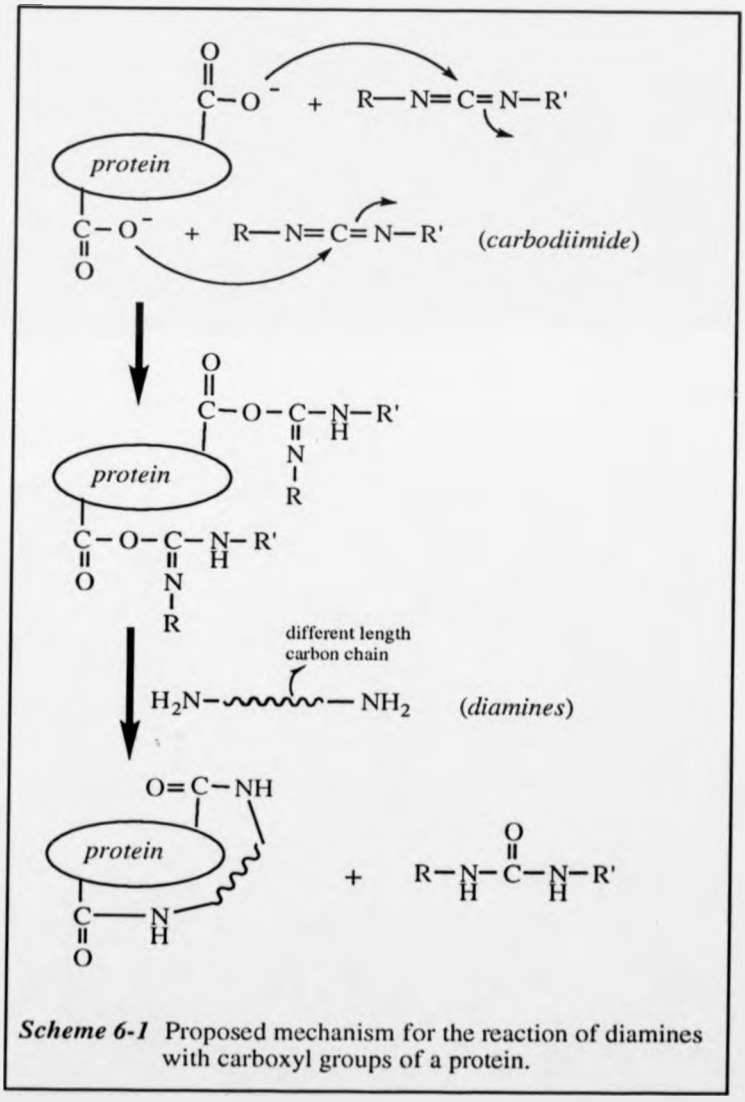
A series of aliphatic diamines (0-6 methylene groups: hydrazine, ethylenediamine, tetramethylenediamine, pentamethylenediamine and hexamethylenediamine) which are commercially available and relatively inexpensive were tried initially. The carboxyl groups of the hydroxylase were first treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at pH 4.5 after which the diamines were introduced under basic conditions to crosslink the activated carboxyl groups in the protein.

Several diamine crosslinking agents were used in an attempt to make an active and thermally stable protein , but these efforts were unsuccessful. Reagents such as hydrazine, ethylenediamine and tetramethylenediamine not only caused complete loss of the enzyme activity but also precipitated the protein. Pentamethylenediamine and hexamethylenediamine treatment resulted in inactive enzyme though no precipitate was observed.

It appeared that crosslinking of the carboxyl groups of the hydroxylase using diamines was unsuitable for this protein and the length of the carbon chain between the groups was not enough to result in an active and thermostable hydroxylase.

It has been established that in all probability the success or failure in increasing the stability of an enzyme by treating it with a bifunctional agent largely depends on the length of the latter and the distance between the centres to be linked on the protein (Torchilin *et al.*, 1978). Each individual protein requires an intramolecular crosslinking

agent of an optimal size. Therefore, a longer length bifunctional agent might be required for the hydroxylase. We decided on the bifunctional reagent polyoxyethylene bis(imidazolyl carbonyl), which reacts with protein amine groups, to investigate whether it would increase the hydroxylase thermostability.



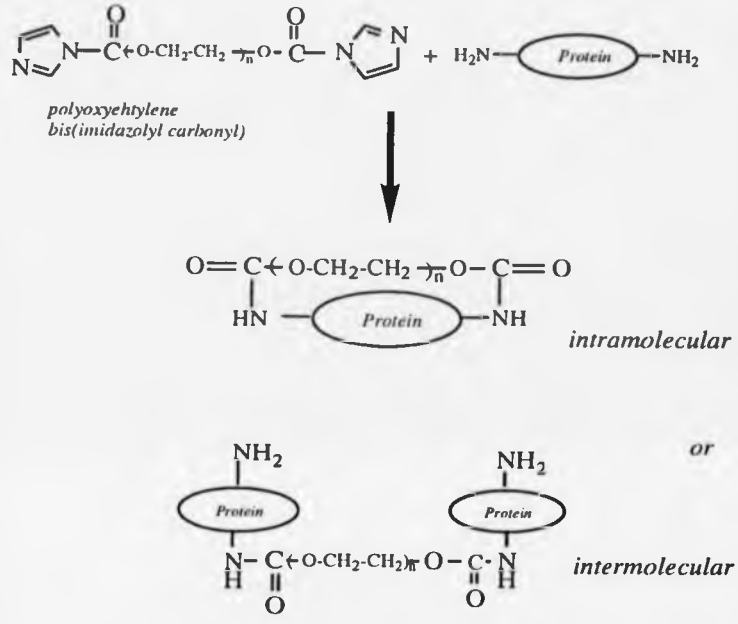
Scheme 6-1 Proposed mechanism for the reaction of diamines with carboxyl groups of a protein.

6.2.2 Polyoxyethylene bis(imidazolyl carbonyl)

The reaction of 1,1-carbonyldiimidazole with hydroxyl groups on agarose to produce a derivative that reacts with nucleophiles was initially reported by Bethell *et al.* in 1979 (Bethell *et al.*, 1979). Later, in 1983 Pizzo and his coworkers (Beauchamp *et al.*, 1983) were the first to utilise this simplified technique for the synthesis of polyethylene glycol (PEG) derivatives (PEG activation) for the study of the effects of PEG-protein adducts on the function, receptor recognition and mechanisms of plasma clearance enzymes such as superoxide dismutase, lactoferrin and α_2-macroglobulin. Since then, a series of PEG derivatives activated by 1,1-carbonyldiimidazole has become commercially available and are constantly used for the study of protein chemistry. The bifunctional reagent polyoxyethylene bis(imidazolyl carbonyl) can react with free amine groups in proteins under appropriate conditions, to form a chemical bridge involving either intra- or intermolecular crosslinking (Scheme 6-2).

In our studies we used commercially available polyoxyethylene bis(imidazolyl carbonyl), molecular weight 3,350, containing approximately (n =) 71 units of -(O-CH_2-CH_2)- between bifunctional groups. The degree of modification is defined as a percentage of the ratio of modified to free amine groups in the protein.

The results showed that with polyoxyethylene bis(imidazolyl carbonyl) (12 mM) to modify the hydroxylase (40 μM, 20 ml) the degree of modification was approximately 22% and the modified protein retained the activity in the H_2O_2-driven assay but no activity could be detected in the native sMMO system with propene as substrate. Analysis of the thermostability by comparison with the non-crosslinked protein using hydrogen peroxide to provide oxygen and electrons showed that the crosslinked hydroxylase thermostability increased significantly (Figure 6-2).



Scheme 6-2. Proposed mechanism for the reaction of polyoxyethylene bis(imidazolyl carbonyl) with the amine groups of a protein.

It was possible that intermolecular crosslinking of the protein molecules might also occur in these experiments, so a minimal concentration (< 2.5 mg/ml, ~10 μM) of proteolysed hydroxylase was reacted with polyoxyethylene bis(imidazolyl carbonyl) to investigate this possibility. The experiments showed that using a five-fold excess of polyoxyethylene bis(imidazolyl carbonyl), the enzyme preparation had the same thermostability and relative activity as that in reaction mixtures containing 40 μM of

proteolysed protein. This was indicative of the fact that under our conditions the interaction of the protein with polyoxyethylene bis(imidazolyl carbonyl) was mainly an intramolecular process.

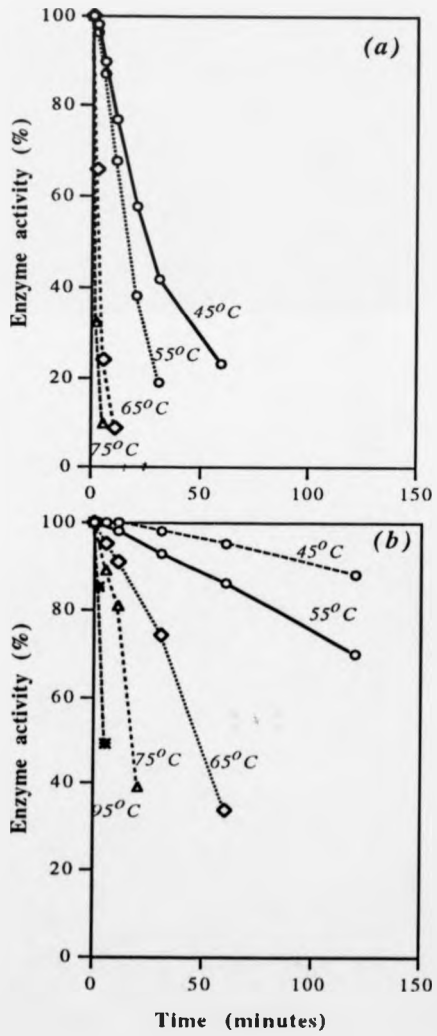


Figure 6-2
Thermostability of proteolysed hydroxylase in the oxidation of propene when using hydrogen peroxide to provide oxygen and electrons.
(a) non-crosslinked.
(b) crosslinked with polyoxyethylene bis(imidazolyl carbonyl).

Note: The hydroxylase was proteolysed by trypsin (2 μg/ mg hydroxylase) at room temperature for 10 minutes.

Analysis the enzyme activity was performed at pH 7.0 (25 mM MOPS), 45°C with propene as substrate, the hydroxylase, 24μM, H_2O_2, 100 mM.

6.3 Effect of different PEG-derivatives

A variety of activated polyethylene glycol (PEG) derivatives have been devised for coupling to protein via amine groups, primarily to lysine ε-amino groups. For example, PEG-derivatives activated with cyanuric chloride (Abuchowski *et al.*, 1977; Jackson *et al.*, 1987; Ljunger *et al.*, 1993), succinimidyl succinate (Abuchowski *et al.*, 1984), 1,1-carbonyldiimidazole (Beauchamp *et al.*, 1983), phenylchloroformates (Veronese *et al.*, 1985), maleic anhydride (Garman and Kalindjian, 1987) and chromophorically labelled 4-fluoro-3-nitrobenzoic acid have been employed to prepare PEG-protein conjugates. Conjugation to the periphery of proteins with PEG-derivatives has proved to be a valuable way of inhibiting interactions with other macromolecules in immunology studies, or of making the protein soluble in organic solvents for chemical analysis.

For our purpose, the use of organic solvents as reaction media could greatly widen the scope of sMMO catalysed conversions. Hydrophobic compounds react much more efficiently in organic than in aqueous media and the use of hydrolytic enzymes to catalyse interesterification reactions, condensation reactions etc. has become possible (Ljunger *et al.* 1993). In most published examples the enzymes have been used in organic media in an insoluble form, either as an enzyme powder or adsorbed on a support material. An alternative method is to modify the enzyme covalently with PEG-derivatives to make it soluble in organic solvents. The PEG-enzyme conjugate can be removed after complete reaction and the product can be isolated. It has been shown that several enzymes can be made soluble in organic media by PEG modification (Matsushima *et al.*, 1984; Takahashi *et al.*, 1984c; 1985a; 1985b; Gaertner and Puigserver, 1988; Wirth *et al.*, 1991; Ljunger *et al.*, 1993).

Methoxypolyethylene glycol is linear, uncharged and available in various sizes. Functionalized PEG is available from commercial sources or can be prepared in the laboratory and the activated PEG easily react with amine residues in proteins. One such derivative is methoxypolyethylene glycol-activated by cyanuric chloride which has

been successfully used for the modification of several enzymes (Abuchowski and Davis, 1979; Takahashi *et al.*, 1984a; Lee *et al.*, 1988; Pina *et al.*, 1989; Gaertner and Puigserver, 1989; souppe *et al.*, 1989; Ljunger *et al.*, 1993). Recently, Wirth *et al.* (1991) using another method for coupling of PEG to protein (with ethanal ω-methoxypolyehtylene glycol) succeeded in modifying horseradish peroxidase so that it was soluble and active in toluene. In this thesis, methoxypolyethylene glycol-activated by cyanuric chloride (PEG-cyanuric chloride, MW 5,000) and ethanal ω-methoxypolyethylene glycol (PEG-aldehyde, MW 5,000) were used to modify the native hydroxylase protein. The activity and the solubility of the modified protein were investigated.

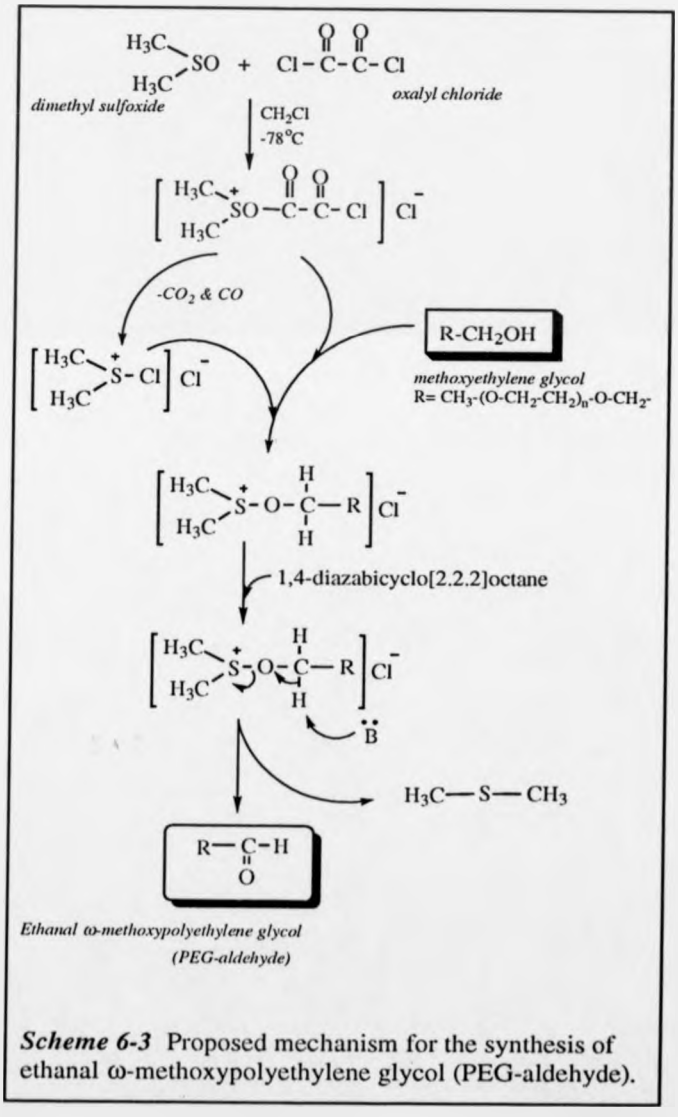
6.3.1 Ethanal ω-methoxypolyethylene glycol (PEG-aldehyde)

A number of methods have been developed for the coupling of PEG to proteins (Veronese *et al.*, 1985; Harris, 1985; Garman and Kalindjian, 1987; Yoshimoto *et al.*, 1987; Pina *et al.* 1989; Souppe *et al.*, 1988; 1989; Urrutigoity and Souppe, 1989; Wirth *et al.*, 1991). In the present investigation, we used commercially available polyethylene glycol monomethyl ethers and the method of Wirth *et al.* (1991) to prepare the corresponding aldehydes, ethanal ω-methoxypolyethylene glycols, and to link them to accessible amine groups in the hydroxylase by reductive amination.

Synthesis

The principle for synthesis of ethanal ω-methoxypolyethylene glycol (PEG-aldehyde) was based on the Moffatt-Swern methods (Mancuso *et al.*, 1978; 1979) of the oxidation of hydroxyl groups (primary or secondary alcohols) to carbonyl groups. The preliminary step, as shown in Scheme 6-3, is activation of dimethyl sulfoxide (Me_2SO) by oxalyl chloride at low temperatures in methylene chloride yielding an unstable intermediate that instantaneously loses CO_2 and CO. The intermediate then reacts rapidly with methoxypolyethylene glycol (MW. 5000) to form the alkoxysulfonium salt that is

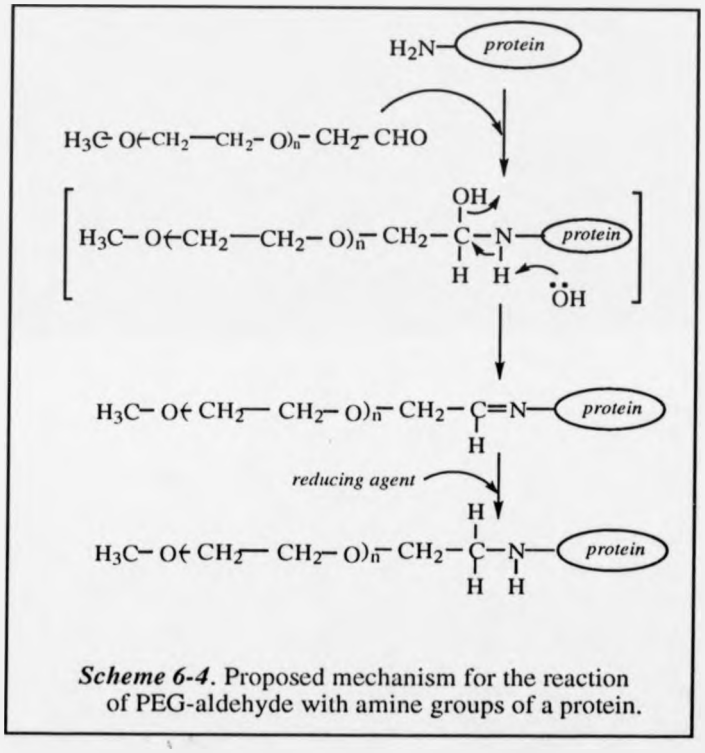
converted to PEG-aldehyde in high yield upon addition of 1,4-diazabicyclo [2.2.2]octane. The amount of aldehyde formed was determined by oxime formation. It was found that the aldehyde content in the PEG-aldehyde thus prepared was approximately 60%, and the aldehydes were used without further purification.



Scheme 6-3 Proposed mechanism for the synthesis of ethanal ω-methoxypolyethylene glycol (PEG-aldehyde).

Modification

PEG-aldehyde will react with accessible amino groups on the protein by reductive amination as shown in Scheme 6-4.



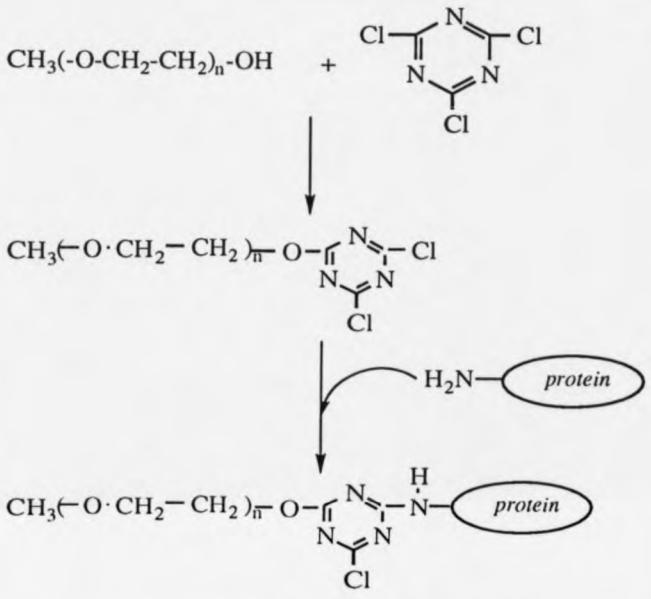
Scheme 6-4. Proposed mechanism for the reaction of PEG-aldehyde with amine groups of a protein.

Initially, the native hydroxylase (30 μM, 10 ml) was incubated with the PEG-aldehydes (20 mM) at pH 8.0, at room teperature for 3 hours, followed then the reductive alkylation of the hydroxylase which could be performed in the presence of sodium borohydride (Means and Feeney, 1968) or sodium cyanoborohydride (Borch ***et al.***, 1971) depending on the molecular weight of PEG used (Wirth, 1991). In our experiments, sodium cyanoborohydride was used since it is a more selective reducing

agent and at pH 8.0, it reduced Schiff bases without affecting the aldehyde groups (Borch *et al.*, 1971). After addition of sodium cyanoborohydride, the incubation was continued for various time intervals (up to 24 hours), which could be effect the degree of the modification. The modified hydroxylase was purified via gel-filtration chromatography, and its solubility in organic solvents and activity in both aqueous and non-aqueous media were determined. The UV-vis spectrum of the hydroxylase modified with PEG-aldehyde showed no change when compared to that of the native form.

6.3.2 Methoxypolyethylene glycol-activated with cyanuric chloride

A number of proteins and enzymes have been modified by the covalent attachment of PEG-derivatives activated with cyanuric chloride. In cyanuric chloride, the chlorine atoms can be mutually activating, so substitution by nucleophilic reagents is the main reaction (Abuchowski *et al.*, 1977). Johnson *et al.* (1974) reported that cyanuric chloride reacted with amine, imine and hydroxyl groups to form stable bonds at approximately 4°C, 25°C and 80°C, respectively, in aqueous solution at pH 9.2. Therefore the modification of the hydroxylase with PEG-cyanuric chloride was carried out initially at 4°C and then at room temperature. Scheme 6-5 outlines the activation of methoxypolyethylene glycol with cyanuric chloride and its attachment to the protein through amine groups. In the present investigation, the commercially available PEG-cyanuric chloride (MW. 5000) (8 mM) was used to modify the hydroxylase protein (24 μM, 10 ml) and the incubation was carried out with various time intervals (up to 5 hours). The modified protein was purified via gel-filtration chromatography, followed by the determination of its solubility in organic solvents and its activity in both aqueous and non-aqueous media. There was no change in the UV-vis spectrum of the hydroxylase modified with PEG-cyanuric chloride compared to that the native protein.



Scheme 6-5. Outlines for the activation of methoxypolyethylene glycol with cyanuric chloride and its attachment to amine groups of a protein.

6.3.3 Modification degree and the solubility

The degree of modification of the hydroxylase protein with both PEG-aldehyde and PEG-cyanuric chloride was determined by the TNBS method of Fields (1972). It was found that the degree of modification was dependent on the incubation time of the polymers with the protein. With an increase of the incubation time the degree of modification could be increased under our conditions.

The degree of modification is defined as a percentage of the ratio of modified to free amine groups in the protein. From the amino acid sequence of the hydroxylase,

there are 120 Lys residues in the protein ($\alpha 2\beta 2\gamma 2$). The TNBS determination showed that (2 μmole of the native protein in the assay, the average OD_{420nm} was 1.8315, the calculation with $\varepsilon = 19{,}200\ M^{-1}cm^{-1}$) there were 48 free amino acids per molecule of the hydroxylase (include the terminal NH_2's if they were not blocked).

The native hydroxylase of sMMO did not show any detectable solubility in organic solvents. After modification, solubility in several organic solvents was observed. As shown in Figure 6-3, the solubility of the modified protein in dichloromethane was increased with increasing degree of modification with both types of PEG- derivatives. Clearly, the attached polymers help the protein to dissolve in non-aqueous media.

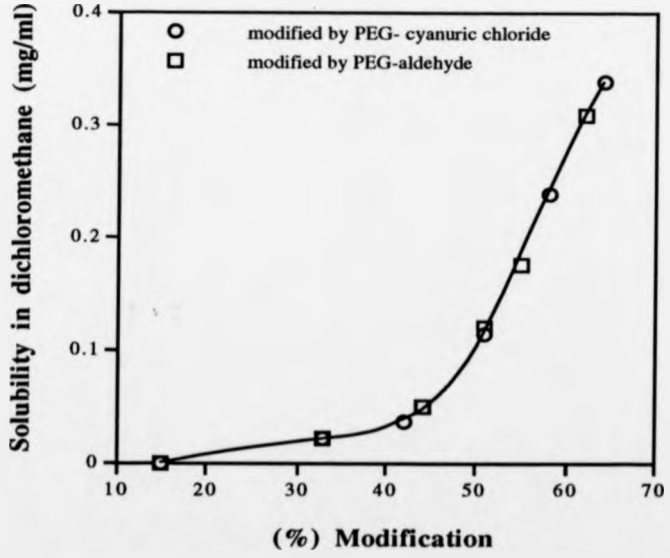


Figure 6-3. Solubility of PEG-modified hydroxylase in dichloromethane as a function of the degree of modification

A comparison of the Fields (1972) and Habeeb (1966) TNBS methods for the assay of the degree of modification of the hydroxylase was also investigated. In Habeeb's method the determination used 0.3 mg/ml TNBS at pH 8.5 and the wavelength used was 335 nm. In Fields' method ~10 mg/ml TNBS was used, the reaction was carried out at a much higher pH value and the wavelength was 420 nm. Recently, Habeeb's method was used by Wirth *et al.* (1991) and they suggested that a long reaction time (4-5 hours) with TNBS was necessary for complete titration.

Our experiments using the two different methods showed that Fields' method was quick and reproducibility was good whereas Habbeb's method not only required a long time (up to 6-8 hours) for TNBS reaction but the data were also not reproducible. Probably, for steric reasons TNBS reacts with amines only partially at pH 8 (Okuyama and Satake, 1960) so Habeeb's method required a long reaction time. In Fields method, measurements was at 420 nm which is removed from the region of absorption by TNBS, thus permitting a high concentration of reagents to be used which shortens the time required for the reaction. Several experiments showed, however, the assay conditions for the modified hydroxylase using Fields' method, required the exclusion of light and at least 15-20 minutes incubation. It appeared that in the light the rate of TNBS reaction with amines was slow and with too short an incubation time (5 minutes) the assay was incomplete.

6.3.4 Activity of the PEG-modified protein

The determination of the catalytic activity of the PEG-modified protein was carried out in both aqueous and nonaqueous media with propene as substrate. For the modified protein in the aqueous assay there was no activity in the complete sMMO system even though the degree of the modification was very low. However, there was activity in the H_2O_2-driven system in aqueous solution at almost same level as that of the native hydroxylase. For PEG-cyanuric chloride-modified protein the activity observed in the aqueous the H_2O_2-driven assay system was independent of the degree of modification.

Both types of modified hydroxylase, with PEG-aldehyde or PEG-cyanuric chloride-protein, had similar catalytic properties, but the catalytic capacity of the PEG-cyanuric chloride-protein was greater than that of PEG-aldehyde-protein. The oxidation of other substrates, such as benzene and methane, was detected using the PEG-cyanuric chloride-modified hydroxylase in the hydrogen peroxide-driven assay system and the amount of product observed was similar to that using unmodified protein.

The estimation of the activity in a non-aqueous medium was dependent on the solubility of the modified protein in the solvent, which in turn was dependent on the degree of modification. When the degree of the modification was lower than 30%, the solubility of the protein in an organic solvent (CH_2Cl_2) was below 0.1 mg/ml and very little activity was detected. An increase in the degree the modification resulted in an enhancement of the solubility of the modified protein in organic solvents and activity was detected. Analysis of the activity of the PEG-aldehyde and the PEG-cyanuric chloride modified proteins, when the degree of modification was 61% and 64% respectively, showed that the modified proteins was able to function in several organic solvents with H_2O_2 as electron and oxygen donor (Table 6-1). The protein modified by both PEG-cyanuric chloride and PEG-aldehyde had almost the same solubility but different activities.

Comparatively, the more successful modification of the hydroxylase, for solubility and function in organic solvents, was by PEG-cyanuric chloride. Soluble MMO catalyses a wide variety of hydrocarbon oxidations and several organic solvents are known to be substrates of the enzyme. The potential oxidation of one solvent (benzene) was investigated. When propene was omitted from the reaction mixture (benzene as solvent), no oxidation product of benzene (to phenol) was observed when hydrogen peroxide was added to a solution of the modified protein. The oxidation of methane catalysed by the PEG-cyanuric chloride-modified protein in dichloromethane was investigated. We assumed that this would be a favourable reaction since methane would be more soluble in non-aqueous than in aqueous solution. Unexpectedly, the oxidation

of methane was not observed when the assay mixture (30 ml) contained 10 mg of PEG-cyanuric chloride-modified hydroxylase and 100 mM hydrogen peroxide.

Table 6-1. Solubility and activity of PEG-modified hydroxylase of sMMO in organic solvents (H_2O_2-driven assay, propene as substrate)

Solvents	PEG-cyanuric chloride		PEG-aldehyde	
	solubility (mg/ml)	activity* (nmol/min)	solubility (mg/ml)	activity* (nmol/min)
Dichloromethane	0.34	43	0.30	19
Toluene	0.15	30	0.14	9
Benzene	0.15	31	0.14	10
Benzene(*no propene*)	0.15	0	0.14	0
Hexane	0.16	33	0.16	14
Ethanol	0.23	35	0.22	16
Ethyl acetate	<0.07	0	<0.07	0

*Analysis was performed with 3 mg of the modified protein and 100 mM hydrogen peroxide, at 45°C for 15 minutes.

6.3.5 Effect of lyophilization

The proteins modified with the PEG-derivatives were lyophilized before analysis of their solubility and activity in organic solvents. Because of concerns that lyophilization of the enzyme might cause a reduction in catalytic activity, the following investigation was undertaken.

Native (unmodified) hydroxylase, in either distilled water or 25 mM MOPS buffer, pH 7.0, was lyophilized. Assay of the activities of the lyophilised protein were carried out in both the sMMO complex and the H_2O_2-driven systems with propene as substrate. As shown in Table 6-2, when the the protein was in buffer solution, the

activity was not affected by the process of lyophilization. However, when the protein was in water, about half the enzyme activity was lost during the process of lyophilization. The presence of salt seems to prevent protein denaturation during freeze drying. So, the modified hydroxylase (PEG-protein) was lyophilized in the buffer solution.

Table 6-2. Effect of lyophilization of native hydroxylase in different solution

	sMMO system* (nmol/min/mg)			Hydroxylase/H_2O_2 system** (nmol/min)			[Fe] *** (μM/μM protein)		
No. experiments	*(1)*	*(2)*	*(3)*	*(1)*	*(2)*	*(3)*	*(1)*	*(2)*	*(3)*
nonlyophilized hydroxylase									
in buffer solution	169,	176,	158	64,	68,	60	1.98,	2.1,	1.95
in water	168,	174,	157	64,	68,	59	1.98,	2.05,	1.94
Lyophilized hydroxylase									
in buffer solution	165,	170,	153	62,	66,	60	1.98,	2.05,	1.90
in water	90,	104,	86	35,	41,	30	1.97,	2.0,	1.90
Retained activity (%)									
in buffer solution	98%,	97%,	97%	97%,	97%,	100%			
in water	54%,	59%,	55%	55%,	60%,	51%			

* 8μM of the hydroxylase, protein B and reductase, reaction was performed at 45°C for 3 mins.

**24μM of the hydroxylase and 100 mM hydrogen peroxide, reaction was performed at 45°C for 15 mins.

*** Fe assay with bathophenanthroline.

After lyophilization, the protein was dissolved in the buffer (25 mM MOPS, pH 7.0) then assay its activity, the protein concentration was measured by BioRad in resolvent.

6.4 Discussion

Stability effected by chemical crosslinking

The hydroxylase crosslinked with polyoxyethylene bis(imidazolyl carbonyl) resulted in approximately a 22% modification of the accessible amine groups in the

protein and greatly enhanced the thermostability of the protein. The observed increase in thermostability is thought to be due to intramolecular "brackets" and cannot be accounted for as a simple chemical modification of the protein. This is inferred from a report that monoamine modification of α-chymotrypsin (which did not involve crosslinking) hardly affected its thermostability (Torchilin *et al.*, 1978). Thus, it is apparent that chemical crosslinking via intramolecular bonds strengthens the compact structure of the protein molecule so that denaturation of the hydroxylase at high temperature is slow. It has been demonstrated that intramolecular disulfide bonds, for example, contribute significant stabilisation energy to proteins in which they occur. Doig and Williams have argued that disulfide bonds destabilize folded structures entropically, but stabilise them enthalpically to a greater extent, resulting in an overall stabilisation of the molecule (Doig and Wiliams, 1991).

The present results have shown that different length of diamines did not stabilise the proteolysed hydroxylase and caused denaturation indicating that activation of carboxyl groups and then crosslinking them was not the reaction of choice for our protein, although this method was successful in the modification of α-chymotrypsin (Torchilin *et al.*, 1978). Clearly, different crosslinking reagents vary significantly in their ability to confer the hydroxylase stability. A major factor in increasing the thermostability of the hydroxylase was indeed to be the length of the intramolecular crosslinks formed.

Solubility and activity in organic solvents effected by chemical modification

Water is a poor solvent for hydrophobic substrates. However, biocatalytic processes are unlike normal chemical processes and conventional biocatalysis like sMMO catalysed reactions, occur in aqueous solutions. This is mainly because nature intended enzymes to be catalytically active in water and organic solvents serve to destroy the catalytic ability of enzymes. If it were possible to dissolve the enzyme and retain its function in organic solvents, the benefit would be the increased solubility of methane

and other hydrophobic sMMO substrates compared to that in water. This could lead to better catalysis by the enzyme, particularly since the overall rate may be limited by substrate accessibility to the enzyme. Also, the oxidation of methane catalysed by the native sMMO and H_2O_2-driven systems involves production of water (eq 1 and 2) and the reaction is normally undertaken in aqueous solution where $[H_2O] = 55$ M.

sMMO system

$$CH_4 + NADH + H^+ + O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CH_3OH + NAD^+ + H_2O \qquad (1)$$

Hydroxylase/H_2O_2 system

$$CH_4 + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CH_3OH + H_2O \qquad (2)$$

It is therefore possible that if the reactions were run in an organic solvent, the reverse reaction will be less favoured ($k_2 << k_1$) and the oxidation of methane to methanol would be even more thermodynamically and kinetically advantageous.

The present results demonstrated that we have covalently modified the hydroxylase of sMMO with PEG-cyanuric chloride and PEG-aldehyde and obtained protein preparations which are soluble and active in organic solvents. The enzymatic oxidation of propene was observed in several organic solvents as shown in Table 6-1. Analysis of the catalytic capacity showed that PEG-cyanuric chloride was a better reagent than PEG-aldehyde for the modification of amine groups in the hydroxylase. The disadvantage of PEG-aldehyde is that the reaction requires a much longer time to reach a high degree of modification and this length of time at room temperature may cause a loss in hydroxylase activity. PEG-cyanuric chloride gave a higher production of modification than did PEG-aldehyde when the both reactions were run up to 5 hours (Figure 6-4).

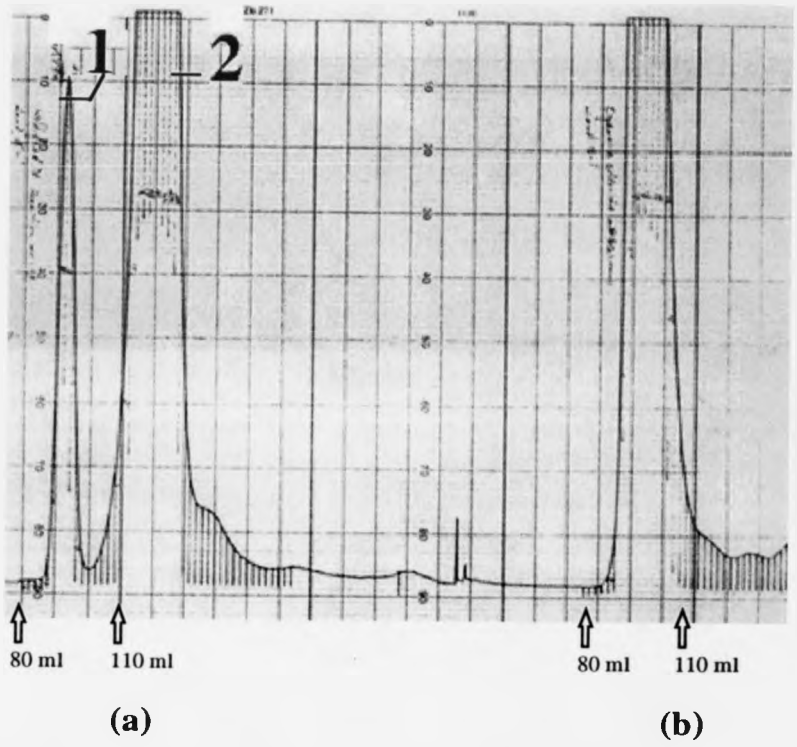


Figure 6-4 Elution protein from Superdex G200 on FPLC of the modified hydroxylase with (a) PEG-aldehyde and (b) PEG-cyanuric chloride

(a) shows separation of the modified(peak 1) from unmodified hydroxylase(peak 2),

(b) shows that the production was completely modifiedwhen using PEG-cyanuric chloride.

Note:4 ml protein was loaded on the column at a concentration of 25 mg/ml and eluted with 25 mM MOPS, pH 7.0, containing 5 mM NaCl. The elution rate was 3 ml/min and the chart speed was 0.05 cm/ml. 4 ml fractions were collected and the scan range was 0 - 2 absorbance units.

This could be due to the reaction rates of the functional groups of the polymers with the amine groups of the protein.

Solvent effect

PEG-modified hydroxylase showed activity in several organic solvents when H_2O_2 was used to provide oxygen and electron equivalents. The oxidation of propene was observed in several organic solvents, but the oxidation of benzene, which is a known substrate of sMMO, was not detected when it was used as the sole substrate and solvent. It is therefore possible that benzene *per se* had an effect on the properties of PEG-modified protein by interaction with amine residues which otherwise would not be affected by this solvent resulting in an inability to catalyse the oxidation of benzene. For example, it is known that the introduction of a PEG chain leads to a decrease in the *p*Ka of some lysine residues in certain proteins (Bruehlman and Verhoek, 1948; Gutbezahl and Grunwald, 1953). At present the differences in the physical properties between the PEG-modified and the native hydroxylase are unclear, so the effect of the medium remains unexplained.

Hydrophobic effect

The PEG-modified hydroxylase showed no ability to catalyse the oxidation of methane in organic solvents. This could be due to a number of reasons. As clearly established earlier (Green and Dalton, 1989; Dalton *et al.*, 1990; Deighton *et al.*, 1991; Wilkins *et al.*, 1992; Dalton *et al.*, 1993a) the oxidation of methane to methanol by native sMMO involves H atom abstraction from the substrate. The energy involved in the activation of a C-H bond in methane (104 kcal/mol) may be realised from the binding energy generated when CH_4 moves from an aqueous environment into the hydrophobic core of the hydroxylase. Methane dissolved in water causes a local rearrangement in the water molecules which gives rise to an ordered shell around the nonpolar molecule. A somewhat simplified explanation is that putting a hydrocarbon molecule into water is

equivalent to making a hole in the water structure. The increase in free energy is dependent on the surface area of the hole. The tendency towards the lowest possible free energy will give a tendency towards reducing the surface area. Therefore hydrocarbon molecules greatly prefer nonpolar environments. It may be that methane can be driven into the hydrophobic region (binding site) of the protein by the regaining of entropy by the ordered water molecules. The gain in energy from the water molecules in this rearrangement, coupled with the displacement of water from the active site (Gaertner and Puigserver, 1988), may contribute energy sufficient to lower the activation energy of methane and permit hydrogen abstraction by the electrophilic Fe-bound oxygen species (Green and Dalton, 1989). When the enzyme is placed in an organic environment, there can be no entropic gain in the alignment of methane to the active site (although some contribution to the binding energy may arise from the displacement of water from the active site) and so there is insufficient energy available to lower the activation energy of methane to a level whereby H atom abstraction can occur (Jiang and Dalton, 1993). The epoxidation of propene, however, may occur by a different mechanism (Wilkins *et al.*, 1992; Jiang *et al.*, 1993) whereby a much lower energy barrier must be overcome (80 kcal/mol for the π bond in propene, cf. 104 kcal/mol for a C-H bond in methane). It is therefore possible that the rearrangement required for binding energy through hydrophobic interactions is obviated in the epoxidation of propene and may explain why this substrate is oxidised in organic solvents and methane is not.

It is also possible that the low concentration of the protein in organic solvents might be insufficient to generate a detectable level of methanol for assay, particularly since it has been shown (Jiang *et al.*, 1993) that relatively high concentrations of enzyme were necessary for the H_2O_2-driven system to catalyse methane oxidation by native hydroxylase.

Polymer and Water effect

Recently, it has been reported that α-chymotrypsin was successfully modified by PEG-cyanuric chloride of molecular weight of 5000 and the modified enzyme could function in organic solvents (Ljunger *et al.*, 1993). In addition, horseradish peroxidase modified by PEG-aldehyde of molecular weight of 5000 was also soluble and functional in toluene, however the lower molecule weight polymers, such as 350 or 1900, were unable render the enzyme soluble in organic solvents (Wirth *et al.*, 1991). In our investigation, the hydroxylase of sMMO modified by both types of PEG-derivative (MW 5000) were also soluble and functional in organic solvents. An interesting question is why enzymes modified by the high molecular weight PEG-derivatives are soluble and functional in organic solvents?

It is found that the relevant high molecular weight PEG-derivatives, such as methoxypolyethylene glycol (MW 5000, $C_{227}H_{454}O_{114}$), are soluble in both aqueous and nonaqueous media since the hydrophobic portions (hydrocarbon chain), ~ 65% of the molecule (MW 5000), can make it soluble in nonpolar solvents and the ~35% polar portions make it soluble in water. Once the polymers are bound to the protein in the modified form, the versatility of the side chain structure of the amino acid in the protein appears to be somewhat different from the native form, particularly the ionic or nucleophilic groups. The unmodified ionic or nucleophilic groups on the surface of the protein and the oxygen atoms in the PEG-polymers will have some water molecules associated with them and the modified protein in a nonaqueous medium would be wearing a water "coat" which covers the protein to prevent contact with the organic solvent, i.e., there may be a water shell surrounding the protein molecule and the polymers may wrap around the protein and render it soluble in organic solvents as shown in Figure 6-5. It is possible that the low molecular weight (short hydrocarbon chain) polymers don't have the capacity to cover the protein molecule surface and a protein modified by these low molecular weight polymers could be insoluble in a nonaqueous medium.

A number of studies (Takahashi *et al.*, 1984; Gaertner and Puigserver, 1988; 1989; Wirth *et al.*, 1991) have showed that the activities of PEG-modified enzymes in organic solvents depend on the concentration of water in the reaction medium. Most investigations indicated that a certain amount of water in the organic solvent is necessary for enzyme activity. In our experiments, the minimum water content in organic reaction media was approximately 0.8%. The amount of water required for activity of our enzyme must be further investigated.

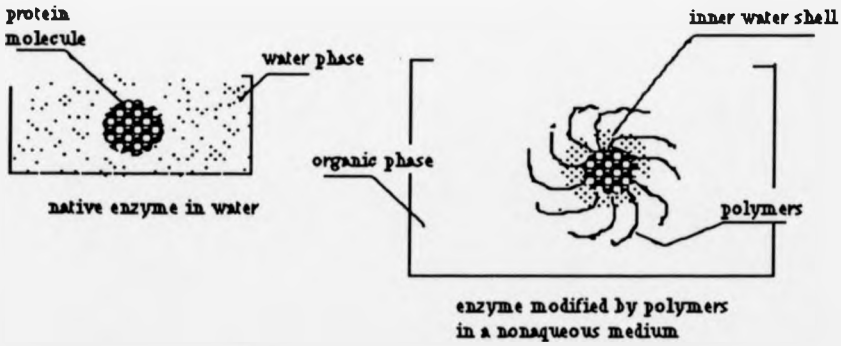


Figure 6-5 Illustration of the modified protein dissolving in a nonaqueous medium.

CHAPTER 7

GENERAL DISCUSSION AND PERSPECTIVES

7.1 The sMMO complex and the H_2O_2-driven systems

It has been demonstrated that soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath) is a three component enzyme (Colby and Dalton, 1978) that catalyses the NAD(P)H_2- and O_2-dependent oxidation of methane to methanol. The hydroxylase is the site of methane activation, the reductase is responsible for electron transfer from NAD(P)H_2 to the hydroxylase and protein B functions as a regulator. The enzyme is quite catholic in nature and is able to insert an atom of oxygen from dioxygen to over 250 different organic compounds. Recently, it has been shown that the enzyme from *Methylosium trichosporium* OB3b (Andersson *et al.*, 1991) and *Methylococcus capsulatus* (Bath) (Jiang *et al.*, 1993) can function via a peroxide shunt in which hydrogen peroxide is used to in replace NAD(P)H_2, O_2 and two of the proteins within the complex. So, in its simplest form, the hydroxylase can oxidise its hydrocarbon substrates using just hydrogen peroxide.

Based on the studies of the substrate specificity (Green and Dalton, 1989b), kinetic (Green and Dalton, 1986, 1989a), and the physicochemical analysis of the active site of the hydroxylase (Woodland and Dalton, 1984, Woodland *et al.*, 1986), and the spin trapping experiments (Deighton *et al.*, 1991; Wilkins *et al.*, 1992), it has been demonstrated that there is a in the similarity reaction mechanism with cytochrome P450 hydroxylase system even though the active sites of the two enzymes are quite

different. That is, both show heterolytic cleavage the O-O bond of the peroxy to form a ferryl species and the resulting ferryl species is highly electrophilic and capable of hydrogen-atom abstraction from substrate as shown in Figure 7-1. However, in the H_2O_2-driven system homolytic cleavage of the peroxy species would be operative, in which the resulting hydroxyl radical would act as a weaker electrophile but may also be able abstract hydrogen from methane. Furthermore, results have shown that oxidation of some substrates may result in a different method of insertion of O atom into C-H bond (Dalton *et al.*, 1993a). For example, oxidation of CO and pyridine into CO_2 and pyridine-N-oxide respectively indicated that there was direct oxygen addition to these substrates. Therefore, the mechanism of action may well be substrate and oxygen donor dependent (Dalton *et al.*, 1993b).

Hydrogen peroxide activation of the hydroxylase in catalysing the hydrocarbon oxidation has been investigated. However, there are still some questions to be answered, i.e., evidence for hydroxyl radical-based Fenton reaction involved in the hydrogen peroxide-driven reaction system has to be proved. Our understanding of this process is far from being complete. The nature of the species generated by chemical interaction between the iron of the active site in the enzyme and the oxidant-hydrogen peroxide is unclear. Perhaps the most intriguing aspects of this reaction is how the activation of the hydrocarbon takes place. The fact that hydrogen peroxide is capable of activation of the hydroxylase in catalysing the hydrocarbon oxidations provides some information to compare with the features of chemical models in mimicking sMMO reactions and our own approach to the study of the O atom source by the labelled oxygen either in air phase or in liquid phase reveals that the enzyme in utilising H_2O_2 as O donor is directly derived from hydrogen peroxide and is unlike Gif chemistry. Therefore, further studies both in chemical model and the enzyme catalysed reactions will be of great benefit to understanding the functionalisation of C-H bond by Nature.

Recent report from Lipscomb's group (Lee *et al.*, 1993) suggested that the enzyme in catalysing hydrocarbon oxidations involved transient intermediates of the sMMO catalytic cycle via direct detection using stopped-flow, freeze quench and chemical quench techniques. This would further support the sMMO reaction mechanism proposed by Green and Dalton (1989) and Fox *et al.* (1990).

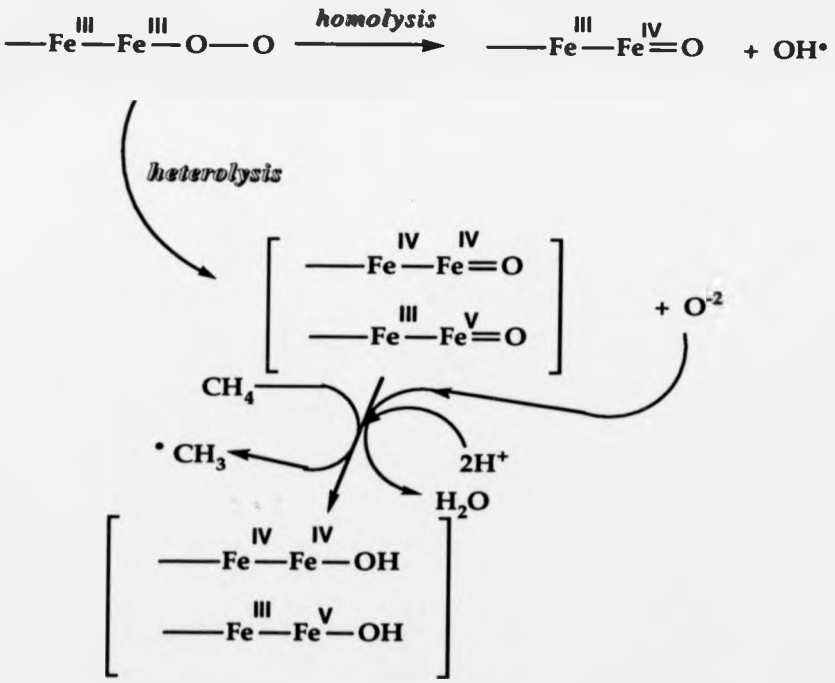


Figure 7-1 Cleavage of iron-peroxy bond in sMMO cycle.

Recently, the studies of the crystal structure of the hydroxylase from *Methylococcus capsulatus* (Bath) have indicated that the two iron atoms are bridged by exogenous hydroxide and acetate ligands and are further coordinated by four glutamate residues, two histidine residues and a water molecule. The binuclear iron centre lies in a hydrophobic active-site cavity for binding methane (Resenzweig *et al.*, 1993). Significantly, this work will be greatly facilitated in construction of a new hydroxylation catalyst based on an understanding of the MMO system. Therefore, a better understanding in both the enzyme catalytic mechanism and the active site would favour the application of the enzyme as a industrial catalyst or aid in the construction of a new catalytic model to achieve the methane conversion by a simple and direct, quick and economic route making more efficient use of natural gas.

7.2 Stabilisation of the enzyme

One of the main priorities of modern biotechnology is obtaining stable or stabilised biocatalysts. The notion of utilising stable enzymes or microorganisms in biotechnological applications has held special appeal (May, 1992). Organic chemists are now employing highly purified enzymes for chiral synthesis of biochemicals, often in partial non-aqueous solvents: stereo- and regio-spcificy of enzymes is of particular value in these syntheses (Cheetham, 1985). For soluble methane monooxygenase the stabilisation of the enzyme, either in a thermostable form or stable in organic solvents, was expected to favour the construction of an industrial-type catalyst. Our effort revealed that the thermostability of the enzyme could be enhanced via chemical crosslinking and the enzyme also could be made soluble in non-aqueous media by chemical modification. Thus, the subsequent stage may be an enhancement of the stability of the enzyme in extreme of pH or pressure. However, empirical rules for selecting chemical reagents for crosslinking are not well defined (Mozhaev, 1993).

These rules may be improved by using computer-aided molecular graphics in the case of proteins with known three-dimensional structures.

A critical aspect of enzyme activity in organic solvents is the role of "bound water"-i.e., whether or not a threshold level of hydration is essential to enzyme activity and the effect of any such "essential water" on turnover rate and specificity (May, 1992). Further study has to be focused on this important issue, which bears directly on the molecular basis for solvent-dependent changes in the enzyme reactivity characteristics.

The direct conversion of methane to methanol by soluble methane monooxygenase at ambient temperature and pressure is significantly advantageous to be a catalyst applying in methane conversion. Therefore, any efforts in increase of the stability of the enzyme will be useful and helpful for this purpose.

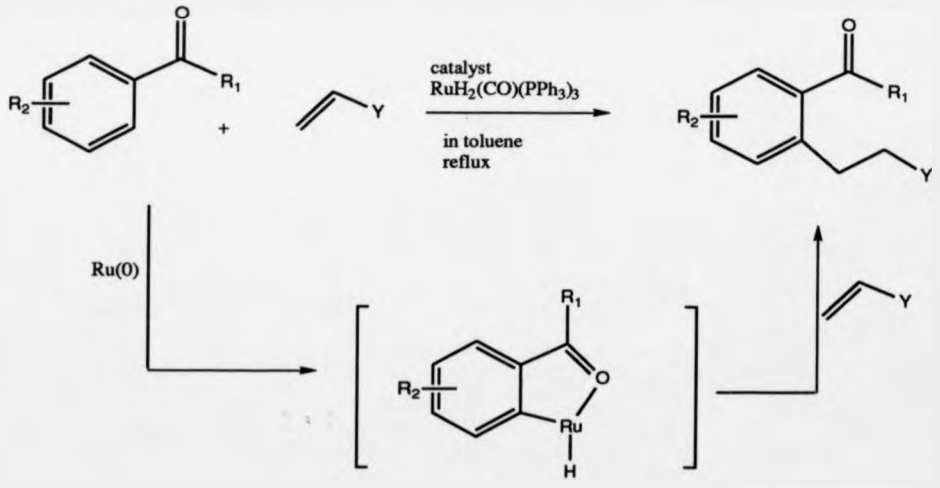
7.3 A perspective

The selective cleavage of carbon-hydrogen bonds in organic compounds is a critical step in many organic syntheses, and is particularly important in the conversion of hydrocarbons to useful organic compounds (Murai *et al.*, 1993). The soluble methane monooxygenase catalyses a wide variety of hydrocarbon oxidations. The catholic catalytic ability is beyond comparison to any chemical catalysts.

The possibility of formation of C-C bond after the direct cleavage of a C-H bond, the initial idea for studying this project, by the sMMO would still be a interesting subject. Recent report by Murai *et al.* (1993) showed a very interesting result that an organometallic ruthenium complex can cleave C-H bonds in a variety of aromatic systems, leading to addition to alkenes by C-C bond formation. The possible mechanisms (Murai *et al.*, 1993) is that the reaction may begin with the coordination of the carbonyl group to the metal. This chelation assistance results in easier, highly site-selective C-H bond cleavage to give the cyclometallated intermediate (Figure 7-2).

Insertion of an olefin into the M-H bond thus formed (or into the M-C bond (Cambie *et al.*, 1992)), followed by reductive elimination, gave the observed products. This would reflect on that if the similar functional metal complex could replace the iron atoms in the active site of the enzyme and the altered protein could show its catalytic function, the C-C bond formation would be not impossible.

Furthermore, the ability of hydrogen peroxide in activation of the hydroxylase to catalyse the hydrocarbon oxidation can give a simplified reaction system in which the cofactors (NADH & O_2) and the other components (reductase & protein B) are not required. Therefore, this might benefit to use it as an industrial catalyst.



Firure 7-2. The Ru-catalysed reaction of aromatic ketones with olefins, showing the cyclometallated intermediate. *(from Murai et al., 1993)*

REFERENCES

Abuchowski, A., Theo, V.E., Palczuk, N.C. and Davis, F.F. (1977) "Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol", *J. Biol. Chem.* **252**, 3578-3581.

Abuchowski, A. and Davis, F.F. (1979) "Preparation and properties of polyethylene glycol-trypsin adducts", *Biochim. Biophys. Acta* **578**, 41-46.

Adachi, S. Nagano, S. Ishimori, K., Watanabe, Y. and Morishima, I. (1993) "Roles of proximal ligand in heme proteins: replacement of proximal histidine of human myoglobin with cysteine and tyrosine by site-directed mutagenesis as models for P450, chloroperoxidase and catalase", *Biochemistry*, **32**, 241-252.

Akent'eva, N.F. and Gvozdev, R.I. (1988) "Purification and physicochemical properties of methane monooxygenase from membrane structures of *Methylococcus capsulatus*,", *Biokhimiya* **53**, 91-96.

Alonso, D.O. and Dill, K.A. (1991) "Solvent denaturation and stabilisation of globular proteins", *Biochemistry* **30**, 5974-5985.

Anderson, K.K., Debey, P. and Balny, C. (1979) "Subzero temperature studies of microsomal cytochrome P450: O-dealkylation of 7-ethoxycoumarin coupled to single turnover", *FEBS Lett.* **102**, 117-120.

Andersson, K.K., Froland, W.A., Lee, S-K. and Lipscomb, J.D. (1991) "Dixoygen independent oxygenation of hydrocarbons by methane monooxygenase hydroxylase component", *New J. Chem.* **15**, 411-415.

Andersson, K.K., Elgren, T.E., Que, L., Jr. and Lipscomb, J.D. (1992) "Accessibility to the active site of methane monooxygenase: the first demonstration of exogenous ligand binding to the diiron cluster", *J. Am. Chem. Soc.* **114**, 8711-8713.

Andréasson, L.E., Brändén, R., Malmström, B.G. and Vänngård, T. (1973) "An intermediate in the reaction of reduced laccate with oxygen", *FEBS Lett.* **32**, 187-189.

Aoki, T., Yamao, Y., Yonemasu, E., Kumasaki, Y. and kako, Y. (1993) "Cross-linking of egg white riboflavin-binding protein by calcium phosphate", *Arch. Biochem. Biophys.* **305**, 242-246.

Augusto, O., Beilan, H.S. and Ortiz de Montellano, P.R. (1982) "The catalytic mechanism of cytochrome P450, spin-trapping evidence for one electron substrate oxidation", *J. Biol. Chem.* **257**, 11288-11295.

Babonneau, M-T., Jacquier, R., Lazaro. R. and Viallefont, P. (1989) "Enzymatic peptide synthesis in organic solvent mediated by modified α-chymotrypsin", *Tetrahedron Lett.* **30**, 2787-2790.

Balasubramanian, P.N. and Bruice, T.C. (1986) "Oxygen-transfer involving non-heme iron, the reaction of (EDTA)Fe^{III} with β-chloroperbenzoic acid", *J. Am. Chem. Soc.* **108**, 5495-5503.

Balch, A.L., Chan, Y.W., Cheng, R.S., LaMar, G.N., Latos, B., Grazyaski, L. and Denner, M.W. (1984) "Oxygenation patterns for iron(II) porphyrins peroxo and ferryl ($Fe^{IV}O$) intermadiates detected by ^1H nuclear magnetic resonance spectroscopy during the oxygenation of (tetramesitylporphyrin)iron(II)", *J. Am. Chem. Soc.* **106**, 7779-7785.

Baldwin, J.E. (1990) "The biosynthesis of penicillins and cephalosporins", *Heterocycl. Chem.* **27**, 71-78.

Bargett, K., Darnall, D.W., Klotz, I.M. and Williams, R.J.P. (1969) *Arch. Biochem. Biophys.* **103**, 419.

Barlow, D.J. and Thornton, J.M. (1983) "Ion-pairs in proteins", *J. Mol. Biol.* **168**, 867-885.

Barton, D.H.R. and Doller, D. (1992) "The selective functionalization of saturated hydrocarbons: Gif chemistry", *Acc. Chem. Rev.* **25**, 504-512.

Barton, D.H.R., Bévière, S.D., Chavasiri, W., Csuhai, E., Doller, D. and Liu, W-G. (1992a) "The functionalization of saturated hydrocarbons. part 20. alkyl hydroperoxides: reaction intermediates in the oxidation of saturated hydrocarbons by Gif-type reactions and mechanistic studies on their formation", *J. Am. Chem. Soc.* **114**, 2147-2156.

Barton, D.H.R., Bévière, S.D., Chavasiri, W., Csuhai, E. and Doller, D. (1992b) "The functionalisation of saturated hydrocarbons. part 21. the Fe(III)-catalysed and the Cu(II)-catalysed oxidation of saturated hydrocarbons by hydrogen peroxide: a comparative study", *Tetrahedron* **48**, 2895-2910.

Barton, D.H.R., Beviere, S.D., Chavasiri, W., Doller, D., Liu, W-G. and Reibenspies, J.H. (1992c) "The functionalization of saturated hydrocarbons. part 22. studies on the iron species present in Gif-type systems", *New J. Chem.* **16**, 1019-1029.

Battioni, P., Renaud, J.P., Bartoli, J.F. and Mansuy, D. (1986) "Hydroxylation of alkanes by hydrogenperoxide: an efficient system using manganese porphyrins and imidazole as catalysts", *J. Chem. Soc., Chem. Commun.* 341-343.

Beauchamp, C.O., Gonias, S., Menapace, O.P. and Pizzo, S.V. (1983) "A new procedure for the synthesis of polyethylene glycol-protein adducts; effects on function, receptor recognition, and clearance of superoxide dismutase, lactoferrin, and α_2-macroglobulin", *Anal. Biochem.* **131**, 25-33.

Beavo, J.A., Rogers, N.L., Crofford, O.B., Hardman, J.G., Sutherland, E.W. and Newman, E.V. (1970) "Effect of xanthine derivatives on lipolysis and on adenosine 3',5'-monophosphate phasphodiesterase activity", *Molecular Pharmacology* **6**, 597-603.

Beers, R.F. and Sizer, I.W. (1952) *J. Biol. Chem.* **195**, 133.

Bentsen, J.G., Lippard, S.J., De Witt, J., Hedman, B., Ericson, A., Hodgson, K.O., Green, J. and Dalton, H. (1989) "ESR and x-ray absorption spectroscopy of protein A of soluble methane monooxygenas", Poster presentation at Metals in Biology Gordon Conference, Ventura, CA, Jan., pp22-27.

Berg, A., Ingelman-sundberrg, M. and Gustafsson, J.-Å. (1979) "Purification and characterization of cytochrome $P\text{-}450_{meg}$", *J. Biol. Chem.* **254**, 5264-5271.

Bethell, G.S., Ayers, J.S., Hancock, W.S. and Hearn, M.T.W. (1979) "A novel method of activation of crosslinked agaroses with 1,1'-carbonyldiimidazole which gives a matrix for affinity chromatography devoid of additional charged groups", *J. Biol. Chem.* **254**, 2572-2574.

Beynon, R.J. and Bond, J.S. (1989) *Proteolytic Enzymes, a practical approach* IRL Press.

Bonfils, C., Debey, P. and Maurel, P. (1979) "Highly purified microsomal cytochrome P450: the oxyferro intermediate stablized at low temperature", *Biochem. Biophys. Res. Commun.* **88**, 1301-1307.

Borch, R.F., Bernstein, M.D. and Durst, H.D. (1971) *J. Am. Chem. Soc.* **93**, 2897-2904.

Borgstrom, B. and Brockman, H.L. (1984) (Ed) *Lipases*, Elsevier, Amsterdam.

Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochem.* **72**, 248-254.

Bredt, D.S. and Snyder, S.H. (1992) "Nitric oxide, a novel neuronal messenger", *Neuron* **8**, 3-11.

Broman, S. (1992) "Crosslinked enzyme crystal show promise for inductrial, clinical uses", *C&EN*, September, 40-43.

Brown, L.R., Strawinski, R.J. and McCleskey, C.S. (1964) "The isolation and characterization of *Methanomonas methanooxidians*" *Can. J. Microbiol.* **10**, 791-799.

Bruehlman, R.J. and Verhoek, F.H. (1948) *J. Am. Chem. Soc.* **70**, 1401-1404.

Burch, R., Squire, G.D. and Tsang, S.C. (1989) "Direct conversion of methane into methanol", *J. Chem. Soc., Faraday Trans. I*, **85**, 3561-3573.

Burch, R., Chalker, S., Loader, P. and Mariscal, R. (1991) "The mechanism of ethane formation on chloride and oxychloride catalysts", *Third european workshop on catalytic methane conversion, 27-29 May, meeting abstracts.*

Burch, R. and Parkyns, N. (1992) "New uses for natural gas", *Chemistry in Britain*, November, 1013.

Burrows, K.J., Cornish, A. Scott, D. and Higgins, I.J. (1984) "Substrate specificities of soluble and particulate methane monooxygenase of *Methylosinus trichosporium* OB3b", *J. Gen. Microbiol.* **130**, 3327-3333.

Bynoe, L.A., Pou, S. Gottsch, J.D. and Rosen, G.M. (1991) "Light-dependent spin trapping of hydroxyl radical from humman erythrocytes", *Biochem. Biophys. Res. Commun.* **179**, 1305-1310.

Cambell, K.D. (1991) "Layered and double perovskites as methane coupling catalysts", In: *Third european workshop on catalytic methane conversion*, 27-29 May, 1991 in France, meeting abstract.

Caraway, K.L., Spoerl, P. and Koshland, Jr., D.E. (1969) *J. Mol. Biol.* **42**, 133-137.

Cardy, D.L.N., Laidler, V., Salmond, G.P.C. and Murrell, J.C. (1991a) "Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylosinus trichosporium* OB3b", *Mol. Microbiol.* **5**, 335-342.

Cardy, D.L.N., Laidler, V., Salmond, G.P.C. and Murrell, J.C. (1991b) "The methane monooxygenase gene cluster of *Methylosinus trichosporium*: cloning and sequencing of the *mmoC* gene", *Arch. Microbiol.* **156**, 477-483.

Chamulitrat, W., Hughes, M.F., Eling, T.E. and Mason, R.P. (1991) "Superoxide and peroxyl radical generation from the reduction of polyunsaturated fatty acid hydroperoxides by soybean lipoxygenase", *Arch. Biochem. Biophys.* **290**, 153-159.

Chan, S.I., Nguyen, H-H.T., Shiemke, A.K. and Lidstrom, M.E. (1993) "Biochemical and biophysical studies toward characterization of the membrane-associated methane monooxygenase", in: *Microbial Growth of C_1 Compounds* (7th International Symposium) Ed by Murrell, J.C. and Kelly, D.P., Intercept Ltd, Athenaeum Press, Newcastle upon Tyne, pp93-107.

Cheetham, P.S.J. (1985) "The application of enzymes in industry", In: *Handbook of enzyme Biotechnology*, 2nd edn, Ed by A. Wiseman, Ellis Horwood, Chichester, pp274-379.

Chothia, C. and Janin, J. (1975) "Principles of protein-protein recognition", *Nature*, **256**, 705-710.

Citterio, A., Minisci, F. and Franchi, V. (1980) *J. Org. Chem.* **45**, 4752-4757.

Colby, J., Dalton, H. and Whittenbury, R. (1975) "An improved assay for bacterial methane monooxygenase: some properties of the enzyme from *Methylomonas methanica*", *Biochem. J.* **151**, 459-462.

Colby, J. and Dalton, H. (1976) "Some properties of a soluble methane monooxygenase from *Methylococcus capsulatus* Strain Bath", *Biochem. J.* **157**, 495-497.

Colby, J., Stirling, D.I. and Dalton, H. (1977) "The soluble methane monooxygenase of *Methylococcus capsulatus* (Bath): its ability to oxygenate n-alkanes, n-alkenes, ethers, and alcyclic, aromatic and heterocyclic compounds", *Biochem. J.* **165**, 395-402.

Colby, J. and Dalton, H. (1978) "Resolution of the methane monooxygenase of *Methylococcus capsulatus* (Bath) into three components", *Biochem. J.* **171**, 461-468.

Colby, J. and Dalton, H. (1979) "Characterization of the second prosthetic group of the flavoenzyme NADH-acceptor reductase (component C) of the methane monooxygenase from *Methylococcus capsulatus* (Bath)", *Biochem. J.* **177**, 903-908.

Colby, J., Dalton, H. and Whittenbury, R. (1979) "Biological and biochemical aspects of microbiol growth on C_1 compounds" *Annnu. Rev. Microbiol.* **33**, 481-517.

Coon, M.J., White, R.E. and Blake, R.C. (1979) "Mechanistic studies with purified liver microsomal cytochrome P450: comparison of O_2 and peroxide-supported hydroxylation reactions", in: *Oxidase and Related Redox Systems* Ed by T.E. King, H.S. Mason and M. Morrison, Pergamon Press, Elmsford, N.Y., pp857-885.

Coon, M.J., Ding, X. Pernecky, S.J. and Vaz, A.D.N. (1992) "Cytochrome P450: progress and predictions", *FASEB J.* **6**, 669-673.

Cornish, A., MacDonald, J., Burrows, K.J., King, T.S., Scott, D. and Higgins, I.J. (1985) "Succinate as *in vitro* electron donor for the particulate methane monooxygenase of *Methylosinus trichosporium* OB3b", *Biotech. Lett.* **7**, 319-324.

Cotton, F.A. and Wilkinson, G. (1988) *Advanced Inorganic Chemistry*, Fifth Edition, John Wiley & Sons, Inc., USA.

Cover, A.E. and Peterson, J.L. (1988) "Remote gas to liquid fuels processing alternatives", *American Institute of Chemical Engineers 1988 Spring National Meeting Session 71B*, Fuels and Chemicals from Natural Gas, March 6-10, New Orleans, LA.

Dalton, H. (1991) "Structure and mechanism of action of the enzyme(s) involved in methane oxidation", in: *Applications of Enzyme Biotechnology*. Ed by Kelly, J.W. and Baldwin, T.O., Plenum Press, New York. pp55-68.

Dalton, H. (1992a) "Methane oxidation by methanotrophs" in: *Methane and Methanol Utilizers*, Ed by Murrell, J.C. and Dalton, H. Plenum Press, New York. pp85-114.

Dalton, H. (1992b) "Biological methane activation-lessons for the chemists", *Catalysis Today* **13**, 455-461.

Dalton, H., Golding, B.T., Waters, B.W., Higgins, R. and Taylor, J.A. (1981) "Oxidations of cyclopropane, methylcyclopropane and arenes with the monooxygenase system from *Methylococcus capsulatus*", *J. Chem. Soc. Chem. Commun.* 482-483.

Dalton, H., Prior, S.D., Leak, D.J. and Stanley, S.H. (1984) in: *Microbial Growth on C-1 Compounds* (4th International Symposium) Ed by Grawford, R.L. and Hanson, R.S., American Society for Microbiology, Washington, D.C., pp75-82.

Dalton, H. and Leak, D.J. (1985) in: Gas Enzymology, Ed. by Degn, H., Cox, R.P. and Toftlund, H., D. Reidel Publishing Co. Dordrecht, Netherlands, pp169-186.

Dalton, H., Smith, D.D.S. and Pilkington, S.J. (1990) "Towards a unified mechanism of biological methane oxidation", *FEMS Microbiology Rev.* **87**, 201-208.

Dalton, H., Wilkins, P.C., Deighton, N., Podmore, I.D. and Symons, M.C.R. (1992) "Electron paramagnetic resonance studies of the mechanism of substrate oxidation by methane monooxygenase" *Faraday Discuss* **93**, 163-171.

Dalton, H., Wilkins, P.C. and Jiang, Y. (1993a) "Structure and mechanism of action of the hydroxylase of soluble methan monooxygenase", in: *Microbial Growth of C_1 Compounds* (7th International Symposium) Ed by Murrell, J.C. and Kelly, D.P., Intercept Ltd, Athenaeum Press, Newcastle upon Tyne, pp65-80.

Dalton, H., Wilkins, P.C. and Jiang, Y. (1993b) "Mechanistic pathways in soluble methane monooxygenase", *Biochim. Soc. Transactions* **21**, 749-752.

Davies, G.E. and Stark, G.R. (1970) "Use of dimethyl suberimidate, a cross-linking reagent in studying the subunit structure of oligomeric proteins", *Proc. Natl. Acad. Sci. U.S.A.* **66**, 651-656.

Davis, J.B., Coty, V.F. and Stanley, J.P. (1964) "Atmospheric nitrogen fixation by methane oxidizing bacteria", *J. Bacteriol.* **88**, 468-472.

Davis, J.C. and Averill, B.A. (1982) "Evidence for a spin-coupled binuclear iron unit at the acitive-site of the purple acid-phosphatase from beef spleen", *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4623-4627.

Davis, K., Cornish, A. and Higgins, I.J. (1987) "Regulation of the intracellular location of methane monooxygenase during growth of *Methylosinus trichosporium* OB3b on methanol", *J. Gen. Microbiol.* **51**, 720-724.

Dawson, J.H., Holm, R.H., Trudell, J.R., Barth, G., Linder, R.E., Bunnenberg, E., Djerassi, C. and Tang, S.C. (1976) *J. Am. Chem. Soc.* **98**, 3707.

Dawson, J.H. (1988) "Probing structure-function relations in heme-containing oxygenases and peroxidases", *Science*, **240**, 433-439.

Deighton, N., Podmore, I.D., Symons, M.C.R., Wilkins, P.C. and Dalton, H. (1991) "Substrate radical intermediates are involved in the soluble methane monooxygenase catalysed oxidations of methane, methanol and acetonitrile", *J. Chem. Soc., Chem. Commun.*, 1086-1088.

Doi, K., Antanaitis, B.C. and Aisen, P. (1988) "The binuclear iron centers of uteroferrin and the purple acid phosphatases *Struct. Bonding*. **70**, 1-26.

Doig, A.J. and Williams, D.H. (1991) "Is the hydrophobic effect stabilizing or destabilizing in proteins? the contribution of disulphide bonds to protein stability", *J. Mol. Biol.* **217**, 389-398.

Dordick, J.S. (1989) "Enzymatic catalysis in monophasic organic solvents", *Enzyme, Micro. Technol.* **11**, 194-209.

Drago, R.S. (1992) "Homogeneous metal-catalyzed oxidations by O_2", *Coord. Chem. Rev.* **117**, 185-213.

Drenth, J., Jansonius, J.N., Koekoek, R. and Wolthers, B.G. (1971) "The structure of papain", *Adv. Protein. Chem.* **25**, 79-115.

Entsch, B., Ballou, D. and Massey, V. (1976) "Flavin-oxygen derivatives involved in hydroxylation by *p*-hydroxbenzonate hydroxylase", *J. Biol. Chem.* **251**, 2550-2563.

Ericson, A., Hedman, B., Hodgson, K.O., Green, J., Dalton, H. Bentsen, J.G. Beer, R.H. and Lippard, S.J. (1988) "Structural characterization by EXAFS spectroscopy of the binuclear iron center in protein A of methane monooxygenase from *Methylococcus capsulatus* (Bath)", *J. Am. Chem. Soc.* **110**, 2330-2332.

Estabrook, R.W., Hildebrandt, A.G., Baron, J. Netter, K.J. and Leibman, K.C. (1971) "New spectral intermediate associated with cytochrome P450 function in liver microsomes", *Biochem. Biophys. Res. Commun.* **42**, 132-139.

Fasco, M.J., Piper, L.J. and Kaminsky, L.S. (1979) "Cumene hydroperoxide-supported microsomal hydroxylations of warfarin-a probe of cytochrome P450 multiplicity and specificity", *Biochem. Pharmacol.* **28**, 97-103.

Fee, J.A. (1980) "Metal ion activation of dioxygen", Ed by T.G. Spiro, John Wiley & Sons Press, New York, pp209.

Fenton, H.J.H. (1894) *J. Chem. Soc.* **65**, 899.

Ferenci, T. (1974) "Carbon monoxide-stimulated respiration in methane-utilizing bacteria", *FEBS Lett.* **41**, 94-98.

Ferjancic, A., Puigserver, A.J. and Gaertner, H.F. (1988) "Unusual specificity of polyethylene glycol-modified thermolysin in peptide-synthesis catalysed in organic solvents", *Biotechnol. Lett.* **10**, 101-106.

Fields, R. (1972) "The rapid determination of amino groups with TNBS", *Methods in Enzymology*, **25B**, 464-468.

Fish, R.H., Fong, R.H., Oberhausen, K.J., Konings, M.S., Vega, M.C., Christou, G., Vincent, J.B. and Buchanan, R.M. (1992) "Biomimetic oxidation studies, 6: synthetic and mechanistic aspects of manganese cluster mediated alkane functionalization reactions", *New J. Chem.* **16**, 727-733.

Fitzpatrick, P.F. and Villafranca, J.J. (1986) "The mechanism of inactivation of dopamine β-hydroxylase by hydrazines", *J. Biol. Chem.* **261**, 4510-4518.

Flicstein, J.M. and Kremer, M.L. (1967) *J. Catal.* **8**, 145-150.

Flory, P.J. (1956) *J. Am. Chem. Soc.* **78**, 5222.

Fontecave, M., gerez, C. Atta, M. and Jeunet, A. (1990) "High valent iron oxo intermediates might be involved during activation of ribonucleotide reductase: single oxygen atom donors generate the tyrosyl radical", *Biochem. Biophys. Res. Commun.* **168**, 659-664.

Foster, J.W. and Davis, R.H. (1966) "A methane dependent coccus with notes on classification and nomenclature of obigate methane utilizing bacteria", *J. Bacteriol.* **91**, 1924-1931.

Foster, N.R. (1985) "Direct catalytic-oxidation of methane to methanol-a review", *Appl. Catal.* **19**, 1-11.

Fox, B.G. and Lipscomb, J.D. (1988a) " Purification of a high specific activity methane monooxygenase hydroxylase component from a type II methanotroph", *Biochem. Biophys. Res. Commun.* **154**, 165-170.

Fox, B.G., Surerus, K.K., Munck, E. and Lipscomb, J.D. (1988b) "Evidence for a m-oxo-bridged binuclear iron clustur in the hydroxylase component of methane monooxygenase", *J. Biol. Chem.* **263**, 10553-10556.

Fox, B.G., Froland, W.A., Dege, J.E. and Lipscomb, J.D. (1989) "Methane monooxygenase from *Methylosinus trichosporium* OB3b", *J. Biol. Chem.* **264**, 10023-10033.

Fox, B.G., Borneman, J.G., Wackett, L.P. and Lipscomb J.D. (1990) "Haloalkene oxidation by soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and envionmental implications", *Biochemistry* **29**, 6419-6427.

Fox, B.G., Liu, Y., Dege, J.E. and Lipscomb, J.D. (1991) "Complex formation between the protein components of methane monooxygenase from Methylosinus trichosporium OB3b, identification of sites of component interaction" *J. Biol. Chem.* **266**, 540-550.

France, J.E., Shamsi, A. and Headley, L.C. (1988) "Oxidative coupling catalyst research for the conversion of natural gas to liquid fuels", *American Institute of Chemical Engineers 1988 Spring National Meeting Session 70A* , Fuels and Chemicals from Natural Gas, March 6-10, New Orleans, LA.

Froland, W.A., Andersson, K.K., Lee, S.-K., Liu, Y. and Lipscomb, J.D. (1991) "Oxygenation by methane monooxygenase: oxygen activation and component interactions", in: *Applications of enzyme biotechnology*, Ed by Kelly, J.W. and Baldwin, T.O., Plenum press, New york and London, pp 39-53.

Froland, W.A., Andersson, K.K., Lee, S.-K., Liu, Y. and Lipscomb, J.D. (1992) "Methane monooxygenase component B and reductase alter the regioselectivity of the hydroxylase component-catalyzed reactions, a novel role for protein-protein interactions in an oxygenase mechanism", *J. Biol. Chem.* **267**, 17588-17597.

Fujisawa, H., Hiromi, K., Uyeda, M., Nozaki, M. and Hayaishi, O. (1971) "Oxygenated form of protocatechuate 3,4-dioxygenase, a non-heme iron-containing dioxygenase as reaction intermediate", *J. Biol. Chem.* **246**, 2320-2321.

Furchgott, R.F. and Zawadzki, J.V. (1980) "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine" *Nature* **288**, 373-376.

Gaertner, H.F. and Puigserver, A.J. (1988) "Peptide synthesis catalysed by polyethylene glycol-modified chymotrypsin in organic solvents", *Proteins: Struct. Funct. Genet.* **3**, 130-137.

Gaertner, H.F. and Puigserver, A.J. (1989) "Determination of serum proteins by means of biuret reaction", *Eur. J. Biochem.* **181**, 207-213.

Garman, A.J. and Kalindjian, S.B. (1987) "The preparation nad properties of novel reversible polymer-protein conjugates", *FEBS Lett.* **223**, 361-365.

Gesser, H.D., Hunter, N.R. and Drakash, C.B. (1985) "The direct conversion of methane to methanol by controlled oxidation", *Chem. Rev.* **85**, 235-244.

Ghrir, R., Becam, A-M. and Lederer, F. (1984) "Primary structure of flavocytochrome b_2 from baker's yeast, purification by reverse-phase high-pressure liquid chromatography and sequencing of fragment α cyanogen bromide peptides", *Eur. J. Biochem.* **139**, 59-74.

Gilson, M.K., Rashin, A., Fine, R. and Honig, B. (1985) "On the calculation of electrostatic interactions in proteins", *J. Mol. Biol.* **183**, 503-516.

Girard, P. and Potier, P (1993) "NO, thiols and disulfides", *FEBS Lett.* **320**, 7-8.

Goldfarb, A.R. (1966) "A kinetic study of the reactions of amino acids and peptides with trinitrobenzenesulfonic acid", *Biochemistry* **5**, 2570-2574.

Green, J. and Dalton, H. (1985) "Protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath): a novel regulatory protein of enzyme activity", *J. Biol. Chem.* **260**, 15795-15801.

Green, J. and Dalton, H. (1986) "Steady-state kinetic analysis of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath)", *Biochem. J.* **236**, 155-162.

Green, J. and Dalton, H. (1988) "The biosynthesis and assembly of protein A of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath)" *J. Biol. Chem.* **263**, 17561-17565.

Green, J. and Dalton, H. (1989 a) "A stopped-flow kinetic study of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath)", *Biochem. J.* **259**, 167-172.

Green, J. and Dalton, H. (1989 b) "Substrate specificity of soluble methane monooxygenase: mechanistic implications", *J. Biol. Chem.* **264**, 17698-17703.

Green, P.N. (1992) "Taxonomy of methylotrophic bacteria", in: *Methane and Methanol Utilizers.* Ed by Murrell, J.C. and Dalton, H. Plenum Press, New York, pp23-84.

Green, W.N. and Ramanathan, R.V. (1988) "Conversion of natural gas to transport fuels", *American Institute of Chemical Engineers 1988 Spring National Meeting Session 70B*, Fuels and Chemicals from Natural Gas, March 6-10, New Orleans, LA.

Groves, J.T. and Van Der Puy, M. (1974) "Stereospecific aliphatic hydroxylation by an iron-based oxidant:, *J. Am. Chem. Soc.* **96**, 5274-5275.

Groves, J.T. and McClusky, G.A. (1976) "Aliphatic hydroxylation via oxygen rebound: oxygen transfer catakyzed by iron", *J. Am. Chem. Soc.* **98**, 859-861.

Groves, J.T., Nemo, T.E. and Myers, R.S. (1979) *J. Am. Chem. Soc.* **101**, 1032-1033.

Groves, J.T. and Nemo, T.E. (1983) "Aliphatic hydroxylation catalyzed by iron porphyrin complexes", *J. Am. Chem. Soc.* **105**, 6243-6248.

Groves, J.T. and Subramanian, D.V. (1984) "Hydroxylation by cytochrome P450 and metalloporphyrin models: evidence for allylic rerrangement", *J. Am. Chem. Soc.* **106**, 2177-2181.

Groves, J.T., Avaria-Neisser, G.E., Fish, K.M., Imachi, M. and Kuczkowski, R.L. (1986) "Hydrogen-deuterium exchange during propylene epoxidation by cytochrome P-450", *J. Am. Chem. Soc.* **108**, 3837-3838.

Guengerich, F.P., Ballou, D.P. and Coon, M.J. (1976) "Spectral intermediates in the reaction of oxygen with purified liver microsomal cytochrome P450", *Biochem. Bophys. Res. Commun.* **70**, 951-956.

Guengerich, F.P. and MacDonald, T.L. (1984) "Chemical mechanisms of catalysis by cytochromes P450-a unified view", *Accs. Chem. Res.* **17**, 9-16.

Guengerich, F.P. (1992) "Cytochrome P450: advance and prospects", *FASEB J.* **6**, 667-668.

Gunsalus, I.C., Meeks, J. Lipscomb, J., Debrunner, P. and Munck, E. (1974) in: *Molecular mechanisms of oxygen activation* , Ed by O. Hayaishi, Academic Press, New York, pp561-614.

Gunter, M. J. and Turner, P. (1991) "Metalloporphyrins as models for the cytochrome P450", *Coord. Chem. Rev.* **108**, 115-161.

Gustafsson, J.-A., Rondahl, L. and Bergman, J. (1979) "Iodosylbenzene derivatives as oxygen donors in cytochrome P450 catalyzed steroid hydroxylations", *Biochemistry* **18**, 865-870.

Gutbezahl, B. and Grunwald, E. (1953) *J. Am. Chem. Soc.* **75**, 559-565.

Habeeb, A.F.S.A. (1966) "Determination of free amino groups in proteins by trinitrobenzenesulfonic acid" *Anal. Biochem.* **14**, 328-336.

Hagen, K.S. and Lachicotte, R. (1992) "Diiron(II) μ-aqua bis(μ-carboxylato) models of reduced dinuclear non-heme iron sites in proteins", *J. Am. Chem.* **114**, 8741-8742.

Halliwell, B. and Gutteridge, J.M.C. (1992) "Biologically relevant metal ion-dependent hydroxyl radical generation an update", *FEBS Lett.* **307**, 108-112.

Hamilton, G.A., Friedman, J.P. and Campbell, P.M. (1966a) *J. Am. Chem. Soc.* **88**, 5266-5268.

Hamilton, G.A., Hanifin Jr. J.W. and Friedman, J.P. (1966b) *J. Am. Chem. Soc.* **88**, 5269-5272.

Hamilton, G.A. (1974) in: *Molecular mechanisms of oxygen activation*, Ed by O. Hayaishi, Academic Press, New York. pp405.

Hanson, R.S. (1992) in: *Methane and Methanol Utilizers*. Ed by Murrell, J.C. and Dalton, H. Plenum Press, New York, pp1-21.

Hamilton, G.A. (1974) "Chemical models and mechanisms for oxygenases", in: *Molecular Mechanisms of Oxygen Activation*, Ed by Hayaishi, O., Academic Press, New York, pp405-448.

Hargreaves, J.S.J., Hutchings, G.J. and Joyner, R.W. (1990) "Control of product selectivity in the partial oxidation of methane", *Nature* **348**, 428-429.

Harris, J.M. (1985) "Laboratory synthesis of polyethylene glycol derivatives", *J. Macromol. Sci. Rev. Macromol. Chem. Phys.* **C25**, 325-373.

Hartman, F.C. and Wold, F. (1967) "Crosslinking of bovine pancreatic ribonuclease A with dimethyl adipimidate", *Biochemistry* **6**, 2439-2448.

Helton, T.E., Gadalla, A.M. and Anthony, R.G. (1988) "Transition metal oxides used in the oxidative coupling of methane", *American Institute of Chemical Engineers 1988 Spring National Meeting Session 71E*, Fuels and Chemicals from Natural Gas, March 6-10, New Orleans, LA.

Hendrich, M.P., Munck, E., Fox, B.G. and Lipscomb, J.D. (1990) "Integer-spin EPR stydies of the fully reduced methane monooxygenase hydroxylase component", *J. Am. Chem. Soc.* **112**, 5861-5865.

Hendrich, M.P., Fox, B.G., Andersson, K.K., Debrunner, P.G. and Lipscomb, J.D. (1992) "Ligation of the diiron site of the hydroxylase component of methane monooxygenase. an electron nuclear double resonance study", *J. Biol. Chem.* **267**, 261-269.

Hibbs, J.B., Jr., Taintor, R.R., Vavrin, Z. and Rachlin, E. (1988) "Nitric oxide: a cytotoxic activated macrophage effector molecule", *Biochem. Biophys. Res. Commun.* **157**, 87-94.

Higgins, I.J. and Quayle, J.R. (1970) "Oxygenation of methane by methane-grown *Pseudomonas methanica* and *Methanomonas methanooxidans*", *Biochem. J.* **118**, 201-208.

Hill, C.L. (1989) in: *Activation and Functionalization of Alkanes*, Wiley, New York, pp253.

Hinsen, W. and Baerns, M. (1983) "Oxidative coupling of methane to C_2-hydrocarbons in the presence of different catalysts", *Chemiker-Zeitung*, **107**, 223-224.

Hiramatsu, M., Velasco, R.D. and Packer, L. (1991) "Decreased carbon centered and hydrogen radicals in skeletal muscle of vitamin supplemented rats", *Biochem. Biophys. Res. Commun.* **179**, 859-864.

Hollenberg, P.F. (1992) "Mechanisms of cytochrome P450 and peroxidase-catalysed xenobiotic metabolism", *FASEB* **6**, 686-694.

Hrycay, E.G. and O'brien, P.J. (1971) "Cytochrome P450 as a microsomal peroxidase utilizing a lipid peroxide substrate", *Arch. Biochem. Biophys.* **147**, 14-27.

Hrycay, E.G. and O'brien, P.J. (1972) "Cytochrome P450 as a microsomal peroxidase in steroid hydrperoxide reduction", *Arch. Biochem. Biophys.* **153**, 480-494.

Hrycay, E.G., Gustafsson, J-Å., Ingelman-Sundberg, M. and Ernster, L. (1975a) "Sodium periodate, sodium chlorite, and organic hydrperoxides as hydroxylatings in hepatic microsomal steroid hydroxylation reactions catalyzed by cytochrome P450", *FEBS Lett.* **56**, 161-165.

Hrycay, E.G., Gustafsson, J-Å., Ingelman-Sundberg, M. and Ernster, L. (1975b) "Sodium periodate, sodium chlorite, organic hydrperoxides, and H_2O_2 as hydroxylating agents in steroid hydroxylation reactions catalyzed by partially purified cytochrome P450", *Biochem. Biophys. Res. Commun.* **66**, 209-216.

Hubley, J.H., Thomson, A.W. and Wilkinson, J.F. (1975) "Specific inhibitiors of methane oxidation in *Methylosinus trichosporium*", *Arch. Microbiol.* **102**, 199-202.

Husain, S.S. and Lowe, G. (1968) *Biochem. J.* **103**, 855.

Hutchings, G.J., Scurrell, M.S. and Woodhouse, J.R. (1987) "The role of surface O^- in the selective oxidation of methane", *J. Chem. Soc., Chem. Commun.* 1388-1392.

Hutchings, G.J., Scurrell, M.S. and Woodhouse, J.R. (1989a) "In Proceedings 1st workshop on catalytic methane conversion, Bochum 1988", *Catal. Today*. **4**, 371.

Hutchings, G.J., Scurrell, M.S. and Woodhouse, J.R. (1989b) "Oxidative coupling of methane using oxide catalysts", *Chem. Soc. Rev.* **18**, 251-283.

Hyman, M.R., Seefeldt, L.C., Morgan, T.V., Arp, D.J. and Mortenson, L.E. (1992) "Kinetic and spectroscopic analysis of the inactivating effects of nitric oxide on the individual components of *Azotobacter vinelandii* nitrogenase", *Biochemistry*, **31**, 2947-2955.

Ignarro, L.J., Lippton, H., Edwards, J.C., Baricos, W.H., Hyman, A.L., Kadowtiz, P.J. and Grutter, C.A. (1981) "Mechanism of vascular smooth muscle relaxation by oganic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates", *J. Pharmacol. Exp. Ther.* **218**, 739-749.

Ignarro, L.J. (1989) "Heme-dependent activation of soluble guanylate cyclase by nitric oxide: regulation of enzyme activity by porphyrins and metalloporphyrins", *Semin. Hematol.* **26**, 63-76.

Ito, T. and Lunsford, J.H. (1985) "Synthesis of ethylene and ethane by partial oxidation of methane over lithium-doped magnesium oxide", *Nature*, **314**, 721-722.

Jackson, C-J.C., Charlton, J.L., Kuzminski, K., Lang, G.M. and Sehon, A.H. (1987) "Synthesis, isolation, and characterization of conjugates of ovalbumin with monomethoxypolyethylene glycol using cyanuric chloride as the coupling agent", *Anal. Biochem.* **165**, 114-127.

Jaenicke, R. (1991) "Protein stability and molecular adaptation to extreme conditions", *Eur. J. Biochem.* **202**, 715-728.

Jiang, Y.and Dalton, H. (1993) "Chemical modification of the hydroxylase of soluble methane monooxygenase gives one form of the protein with significantly increased thermostability and another that functions well in organic solvents", in press.

Jiang, Y., Wilkins, P.C. and Dalton, H. (1993) "Activation of the hydroxylase of sMMO from *methylococcu capsulatus* (Bath) by hydrogen peroxide", *Biochim, Biophys. Acta*. **1163**, 105-112.

Johnson, D., Mendicino, J., Cormier, M. and Travis, J. (1974) "Cyanuric chloride: a new coupling reagent for affinity chromatography", *Fed. Proc.* **33**, 1502, abstract 1572.

Jolley, R.L., Evans, L.H., Makino, N. and Mason, H.S. (1974) "Oxytyrosinase", *J. Biol. Chem.* **249**, 335-345.

Jones, C.A., Leonard, J.J. and Sofranco, J.A. (1984) *US Patent* 4,443,644.

Judd, R.W., reynolds, T.J. and Komodromos, C. (1991) "Mangansese based alkali chloride catalysts for oxidative coupling", In: *Third european workshop on catalytic methane conversion*, 27-29 May, 1991 in France, meeting abstract.

Kadlubar, F.F., Morton, K.C. and Ziegler, D.M. (1973) "Microsomal-catalyzed hydroperoxide-dependent c-oxidation of amines", *Biochem. Biophys. Res. Commun.* **54**, 1255-1261.

Keevil, T. and Mason, H.S. (1978) "Molecular oxygen in biological oxidations-an overview", *Methods in Enzymology*, **LII**, part c, 3-40.

Keim, W. (1986) "Industrial chemicals via C1 process", *ACS Symp.ser.*, **328**, 3.

Keller, G.E. and Bhasin, M.M. (1982) "Synthesis of ethylene via oxidative coupling of methane, 1. determination of active catalysts", *J. Catal.* **73**, 9-19.

Kellis, J.T., Jr., Nyberg, K., Sali, D. and Fersht, A.R. (1988) "Contribution of hydrophobic interactions to protein stability", *Nature*, **333**, 784-786.

Kennedy, C.H. and Mason, R.P. (1990) "A reexamination of the cytochrome P450-catalyzed free radical production from a dihydropyridine-evidence of trace transition metal catalysis", *J. Biol. Chem.* **265**, 11425-11428.

Khan, S.A. and Halling, P.J. (1992) "Polyethylene glycol-modified subtilisin forms microparticulate suspensions in organic solvents", *Enzyme Microb. Technol.* **14**, 96-100.

Kitajima, N., Fukui, H. and Moro-oka, Y. (1988) "A model for methane monooxygenase: dioxygen oxidation of alkanes by use of a μ-oxo binuclear iron complex", *J. Chem. Soc., Chem. Commun.* 485-486.

Kivirikko, K.I., Myllylä, R., Pihlajaniemi, T. (1989) "Protein hydroxylation: propyl 4-hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit", *FASEB* **3**, 1609-1617.

Kiuchi, K., Kiuchi, K., Titani, K., Fujita, K., Suzuki, K. and Nagatsu, T. (1991) "Limited proteolysis of tyrosine hydroxylase by Ca^{2+}-activated neutral protease (calpain)", *Biochemistry*, **30**, 10416-10419.

Kiwi, J., Thampi, K.R. and Grazel, M. (1990) "Molybdate compounds as active and long-living methane dimerisation catalysts", *J. Chem. Soc., Chem. Commun.* 1690-1692.

Knight, C. and Perkins, M.J. (1991) "Concerning the mechanism of 'Gif' oxidations of cycloalkanes", *J. Chem. Soc., Chem. Commun.* 925-927.

Kohno, M., Yamada, M., Mitsuta, K., Mizuta, Y. and Yoshihawa, T. (1991) "Spin-trapping studies on the reaction of iron complexes with peroxides and the effects of water-soluble antioxidants", *Bull. Chem. Soc. Jpn.* **64**, 1447-1453.

Korzekwa, K.R., Swinney, D.C. and Trager, W.F. (1989) "Isotopically labeled chlorobenzenes as probes for the mechanism of cytochrome P450 catalyzed aromatic hydroxylation", *Biochemistry* **28**, 9019-9027.

Kraut, J. (1977) "Serine proteases: structure and mechanism of catalysis", *Ann, Rev. Biochem.* **46**, 331-358.

Kurusu, Y. and Neckers, D.C. (1991) "Functionalization of silica gel: application for the catalytic oxidation of alkanes", *J. Org. Chem.* **56**, 1981-1983.

Labeque, R. and Marnett, L. (1989) "Homolytic and heterolytic scission of organic hydrperoxides by (meso-tetraphenylporphinato)iron(III) and its relation to olefin epoxidation", *J. Am. Chem. Soc.* **111**, 6621-6627.

Ladd, D.L. and Snow, R.A. (1993) "Reagents for the preparation of chromophorically labeled polyethylene glycol-protein conjugates", *Anal Biochem.* **210**, 258-261.

Laemmli, U.K. (1970) "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature*, **227**, 680-685.

Large, P.J. and Bamforth, C.W. (1988) *Methylotrophy and Biotechnology*, Longman, Wiley, New york.

Lawrene, A.J. and Quayle, J.R. (1970) "Alternative carbon assimilation pathways in methane utilizing bacteria", *J. Gen. Microbiol.* **63**, 371-374.

Leadbetter, E.R. and Foster, J.W. (1959) "Incorporation of molecular oxygen in bacterial cells utilizing hydrocarbons for growth", *Nature* **184**, 1428-1429.

Leak, D.J., Aikens, P.J. and Seyed-Mahmoudian, M. (1992) "The microbial production of epoxides", *Trends in Biotechnol.* **10**, 256-261.

Lee, H., Takahashi, K., Kodera, Y., Owada, K., Tsuzuki, T., Matsushima, A and Inada, Y. (1988) *Biotechnol. Lett.* **10**, 403-407.

Lee, S-K., Nesheim, J.C. and Lipscomb, J.D. (1993) "Transient intermediates of the methane monooxygenase catalytic cycle", *J. Biol. Chem.* **268**, 21569-21577.

LeGall, J., Prickril, B.C., Moura, I., Xavier, A.V., Moura, J.J.G. and Huynh, B.H. (1988) "Isolation and characterization of rubrerythrin, a non-heme iron protein from *Desulfovibrio vulgaris* that contains rubredoxin centers and a hemerythrin-like binuclear iron cluster", *Biochemistry* **27**, 1636-1642.

Lewin, B. (1990) Ed, *GENS IV*, Oxford University Press and Cell Press.

Lichtenberger, R., Nastainczyk, W. and Ullrich, V. (1976) "Cytochrome P450 as an oxene transferase", *Biochem. Biophys. Res. Commun.* **70**, 939-946.

Lidstrom, M.E. and Stirling, D.I. (1990) "Methytotrophs: genetics and commercial applications", *Annu. Rev. Microbiol.* **44**, 27-58.

Lippard, S.J. (1993) "Oxygen activation by non-heme iron complexes and proteins", *Lecture on ICBIC 6th, at University of California San Diego, U.S.A.*

Liu, A.-M., Li, S.-B., Miao, D.-X., Yu, W.-L., Zhang, F. and Su, P. (1991) "Isolation and purification of methane monooxygenase from *Methylomonas* sp. GYJ3", *Chinese Chem. Lett.* **2**, 419-422.

Liu, K.E., Johnson, C.C., Newcomb, M. and Lippard, S.J. (1993) "Radical clock substrate probes and kinetic isotope effect studies of the hydroxylation of hydrocarbons by methane monooxygenase", *J. Am. Chem. Soc.* **115**, 939-947.

Ljunger, G., Adlercreutz, P. and Mattiasson, B. (1993) "Reaction catalyzed by PEG-modified α-chymotrypsin in organic solvents. Influence of water contene and degree of modification", *Biocatalysis*, **7**, 279-288.

Lund, J. and Dalton, H. (1985) "Further characterisation of the FAD and Fe_2S_2 redox centres of component C, the NADH: acceptor reductase of the soluble methane monooxygenase of *Methylococcus capsulatus* (Bath)", *Eur. J. Biochem.* **147**, 291-296.

Lund, J., Woodland, M.P. and Dalton, H. (1985) "Electron transfer in the soluble methane monooxygenase of *Methylococcus capsulatus* (Bah)", *Eur. J. Biochem.* **147**, 297-305.

Lunsford, J.H. (1990) "The role of sodium-carbonate and oxides supported on lanthanide oxides in the oxidative dimerization of methane", *J. Catal.* **126**, 291-298..

Lunsford, J.H. (1991) "The oxidative coupling of methane: an example of coupled heterogeneous and homogeneous reactions", *Abstract in Third European Workshop on catalytic Methane Conversion*, 27-29 May, in France.

Lynch, J.B., Juarez-Garcia, C., Munck, E. and Que, L. Jr. (1989) "Mossbauer and EPR studies of the binuclear iron center in ribonucleotide reductase from *Escherchia coli*, a new iron-to-protein stoichiometry", *J. Biol. Chem.* **264**, 8091-8096.

Mancuso, A.J., Huang, S-L. and Swern, D. (1978) "Oxidation of long-chain and related alcohols to carbonyls by dimethyl sulfoxide 'activated' by oxalyl chloride", *J. Org. Chem.* **43**, 2480-2482.

Mancuso, A.J., Brownfain, D.S. and Swern, D. (1979) "Structure of dimethyl sulfoxide-oxalyl chloride reaction product. Oxidations of heteroaromatic and diverse alcohols to carbonyl compounds", *J. Org. Chem.* **44**, 4148-4150.

Mansuy, D., Battioni, P. and Renaud, J.-P. (1984) "In the presence of imidazole, iron- and manganese-porphyrins catalyze the epoxidation od alkenes by alkyl hydroperoxides", *J. Chem. Soc. Chem. Commun.* 1255-1257.

Mansuy, D. (1987) "Cytochrome P450 and synthetic models", *Pure Appl. Chem.* **59**, 759-770.

Mansuy, D. and Battioni, P. (1989) "Alkane functionalization by cytochromes P450 and By model systems using O_2 or H_2O_2", in: *Activation and Functionalization of Alkanes*, Ed by Hill, C.L., John Wiley and Sons, New York, pp195-218.

Mathews, C.K. and van Holde, K.E. (1990) Ed, *Biochemitry*, The Benjamin/Cummings Publishing Company, Inc.

Martinek, K. and Torchilin, V.P. (1988) "Stabilization of enzymes by intramolecular crossinking using bifunctional reagents", *Methods in Enzymol.* **137**, 615-626.

Matsushima, A., Okada, M. and Inada, Y. (1984) "Chymotrypsin modified with polyethylene glycol catalyzes peptide synthesis reaction in benzene", *FEBS Lett.* **178**, 275-277.

May, S.W. (1992) "Biocatalysis in the 1990s: A perspective", *Enzyme Microb. Technol.* **14**, 80-84.

McCarty, J.G., Quinlan, M.A. and Sancier, K.M. (1988) "Oxidative methane dimerisation by basic oxide catalysts", *American Institute of Chemical Engineers 1988 Spring National Meeting Session 70C*, Fuels and Chemicals from Natural Gas, March 6-10, New Orleans, LA.

McCord, J.M. and Fridovich, I.J. (1969) "Superoxide dismutase, an enzymic function for erythrocuprein (hemocuprein)", *J. Biol. Chem.* **244**, 6049-6051.

McMurray, T.J. and Groves, J.T. (1986) "Metalloporphyrin models for cytochrome P450", in: *Cytochrome P450, Structure, Mechanism and Biochemistry*", Ed by Ortiz de Montellano, P.R., Plenum Press, New York, pp1-28.

Means, G.E. and Feeney, R.E. (1968) "Reductive alkylation of amino groups in proteins", *Biochemistry* **7**, 2192-2201.

Moore, S and Stein, W.H. (1954) *J. Biol. Chem.* **211**, 907.

Morrison, R.T. and Boyd, R.N. (1987) Ed, *Organic chemistry*, Allyn and Bacon, Inc. U.S.A.

Mottley, C., Robinson, R.E. and Mason, R.P. (1991) "Free radical formation in the oxidation of malondialdehyde and acetylacetone by peroxidase enzyme", *Arch. Biochem. Biophys.* **289**, 153-160.

Mozhaev, V.V., Shikshnis, V.A., Torchilin, V.P. and Martinek, K. (1983) "Operational stability of copolymerized enzymes at elevated temperatures", *Biotechnol. Bioeng.* **25**, 1937-1946.

Mozhaev, V.V. and Martinek, K. (1984) "Structure stability relationships in proteins-new approaches to stabilizing enzymes", *Enzyme Microb. Technol.* **6**, 50-65.

Mozhaev, V.V., Berezin, I.V. and Martinek, K. (1988) "Reaction of immobilized enzymes", *CRC Crit. Rev. Biochem.* **23**, 235-281.

Mozhaev, V.V. (1993) :Mechanism-based strategies for protein thermostabilization", *TIBTECH*, **11**, 88-95.

Muhoberac, B.B., Wharton, D.C., Babock, L.M., Harrington, P.C. and Wilkins, R.G. (1980) "EPR spectroscopy of semi-methemerythrin", *Biochim. Biophys. Acta.* **626**, 337-345.

Mullens, I.A. and Dalton, H. (1987) "Cloning of the gamma-subunit methane monooxygenase from *Methylococcus capsulatus*", *Bio/Technology* **5**, 490-493.

Murad, F. (1986) "Cyclic guanosine monophosphate as mediator of vasodilation", *J. Clin. Invest.* **78**, 1-5.

Murai, S, Kakiuchi, F., Sekine, S., Tanaka, Y., Kamatani, A., Sonoda, M. and Chatani, N. (1993) "Efficient catalytic addition of aromatic carbon-hydrgen bonds to olefins", *Nature*, **366**, 529-531.

Murch, B.P., Bradley, F.C. and Que, L.Jr., (1986) "A binuclear iron peroxide complex capable of olefin epoxidation", *J. Am. Chem. Soc.* **108**, 5027-5028.

Murrell, J.C. (1992) "Genetics and molecular biology of methanotrophs", *FEMS Microbiol. Rev.* **88**, 233-248.

Nakajima, T., Uchiyama, H., Yagi, O. and Nakahara, T. (1992) "Purification and properties of a soluble methane monooxygenase from *Methylocystis* sp. M", *Biosci. Biotech. Biochem.* **56**, 736-740.

Nam, W., Ho, R. and Valentine, J.S. (1991) "Iron-cyclam complexes as catalysts for the epoxidation of olefins by 30% aqueous hydrogen peroxide in acetonitrile and methanol", *J. Am. Chem. Soc.* **113**, 7052-7054.

Nash, T. (1953) "The colorimetric estimation of formaldehyde by means of the hantzsch reaction", *Biochem. J.* **55**, 416-421.

Nishida, Y., Nasu, M. and Akamatsu, T. (1992) "Reaction between binuclear iron(III) compounds and DMPO (5,5-dimethyl-3,4-dihydropyrroles N-oxide)", *J. Chem. Soc., Chem. Commun.* 93-94.

Nordblom, G.D., White, R.E. and Coon, M.J. (1976) "Studies on hydroperoxide-dependent substrate hydroxylation by purified microsomal cytochrome P450", *Arch. Biochem. Biophys.* **175**, 524-533.

Nordlund, P., Sjoberg, B-M. and Eklund, H. (1990) "The three-dimensional structure of the free radical protein of ribonucleotide reductase", *Nature* **345**, 593-598.

Nordlund, P., Dalton. H. and Eklund, H. (1992) "The active site structure of methane monooxygenase is closely related to the binuclear iron center of ribonucleotide reductase", *FEBS Lett.* **307**, 257-262.

Noronha-Dutra, A.A., Epperlein, M.M. and Woolf, N. (1993) "Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric-mediated killing", *FEBS Lett.* **321**, 59-62.

Okuyama, T. and Satake, K. (1960) *J. Biochem. (Tokyo)* **47**, 454.

Olah, G.A., Sommer, J. and Namanworth, E. (1967) "Stable carbonium ions. XLI. Protonated aliphatic alcohols and their cleavage to carbonium ions", *J. Am. Chem. Soc.* **89**, 3576-3581.

Olsbye, U., Desgrandchamps, G., Jens, K-J. and Kolboe, S. (1991) "A kinetic study of the oxidative coupling of methane over a 25% $BaCO_3/La2O_3$ catalyst", *Third european workshop on catalytic methane conversion, 27-29 May, meeting abstracts.*

Ortiz de Montellano, P.R., Augusto, O, Viola, F. and Kunze, K.L. (1983) "Carbon radicals in the metabolism of alkyl hydrazines" *J. Biol. Chem.* **258**, 8623-8629.

Ortiz de Montellano, P.R. (1986) "Oxygen activation and transfer", in: *Cytochrome P450, Structure, Mechanism and Biochemistry*, Ed by Ortiz de Montellano, P.R., Plenum Press, New Youk, pp.217-271.

Otsuka, K. and Nakajima, T. (1987) "Oxidative coupling of methane over samarium oxides using nitrous oxide as the oxidant", *J. Chem. Soc., Faraday Trans.* **83**, 1315.

Otsuka, K., Uragami, Y. and Hatano, M. (1991) "The oxidative coupling of methane in the presence and absence of catalyst", *Third european workshop on catalytic methane conversion, 27-29 May*, meeting abstracts.

Packer, L., Valenza, M., Serbinova, E., Starke-Reed, P. Frost, K. and Kagan, V. (1991) "Free radical scavenging is involved in the protective effect of L-propionyl-carnitine against ischemia-reperfusion injury of the heart", *Arch. Biochem. Biophys.* **288**, 533-537.

Padbury, G. and Sligar, S.G. (1988) "Ferric bleomcin catalyzed reduction of 10-hydroperoxy-8,12-octadecadienoic acid: evidence for homolytic O-O bond scission", *Biochemistry*, **27**, 7846-7852.

Palmer, R.M.J., Ferridge, A.G. and Moncada, S. (1987) "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor", *Nature* **327**, 524-526.

Park, S., Hanna, M.L., Taylor, R.T. and Droege, M.W. (1991) "Batch cultivation of *Methylosinus trichosporium* OB3b: I. production of soluble methane monooxygenase", *Biotechnol. Bioeng.* **38**, 423-433.

Park, S., Shah, N.N., Taylor, R.T. and Droege, M.W. (1992) "Batch cultivation of *Methylosinus trichosporium* OB3b: II. production of particulate methane monooxygenase", *Biotechnol. Bioeng.* **40**, 151-157.

Patel, R.N. and Savas, J.C. (1987) " Purification and properties of the hydroxylase component of methane monooxygenase", *J. Bacteriol.* **169**, 2313-2317.

Penner-Hahn, J.E., McMurray, J.T., Rnner, M., Latos-Grazynsky, L., Eble, K.S., Davis, I.M., Balch, A.L., Groves, J.T., Dawson, J.H. and Hodgson, K.O. (1983) "X-ray absorption spectroscopic studies of high valent iron porphyrins", *J. Biol. Chem.* **258**, 12761-12764.

Penner-Hahn, J.E., McMurray, J.T., Rnner, M., Eble, K.S., Davis, I.M., Balch, A.L., Groves, J.T., Dawson, J.H. and Hodgson, K.O. (1986) *J. Am. Chem. Soc.* **108**, 7819.

Percival, M.D., Denis, D., Riendeau, D. and Gresser, M.J. (1992) "Inactivation of the mechanism of non-turnover-dependent inactivation of purified human 5-lipoxygenase, inactivation by H_2O_2 and inhibition by metal ions', *Eur. J. Biochem.* **210**, 109-117.

Periana, R.A., Taube, D.J., Evitt, E.R., Loffler, D.G., Wentrcek, P.R., Voss, G. and Masuda, T. (1993) "A mercury-catalysed, high-yield system for the oxidation of methane to methanol", *Sciene*, **259**, 340-343.

Perfetti, R.B., Anderson, C.D. and Hall, P.L. (1976) "The chemical modification of papain with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide", *Biochemistry* **15**, 1735-1743.

Peterson, J.A., Ishimura, Y. and Griffin, B.W. (1972) "*Pseudomonas putida* cytochrome P450: characterization of an oxyhenated form of the hemoprotein", *Arch. Biochem. Biophys.* **149**, 197-208.

Peterson, L., Graslund, A., Ehrenberg, A., Sjoberg, B.M. and Reichard, P. (1980) "The iron center in ribonucleotide reductase from *Escherichia coli*", *J. Biol. Chem.* **255**, 6706-6712.

Pilkington, S.J. and Dalton, H. (1990) "Soluble Methane Monooxygenase from *Methylococcus capsulatus* Bath", *Methods in Enzymology*, **188**, 181-190.

Pilkington, S.J., Salmond, G.P.C., Murrell, J.C. and Dalton, H. (1990) "Identification of the gene encoding the regulatory protein B of soluble methane monooxygenase", *FEMS Microbiology Lett.* **72**, 345-348.

Pilkington, S.J. and Dalton, H. (1991) "Purification and characterization of the soluble methane monooxygenase from *Methylosinus sporium* 5 demonstrates the highly conserved naure of this enzyme in methanotrophs", *FEMS Microbiol. Lett.* **78**, 103-108.

Pina, C., Clark, D. and Blanch, H. (1989) *Biotechol. Techniques* **3**, 333-338.

Plapp, B.V., Moore, S. and Stein, W.H. (1971) "Activity of bovine pancreatic deoxyribonuclease A with modified amino groups", *J. Biol. Chem.* **246**, 939-945.

Priestley, N.D., Floss, H.G., Froland, W.A., Lipscomb, J.D., Williams, P.G. and Morimoto, H. (1992) "Cryptic stereospecificity of methane monooxygenase", *J. Am. Chem. Soc.* **114**, 7561-7562.

Prince, R.C., George, G.N., Savas, J.C., Cramer, S.P. and Patel, R.N. (1988) "Spectroscopic properties of the hydroxylase of methane monooxygenase", *Biochem. Biophys. Acta.* **952**, 220-229.

Prior, S.D. and Dalton, H. (1985a) "Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath)", *FEMS Microbiol. Lett.* **29**, 105-109.

Prior, S.D. and Dalton, H. (1985b) "The effect of copper ions on membrane content and methane monooxygenase activity in methanol-grown cells of *Methylococcus capsulatus* (Bath)", *J. Gen. Microbiol.* **131**, 155-163.

Quayle, J.R. (1987) "An eightieth anniversary of the study of C_1 metabolism" in: *Microbiol Growth on C_1-Compounds*, Ed by Van Verseveld, H.W. and Duine, J.A., Martinus Nijhoff, Dordrecht, pp1-5.

Raag, R. and Poulos, T.L. (1989) "The structural basis for substrate-induced changes in redox potential and spin equilibrium in cytochrome $P450_{CAM}$", *Biochemistry* **28**, 917-922.

Rahimtula, A.D. and O'Brien, P.J. (1974) "hydrperoxide catalyzed liver microsomal aromatic hydrxylation reactions involving cytochrome P450", *Biochem. Biophys. Res. Commun.* **60**, 440-447.

Rardin, R.L., Tolman, W.B. and Lippard, S.J. (1991) "Monodentate carboxylate complexes and the carboxylate shift: implications for polymetalloprotein structure and function", *New J. Chem.* **15**, 417-430.

Rataj, M.J., Kauth, J.E. and Donnelly, M.I. (1991) "Oxidation od deuterated compounds by high specific activity methane monooxygenase from *Methylosinus trichosporium*", *J. Biol. Chem.* **266**, 18684-18690.

Ribbons, D.W. and Michalover, J.L. (1970) "Methane oxidation by cell-free extracts of *Methylococcus capsulatus*", *FEBS Lett.* **11**, 41-44.

Ribbons, D.W. (1975) "Oxidation of C_1 compounds by particulate fractions from *Methylococcus capsulatus*: distribution and properoties of methane dependent reduced nicotinamide adenine dinucleotide oxidase (methane hydroxylase)", *J. Bacteriol.* **122**, 1351-1363.

Roberts Jr. J. L., Morrison, M.M. and Sawyer, D.T. (1978) "Base-induced generation of superoxide ion and hydroxyl radical from hydrogen peroxide", *J. Am. Chem. Soc.* **100**, 329.

Roguleva, V.G., Nikiphorova, M.A., Maksimov, N.G. and Anshits, A.G. (1991) "Oxidative coupling of methane over Li/CaO catalysts using O_2 and N_2O as oxidants",In: *Third european workshop on catalytic methane conversion*, 27-29 May, 1991 in France, meeting abstract.

Rosen, H. (1957) *Arch. Biochem. Biophys.* **67**, 10.

Rosenzweig, A.C., Frederick, C.A. and Lippard, S.J. (1992) "Crystallization and preliminary X-ray analysis of the methane monooxygenase hydroxylase protein from *Methylococcus capsulatus* (Bath)", *J. Mol. Biol.*. **227**, 583-585.

Rosenzweig, A.C., Frederick, C.A., Lippard, S.J. and Nordlund, P. (1993) "Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane", *Nature*, **366**, 537-543.

Ross, J.R.H., Van Ommen, J.G., seshan, K., Korf, S.J., Swaan, H.M., McNamara, D. and Fornasari, G. (1991) "Niobium oxide as promotor in alkali-doped catalysts for the oxidative coupling of methane", In: *Third European Workshop on Catalytic Methane Conversion*, 27-29 May, 1991 in France, meeting abstract.

Ruzicka, F., Huang, D.-S., Donnelly, M. and Frey, P.A. (1990) "Methane monooxygenase catalyzed oxygenation of 1,1-dimethylcyclopropane. Evidence for radical and carbocationic intermediates", *Biochemistry*, **29**, 1696-1700.

Sahlin, M., Sjöberg, B.-M., Backes, G., Loehr, T. and Sanders-Loehr, J. (1990) "Activation of the iron-containing B2 protein of ribonucleotide reductase by hydrogen peroxide", *Biochem. Biophys. Res. Commun.* **167**, 813-818.

Sawyer, D.T., Kang, C. Llobet, A. and Redman, C. (1993) "Fenton reagents (1:1 $Fe^{II}L_x$/HOOH) react via [$L_xFe^{II}OOH(BH^+)$] (1) as hydroxylases (RH---ROH), not as generators of free hydroxyl radicals (HO.)", *J. am. Chem. Soc.* **115**, 5817-5818.

Schulz, G.E. and Schirmer, R.D. (1979) *Priniciple of protein structure*. Springer-Verlag, Berlin.

Sen, R.K., Zagal, J. and Yenger, E. (1977) "The electrocatalysis of molecular oxygen reduction", Inorg. Chem. **16**, 3379-3383.

Serrano, L., Bycroft, M. and fersht, A.R. (1991) "Aromatic-aromatic interactions and protein stability, inrestriction by double-mutant cycles", *J. Mol. Biol.* **218**, 465-475.

Sharrock, M., Munck, E., Debrunner, P.G., Marshall, V., Lipscomb, J.D. and Gunsalus, I.C. (1973) "Mössbauer studies of cytochrome $P450_{cam}$", *Biochemistry* **12**, 258-265.

Sharrock, M., Debrunner, P.G., Schulz, C., Lipscomb, J.D., Marshall, V. and Gunsalus, I.C. (1976) "Cytochrome $P450_{cam}$ and its complexes: Mössbauer parameters of the heme iron", *Biochim. Biophys. Acta*. **420**, 8-26.

Shah, N.N., Park, S., Taylor, R.T. and Droege, M.W. (1992) "Cultivation of *Methylosiuns trichosporium* OB3b:III. production of particulate methane monooxygenase in continuous culture", *Biotechnol. Bioeng*. **40**, 705-712.

Shaked, Z. and Wolfe, S. (1988) "Stabilization of pyranose 2-oxidase and catalase by chemical modification", *Methods in Enzymology*, **137**, 599-615.

Sharp, K.A., Nicholls, A., Friedman, R. and Honig, B. (1991) "Extracting hydrophobic free energies from experimental data: relationship to protein folding and theoretical models", *Biochemistry*, **30**, 9686-9697.

Shepelev, S.S. and Ione, K.G. (1983) *React. Kinet. Catal. lett*. **23**, 323.

Sheu, C. and Sawyer, D.T. (1990) "Activation of dioxygen by bis[(2-carboxy-6-carboxylato)pyridine] iron(II) for the bromination (via $BrCCl_3$) and monooxygenation (via PhNHNHPh) of saturated hydrocarbons: reaction mimic for the methane monooxygenase proteins", *J. Am. Chem. Soc*. **112**, 8212-8214.

Shi, X. and Dalal, N.S. (1991) "Flavoenzymes reduce vanadium(V) and molecular oxygen and generate hydroxyl radical", *Arch. Biochem. Biophys*. **289**, 355-361.

Shilov, A.E. (1984) *Activation of Saturated Hydrocarbons by Transition Metal Complexes*, Ed by D. Reidel, Dordrecht.

Shilov, A.E. (1989) in: *Activation and Functionalization of Alkanes*, Ed by Hill, C.L., Wiley, New York.

Sinha, B.K. (1983) "Enzymeatic activation of hydrazine derivatives-a spin-trapping study", *J. Biol. Chem.* **258**, 796-801.

Sligar, S.G., Kennedy, K.A. and Pearson, D.C. (1980) In *Oxidases and related redox systems*, Ed by T.E. King, H.S. Mason and M. Morrison. Baltimore: Univ. Park Press.

Smith, D.D.S. and Dalton, H. (1989) "Solubilisation of methane monooxygenase from *Methylococcus capsulatus* (Bath)", *Eur. J. Biochem.* **182**, 667-671.

Smith, D.D.S. and Dalton, H. (1992) "Evidence for two histidine ligands at the diiron site of methane monooxygenase", *Eur. J. Biochem.* **210**, 629-633.

Smith, J.R.L. and Sleath, P.R. (1983) "Model systems for cytochrome P450 dependent monooxygenase, 3. the stereochemistry of hydroxylation of *cis*-decahydronaphthalene and *trans*-decahydronaphthalene by chemical-models for cytochromes P450 dependent monooxygenase", *J. Chem. Soc. Perkin Trans II*, 1165-1169.

Solymosi, F., Tombacz, I. and Katson, G. (1985) "Partial oxidation of methane by nitrous oxide over B1203-SNo2", *J. Chem. Soc., Chem. Commun.* 1455-1456.

Souppe, J., Urrutigoity, M. and Levesoue, G. (1988) "Application of the reaction of dithioesters with ε-amino groups in lysine to the chemical modification of proteins", *Biochim. Biophy. Acta.* **957**, 254-257.

Souppe, J., Urrutigoity, M. and Levesoue, G. (1989) "Application of the reaction of dithioesters with ε-amino groups in lysine to the chemical modification of proteins", *New J. Chem.* **12**, 503-506.

Springborg, J., Wilkins, P.C. and Wilkins, R.G. (1989) "Kinetics and thermodynamics of the reaction of deoxyhemerythrin with nitric oxide", Acta. Chemca. Scandinavica. **43**, 967-974.

St. Clair, N.L. and Navia, M.A. (1992) "Crosslinked enzyme crystals as robust biocatalysts", *J. Am. Chem. Soc.* **114**, 7314-7316.

Stainthorpe, A.C., Murrell, J.C., Salmond, G.P.C., Dalton, H. and Lees, V. (1989) "Molecular analysis of methane monooxygenase from *Methylococcus capsulatus* (Bath)", *Arch Microbiol.* **152**, 154-159.

Stanley, S.H. and Dalton, H. (1982) "Role of ribulose-1,5-biphosphate carboxylase/oxygenase in *Methylococcus capsulatus* (Bath)", *J. Gen. Microbiol.* **128**, 2927-2935.

Stanley, S.H., Prior, S.D., Leak, D.J. and Dalton, H. (1983) "Copper stress underlies the fundamental change in intracellular location of methane monooxygenase in methane-oxidizing organism: studies in bath and continuous cultures", *Biotech. Lett.* **5**, 487-492.

Stanley, S.H. and Dalton, H. (1992) "The biotransformation of propylene to propylene oxide by *Methylococcus capsulatus* (Bath): 1. optimization of rates", *Biocatalysis* **6**, 163-175.

Stassinopoulos, A. and Caradonna, J.P. (1990) "Binuclear non-heme iron oxo transfer analogue reaction system: observations and biological implications", *J. Am. Chem. Soc.* **112**, 7071-7073.

Stearns, R.A. and Ortiz de Montellano, P.R. (1985) "Cytochrome P450 catalyzed oxidation of quadricyclane evidence for a radical cation intermediate", *J. Am. Chem. Soc.* **107**, 4081-4082.

Stigter, D. and Dill, K.A. (1990) "Charge effects on folded and unfolded proteins", *Biochemistry*, **29**, 1262-1271.

Stirling, D., Colby, J. and Dalton, H. (1979) "A comparison of the substrate and electron-donor specificities of the methane monooxygenase from three strains of methane-oxidizing bacteria", *Biochem. J.* **177**, 361-364.

Stirling, D. and Dalton, H. (1980) "Oxidation of dimethyl ether, methylformate and bromomethane by *Methylococcus capsulatus* (Bath)", *J. Gen. Microbiol.* **116**, 277-283.

Stubbe, J. (1990) "Ribonucleotide reductases: amazing and confusing", *J. Biol. Chem.* **265**, 5329-5332.

Suga, A., Sugiyama, T., Otsuka, M., Ohno, M., Sugiura, Y. and Maeda, K. (1991) "Synthetic studies on antitumor antibiotic bleomycin XXXI. oxidation of alkenes by a chiral non-porphyrinic oxidizing catalyst based on the bleomycin-iron(II) complex", *Tetrahedron* **47**, 1191-1204.

Swaan, H.M., Toebes, A., Seshan, K., Van Ommen, J.G. and Ross, J.R.H. (1991) "The kinetic and mechanistic aspects of the oxidative dehydrogenation of ethane over Li/Na/MgO catalysts", *Third european workshop on catalytic methane conversion, 27-29 May,* meeting abstracts.

Tabushi, I. (1988) "Reductive dioxygen activation by use of artificial P450 systems", *Coord. Chem. Rev.* **86**, 1-42.

Takeda, K., Tezuka, C., Fukuoka, S. and Takahara, Y. (1976) "Role of copper ions in methane oxidation by *Methanomonas margaritae*", *J. Ferm. Technol.* **54**, 557-562.

Takahashi, K., Ajima, A., Yoshimoto, T. and Inada, Y. (1984a) "Polyethylene glycol-modified catalase exhibits unexpectedly high activity in benzene", *Biochem. Biophys. Res. Commun.* **125**, 761-766.

Takahashi, K., Nishimura, H., Yoshimoto, T., Saito, Y. and Inada, Y. (1984b) "A chemical modification to make horseradish peroxidase soluble and active in benzene", *Biochem. Biophys. Res. Commun.* **121**, 261-265.

Takahashi, K., Nishimura, H., Yoshimoto, T., Okada, M., Ajima, A, Matsushima, A, Tamaura, Y., Satio, Y. and Inada, Y. (1984c) "Polyethylene glycol-modified enzymes trap water on their surface and exert enzymic activity in organic solvents", *Biotechnol. Lett.* **6**, 765-770.

Takahashi, K., Kodera, Y., Yoshimoto, T., Ajima, A., Matsushima, A. and Inada, Y. (1985a) "Ester-exchange catalysed by lipase modified with polyethylene glycol", *Biochem. Biophys. Res. Commun.* **131**, 532-536.

Takahashi, K., Ajima, A., Yoshimoto, T., Okada, M., Matsushima, A. and Tamaura,Y. (1985b) "Chemical reactions by polyethylene glycol modified enzymes in chlorinated hydrocarbons", *J. Org. Chem.* **50**, 3414-3415.

Tamura, M., Tamura, T., Burnham, D.N., Uhlinger, D.J. and Lambeth, J.D. (1989) "Stabilization of the superoxide generating respiratory burst oxidase of human neutrophil plasma membrane by crosslinking with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide", *Arch. Biochem. Biophys.* **275**, 23-32.

Tanford, C. (1980) *The hydrophobic effects: formation of micelles and biological membranes*, 2nd Ed. John Wiley & Sons, New York.

Tatsumoto, K., Oh, K.K., Baker, J.O. and Himmel, M.E. (1990) *Applied Biochem. Biotechnol.* **20/21**, 293-308.

Taylor, S., Dalton, H. and Dow, C.S. (1980) "Purification and initial characterization of ribulose 1,5-bisphosphate carboxylase from *Methylococcus capsulatus* (Bath)", *FEMS Microbiol. Lett.* **8**, 157-160.

Tolman, W.B., Liu, S., Bentsen, J.G. and Lippard, S.J. (1991) "Models of the reduced forms of polyiron-oxo proteins: an asymmetric, triply carboxylate bridged diiron(II) complex and its reaction with dioxygen", *J. Am. Chem. Soc.* **113**, 152-164.

Tong, Y., Rosynek, M.P. and Lunsford, J.H. (1989) "Secondary reactions of methyl radicals with lanthanide oxides: their role in the selective oxidation of methane", *J. Phys. Chem.* **93**, 2896.

Tong, Y., Rosynek, M.P. and Lunsford, J.H. (1990) "The role of sodium carbonate and oxides supported on lanthanide oxides in the oxidative dimerization of methane", *J. Catal.* **126**, 291.

Tonge, G.M., Harrison, D.E.F., Knowles, G.J. and Higgins, I.J. (1975) "Properties and partial purification of the methane-oxidizing enzyme system from *Methylosinus trichosporium*", *FEBS Lett.* **58**, 293-299.

Tonge, G.M., Harrison, D.E.F. and Higgins, I.J. (1977) "Purification and properties of the methane monooxygenase enzyme system from *Methylosinus trichosporium* OB3b", *Biochem. J.* **161**, 333-344.

Torchilin, V.P., Maksimenko, A.V., Smirnov, V.N., Berezin, I.V., Klibanov, A.M. and Martinek, K. (1978) "The principles of enzyme stabilization, III. the effect of the length of intramolecular crosslinkages on thermostability of enzymes", *Biochim. Biophys. Acta.* **522**, 277-283.

Torchilin, V.P., Trubetskoy, V.S. and Martinek, K. (1983) "Stabilization of enzyme subunits by intergubunit crosslinking with bifunctional reagents: studies with glyceraldehyde-3-phosphate dehydrogenase", *J. Mol. Catal.* **19**, 291-307.

Traylor, T.G. and Sharma, V.S. (1992) "Why NO?" *Biochemistry* **31**, 2847-2849.

Tsien, H.C., Brusseau, G.A., Hanson, R.S. and Wackett, L.P. (1989) "Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b", *Appl. Enviro. Microbiol.* **55**, 3155-3161.

Tube, H. (1965) *J. Gen. Physiol.* **49**, 24.

Tyson, C.A., Lipscomb, J.D. and Gunsalus, I.C. (1972) "The roles of putidaredoxin and $P450_{cam}$ in methylene hydroxylation", *J. Biol. Chem.* **247**, 5777-5784.

Ullrich, V., Ruf, H.H. and Wende, P. (1977) "The strucyure and mechanism of cytochrome P450", *Croat. Chem. Acta.* **49**, 211.

Ullrich, V. (1980) *J. Mol. Catal.* **7**, 159.

Ullrich, V., Castle, L. and Weber, P. (1981) "Spectral evidence for the cytochrome P450 nature of postacyclin synthetase", *Biochem. Pharmacol.* **30**, 2033-2036.

Urrutigoity, M. and Souppe, J. (1989) "Biocatalysis in organic solvents with a polymer-bound horseradish peroxidase", Biocatalysis **2**, 145-149.

Vallee, B.L., Galdes, A., Auld, D.S. and Riordan, J.F. (1983) In Zinc Enzymes. Ed by Spiro, T.G., Wiley, New York, pp27.

Vaska, L. (1976) "Dioxygen-metal complexes toward a unified view", *Acc. Chem. Res.* **9**, 175-178.

Vaz, A.D.N., Roberts, E.S. and Coon, M.J. (1991) "Olefin formation in the oxidative deformylation of aldehydes by cytochrome p450: mechanistic implications for catalysis by oxygen-derived peroxide", *J. Am. Chem. Soc.* **113**, 5886-5887.

Vermeiren, W. (1991) "Oxidative coupling of methane into higher hydrocarbons", *Thesis of Phd*, Katholieke Universiteit Leuven, Belgium.

Veronese, F.M., Largajolli, R., Boccu, E., Benassi, C.A. and Schiavon, O. (1985) *Appl. Biochem. Biotechnol.* **11**, 141-152.

Vincent, J.B., Huffman, J.C., Christou, G., Li, Q., Nanny, M.A., Hendrickson, D.N., Fong, R.H. and Fish, R.H. (1988) "Modeling the dinuclear site of iron biomolecules: synthesis and properties of $Fe_2O(OAc)_2Cl_2(bipy)_2$ and its use as an alkane activation catalyst", *J. Am. Chem. Soc.* **110**, 6898-6900.

Walling, C. and Goosen, A. (1973) *J. Am. Chem. Soc.* **95**, 2987-2991.

Walling, C. (1975) "Fenton's reagent revisited" *Acc. Chem. Res.* **8**, 125-131.

Walsh, K.A. (1986) *Methods in Protein Sequence Analysis*. Humana Press, Clifton, N.J.

Wang, D, Wilson, G. and moore, S. (1976) "Preparation of crosslinked dimers of pancreeeatic ribonuclease", *Biochemistry* **15**, 660-665.

Wang, D. and Moore, S. (1977) "Polyspermine-ribonuclease prepared by crosslinkage with dimethyl suberimidate", *Biochemistry* **16**, 2937-2942.

Wang, P., Hill, T.G., Wartchow, C.A., Huston, M.E., Oehler, L.M., Smith, M.B., Bednarski, M.D. and Callstrom, M.R. (1992a) "New carbohydrate-based materials for the stabilization of proteins", *J. Am. Chem. Soc.* **114**, 378-380.

Wang, J. Wu, L-H. and Angnes, L. (1991) "Organic-phase enzymatic assays with ultramicroelectrodes", *Anal. Chem.* **63**, 2993-2996.

Wang, J. Naser, N. Kwon, H-S. and Cho, M.Y. (1992b) "Tissue bioelectrode for organic-phase enzymatic assays", *Anal. Chem. Acta.* **264**, 7-12.

Weast, R.C. (1985) in: *Handbook of chemistry and physics, 65th edn.* Ed by R.C. Weast, Chemical Rubber Publishing Co., Cleveland.

West, C.A., Salmond, G.P.C., Dalton, H. and Murrell, J.C. (1992) "Functional expression in *Escherichia coli* of protein B and C from soluble methane monooxygenase of *Methylococcus capsulatus* (Bath)", *J. Gen. Microbiol.* **138**, 1301-1307.

White, R.E. and Coon, M.J. (1980) "Oxygen activation by cytochrome P450", *Annu. Rev. Biochem.* **49**, 315-356.

White, F. and Olsen, K.W. (1987) "Effects of crosslinking on the thermal stability of hemoglobin, I. the use of bis(3,5-dibromosalicyl) fumarate", *Arch. Biochem. Biophys.* **258**, 51-57.

Whittenbury, R. and Dalton, H. (1981) "The methylotrophic bacteria", in: *The Procaryotes*. Ed by Starr, M.P., Stolph, H., Truper, H.G., Balowes, A. and Schlegel H.G., Springer-Verlag, Berlin, pp894-902.

Whittenbury, R., Phillips, K.C. and Wilkinson, J.F. (1970) "Enrichment, isolation and some properties of methane-utilizing bacteria", *J. Gen. Microbiol.* **61**, 205-218.

Wirth, P., Souppe, J., Tritsch, D. and Biellmann, J-F. (1991) "Chemical modification of horseradish peroxidase with ethanal-methoxypolyethylene glycol: solubility in organic solvents, activity, and properties", *Bioorganic Chem.* **19**, 133-142.

Wilkins, P.C. and Wilkins, R.G. (1987 a) "The coordination chemistry of the binuclear iron site in hemerythrin", *Coord. Chem. Rev.* **79**, 195-214.

Wilkins, P.C. and Wilkins, R.G. (1987 b) "Acid-assisted anion interaction with deoxyhemerythrin", *Biochim. Biophys. Acta.* **912**, 48-55.

Wilkins, P.C., Dalton, H., Podmore, I.D., Deighton, N. and Symons, M.C.R. (1992) "Biological methane activation involves the intermediacy of carbon-centered radicals", *Eur. J. Biochem.* **210**, 67-72.

Wilkins, R.G. (1992) "Binuclear iron centres in proteins", *Chem. Soc. Rev.* **21**, 171-178.

Williams, D.L.H. (1985) "S-nitrosation and the reactions of S-nitroso compounds", *Chem. Soc. Rev.* **14**, 171-196.

Wilshire, J.P. and Sawyer, D.T. (1979) "Redox chemistry of dioxygen species", *Acc. Chem. Res.* **12**, 105-110

Wirth, P., Souppe, J., Tritsch, D. and Biellmann, J-R. (1991) "Chemical modification of horseradish peroxidase with ethanal-methoxypolyethylene glycol, solubility in organic solvents, activity and properties", *Bioorg. Chem.* **19**, 133-142.

Wong, S.S. (1991) *Chemistry of protein conjugation and crosslinking*, CRC press, Boca Raton, FL.

Wong, S.S. and Wong, L-J.C. (1992) "Chemical crosslinking and the stabilization of proteins and enzymes", *Enzyme Microb. Technol.* **14**, 866-874.

Wong, S.S., Iosiewicz, M. and Wong, L-J. (1992) in: *Biocatalyst Design for stability and specificity*, Ed by M.E. Himmel and G. Georgiou, Am. Chem. Soc., Washington, D.C.

Woodland, M.P. and Dalton, H. (1984) "Purification and characterization of component A of the methane monooxygenase from *Methylococcus capsulatus* (Bath)", *J. Biolog. Chem.* **259**, 53-59.

Woodland, M.P., Patil, D.S., Cammack, R. and Dalton, H. (1986) "ESR studies of protein A of the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath)", *Biochim. Biophy. Acta.* **873**, 237-242.

Yamahuchi, K., Watanabe, Y. and Morishima, I. (1992) "Direct observation of push effect on the heterolytic O-O bond cleavage reaction of acylperoxo-iron(III) porphyrin adducts", *J. Chem. Soc., Chem. Commun.* 1709-1710.

Yamazaki, I. and Piette, L.H. (1991) "EPR spin-trapping study on the oxidizing species formed in the reaction of the ferrous ion with hydrogen peroxide", *J. Am. Chem. Soc.* **113**, 7588-7593.

Yang, T. and Olsen, K.W. (1988) "Effects of crosslinking on the thermal stability of hemoglobins, II. the stabilization of met-, Cyanomet- and Carbonmonoxyhemoglobins A and S with bis(3,5-dibromosalicyl) fumarate", *Arch. Biochem. Biophys.* **261**, 283-290.

Yang, T. and Olsen, K.W. (1991) "Thermal stability of hemoglobin crosslinked in the T-state by bis(3,5-dibromosalicyl) fumarate", *Biochem. Biophys. Res. Commun.* **174**, 518-523.

Yoshimoto, T., Takahashi, K., Nishimura, H., Ajima, A., Tamaura, Y. and Inada, Y. (1984) "Modified lipase having high stability and various enzymic activities in benzene and its re-use by recovering from benzene solution", *Biotechnol. Lett.* **6**, 337-340.

Yoshimoto, T., Ritani, A., Ohwada, K., Takahashi, K., Kodera, Y., Matsushima, A., Satio, Y. and Inada, Y. (1987) "Polyethylene glycol derivative-modified cholesterol oxidase soluble and active in benzene", *Biochem. Biophys. Res. Commun.* **148**, 876-882.

Zale, S.E. and Klibanov, A.M. (1983) "On the role of reversible denaturation (unfolding) in the irreversible thermal inactivation of enzymes", *Biotechnol Bioeng.* **25**, 2221-2230.

Zecherle, G.N., Oleinikov, A. and Traut, R.R. (1992) "The C-terminal domains of *E.coli* ribosomal protein L7/L12 can occupy a location near the factor-binding domain of the 50S subunit as shown by crosslinking with N-[4-(p-azidosalicylamido)butyl]-3-(2'-pyridyldithio)propionamide", *Biochemistry*, **31**, 9526-9532.

Ziering, D.L., Pascal, Jr., R.A. (1990) "Mechanism-based inhibition of bacterial γ-butyrobetaine hydroxylase", *J. Am. Chem. Soc.* **112**, 834-838.

Zhen, K.J., Khan, M.M., Mak, C.H., Lewis, K.B. and Somorjai, G.a. (1985) "Partial oxidation of methane with nitrous oxide-over V205-S102 catalyst", *J. Catal.* **94**, 501-507.

Zhou, N.E., Kay, C.M. and Hodges, R.S. (1992) Synthetic model proteins: positional effects of interchain hydrophobic interactions on stability of two-stranded α-helical coiled-colis", *J. Biol. Chem.* **267**, 2664-2670.

APPENDIX 1

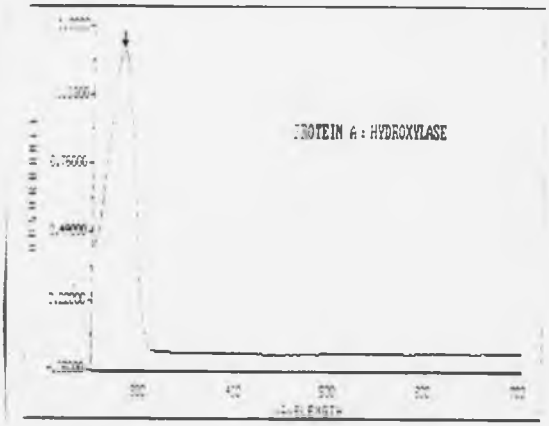
Problems in the direct conversion of methane

Reaction	Thermochemistry H (KJ/Mol)	G (KJ/Mol)
$2CH_4 \longrightarrow C_2H_6 + H_2$	73.8	71.7 (at 1000°K)
$CH_4 \longrightarrow CH_3 + H$	103	
$CH_3 \longrightarrow CH_2 + H$	105	
$CH_2 \longrightarrow CH + H$	105	
$CH \longrightarrow C + H$	80	

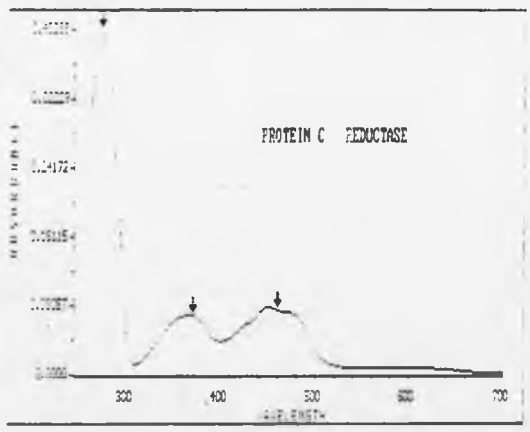
"Oxidative coupling of methane", 1988 Spring National Meeting abstracts.

APPENDIX 2

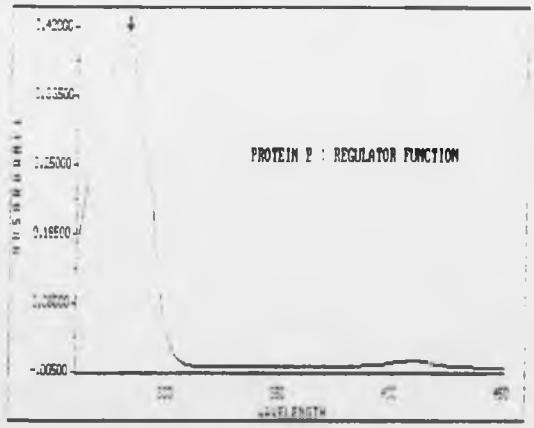
The spectra of the hydroxylase, reductase and protein B of sMMO from *Methylococcus capsulatus* (Bath)



Hydroxylase



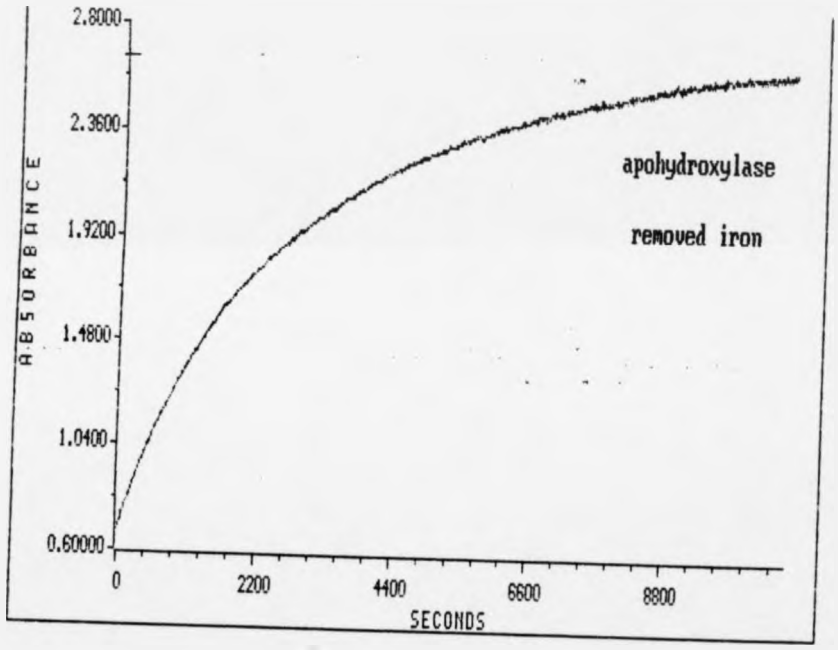
Reductase



Protein B

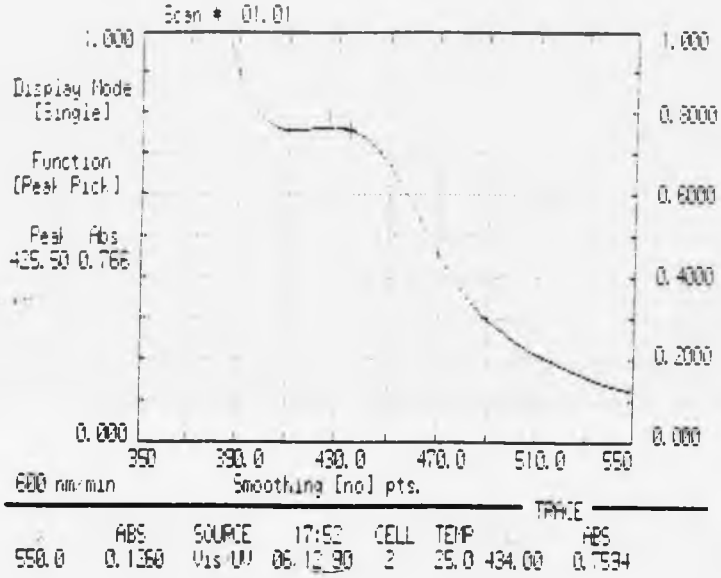
APPENDIX 3

A spectrum for preparation of apohydroxylase removing iron atoms from the protein

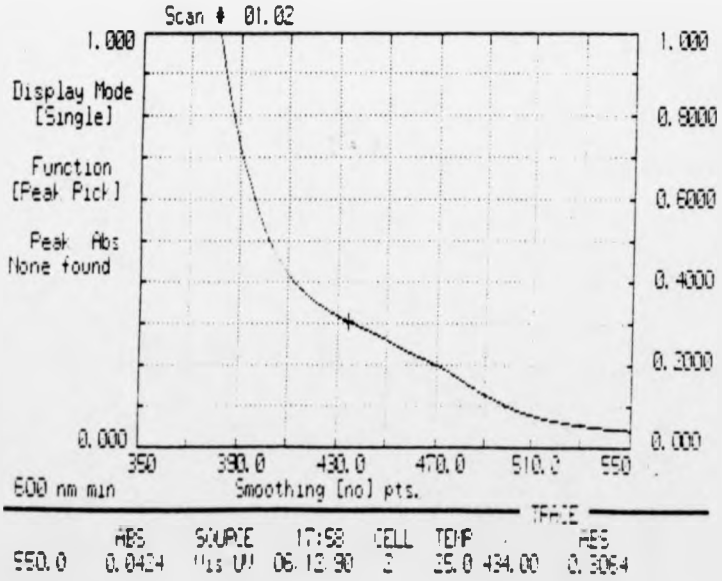


APPENDIX 4

The spectra of preparation of NO-saturated solution



NO_2 peak observed



no NO_2 peak observed

APPENDIX 5

Physical constants of oxygen (O_2)

MW = 32

Density or specgravity:	gas 1.429 ^{0} g/l
	liq 1.149 $^{-183}$ g/l
Melting point °C	-218 4
Boiling point °C	-182 962

Solubility in grams per 100 cc.

Cold water	4.89 ^{0} cm^3
	3.16 ^{25} cm^3
Hot water	2.46 ^{50} cm^3
	2.30 ^{100} cm^3
Alcohol	2.78 ^{25} cm^3

"Handbook of chemistry and physics" pp.B-112, 67th Edition (1986-1987), CRC Press.

APPENDIX 6

Some physical constants of NO, NO_2 and N_2O

NO

Mononitrogen monoxide; nitrogen monoxide
NO, mol wt 30.01; N 46.68%, O 53.32%. Colorless gas, mp. -163.6°; bp. -157.7°; d-150.2(liq) 1.27; d(gas) 1.04 (air=1); trouton constant 27.1; contains an odd number of electrons and is paramagnetic.
Solubility in water (ml/100 ml, 1 atm): 4.6 (20°C); 2.37 (60°C).

NO_2

Nitrogen dioxide
NO_2, mol wt 46.01; N 30 45%, O 69.55%. Reddish-brown gas, mp. -9.3°; bp. 21.15°; d_4^{20}(liq) 1.448; d(gas) 1.58 (air=1); $d_{gas}^{21.3}$ 3.3 g/liter.

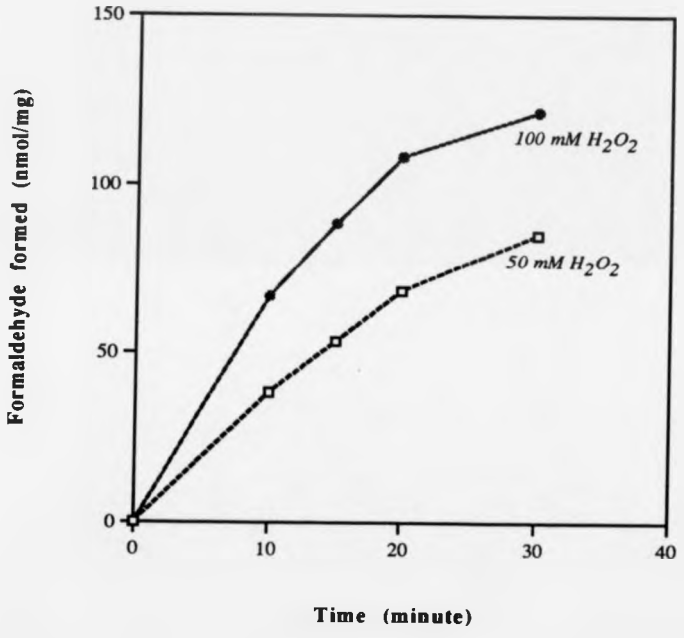
N_2O

Dinitrogen monooxide; laughing gas; hyponitrous acid; factitious air.
N_2O, mol wt 44.02; N 63.65%, O 36.35%. Colorless gas, mp. -90.81°; bp_{760} -88.46°. Trouton constant 21.4. d^{-89} (liq) 1.226; d(S.T.P.) 1.976; d(gas) 1.53 (air=1). At 20° and 2 atm one liter of the gas dissolves in 1.5 liters of water. Freely sol in sulfuric acid. Sol in alcohol, ether, oils.

"The Merck Index", Tenth Edition, 1983.

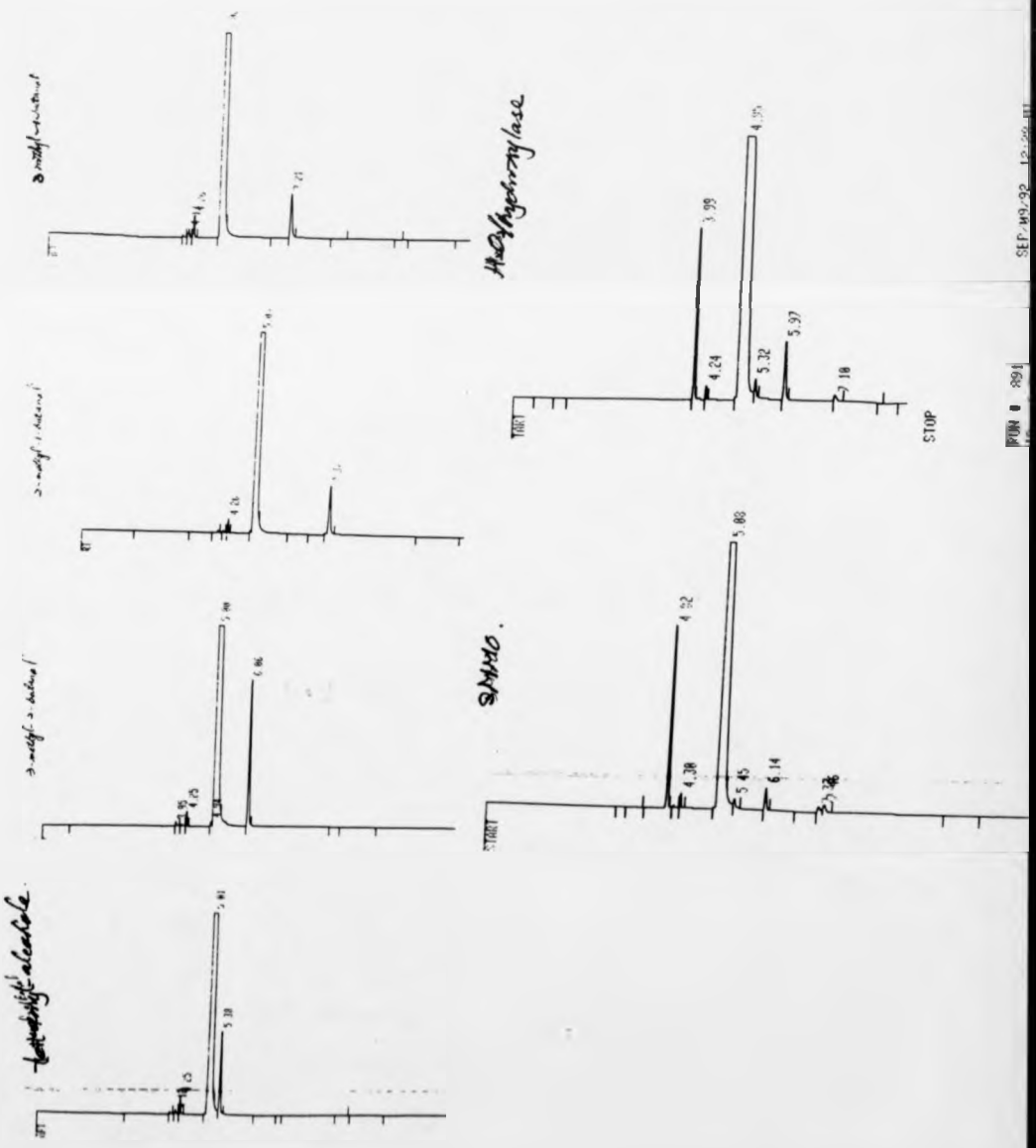
APPENDIX 7

The time course of methanol oxidation by the hydroxylase/H_2O_2 system.



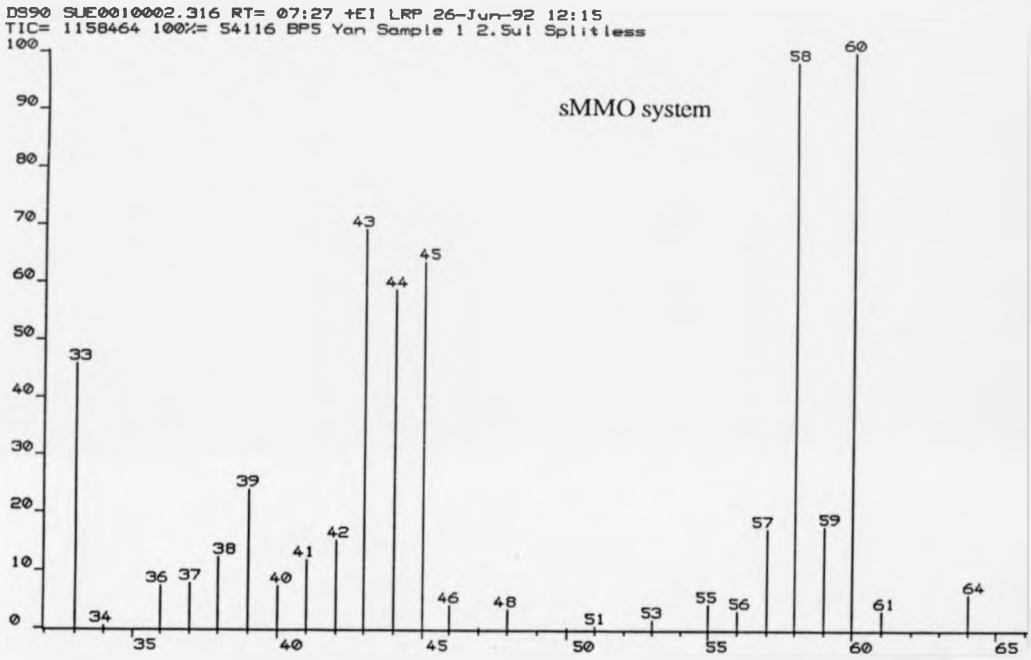
APPENDIX 8

The standards of 3-methyl-1-butanol, 2-methyl-1-butanol, 3-methyl-2-butanol and tert-amyl alcohol and the oxidation of 2-methylbutane by the sMMO complex and the hydroxylase/H_2O_2 system from GC (BP-5 column) assay.

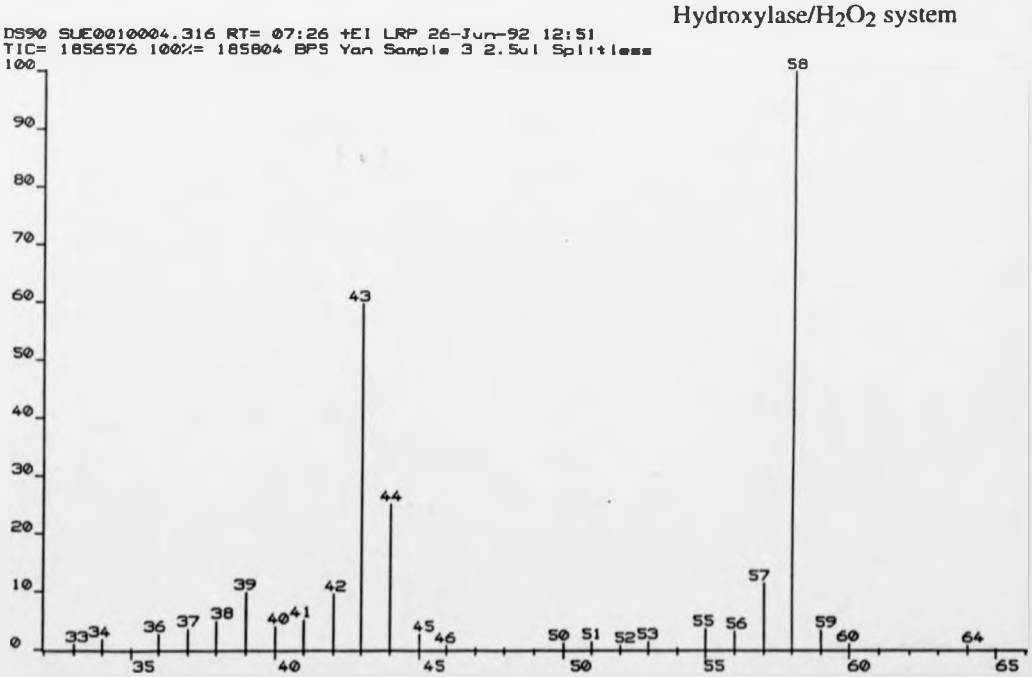


APPENDIX 9

The mass spectra of oxidation of propene by the sMMO and the hydroxylase/H_2O_2 systems in the presence of $^{18}O_2$.

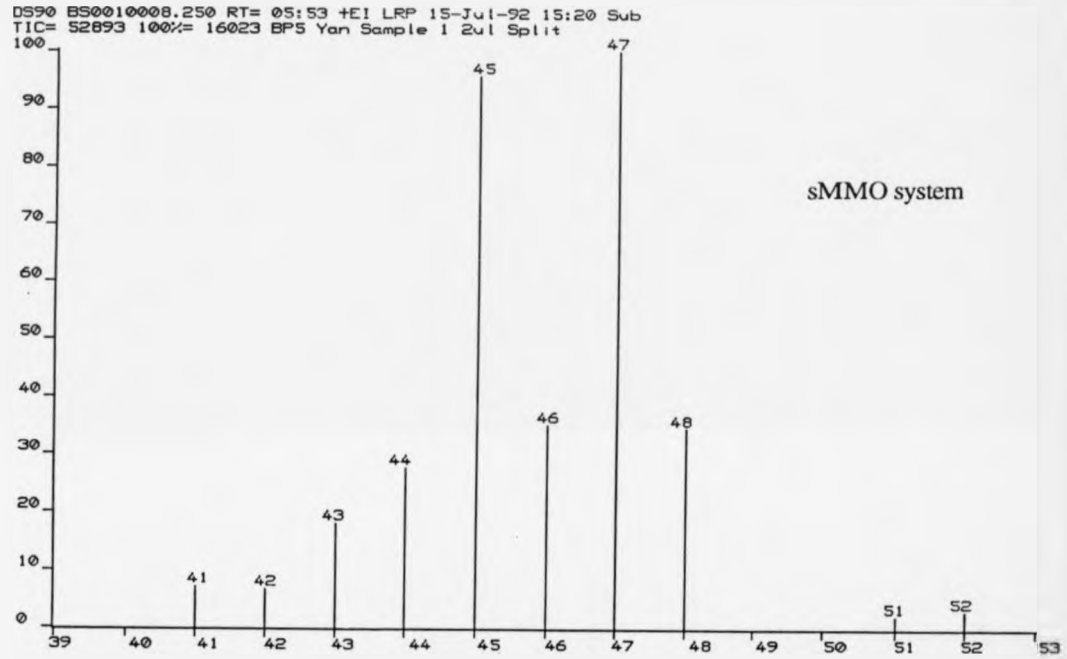


Hydroxylase/H_2O_2 system

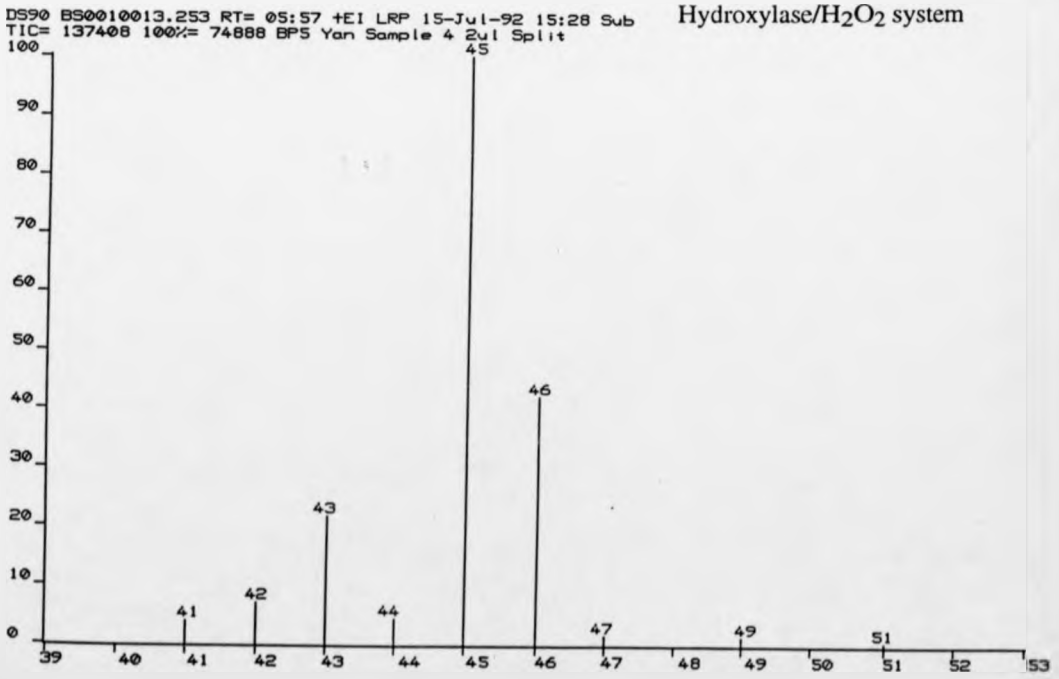


APPENDIX 10

The mass spectra of oxidation of ethane by the sMMO and the hydroxylase/H_2O_2 systems in the presence of $^{18}O_2$.



sMMO system



Hydroxylase/H_2O_2 system

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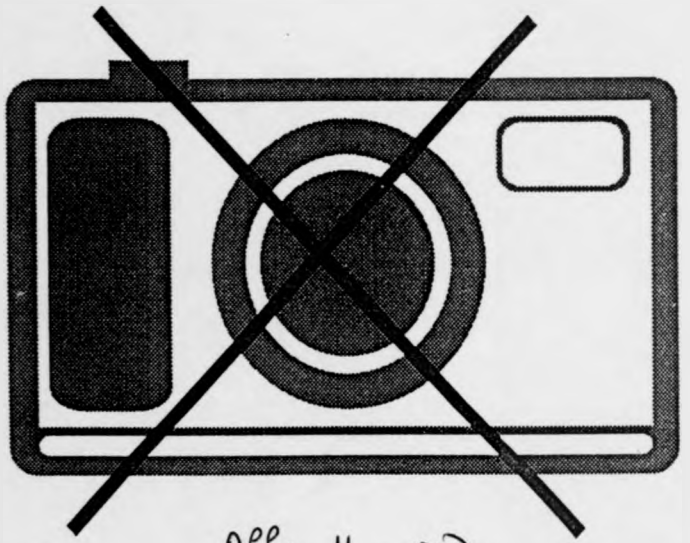

Appx II – END.