The soluble methane monooxygenase and ammonia oxidation in the obligate methanotroph Methylosinus trichosporium (OB3b).

By

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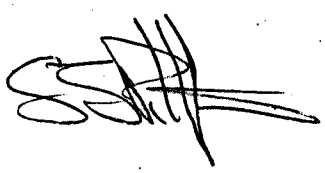
To my Mother, Father and Dave

without whom none of this would have been possible.

Declaration.

The work contained in this thesis is the result of original research conducted by myself under the supervision of Prof. H. Dalton. All sources of information have been specifically acknowledged by means of reference. None of the work contained in this thesis has been used in any previous application for a degree.



S. J. Pilkington

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Summary.

The aim of this project was to isolate and characterise the soluble methane monooxygenase (MMO) from the obligate methanotroph Methylosinus trichosporium (OB3b) and to investigate its role in the oxidation of ammonia.

The nature and location of the MMO was shown to be dependent on the availability of copper to the organism. Cells grown in chemostat culture with copper in excess produced a particulate MMO, whereas under conditions of copper stress a soluble MMO is produced. This response was independent of the carbon and energy source used for growth (methane or methanol).

The soluble MMO was separated into two fractions by DEAE ion exchange chromatography. Each had no MMO activity when assayed individually but had MMO activity when assayed in combination. Fraction A consisted of material that failed to bind to DEAE cellulose and from it component A of the soluble MMO was purified. Component A had an M_r of 230000 and consisted of three subunits α, β and γ of M_r 54000, 40000 and 18500 respectively, suggesting a $\alpha_2\beta_2\gamma_2$ subunit structure. Component A could replace component A of the soluble MMO of Methylococcus capsulatus (Bath) in assays of pure components of the soluble MMO from this organism and was therefore identified as the hydroxylase component of the enzyme.

Fraction C consisted of material eluted from DEAE cellulose by 0.3 M NaCl, from it component C of the soluble MMO was partially purified. Component C was purified to a point where it consisted predominantly of two proteins of M_r 38000 and 58000. Component C could replace component C of the soluble MMO of Methylococcus capsulatus (Bath) in MMO assays of pure components of the soluble MMO from this organism and was therefore identified as the NADH:acceptor reductase component of the enzyme.

The presence of a third component (component B) of the soluble MMO essential for MMO activity was demonstrated. Component B was not purified or isolated from components A or C but it was shown to be analogous to component B of the soluble MMO of Methylococcus capsulatus (Bath).

The close functional and physicochemical similarity between the components of the soluble MMOs from Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) is discussed, as is the distinct difference between the soluble and particulate MMOs from Methylosinus trichosporium (OB3b).

The soluble MMO was shown to oxidise ammonia to hydroxylamine in that: 1. ammonia oxidation required the presence of NAD(P)H for activity as does the soluble MMO; 2. ammonia oxidation was inhibited by acetylene and 8-hydroxyquinoline, specific inhibitors of the soluble MMO, 3. Ammonia oxidation required the presence of both DEAE fractions of the soluble MMO for activity, and 4. ammonia oxidation activity was always associated with the soluble MMO and was never present in extracts lacking soluble MMO activity. Hydroxylamine inhibits the soluble MMO (50% at 1 mM) and this was identified as a cause of the cessation of maximum ammonia oxidising activity after 1 minute in vitro. Only low levels of hydroxylamine oxidoreductase activity were measured in vitro (> 1 nmol/min/mg) and activity failed to be stimulated by the addition of a number of electron donors.

Contents.

List of figures.

List of tables.

Abbreviations.

AMP	Adenosine monophosphate
AMS	Ammonium minimal salts
APBA	Aminophenylboronic acid
ATP	Adenosine triphosphate
DCPIP	Dichloroindolphenol
DEAE	Diethylamino Ethyl
DDTC	Diethyldithiocarbamate
DTT	Dithiothrietol
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
FAD	Flavin adenine nucleotide
GS/GOGAT	Glutamine synthetase / glutamate synthetase
HAO	Hydroxylamine oxidoreductase
HPGPC	High pressure gel permeation chromatography
HPLC	High pressure liquid chromatography
MDH	Methanol dehydrogenase
MMO	Methane monooxygenase
Mr	Molecular radius
MS	Minimal salts
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMS	Nitrate minimal salts
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethyleneglycol
PEP	Phosphoenolpyruvate
PHB	Poly-β-hydroxybutyrate

PMS	Phenazine methosulphate
PMSF	Phenylmethylsulphonylfluoride
RMP	Ribulose monophosphate
RuBP	Ribulose bisphosphate
SDS	Sodium dodecyl sulphate
TCA	Tricarboxylic acid
UV	Ultra violet

Section 1. Introduction.

A.1 Definition of C-1 Utilization.

Methylosinus trichosporium, the subject of this thesis, is a Gram negative aerobic methane oxidizing rod and is a member of a group of organisms known as C-1 utilizers. C-1 utilizing microorganisms are those that have the ability to use C-1 compounds as their sole carbon and energy source.

C-1 compounds are defined as those compounds that are more reduced than carbon dioxide and contain no carbon to carbon bonds, e.g. methane, methanol, S-methyl and N-methyl compounds. Of these compounds methane is the most abundant in nature.

Two groups of these organisms have been identified:

a) The Methylotrophs, as defined by Colby and Zatman (1973), and Quayle and Ferenci (1978), are those organisms that can obtain their energy from the oxidation of C-1 compounds and assimilate carbon as formaldehyde or a mixture of formaldehyde and carbon dioxide.

b) C-1 utilizing autotrophs, as defined by Schlegel (1975), and Quayle and Ferenci (1978), as organisms that can oxidise C-1 compounds to carbon dioxide (in most cases providing energy for growth) and then assimilate the carbon dioxide formed.

It is not the intention here to give a comprehensive review of the physiology and biochemistry of C-1 utilizers, this area has been extensively covered by a number of recent

reviews (Colby, et al.,1979; Higgins et al., 1981b; Anthony, 1982.). Therefore this section of the introduction will primarily be concerned with the physiology and biochemistry of obligate methanotrophs with particular reference to Methylosinus trichosorium (OB3b).

A.2 Occurrence, diversity, ecology and history of the isolation of obligate methanotrophs.

It has been estimated that 50% of the total organic carbon degraded by anaerobic microflora is converted into methane, a total of 1-4 x 10^{15}g of atmospheric methane produced per year (Ehhalt, 1976). Methane produced by non-biological processes has been estimated to be 20-100% of that produced by biological processes (Gold 1979).

With such a large amount of methane produced it is not suprising that organisms have developed the ability to utilize methane as both a carbon and an energy source. The occurrence of these bacteria is widespread. Heyer (1977) found that 90% of 250 samples from a wide range of natural sources contained methane oxidising bacteria. Only samples from the acid soils of heathland and coniferous woods failed to yield methanotrophs. In most habitats where methane diffuses into an aerobic environment, populations of methanotrophs can be found; e.g. soils, surface layers of sediments and natural waters.

One environment that has been extensively studied is that of dimictic lakes. Dimictic lakes during the summer

stratify to form three distict layers. The hypolimnion which is a cool oxygen depleted bottom layer, the metalimnion which is narrower and an area of rapidly decreasing temperature with depth, and the epilimnion, a warm aerobic surface layer. Nearly all methane oxidation by microorganisms is confined to the lower part of the metalimnion where the oxygen tension is very low (Rudd and Hamilton 1975). However in Autumn when the stratification of the lakes breaks down, methane oxidation is observed throughout the water column. For a comprehensive review of the production and oxidation of methane in aquatic environments see Rudd and Taylor (1980).

Considering the widespread natural occurrence of methane-oxidising bacteria it is perhaps surprising that up until a few years ago only a small number of methanotrophs had been isolated. The first well characterised organism was isolated by Söhngen (1906) and named by him Bacillus methanicus. This organism was renamed Pseudomonas methanica, and finally renamed by Foster and Davies (1966) as Methylomonas methanica. Despite vigorous attempts to isolate methanotrophs only two other species were well defined by 1970, Methylomonas methanooxidans (Stocks and McCleskey 1964) and Methylococcus capsulatus (Foster and Davies 1966).

It was not until Whittenbury et al., (1970b) initiated their study of methane oxidisers that a large number (over 100 different stains) of methanotrophs were isolated. The success of Whittenbury and his colleagues in isolating new species of methanotrophs depended on the short enrichment

time employed (3-4 days) which limited losses due to predation and overgrowth by bacteria growing on substrates other than methane. Whittenbury et al., classified their isolates into five groups; Methylosinus, Methylocystis, Methylomonas, Methylobacter, and Methylococcus and further divided them into fifteen subgroups. Since 1970 there have been a number of reports of the isolation of new groups of methylotrophs (Hazeu 1975; Malashenko et. al., 1976). Methylosinus trichosporium (OB3b) was one of Whittenbury's original isolates.

A.3 Morphology and Classification.

The studies by Whittenbury and his colleages (Whittenbury et al., 1970ab; Davies and Whittenbury 1970) on methanotrophs provide a basis for their classification. All of the one hundred and more strains isolated were obligate methanotrophs, aerobic Gram negative, catalase positive bacteria. Many isolates formed heat resistant spores (Whittenbury et al., 1970a). Examination under the electron microscope of sections of methanotrophs revealed a complex structure of intracytoplasmic membranes resembling those already observed in nitrifying and photosynthetic bacteria (Procter et al., 1969; Davies and Whittenbury 1970; Smith et al., 1970). On the basis of their membrane arrangments Davies and Whittenbury (1970) divided the methanotrophs into two groups. Type 1 were described as having bundles or disc shaped membrane vesicles distributed throughout the cell.

Type 2 organisms possessed layers of paired membranes situated around the periphery of the cell. Recently it has been demonstrated that the conditions of growth determine the intracytoplasmic membrane content of certain methanotrophs. Notably, the availability of copper to cells. This also affects the type and position of the methane monooxygenase (MMO) in these bacteria and will be discussed later (Section A.4.1.2b).

Lawrence and Quayle (1970) working on the carbon assimilation pathways in a number of methanotrophs, found a correlation between the type of membrane arrangement and the method employed for carbon assimilation. Type 1 organisms incorporated carbon at the level of formaldehyde using the ribulose monophosphate cycle, type 2 organisms used the serine pathway. Davey et al., (1972), discovered further biochemical divisions between the two groups while examining the activities of key enzymes in cell free extracts of various methanotrophs. Type 2 methanotrophs had a complete set of enzymes for the tricarboxylic acid (TCA) cycle, whereas the type 1 methanotrophs lacked α-ketoglutarate dehydrogenase. An NADP-specific gluconate-6-phosphate dehydrogenase was only detected in type 1 and not type 2 methanotrophs.

Colby et al.,(1979) have recently expanded this classification scheme to accommodate two subgroups in each of the type 1 and type 2 groups of methanotrophs (FIG 1.A.1.). Of type 1 methanotrophs subgroup A consists of bacteria with a DNA base ratio of 50-54 mol % guanine plus cytosine (G+C) and do not fix carbon dioxide

Figure 1.A.1.

Tentative classification scheme for methane-oxidizing bacteria[a]

Determinants	Type I		Type II	
Membrane arrangement	Bundles of vesicular discs		Paired membranes around cell periphery	
Resting stages	Cysts (*Azotobacter*-like)		Exospores or lipid cysts	
Major carbon assimilation pathway	RuMP (hexulosephosphate synthase+)		Serine pathway (hydroxypyruvate reductase +; hexulosephosphate synthase -)	
TCA cycle	Incomplete (2-oxoglutarate dehydrogenase negative)		Complete	
Nitrogenase	Some[+]		+	
Predominant fatty acid C chain length	16		18	
	Subgroup A	Subgroup B	Subgroup obligate[b]	Subgroup facultative[c]
Presence of RuMP carboxylase	-	+	-	
DNA base ratio (%G+C)	50-54	62.5	62.5+ (where tested)	
Isocitrate dehydrogenase	NAD or NADP dependent	NAD dependent	NADP dependent	
Cell shape	Rod & ? coccus	Coccus	Rod and vibrio	
Growth at 45°C	Some +	+	-	
Presence of glutamate dehydrogenase when grown on ammonia	present	absent (uses alanine dehydrogenase)	absent (uses GS/GOGAT)	
Examples	*Methylomonas methanica* and *Methylomonas albus*	*Methylococcus capsulatus*	*Methanomonas methano-oxidans*, *Methylosinus trichosporium* (both obligate) and *Methylobacterium organophilum* (facultative)	

[a] Not all strains classifiable into type I and type II have been shown to possess all the biochemical characteristics outlined in this scheme.

[b] Use methanol and formaldehye as carbon and energy source, but not C_2+ compounds.

[c] Use variety of organic compounds, e.g. glucose as carbon and energy source.

autotrophically. Methylomonas methanica and Methylomonas albus are examples of such organisms. Subgroup B includes bacteria with a DNA base ratio of 62.5 mol % G+C which possess the ribulose monophosphate cycle for carbon assimilation, as do all type 1 methanotrophs. However they also possess some of the enzymes of the serine pathway and Benson-Calvin cycle (phosphoribulokinase and ribulose bisphosphate carboxylase). It is unclear how these pathways contribute to the overall carbon assimilation of these bacteria (Taylor et al.,1980; 1981; Stanley and Dalton 1982). Methylococcus capsulatus (Bath) is an example of such an organism and may well represent a third major type of methanotroph, the so called type X (Whittenbury and Dalton 1981).

The type 2 methanotrophs have been expanded to include the facultative methanotrophs into a subgroup B. Facultative methanotrophs are organisms able to grow on multi-carbon substrates as well as methane. Reports by Patt et al., (1974) and Patel et al., (1978b), describe two strains of a similar organism of a new species Methylobacterium. These organisms are reported to grow on methane, and glucose, ethanol, acetate, and succinate in the absence of methane. Zhao and Hanson (1984) have recently reported the isolation of a facultative methanotroph which would grow in liquid media containing glucose and caesin hydrolysate in the absence of methane, as well as methane and methanol. It possesed a complete TCA cycle, 3-hexulose phosphate synthase (an enzyme of the ribulose monophosphate pathway) and arrays of intracytoplasmic membranes typical of a type 1 methanotroph.

There is now a concensus of opinion that the classification scheme proposed by Whittenbury et al., (1970a,b) should form the basis of any formal taxomony of the methanotrophs. A recent study of the phospholipid, DNA homology and protein patterns as taxanomically useful characteristics, produced results that coincided with the classification scheme of Whittenbury and his collaborators (Galchenko and Andreev 1984).

A.4 Physiology and Biochemistry of Methanotrophs

A.4.1 Carbon Metabolism.

Methanotrophs have the ability to oxidise methane completely to carbon dioxide (FIG 1.A.2.). Methane is first oxidised to methanol via the action of an MMO, methanol is then further oxidised to formaldehyde by a methanol dehydrogenase, and it is at this level that there is a branch in the pathway. Formaldehyde can either be assimilated in the cell to form cell carbon or further oxidised in dissimilatory reactions via formate to carbon dioxide to provide the cell with energy for its assimilatory pathways. Both assimilatory and dissimilatory pathways occur simultaneously in the cell.

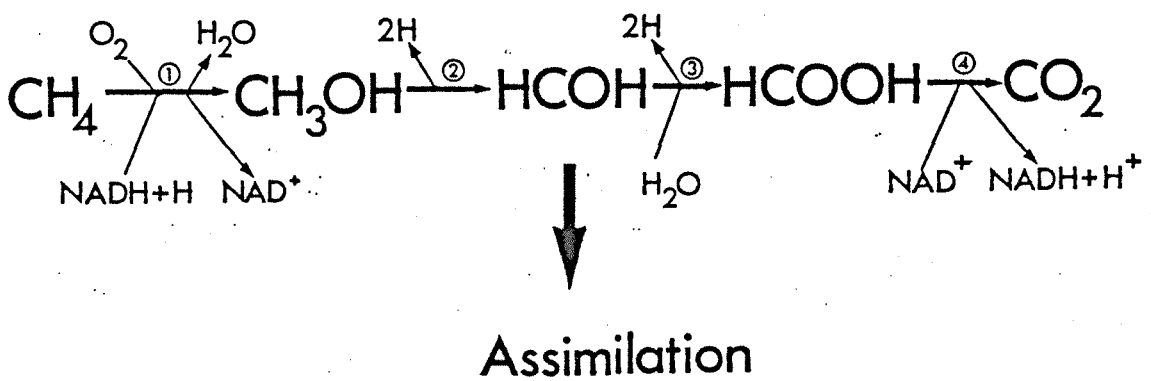


Figure 1.A.2. Pathway for the oxidation of methane to carbon dioxide.

1) Methane monooxygenase. 2) Methanol dehydrogenase.

3) Formaldehyde dehydrogenase. 4) Formate dehydrogenase.

A.4.1.1 Carbon Assimilation Pathways

This aspect of the biochemistry of methanotrophs is not directly related to the work contained in this thesis and so only a brief outline is presented here. For a more comprehensive review the reader is refered to recent reviews by Quayle (1972), Colby *et al.*, (1979), Higgins *et al.*, (1981b) and Anthony (1982).

As already stated the obligate methanotrophs incorporate carbon at the oxidation level of formaldehyde. Type 1 methanotrophs all use the ribulose monophosphate cycle and type 2 methanotrophs the serine pathway to fix formaldehyde.

A.4.1.1a Ribulose monophosphate cycle

The ribulose monophosphate cycle (FIG 1.A.3.) was first proposed by Kemp and Quayle (1967) after radio-isotope studies on C-1 incorporation in *Methylomonas methanica*. The overall effect of the cycle is to synthesise a C-3 compound (either pyruvate or dihydroxyacetone phosphate) from three molecules of formaldehyde. The cycle can conveniently be split into three stages: fixation, cleavage and rearrangement.

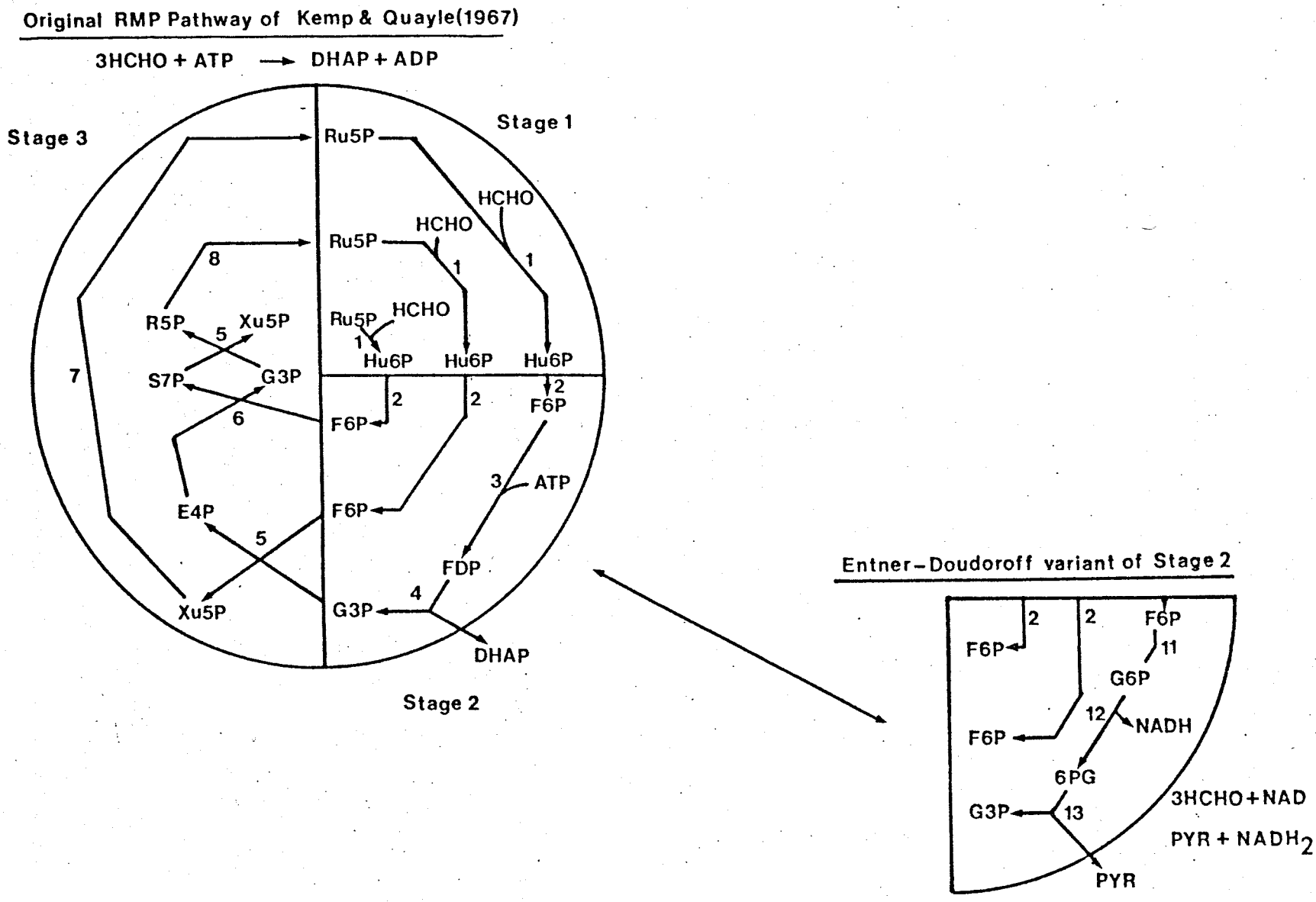
Fixation: by the action of hexulose phosphate synthase three molecules of formaldehyde are condensed with three molecules of ribulose-5-phosphate to give three molecules of hexulose-6-phosphate. This stage is common to all obligate methylotrophs.

Cleavage: one molecule of hexulose-6-phosphate is

Figure 1.A.3. The ribulose monophosphate pathway.

Ru5P	Ribulose-5-phosphate
Hu6P	D-erythro-3-hexulose-6-phosphate
F6P	Fructose-6-phosphate
FDP	Fructose-1,6-diphosphate
G3P	Gyceraldehyde-3-phosphate
E4P	Erythrose-4-phosphate
S7P	Sedoheptulose-7-phosphate
SDP	Sedoheptulose-1,7-diphosphate
R5P	Ribose-5-phosphate
G6P	Glucose-6-phosphate
6PG	6-phosphogluconate
PYR	Pyruvate

1. 3-hexulosephosphate synthase
2. Phospho-3-hexuloisomerase
3. 6-phosphofructokinase
4. Fructose diphosphate aldolase
5. Transketolase
6. Transaldolase
7. Ribulose phosphate epimerase
8. Ribulose phosphate isomerase
11. Glucose phosphate isomerase
12. Glucose-6-phosphate dehydrogenase
13. 6-phosphogluconate dehydratase plus phospho-2-keto-3-deoxygluconate aldolase


Original RMP Pathway of Kemp & Quayle(1967)
3HCHO + ATP → DHAP + ADP
Stage 3
Stage 1
Stage 2
Ru5P
HCHO
R5P
Xu5P
S7P
G3P
E4P
Hu6P
F6P
FDP
ATP
DHAP
Xu5P
Entner-Doudoroff variant of Stage 2
F6P
G6P
NADH
6PG
G3P
PYR
3HCHO+NAD
PYR + NADH2

isomerised to fructose-6-phosphate and this is then split into two C-3 molecules. This being achieved either by the enzymes of the glycolytic sequence or by the Entner-Doudoroff pathway enzymes. Methylococcus capsulatus (Bath) contains both sets of enzymes. However fructose bisphosphate aldolase was not at very high activities in this organism, so clevage of hexulose-6-phosphate and the production of glyceraldehyde-3-phosphate is thought to occur via Entner- Doudoroff pathway enzymes.

Rearrangement: this stage acts to regenerate the three molecules of ribulose-5-phosphate from two molecules of hexulose-6-phosphate and one molecule of glyceraldehyde-3-phosphate, for the completion of the cycle, in a series of reactions similar to those of the ribulose bisphosphate cycle. As in cleavage there are two variants. In methanotrophs these interconversions are carried out by a series of transaldolase and transketolase reactions. In some methylotrophs the rearrangements involve sedoheptulose biphosphatase and fructose bisphosphate aldolase.

A.4.1.1b The Serine Pathway

The serine pathway (FIG 1.A.4.) was proposed by Lawrence and Quayle (1970) after radio-isotope studies of C-1 incorporation in the facultative methylotroph Pseudomonas AM1. The overall result of the pathway is to incorporate two molecules of formaldehyde and one molecule of carbon dioxide into a C-3 compound, 3-phosphoglycerate.

Two molecules of serine are formed from two molecules of glycine plus two molecules formaldehyde by the action of

Figure 1.A.4.

The Serine Pathway.

From Colby et al., 1979.

a, Serine transhydroxymethylase.

b, Serine glyoxylate amino-trasferase.

c, Hydroxypyruvate reductase.

d, Glycerate kinase.

e, Phosphopyruvate hydratase.

f, Phosphoenol-pyruvate carboxylase.

g, Malate dehydrogenase.

h, Malate thiokinase.

i, Malyl-CoA lyase.

j, Isocitrate lyase.

----, Unknown reactions.

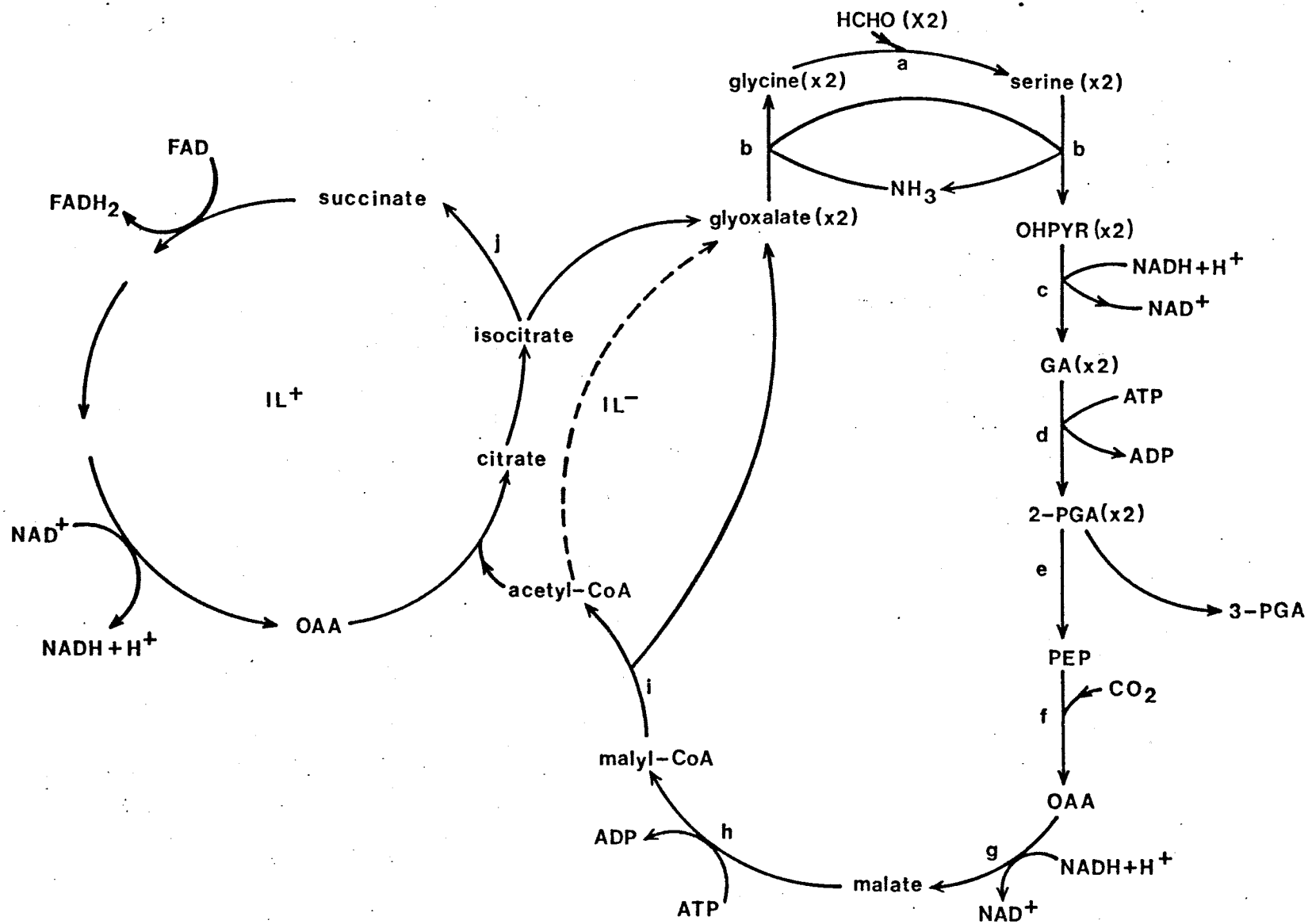
OHPYR, Hydroxypyruvate.

GA, Glycerate.

PGA, Phosphoglycerate.

PEP, Phosphoenol pyruvate.

OAA, Oxaloacetate.


HCHO (X2)
a
glycine(x2)
serine (x2)
b
b
NH3
glyoxalate (x2)
OHPYR (x2)
NADH+H+
c
NAD+
GA(x2)
ATP
d
ADP
2-PGA(x2)
e
3-PGA
PEP
CO2
f
OAA
g
NADH+H+
NAD+
malate
h
ADP
ATP
malyl-CoA
i
acetyl-CoA
citrate
isocitrate
j
succinate
FAD
FADH2
IL+
IL−
NAD+
NADH+H+
OAA

serine transhydroxymethylase. By a series of reactions serine is converted into 2-phosphoglycerate, which is isomerised to 3-phosphoglycerate for assimilation into cell carbon or converted into phosphoenol pyruvate (PEP). PEP carboxylase catalyses the carboxylation of PEP with carbon dioxide to form oxaloacetate, which subsequently forms malyl-CoA. It is at this point, in the regeneration of glycine from malyl-CoA that the two variants of this pathway occur. In organisms possessing isocitrate lyase, malyl-CoA is cleaved to yield glyoxylate and acetyl-CoA. The acetyl-CoA is then converted to glyoxylate via a cycle of reactions involving isocitrate lyase. In organisms lacking this enzyme the route of regeneration of glyoxylate from acetyl-CoA is yet to be elucidated. A recent report implicated the involvment of homoisocitrate lyase and a homoisocitrate/glyoxylate cycle was proposed for the regeneration of glyoxylate from acetyl-CoA (Kortstee 1980). However much doubt has been expressed on the operation of this cycle due to the failure of other workers to repeat this work (Bellion et al., 1981).

A.4.1.1c Other carbon assimilation pathways.

The apparent sharp division between type 1 and type 2 methanotrophs with respect to their carbon assimilation pathways, has recently been challenged by evidence that suggests that certain methanotrophs may use more than one pathway for C-1 assimilation. Possibly the best example of such an organism is Methylococcus capsulatus (Bath).

Methylococcus capsulatus (Bath), a type 1 methanotroph, appeared to use only the ribulose monophosphate cycle for C-1 assimilation (Strøm et al., 1974). However it has now been shown to possess hydroxypyruvate reductase, a key enzyme in the serine pathway, albeit at low levels (Reed 1976). Furthermore, the presence of ribulose 1-5 bisphosphate carboxylase and phosphoribulokinase in this organism (Taylor 1977), has led to speculation on its role as an alternative cleavage route for C-6 molecules in the ribulose monophosphate pathway for the production of glyceraldehyde-3-phosphate. Attempts to grow Methylococcus capsulatus (Bath) autotrophically in the presence of a suitable energy source have failed (Taylor 1979. Stanley and Dalton 1982). The activities of phosphofructokinase and frutose bisphosphate aldolase are low in this organism, so the main pathway for the production of glyceraldehyde-3-phosphate is thought to be via Entner-Doudoroff enzymes. (Taylor 1979; Quayle 1979; Stanley and Dalton 1982).

There is some evidence for the simultaneous functioning of two complete C-1 assimilating pathways in microorganism (Levering et al., 1981) and further work on the alternative C-1 assimilation pathways in type 1 methanotrophs will hopefully explain their role and importance in the overall carbon metabolism of these organisms.

A.4.1.2 Methane Oxidation.

A.4.1.2a Isolation, purification and characterisation of the methane monooxygenase.

The first report of a cell-free methane-oxidising system came from Ribbons and Michalover (1970) using particulate (membrane) preparations of Methylococcus capsulatus (Texas). This was followed by work, again in particulate preparations on Methylomonas methanica (Ferenci 1974; Ferenci et al., 1975.) who measured methane stimulated disappearance of oxygen and NADH. However no methanol accumulation was demonstrated, presumably due to the presence of methanol dehydrogenase and formaldehyde dehydrogenase. Colby et al., (1975) demonstrated the NADH dependent disappearance of bromomethane in a particulate cell free extract of Methylomonas methanica. Bromomethane is a substrate analogue of methane that is oxidised by the MMO. These reports were followed by descriptions of cell free methane-oxidising activity in Methylosinus trichosporium (Tonge et al., 1975.) and Methylococcus capsulatus (Bath), (Colby and Dalton 1976), and it is in these two organisms where the methane-oxidising complex has been purified and studied in detail.

Methylococcus capsulatus (Bath).

A soluble methane monooxygenase (MMO) from Methylococcus capsulatus (Bath) was resolved into three fractions A, B and C by DEAE ion exchange chromatography (Colby and Dalton 1978). Subsequently it has been shown that each fraction contains a single protein essential for in vitro MMO activity. Early reports that fraction B was not essential but merely stimulated MMO activity was explained by contamination of fraction A with fraction B (Colby and Dalton 1976, 1978).

All three components of the soluble MMO from Methylococcus capsulatus (Bath) have now been purified and characterised. Protein A has a relative molecular mass (M_r) of 210000 and is composed of three subunits α, β and γ, of M_r 54000, 42000 and 17000 respectively, indicating that the holoprotein consists of two of each of the three subunits (Woodland and Dalton 1983). The protein is colourless even at concentrations of 50 mg/ml and contains 2.3 ± 0.7 moles of non-haem iron and 0.2-0.5 moles of zinc per mole of protein A, as measured by atomic absorption spectroscopy (Woodland and Dalton 1984a). No acid-labile sulphide was detected (Woodland and Dalton 1984a), though 2 moles per mole protein were reported previously (Dalton 1980). Protein A has no discernible independent catalytic activity. However protein A is believed to be the component responsible for substrate binding. A large change in the ESR spectrum of reduced protein A is observed in the

presence of a substrate (Dalton 1980, Woodland and Dalton 1984a).

Protein B is a colourless protein of M_r 16000 consisting of a single polypeptide. It is devoid of prosthetic groups and like protein A has no discernable independent catalytic activity (Green and Dalton 1985).

Protein C is an iron-sulphur flavoprotein of M_r 38000, consisting of a single polypeptide. It contains one flavin adenine dinucleotide (FAD) molecule and one Fe_2S_2 centre per molecule of C (Colby and Dalton 1978, 1979, Lund 1983). Protein C unlike proteins A or B has a measurable independent catalytic activity. It catalyses the transfer of electrons from NAD(P)H to a variety of electron acceptors such as: DCPIP, potassium ferricyanide, oxygen and protein A, i e., it acts as an NAD(P)H: acceptor reductase.

The recent development of purification procedures for all three proteins has enabled the investigation of the mechanism of methane oxidation by the reconstituted complex. Protein C as already stated can transfer electrons from NADH to a wide range of electron acceptors including protein A. By the use of electronic and electron paramagnetic resonance (EPR) spectroscopy, the ordering of the redox couples of the FAD and Fe_2S_2 redox centres was carried out (Lund and Dalton 1985). The redox centres can exist in odd electron forms, $0e^{-1}$ (oxidised), $1e^{-1}$ (semiquinone), $2e^{-1}$ (mostly semiquinone and reduced Fe_2S_2), and $3e^{-1}$(dihydroquinone and reduced Fe_2S_2). This ability suggests a role for protein C as a $2e^{-1}/1e^{-1}$ transformase, electron pairs from NADH being split up and donated to

protein A as single electrons of equal redox potential. FAD appears to interact with NADH, transfering single electrons onto Fe_2S_2 which donates them to protein A (Lund and Dalton 1985, Lund Woodland and Dalton 1985).

Protein C can therefore pass electrons singly from NADH to protein A at constant redox potential. These electrons can then be used to reduce oxygen to water resulting in an NADH:oxidase activity for the protein A plus C complex (Lund Woodland and Dalton 1985). This occurs in the absence of protein B, and so electron flow is independent of the presence of B, B does however act to shutdown this electron flow (Green and Dalton 1985). On the addition of a suitable substrate, electron flow and oxygen uptake are immediately stimulated, the complete monooxygenase complex being active. The addition of substrate to proteins A and C has no effect on the flow of electrons. Protein B therefore appears to act to couple the flow of electrons from NADH through protein C to protein A to the oxidation of substrate, switching the enzyme complex from an oxidase to an oxygenase (Green and Dalton 1985).

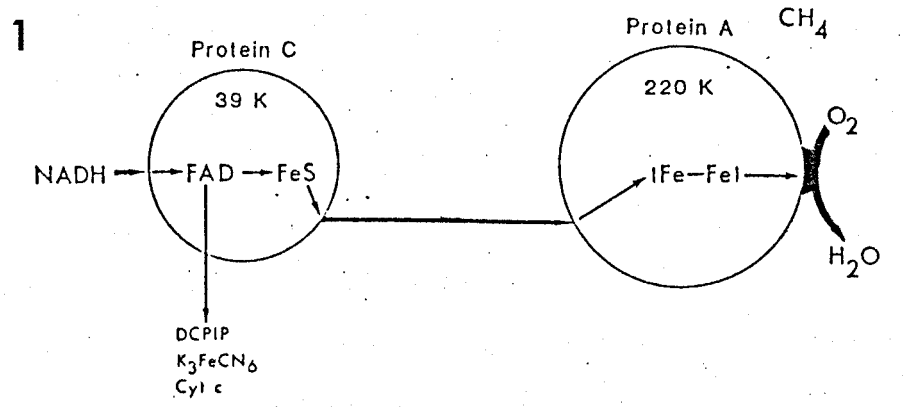
The roles of the three component proteins of the soluble MMO of Methylococcus capsulatus (Bath) have now been elucidated. In summary, the FAD group of protein C interacts with NADH picking up two electrons. These can pass singly to the Fe_2S_2 centre of the protein or can directly reduce a number of electron acceptors. From the Fe_2S_2 centre of protein C electrons pass singly to protein A. In the absence of protein B these electrons reduce oxygen to water. In the presence of protein B electron flow

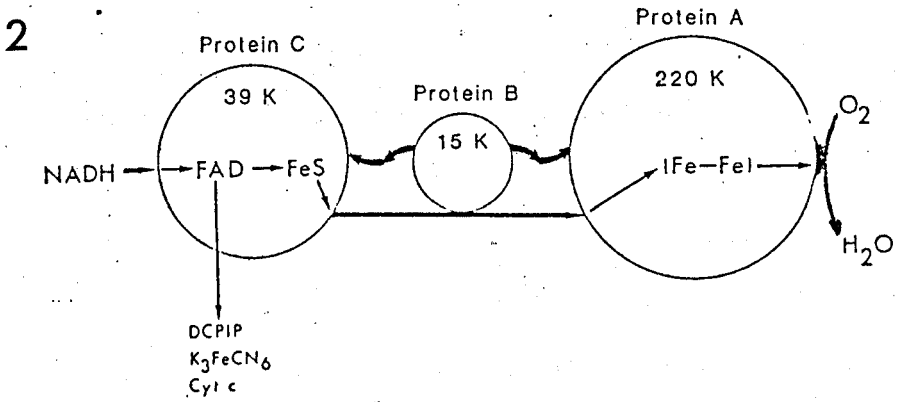
only occurs in the presence of a suitable substrate. Electrons pass from protein C to protein A where the oxygenase reaction is catalysed (Mechanism summarised FIG 1.A.5).

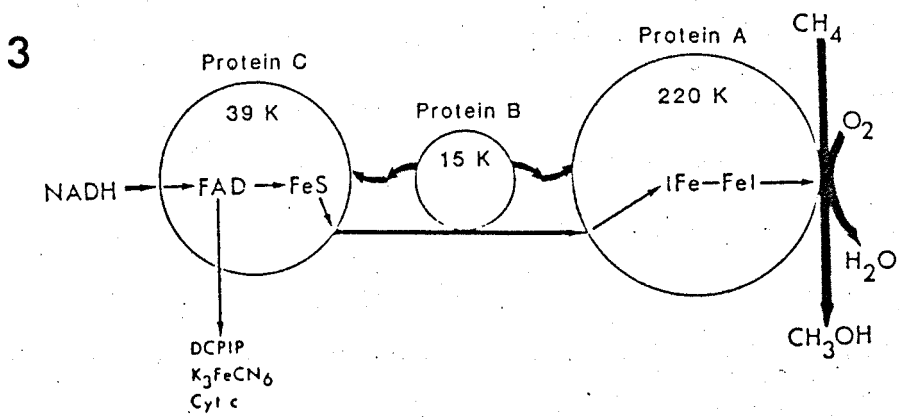
The B protein of the soluble MMO of Methylococcus capsulatus (Bath) acts to couple the flow of electrons to the oxidation of substrate, converting the enzyme from an oxidase to an oxygenase. The enzyme is not simply switched off but uncoupling leads to the wasteful oxidation of NADH, a co-factor thought to be limiting in these organisms (Anthony 1982). It has been suggested that the ability of the soluble MMO to uncouple may be a product of the cells need to regulate the relative levels of NADH and NAD^+ (Green and Dalton 1985). The toxic intermediate formaldehyde has been shown to accumulate in cells of Methylosinus trichosporium (OB3b) that contain particulate MMO when growing on methanol. Cells containing soluble MMO did not contain significant levels of formaldehyde (Cornish et al., 1984). It was suggested that the uncoupled soluble MMO acts as an NADH oxidase providing sufficient NAD^+ for the further oxidation of formaldehyde. The particulate MMO appears to obtain its reducing equivalents from non-NAD^+ linked dehydrogenases and therefore would not act efficiently as an NADH oxidase (Leak and Dalton 1983). Formaldehyde has been shown to uncouple the soluble MMO of Methylococcus capsulatus (Bath), though at high, possibly non-physiological levels (Green and Dalton 1985).

Figure 1.A.5. Mechanism of the soluble MMO from Methylococcus capsulatus (Bath).

1. Components A and C catalyse the four electron reduction of oxygen to water in the presence or absence of a hydroxylatable substrate.
2. The addition of component B switches the enzyme from an oxidase to an oxygenase. In the absence of a hydroxylatable substrate electron flow between components A and C is shut down preventing the reduction of oxygen to water.
3. The addition of a hydroxylatable substrate (methane) to the complete soluble MMO complex restores electron flow between components A and C and the oxygenase reaction is catalysed to the complete exclusion of the oxidase reaction.


1
Protein C
39 K
Protein A
220 K
CH4
NADH
FAD
FeS
IFe–FeI
O2
H2O
DCPIP
K3FeCN6
Cyt c


2
Protein C
39 K
Protein B
15 K
Protein A
220 K
NADH
FAD
FeS
IFe–FeI
O2
H2O
DCPIP
K3FeCN6
Cyt c


3
Protein C
39 K
Protein B
15 K
Protein A
220 K
CH4
NADH
FAD
FeS
IFe–FeI
O2
H2O
CH3OH
DCPIP
K3FeCN6
Cyt c

Methylobacterium sp. Strain CRL-26.

A soluble MMO has been purified and characterised from Methylobacterium sp. Strain CRL-26 (Patel et al., 1982, Patel 1984). It was resolved into three fractions A, B and C by DEAE ion exchange chromatography in an identical manner to the soluble MMO of Methylococcus capsulatus (Bath). The initial report stated that all three fractions were required for maximum activity (Patel et al ., 1982). However subsequently it was reported that fraction B was not required for MMO activity (Patel 1984).

Protein A has a M_r of 220000 and is composed of three subunits α , β and γ , of M_r 60000, 40000 and 20000 respectively, indicating that the holoenzyme consists of two of each of the three subunits. It contains 2.8± 0.2 moles of iron per mole of protein. Protein C consists of a single polypeptide chain of M_r 40000 and contains one FAD molecule and one Fe_2S_2 centre per mole of protein.

This soluble MMO is obviously closely related to that of Methylococcus capsulatus (Bath). The apparent lack of a requirement for a regulatory protein B for monooxygenase activity is a major difference. It will be interesting when mechanistic studies, analogous to those carried out on the soluble MMO of Methylococcus capsulatus (Bath), are performed on this system, confirming or otherwise its independence from a regulatory B protein.

Organism SB1.

A soluble MMO was resolved into two fractions from the type 2 obligate methanotroph organism SB1, using DEAE ion exchange chromatography, both components were required for activity (Allen et al., 1984). The presumed oxygenase component A, was purified to homogeneity, had a M_r of 260000 and is composed of four subunits of M_r 60000, each containing 0.5 g atom iron per mole protein. Visible and EPR spectra confirm the protein to be an iron-sulphur protein. The second component, presumed analagous to protein C of the soluble MMO of Methylococcus capsulatus (Bath), was not purified to homogeneity and so the absence of a third component cannot be unequivocally stated. The subunit M_r of the C component was estimated to be 48000. Visible spectra indicated the presence of a flavin and this fraction possessed NADH:acceptor reductase activity.

Methylosinus trichosporium (OB3b).

a) Soluble.

There has only been a single report on the resolution of a soluble MMO from Methylosinus trichosporium (OB3b) into its component proteins. The studies by Stirling and Dalton (1979) indicated that crude extracts containing MMO activity could be resolved into two components by DEAE ion exchange chromatography. Fraction 1 consisting of material that does not bind to DEAE cellulose, and fraction 2 consisting of the eluate from DEAE cellulose when washed with 20 mM sodium

phosphate buffer containing 0.5M sodium chloride. Neither fraction contained any MMO activity alone, however 25% of the cell free activity was measured when both fractions were assayed together. Full activity was restored to fraction 1 by the addition of DEAE fractions B and C from the soluble MMO of Methylococcus capsulatus (Bath). No complementation of activity was observed with fraction 2 and any components of the soluble MMO of Methylococcus capsulatus (Bath).

These results indicated a similarity between fraction 1 of Methylosinus trichosporium (OB3b) and protein A of the soluble MMO of Methylococcus capsulatus (Bath), the hydroxylase component. Further evidence for similarity between the two components is evident when cell free extracts of Methylosinus trichosporium (OB3b) containing soluble MMO are compared to similar extracts of Methylococcus capsulatus (Bath) as analysed on SDS polyacrylamide gels. The three subunits of Protein A appear as distinct bands, this protein has been estimated to constitute up to 30% of the total soluble protein (Woodland and Dalton 1984). Crude cell free extracts of Methylosinus trichosporium (OB3b) grown under conditions where the soluble MMO was present contained three prominent bands that roughly corresponded (M_r of 53000, 45000 and 23000) to the three subunits of fraction A (Burrows et al., 1984). These bands disappeared when the production of the soluble MMO was repressed (Section A.4.1.2b).

Methylosinus trichosporium (OB3b).

b) Particulate.

A particulate MMO from Methylosinus trichosporium (OB3b) was solubilised from membrane preparations following incubation in the presence of phospholipase-D (Tonge et al., 1975, 1977). This solubilised fraction was further resolved into two fractions by ultrafiltration over a PM10 membrane. From the residue fraction a protein (Protein 1) was purified, with a M_r of 47000 and which contained one atom of copper per protein molecule. From the filtrate protein 2 was purified, it had a M_r of 9400 and no detectable iron or copper. The presence of the copper atom and changes in the EPR copper signal, on the addition of substrate to the reconstituted complex, led Tonge et al., (1977) to propose that protein 1 may be the monooxygenase component responsible for binding oxygen.

For the maximum particulate MMO activity the presence of a soluble carbon monoxide-binding cytochrome c was required. In such preparations ascorbate (1.5mM) could act as an alternative electron donor to NADH, as could methanol in crude extracts that contained methanol dehydrogenase (Tonge et al., 1975). The purified cytochrome c_{CO} has a M_r of 13000, one iron and 0.3-0.8 atoms of copper per molecule of protein, it had NADH/ascorbate oxidase activity and because of this it was proposed to be the reductase component, the immediate electron donor to the particulate MMO (Tonge et al., 1977). However due to its ability to bind not only oxygen and carbon monoxide but also methane

and ethane (Hammond et al., 1979) the possibility of the cytochrome c_{co} being the monooxygenase component was not discounted. However as previously reported the cytochrome c_{co} was not an absolute requirement for particulate MMO activity, activity proceeding at one tenth maximum in its absence. In a later report (Scott et al., 1981a) the addition of cytochrome c_{co} to MMO active crude particulate preparations failed to result in ascorbate-linked MMO activity. Cytochrome c_{co} plays no part in the soluble MMO (Scott et al., 1981a) and its role, if any, in the particulate MMO as an electron donor, is as yet unclear.

A.4.1.2b Soluble and particulate MMO

The enzyme described and purified by Higgins's group from Methylosinus trichosporium was associated with the particulate fraction of the cell free extract. Dalton's group at Warwick University however observed and partially purified a soluble MMO from Methylosinus trichosporium, which had properties similar to the enzyme purified from Methylococcus capsulatus (Bath) (Stirling and Dalton; 1979).

The controversy over these apparently contradictory reports has only recently been resolved by the realisation that the MMO from certain methanotrophs can exist in either a particulate or a soluble form, depending on the conditions under which the organism is grown (Scott et al., 1981ab; Stanley et al., 1983).

Methylosinus trichosporium grown in a chemostat under

oxygen limiting conditions had 100% particulate MMO activity, under nitrate limitation 100% soluble MMO was observed. Limitation by methane produced a mixture of soluble and particulate activities, 78% and 22% respectively (Scott et al., 1981a,b).

Recent studies on Methylococcus capsulatus (Bath) (Stanley et al., 1983) showed that the intracellular location of the MMO depended on the availability of copper and was not dependent on either methane or nitrate limitation. Particulate MMO was observed under conditions with copper in excess, whereas under conditions of copper stress a soluble MMO was found. The two activities were not mutually exclusive and conditions under which both occur could be stably maintained.

The availability of copper has also been shown to control the location of the MMO in Methylosinus trichosporium (Stanley et al., 1982, Burrows et al., 1984). Previous reports linking oxygen, methane and nitrate limitations with the location of the MMO ignored the variation in the cell density of the chemostat cultures used. When this was done, it could be seen that these results were attributable to copper availability.

Both particulate and soluble MMO have been observed in Methylobacterium sp. Strain CRL-26 (Patel et al., 1982). Particulate MMO was present in organisms grown in shake flasks, presumably at low cell density and soluble MMO in fermenter cultures at high cell densities. The effect of copper on the location of the MMO in this organism has yet to be studied, though from the above results it would appear

that it may be similar to Methylococcus capsulatus (Bath).

Concomitant with particulate MMO production is the production of the characteristic extensive membrane arrangements found in methanotrophs. Organisms growing under conditions where the soluble MMO is expressed have a far less extensive system of internal membranes, the "vesicular" membrane arrangement (Scott et al., 1981ab. Cornish et al., 1985. Prior and Dalton 1985).

Not all strains of methanotrophs have the ability to avoid copper limitation and presumably also lack the ability to produce a soluble MMO. Methylocystis parvus (OBBP), Methylomonas albus (BG8) and Methanomonas margaritae are examples of such organisms (Dalton et al., 1984. Takeda et al., 1976). There appears to be no obvious type specific quality to this characteristic. Both type 1 and type 2 methanotrophs have been shown to produce both soluble and particulate MMO and other strains lack the ability to avoid copper limitation.

The soluble MMO appears to be more stable than the particulate MMO and therefore easier to purify and characterise. There has only been a single particulate MMO purified and characterised, although recently this procedure has failed to yield active enzyme (Higgins et al., 1981). In the absence of a repeatable method for the isolation of a particulate MMO, it is difficult to assess whether or not the soluble and particulate MMOs have any components in common or whether they are distinct, unrelated enzymes. However some insight can be gained from the information already available.

The soluble MMO of Methylococcus capsulatus (Bath) is made up of three components, protein A the oxygenase, protein B a regulatory coupling effector and protein C an NADH:acceptor reductase. The particulate MMO appears to accept its electrons, not directly from NADH but via an electron transport chain (Section A.4.1.2c). Protein C of the soluble MMO would therefore be superfluous to the particulate MMO. There is no evidence for the equivalent of protein B of the soluble MMO within the particulate MMO and for reasons discussed previously, the suggested function of protein B may not act efficiently in the particulate MMO system (Section A.4.1.2a).

Protein A can constitute up to 30% of the soluble protein of the cell (Woodland and Dalton 1984), producing very prominant bands on SDS: polyacrylamide gels corresponding to the three subunits. These bands disappeared when cells were induced to produce particulate MMO (Stanley et al., 1983. Cornish et al., 1984). New bands appeared in particulate extracts of cells with the particulate MMO, one of particular interest is a high molecular weight copper containing protein (Dalton et al., 1984), however there were no proteins equivalent to the subunits of protein A of the soluble MMO. Finally, the one purified particulate MMO system, that from Methylosinus trichosporium (OB3b) bears no resemblance to the purified soluble MMO from Methylococcus capsulatus (Bath), an enzyme known to be similar to that found in Methylosinus trichosporium (OB3b) (Stirling and Dalton 1979).

So it would appear that the soluble MMO and particulate

MMO are very different. However not until both enzymes are purified and characterised from the same organism and protein sequencing data is available will we know if these two enzymes bear any resemblance at all.

A.4.1.2c Electron donation to the methane monooxygenase.

The lack of understanding of the effect of growth conditions on the nature of the MMO in certain strains of methanotrophs, has also led to confusion over the source of electrons for the enzyme. However it is now clear that the only known source of electrons for the soluble MMO is NAD(P)H (Colby and Dalton 1978. Scott et al., 1981. Patel et al., 1982). The purified soluble MMO of Methylococcus capsulatus (Bath) has been shown to have a component that directly interacts with NADH, passing electrons to the oxygenase component of the enzyme (Lund et al., 1985).

The provision of electrons to the particulate MMO appears to be more complicated. The first evidence for a difference in the provision of electrons to the particulate MMO as compared to the soluble MMO was that the particulate MMO was shown to be susceptible to a wide range of inhibitors, notably a number of electron transport inhibitors, whereas the soluble MMO was only inhibited by 8-hydroxyquinoline, a chelating agent, and ethyne (acetylene), a suicide substrate for the enzyme. The particulate MMO could accept electrons from NAD(P)H in vitro (Scott et al., 1981. Stanley et al., 1983). Reports of in vitro ascorbate

and methanol:methanol dehydrogenase (MDH) -linked particulate MMO activity have not been substantiated (Tonge et al., 1975,1977. Scott et al., 1981. Dalton et al., 1984). However in vivo ethanol can act as an electron donor for the particulate MMO in Methylococcus capsulatus (Bath) and Methylomonas methanica (Ferenci et al., 1975. Leak and Dalton 1983), ethanol being oxidised to acetaldehyde by the MDH, producing electrons which could either flow directly to the particulate MMO or by reverse electron transport, produce NADH which would then donate its electrons to the particulate MMO. Recently Cornish et al., (1985), demonstrated succinate-linked NADH-independent particulate MMO activity in cell free extracts of Methylosinus trichosporium (OB3b). Along with the above evidence this suggests that the particulate MMO can obtain electrons in vivo from the respiratory chain and NAD^+-independent dehydrogenases such as MDH. This theory has also been invoked to explain the increased yields of methanotrophs observed in cells containing the particulate MMO compared with those containing the soluble MMO (Leak et al., 1985). A candidate for the immediate electron donor to the particulate MMO is the cytochrome c_{co}, which greatly stimulated the particulate MMO activity when added in a purified form to extracts of Methylosinus trichosporium (OB3b) (Tonge et al., 1975). Cytochrome c_{co} is known to be involved in the oxidation of methanol in methylotrophs (Beardmore-Gray et al., 1983; Section A.4.1.3). A scheme has been proposed, where cytochrome c_{co} is reduced in the oxidation of methanol to formaldehyde, and then donates

these electrons to the particulate MMO for the oxidation of methane to methanol (Anthony 1982). However for reasons discussed previously the role of cytochrome c_{co} in the provision of electrons for the particulate MMO still remains unclear (Section A.4.1.2a).

A.4.1.2d Substrate specificity.

One of the major incentives to research in the biochemistry of methylotrophs is the industrial exploitation of the MMO's unusual lack of substrate specificity. MMO from several species of methanotrophs have been shown to insert an oxygen atom into a wide range of substrates. These include, carbon monoxide (Ferenci 1974), ammonia (Dalton 1977), n-alkanes, n-alkenes, dimethyl and diethyl ether, alycyclic and aromatic hydrocarbons (Colby et al., 1977; Stirling et al., 1979; Higgins et al., 1980.). Oxidation by MMO can produce a molecule that is not further metabolised such as 1-2 epoxypropane from the oxidation of propene or a molecule that is further oxidised by other oxidoreductases such as benzyl alcohol from the oxidation of toluene, which is further oxidised to benzoic acid.

The particulate MMO from a number of organisms oxidises a more restricted range of compounds than the soluble MMO. The particulate MMO can oxidise n-alkanes and n-alkenes but not aromatics or alicyclics (Stirling et al., 1979. Burrows et al., 1984). Whether this is due to differences in the active sites of the enzymes or inhibition by co-metabolites

of the electron transport chain associated with the particulate MMO, is yet to be resolved. For a comprehensive review of the oxidation of hydrocarbons by methanotrophs see Dalton (1980).

A.4.1.3. Methanol Oxidation

A broad specificity NAD(P)-independent methanol dehydrogenase is common to all methane-oxidising bacteria so far studied (Wadzinski and Ribbons, 1975a. Patel and Felix, 1976.), an enzyme first characterised by Anthony and Zatman (1964) from the methanol-utilizing Pseudomonas sp M27. The methanol dehydrogenase from these sources has an M_r of 120000 and is comprised of two equal subunits of M_r 60000. *in vitro*, the enzyme activity can be assayed by coupling it to the reduction of phenazine methosulphate (PMS) in the presence of ammonium ions at pH 9.0. *in vivo*, the enzyme is thought to be coupled to the electron transport chain at the level of cytochrome *c* (Duine and Frank 1981b). However only recently has this activity been demonstrated *in vitro* using anaerobically prepared enzyme and cytochrome *c* (Beardmore-Gray *et al.*, 1983).

Methanol dehydrogenase is found in the soluble fraction, released from the membrane by cell breakage (Wadzinski and Ribbons 1975 . Alefounder and Ferguson (1981) investigated the methanol dehydrogenase in Paracoccus denitrificans a facultative chemoautotroph able to grow on methanol and found it to be soluble and located on the

periplasmic side of the membrane. Methanol dehydrogenase as well as both cytochrome c's (low and high isoelectric point forms) found in Methylophilus methylotrophus are found almost exclusively in the periplasmic space (Jones et al., 1982). Methanol dehydrogenase has been shown to pass electrons only to the low isoelectric point form of cytochrome c (Beardmore-Gray et al.,1983). Formaldehyde is also a substrate for methanol dehydrogenase catalysing its oxidation to formate.

Recently the nature of the novel prosthetic group of the methanol dehydrogenase has been determined to be a pyrrolo-quinoline quinone (Duine et al., 1980), though novel at the time it has subsequently been found in a number of other dehydrogenases, glucose dehydrogenase (Duine et al., 1979) alcohol dehydrogenase (Duine and Frank 1981 and aldehyde dehydrogenase (Ameyama et al., 1981).

A.4.1.4 Formaldehyde and Formate Oxidation

There are three possible routes of formaldehyde oxidation in obligate methanotrophic bacteria. The first is oxidation by formaldehyde dehydrogenase to give formate. There exist two forms of this enzyme, an NAD(P)-linked and a NAD(P)-independent form. Stirling and Dalton (1978) purified a NAD(P) linked formaldehyde dehydrogenase from Methylococcus capsulatus (Bath) of M_r 115,000. Formate so formed is further oxidised by a NAD(P)-linked formate dehydrogenase to carbon dioxide (Stirling and Dalton 1978).

The second route is via a cyclic series of reactions involving hexulose phosphate synthase which yields carbon dioxide and two molecules of NAD(P) (Strøm et al., 1974).

In addition to oxidising methanol, methanol dehydrogenase will also oxidise formaldehyde to formate in vitro, whether or not it functions in vivo, in this way is not known.

A.4.2. Nitrogen Metabolism

The obligate methanotrophs have the ability to utilize nitrate and ammonia as nitrogen sources and some can fix dinitrogen.

A.4.2.1 Ammonia Assimilation.

The only detailed study of nitrogen metabolism has come from Murrell (1981), Murrell and Dalton (1983a,b), who studied the nitrogen assimilation pathways in Methylomonas methanica, Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath).

All obligate methanotrophs were shown to metabolise ammonia via the glutamine synthetase / glutamate synthase (GS/GOGAT) pathway. However type 1 organisms also possess other ammonia assimilating systems, the pathway of ammonia assimilation being determined by the fixed nitrogen source present. Type 2 organisms assimilate ammonia exclusively via the GS/GOGAT pathway whether grown with ammonia, nitrate or

dinitrogen.

During growth on nitrate type 1 organisms possessed high levels of GS/GOGAT activities with little or no alanine dehydrogenase or glutamate dehydrogenase activity. Growth with ammonia repressed GS/GOGAT pathway enzymes and ammonia was assimilated by alanine dehydrogenase and glutamate dehydrogenase.

A.4.2.2 Nitrogen Fixation.

A survey of a wide range of obligate methanotrophs for their ability to fix nitrogen once again indicated a fundamental difference between type 1 and type 2 methanotrophs (Murrell 1981; Murrell and Dalton 1983b). Of seven type 1 organisms only Methylococcus capsulatus (Bath) was shown to fix nitrogen. All type 2 organisms tested were found to fix nitrogen.

Methylococcus capsulatus (Bath) as already stated has a number of characteristics which separate it from other type 1 methanotrophs. Its ability to fix nitrogen provides further support for its inclusion in a separate group, the so called type X methanotrophs (Whittenbury and Dalton 1981).

A.4.2.3 Ammonia Oxidation.

The first report of ammonia oxidation by a methanotroph was by Hutton and Zobell (1953), who reported the production of nitrite in media containing ammonia as the nitrogen source by an unidentified type 1 methanotroph. Whittenbury et al., (1970b) found all their isolates formed non-inhibitory levels of nitrite when grown on an ammonia minimal salts medium. Subsequently there have been a number of short reports concerning ammonia oxidation by methanotrophs (Colby et al., 1975; Ferenci et al., 1975. Drozd 1980). The most comprehensive work to date has been that of O'Neil and Wilkinson (1977) working with whole cells of Methylosinus trichosporium (OB3b), Dalton (1977) and Pilkington (1983), working with cell-free extracts of Methylococcus capsulatus (Bath), and Sokolov et al., (1980; 1981) working with cell-free extracts of Methylococcus thermophilus, who concentrated on the oxidation of hydroxylamine.

O'Neil and Wilkinson (1977), working with whole cell suspensions of Methylosinus trichosporium (OB3b) measured ammonia oxidation and hydroxylamine oxidation by the measurement of oxygen uptake and nitrite formation. Ammonia oxidation was 4% of the methane oxidation rate as measured by oxygen uptake. Measurements of ammonia disappearance, nitrite appearance and oxygen consumption were consistent with the stoichiometry:

$$NH_4^+ + 1\tfrac{1}{2}\, O_2 \longrightarrow NO_2^- + 2H^+ + H_2O$$

The same as that established for the autotrophic

ammonia-oxidising bacteria (Hofman and Lees 1953).

A number of compounds were shown to stimulate the oxidation of ammonia but not hydroxylamine, including; formate, formaldehyde, pyruvate, acetate and methanol. The effect of methane on the oxidation of ammonia was complex, at low levels methane stimulated ammonia oxidation, whereas at high levels it was inhibitory. The Km for ammonium ion (NH_4^+) at pH 6.5 was 4.1 mM and at pH 7.5 was 0.6 mM. Recalculating the Km in terms of ammonia (NH_3) concentration (using the Henderson-Hasselbach equation) gives essentially similar values of 7.4 and 10.4 micromolar respectively. The competitive inhibition of methane oxidation by ammonium ion also rises with pH. Both these results suggest that ammonia and not ammonium ion is the substrate for the enzyme.

Dalton (1977), working with soluble extracts of Methylococcus capsulatus (Bath), presented strong evidence in support of the hypothesis that the MMO was responsible for the oxidation of ammonia to hydroxylamine. This was presented as four main points; 1) Both methane and ammonia oxidation require the presence of reduced pyridine nucleotides and oxygen for activity. 2) The oxidation of methane to methanol and ammonia to hydroxylamine were specifically inhibited by acetylene, 8-hydroxyquinoline and methanol. 3) Neither methane nor ammonia oxidation were inhibited by thiourea, hydrazine, diethyldithiocarbamate (DDTC) and α,α'-dipyridyl. 4) Methane was a good inhibitor of ammonia oxidation.

Pilkington (1983) using purified components of the soluble MMO of Methylococcus capsulatus (Bath), showed

unequivocally that ammonia oxidation was catalysed by the soluble MMO and also presented evidence that suggested that the particulate MMO also catalysed this reaction. The pH optimum for the reaction catalysed by the soluble MMO was 7.6, a higher value than that reported for the oxidation of other substrates (Colby and Dalton 1976). Unlike the system in Nitrosomonas (Suzuki et al., 1974) the Km with respect to both ammonia (NH_3) and the ammonium ion (NH_4^+) rose with pH, being 0.18mM and 32.5mM at pH 7.0, and 0.73mM and 41.2mM at pH 7.5, respectively (Dalton 1977, Pilkington 1983). Hydroxylamine, the product of the oxidation of ammonia, was a strong inhibitor of the soluble MMO (49% at 1mM) and its accumulation was identified as the cause of the cessation of ammonia oxidation activity after one minute in assays of crude or partially purified soluble MMO where the hydroxylamine oxidoreductase was inactive.

In whole cell suspensions of Methylococcus NCIB 11083 a lower Km for the ammonium ion of 2.8 mM at pH 7.0 was observed, and hydroxylamine oxidation was partially competitive with methanol oxidation in whole cells (Drozd 1980).

Recently there have been a number of reports on the inhibition of ammonia and methane oxidation in the autotrophic ammonia-oxidising bacteria and methanotrophs by nitrapyrin (2-chloro-6-(trichloromethyl) pyridine) and other pyridine derivatives (Topp and Knowles 1982, 1984; Salvas and Taylor 1984). The aim of this work has been to try to provide the means to differentiate between ammonia oxidation carried out by the autotrophic ammonia-oxidisers and ammonia

oxidation carried out by the methanotrophs in the environment. Only picolinic acid (2-carboxy pyridine) showed any specificity, inhibiting ammonia oxidation by methanotrophs but not by the autotrophic ammonia-oxidisers (Salvas and Taylor 1984).

A.4.2.4 Hydoxylamine Oxidation

Dalton (1977) showed that hydroxylamine was an intermediate in the oxidation of ammonia to nitrite in Methylococcus capsulatus (Bath). From inhibitor profiles of methane, methanol and hydroxylamine oxidation it was deduced that the enzyme responsible for hydroxylamine oxidation was neither MMO or methanol dehydrogenase. A purified preparation of methanol dehydrogenase showed no hydroxylamine oxidoreductase (HAO) activity nor did methanol inhibit this activity in crude soluble extracts. HAO activity was stimulated by the presence of phenazine methosulphate (PMS). Pilkington (1983) partially purified the HAO from Methylococcus capsulatus (Bath) separating it from both soluble MMO and methanol dehydrogenase. It was associated with the membrane and had hydroxylamine-dependent cytochrome c reductase activity, although cytochrome c was unable to act as an electron acceptor for hydroxylamine oxidation.

Using crude soluble extracts of Methylococcus thermophilus, Sokolov et al., (1980) showed that the HAO and methanol dehydrogenase activities could be separated by ion exchange chromatography. The pH optimum for HAO was 9.0 and

associated with it was hydroxylamine cytochrome c oxidoreductase activity that was inhibited by cyanide. The differential absorption spectra of their preparation was characterised by absorption bands at 552 nm, 523 nm and 419 nm corresponding to the alpha, beta and gamma peaks of cytochrome c. Shoulders at 557 nm and 531 nm corresponded to the alpha and beta peaks of cytochrome b.

Subsequently Sokolov et al., (1981) further purified the HAO and methanol dehydrogenase along with a number of cytochrome components from Methylococcus thermophilus. From the properties presented they proposed a scheme for electron transport during methanol and hydroxylamine oxidation (FIG 1.A.6.). For hydroxylamine oxidation this involved the passage of four electrons from hydroxylamine to a cytochrome c554 via HAO, which were then passed on to a terminal cytochrome a oxidase. Previously it had been shown that ATP, and, via reverse electron flow, NADH could be synthesised, coupled to the flow of electrons from hydroxylamine to the terminal oxidase (Malashenko et al., 1979).

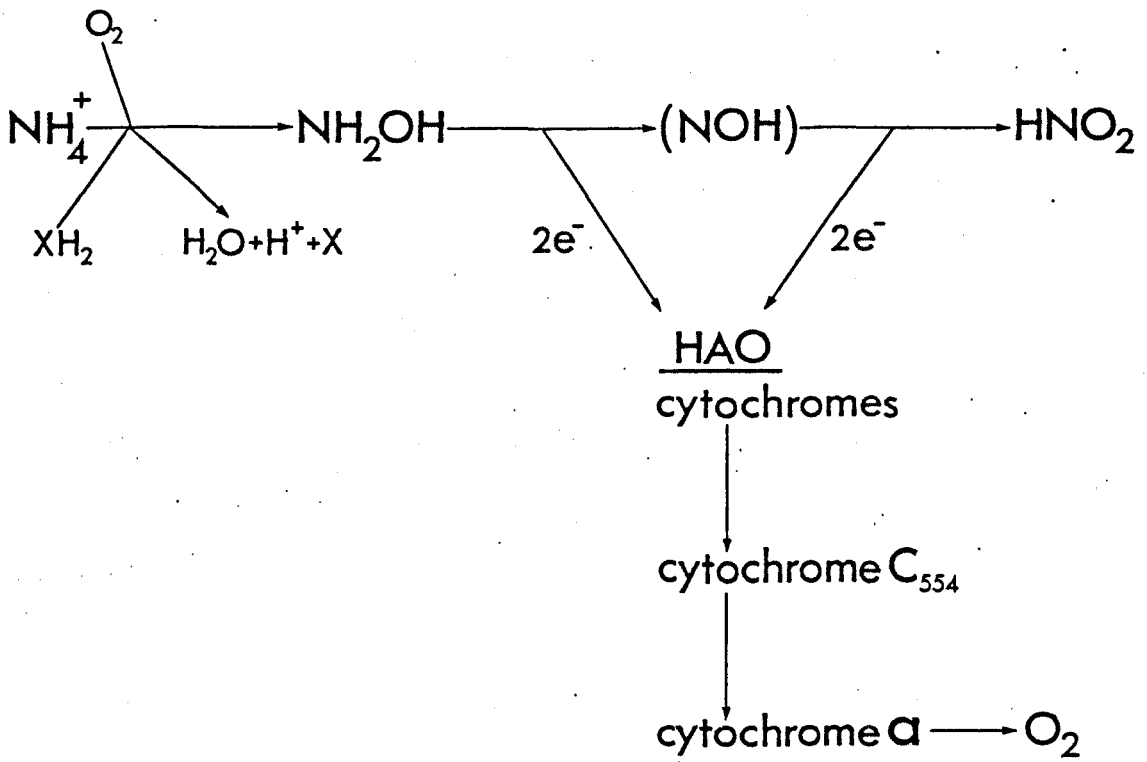


Figure 1.A.6. Proposed electron transport during the oxidation of ammonia by Methylococcus thermophilus. Taken from Sokolov et al., 1981.

X -electron donor for methane monooxygenase.

B. Ammonia oxidation in the chemolithotrophic ammonia oxidising bacteria.

Ammonia is oxidised by both heterotrophic and autotrophic bacteria, as well as some methylotrophic bacteria. In this section it is my intention to restrict discussion to the ammonia-oxidising chemolithotrophic bacteria typified by Nitrosomonas europeae, largely because there appears to be a number of similarities in the oxidation of ammonia in this organism and the methanotrophs. For discussion of heterotrophic ammonia oxidation the reader is refered to a number of other papers (Hirsch et al., 1961; Verstraete and Alexander 1972 a,b).

B.1. The Nitrobacteriaceae.

Autotrophic nitrification is associated with the family Nitrobacteriaceae. All organisms from this group are capable of deriving all their energy for growth from the oxidation of either ammonia or nitrite. These organisms were first described by Winogradsky (1890) in his classical early studies of the nitrifying bacteria.

Bergey's manual (1984), lists four genera which oxidise ammonia, these being; Nitrosomonas, Nitrosospira, Nitrosococcus and Nitrosolobus. Of these Nitrosomonas is the most commonly isolated and presumed to be the most common in nature. This is a very large assumption to make,

the procedure employed in the isolation of any bacterium will tend to select from a group of metabolically similar organisms, that organism which can best survive and grow under the conditions of isolation, not necessarily that organism which predominates in the natural environment (Belser and Schmidt, 1978).

However Nitrosomonas has been isolated from a wide variety of terrestrial and aquatic environments and may well be the dominant organism in at least some of these, and it is the organism on which the bulk of the research on the biochemistry and physiology of autotrophic ammonia oxidation has been undertaken.

B.2. Ammonia oxidation.

B.2.1. Enzymology.

The overall reaction for the oxidation of ammonia to nitrite carried out by Nitrosomonas is:

$$NH_4^+ + 1\tfrac{1}{2}\ O_2 \longrightarrow NO_2^- + 2H^+ + H_2O$$

hydroxylamine was shown to be the first stable intermediate (Lees, 1952. Hoffman and Lees, 1953.). In the above equation the substrate is written as the ammonium ion (NH_4^+), although measurements on the rate of ammonia utilization and Km values of whole cell ammonia oxidation at various pH values suggest that ammonia (NH_3) is the actual substrate (Suzuki et al., 1974, Drozd, 1976).

The first step of ammonia oxidation is normally written:

$$NH_3 + O_2 + XH_2 \longrightarrow NH_2OH + H_2O + X$$

although there is some degree of uncertainty as to the exact mechanism of the ammonia oxidation. The above reaction is catalysed by an enzyme variously called ammonia hydroxylase, ammonia oxygenase, or ammonia monooxygenase. Whole cell studies of ammonia oxidation by Nitrosomonas showed a small incorporation of $^{18}O_2$ into nitrite (Rees and Nason 1966) which suggested direct incorporation of oxygen into ammonia at some stage of its oxidation though not necessarily in the initial reaction. ^{15}N and ^{18}O tracer experiments using highly enriched ^{15}N-ammonium chloride and ^{18}O-dioxygen have now established that the production of hydroxylamine from ammonia involves a monooxygenase-type reaction by way of direct insertion of one atom from dioxygen, the other presumably being reduced to water (Hollocher et al., 1981).

Little is known about the initial oxidation of ammonia due to the difficulty in preparing active ammonia-oxidising cell free extracts of Nitrosomonas (Watson et al., 1970, Suzuki and Kwok, 1970). Recently however Suzuki et al.,(1981) have described conditions under which active ammonia-oxidising membrane preparations can be made from whole cells of Nitrosomonas. Bovine serum albumin, spermine or magnesium chloride were required for ammonia oxidation and the concentration of phosphate determined the

effectivness of these activators. Phosphate could be replaced by chloride, sulphate or nitrate so its effect was proposed to be ionic, although its mechanism was not clear. The cell free extract obtained oxidised ammonia to nitrite, a functional separation of the ammonia monooxygenase from the HAO has yet to be achieved.

Suzuki and Kwok (1981) resolved this extract into three components each required for ammonia oxidation. A membrane fraction containing cytochrome a1 and c type cytochromes, a fraction with hydroxylamine cytochrome c reductase activity and a cytochrome c fraction. Ammonia oxidation activity, as measured by ammonia-dependent oxygen uptake and nitrite formation, could be reconstituted by the combination of these three fractions, however more consistent results were obtained by the addition of Nitrosomonas cytochrome c554 purified earlier by Yamanaka and Shinra, (1974), which increased the hydroxylamine cytochrome c reductase activity. Addition of Nitrosomonas cytochrome c552 also purified earlier by Yamanaka and Shinra (1974), was required for the oxidation of hydroxylamine to nitrite. Cytochrome c554 probably acts to mediate electron transfer between hydroxylamine oxidation and ammonia oxidation (Suzuki and Kwok, 1981).

Ammonia oxidation is known to be inhibited by a wide range of metal-binding agents, uncouplers of oxidative phosphorylation, carbon monoxide and electron acceptors such as PMS. The electron acceptors probably oxidise the electron donor, XH_2, while the uncouplers of oxidative phosphorylation may act to destroy membrane potential.

Inhibition by chelating agents such as thiourea suggests the involvememnt of a metal ion such as copper (Shears and Wood 1985).

Further evidence for the involvement of copper comes from the discovery that ammonia oxidation in whole cells of Nitrosomonas is photosensitive to near U.V. light (Hooper and Terry 1974; Shears and Wood 1985). Illumination also causes bleaching in the absorption spectrum around 380nm, along with changes in the cytochrome gamma-band (Soret) region. Similar sensitivity to U.V. light was seen with the copper-containing proteins haemocyanin and tyrosinase and was attributed to the liberation of a highly reactive superoxide ion at the active site. Tyrosinase is the generic name for a group of copper proteins that typically have both catechol oxidase and monophenol monooxygenase activities. From their results Shears and Wood (1985) suggested that the ammonia monooxygenase contained two copper atoms at its active site and had a three stage catalytic cycle analogous to that of tyrosinase.

Recently Hyman and Wood (1985) demonstrated that acetylene was a suicide substrate for ammonia monooxygenase. Acetylene oxidation is thought to yield an unsaturated epoxide (oxirene) which being highly reactive and unstable, covalently bonds to the ammonia monooxygenase by either, reacting with the prosthetic group of the enzyme in an analogous way to the mechanism proposed by Ortiz de Montellano and Kunze (1980) for the inactivation of cytochrome P-450 or, may attack one or more of the amino acid side chains that bind the copper (Hyman and Wood 1985).

Incubation of cells with ^{14}C-acetylene caused the labelling of a single membrane polypeptide of M_r 28000. This provides the first identification of a constituent polypeptide of the ammonia monooxygenase.

B.2.2. Substrate specificity.

As in the methanotrophs, whole cells of Nitrosomonas have recently been shown to oxidise a wide range of substrates. These include methane which is oxidised to methanol and carbon dioxide (Hyman and Wood, 1983; Jones and Morita, 1983), ethylene to ethylene oxide (Hyman and Wood 1984), carbon monoxide to carbon dioxide (Jones and Morita 1984), propylene to propylene oxide, benzene to phenol and cyclohexane to cyclohexanol (Drozd, 1980). Acetylene a potent inhibitor of the MMO, has also been shown to strongly inhibit the ammonia monooxygenase (Hynes and Knowles, 1982). Obviously the non-specific nature of the ammonia monooxygenase is similar to that of the MMO. The oxidation of methane is therefore of particular interest.

Methane was shown to be a competitive inhibitor of ammonia oxidation in Nitrosomonas (Suzuki et al., 1976). Hyman and Wood (1983) demonstrated the production of methanol from methane in whole cells of Nitrosomonas, this oxidation was inhibited by the same spectrum of inhibitors as ammonia oxidation. This evidence led Hyman and Wood (1983) to conclude that the ammonia monooxygenase also catalysed the oxidation of methane to methanol. Jones and Morita (1983) examined a range of ammonia-oxidising and

nitrite-oxidising bacteria for their ability to oxidise 14C-methane. All the ammonia oxidisers, but none of the nitrite oxidisers tested, had the ability to oxidise methane to carbon dioxide and incorporate various amounts of carbon into cellular components. The addition of ammonia (10 ppm) stimulated both carbon dioxide formation and cellular incorporation of carbon derived from methane, as did the presence of nitrate, nitrite and yeast extract. Ammonia may activate the enzyme system, leading to increased levels of methane oxidation. However levels of ammonia above 10 ppm decreased the rate of methane oxidation. Stimulation by nitrate, nitrite and ammonia may also be caused by the presence of a nitrogen source in the assay (Jones and Morita 1983).

B.3. Hydroxylamine Oxidation

Unlike the ammonia monooxygenase the hydroxylamine oxidoreductase (HAO) from Nitrosomonas europeae has been purified and extensively characterised (Hooper and Nason 1965). HAO had an M_r of 220000 and an alpha3, beta3 subunit structure (Terry and Hooper 1981). The alpha subunit had an M_r of 63000 and contains six c-type haems and a single P460 haem, this being a novel CO-binding haem unique to the ammonia-oxidising autotrophs. The beta subunit was a mono-haem cytochrome c and had an M_r of 11000.

HAO is a soluble, extracytoplasmic (periplasmic) enzyme (Hooper et al., 1972. Olson and Hooper, 1983) which, in

vitro, requires the presence of a suitable electron acceptor (PMS, cytochrome *c*) to catalyse the two electron dehydrogenation of hydroxylamine to a compound at the oxidation state of nitroxyl (HNO). This is further oxidised to nitrite by HAO. The enzyme will also catalyse the oxidation of hydrazine to dinitrogen (N_2) and the hydroxylamine-dependent reduction of cytochrome *c*. Experimentally, in whole cells 100% of the hydroxylamine oxidised is converted to nitrite, however in cell extracts and purified preparations of HAO less than 50% of the hydroxylamine oxidised appeared as nitrite (Hooper *et al.*, 1977) and although nitrite was thought to be the end product of ammonia oxidation in *Nitrosomonas*, nitrate as well as nitrite was produced in roughly equimolar amounts by partially purified preparations of HAO (Hooper *et al.*, 1977). The capacity of the HAO *in vitro*., to produce nitrate results not from the addition of an artificial electron acceptor such as PMS but from a change in the enzyme itself or from its association with other enzymes upon cell breakage (Hooper *et al.*, 1977). In the presence of diethyldithiocarbamate (DDTC), nitrite alone is produced which is coupled to the oxidation of DDTC (Hooper *et al.*, 1977). DDTC may act by either: a) reducing a reactive form of oxygen generated during the oxidation of hydroxylamine, thus preventing the oxidation of an intermediate to nitrate; or b) reducing a nitrogen-containing intermediate of nitrate to a nitrogen compound which is subsequently oxidised to nitrite. The production of nitrate may well be the result of chemical reactions between enzymatically produced nitric

oxide with oxygen and water, or the oxidation of hydroxylamine may result in the activation of an oxygen atom which reacts with an N-containing intermediate such as nitroxyl (HNO) or N_2O_4.

Nitrous oxide can also be a product of the oxidation of ammonia and hydroxylamine by whole cells of Nitrosomonas (Richie and Nicholas, 1972). It's production has also been demonstrated in cell-free extracts with hydroxylamine as the oxidisable substrate. Recently by the use of ^{18}O ^{15}N labelled hydroxylamine and analysis of the products by NMR, the origin of the second oxygen in nitrite was found to be water, showing the HAO to be a dehydrogenase rather than an oxygenase (Anderson and Hooper 1983). Previous attempts to determine the source of oxygen were hampered by a rapid exchange between the oxygens of nitrite and water catalysed by Nitrosomonas (Anderson et al.,1982).

B.4. Mechanism for the oxidation of ammonia to nitrite.

It is now possible to construct a scheme for the oxidation of ammonia to nitrite (FIG 1.B.1.) centering around the functioning of a periplasmic dehydrogenase (HAO) and an inner membrane-bound terminal oxidase (cytochrome a1) (Hooper et al 1984). As already stated, Suzuki and Kwok (1981) reported the isolation of an active membrane-bound ammonia-oxidising system, which required the addition of purified cytochrome c554. Subsequently carbon monoxide- and ammonia-dependent re-oxidation of cytochrome c554 and oxygen uptake has been demonstrated using this system (Tsang and

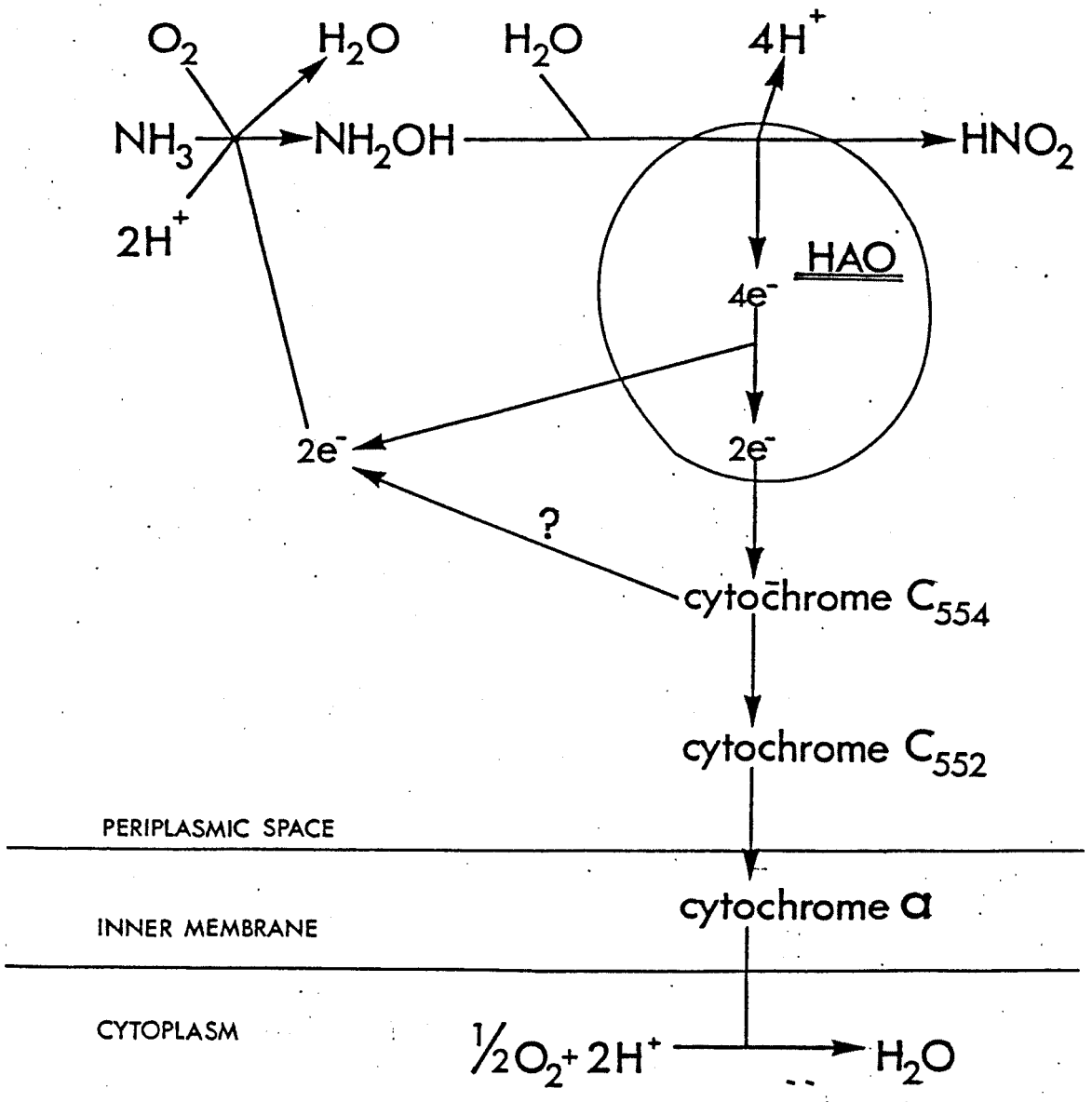


Figure 1.B.1. Proposed electron transport during the oxidation of ammonia in Nitrosomonas.

Taken from Hooper et al.,(1984a)

Suzuki, 1982). This suggests the operation of a carbon monoxide and ammonia oxidase activity utilizing electrons from cytochrome c554. EPR spectroscopy studies of this cytochrome have shown it to be capable of acting as an electron donor to an oxygenase or it may itself be an oxygenase (Anderson et al., 1982).

Electrons from the oxidation of hydroxylamine are thought to pass to a membrane-bound terminal oxidase, cytochrome a1, for the establishment of a proton gradient for the synthesis of ATP. Cytochrome a1 has been purified and characterised (Erickson et al.,1972). HAO being both soluble and periplasmic would seem to act as a proton-releasing dehydrogenase with concomitant trans-membrane movement of electrons to the terminal oxidase which consumes cytoplasmic protons for the establishment of a proton gradient (Olson and Hooper, 1983. Hooper et al., 1984). The natural electron acceptors for HAO are unknown, however in the presence of hydroxylamine and Nitrosomonas cytochrome c554, HAO catalysed the reduction of Nitrosomonas cytochrome c552. It would therefore be logical to assume that electrons from hydroxylamine pass from HAO to cytochrome c552 via cytochrome c554 and onto the terminal oxidase, cytochrome a1. The path of electrons through the HAO has recently been determined (Hooper et al., 1984). Electrons pass from hydroxylamine to the HAO-P460 centre to the HAO-cytochrome c haems and onto cytochrome c554.

Olson and Hooper (1983) point out that this type of scheme, involving a periplasmic dehydrogenase and a membrane bound terminal oxidase, is applicable to the oxidation of a

number of other small molecules in other organisms where, in contrast to many cytoplasmic oxidations, products are not necessarily further metabolised for energy yielding or biosynthetic reactions. Evidence of substrate oxidation on the periplasmic side of the membrane has been found in the oxidation of a number of substrates including methanol (Alefounder and Ferguson 1981).

Section 2. Materials and Methods.

A. Organism.

The organisms used in these studies were *Methylosinus trichosporium* (OB3b) and *Methylococcus capsulatus* (Bath) obligate methanotrophs originally isolated by Whittenbury *et al.*, (1970b).

B. Media.

Minimal salts medium (Whittenbury *et al.*, 1970b) was used throughout these studies for the routine growth of the organisms in flasks, 2.0 l chemostats and 100 l batch cultures. In flasks and on plates the standard medium was used with nitrate (NMS) as the nitrogen source. In 2 l chemostat culture nitrate, ammonium, or gaseous nitrogen were used as the nitrogen source and the copper level of the mediumwas varied between 0 - 1.2 mg/l copper sulphate as indicated in the text. 100 l batch cultures were grown on minimal mediumwith no added copper and ammonium as nitrogen source.

For solid medium 15 g/l Difco bacto-agar was added to NMS prior to sterilisation.

C. Growth and maintenance

Methylosinus trichosporium (OB3b) and *Methylococcus capsulatus* (Bath) were maintained by subculturing on NMS

plates every two weeks. The plates were incubated at 30°C and 45°C respectively in an anaerobic jar flushed with methane from a football bladder to give a concentration of 50% (v/v) methane in air.

Growth in continuous culture at 30°C was performed in a 2.0 l working volume LH 1000 fermenter (LH Engineering Ltd, Stoke Poges, Bucks, U.K.) on NMS, with methane (approximately 10-20% v/v in air) or methanol (1% w/v medium) as the carbon source. Dilution rate was maintained at 0.05 h^{-1} except where indicated. The pH value of the culture was maintained at 6.8 by automatic titration with 1.0 M HCl and/or 1.0 M NaOH.

Whole cells of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) for the preparation of large quantities of cell free extract, used in the purification of components of the soluble MMO, were grown in a 100 l batch fermenter (LH Engineering Ltd). 100 l of ammonium minimal salts medium (AMS) was maintained at either 30°C or 45°C and pH 6.8, and was inoculated with 500 ml of culture from a 2.0 l chemostat. Methane was used as the carbon source and the oxygen tension (pO_2) of the culture was maintained below 10% by the use of an LKB 1601 Ultraferm pO_2 controller and valve (LKB, Bromma, Sweden). Over a period of 24-48 h the optical density ($A_{540\ nm}$) of the culture increased to between 8 and 14, at which point the culture was harvested using a Westfalia continuous centrifuge (Westfalia Separator Ltd, Wolverton, Bucks, U.K.). The centrifuge was connected to the outflow of the fermenter via a stainless steel cooling coil immersed in ice. The cells were washed once with ice

cold 20 mM sodium phosphate buffer pH 7.0. Cells of Methylosinus trichosporium (OB3b) were then drop frozen into liquid nitrogen and stored at -80^{0}C until required. Cells of Methylococcus capsulatus (Bath) were resuspended in buffer containing 5 mM sodium thioglycollate ready for extract preparation. For consecutive batches of organisms approximately 5 l of culture was left in the vessel as the inoculum for the following run. Sterilisation of the vessel was by steam at 15 PSI for 3 hrs and sterilisation of the media was by filtration (0.2 micrometer) through a Sartobran filtration capsule (Sartorius Instruments Ltd. Sutton. Surrey).

D. Culture Purity.

The purity of all the cultures was checked by plating out on nutrient agar plates and incubating these at 30^{0}C and 45^{0}C for 48 h. The presence of heterotrophic bacteria was revealed by growth of colonies on these plates. Methanotrophs fail to grow under these conditions. The presence of other methanotrophs as contaminants was checked by plating out on NMS plates and incubating these at 30^{0}C and 45^{0}C under a methane atmosphere as previously described. Contaminants were identified by their different colony morphology to the methanotroph being cultivated. Chemostat cultures were examined daily for purity by phase contrast microscopy using an Olympus X-Tr stereoscopic microscope.

E. Preparation of cell free extracts.

Whole cells of Methylosinus trichosporium (OB3b) grown in chemostat or 100 l batch culture were centrifuged at 10000 g for 10 minutes, washed once with, and resuspended in 20 mM sodium phosphate buffer pH 7.0 containing 5 mM magnesium chloride and freshly added 1 mM dithiothreitol and 1 mM phenylmethylsulphonylfluoride (PMSF). Crude cell extracts were prepared by two passages through a pre-cooled French pressure cell (American Instrument Company, Maryland, U.S.A) at 137 MPa. Unbroken cells and debris were removed by centrifugation at 32000 g for 15 minutes. 1% protamine sulphate (w/v) was added as a 10% solution and the extract incubated with stirring for 10 minutes at 4^{0}C. The extract was then further centrifuged at 150000 g for 60 minutes to yield soluble (S_{150}) and particulate (P_{150}) extracts, which were drop frozen in liquid nitrogen and stored at -80^{0}C.

Soluble extracts of Methylococcus capsulatus (Bath) from the 100 l fermenter were prepared as described by Colby et al., (1977). Crude extracts were prepared by a single passage of the cell suspension through a French pressure cell at 137 MPa, followed by centrifugation at 80000 g for one hour and yielding a soluble crude extract (S80). All extracts were frozen by dropwise addition to liquid nitrogen and stored at -80^{0}C until required.

F. Purification of components of the soluble MMOs of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) from soluble cell free extracts.

All procedures described below were carried out at 4 °C. All fractions were frozen by dropwise addition to liquid nitrogen and stored at -80 °C until required.

F.1 Methylococcus capsulatus (Bath).

F.1.1 DEAE cellulose ion exchange chromatography.

Resolution of the MMO into three components was carried out as described by Colby and Dalton (1978). Soluble extract (350 ml) was thawed and placed on a 4.5 x 7.0 cm column of DEAE cellulose equilibrated with 20 mM Tris/HCl, 5 mM sodium thiogylcollate buffer pH 7.0. Material not binding to the column packing was eluted with the above buffer yielding fraction A. The column was then eluted with successive batches of the same buffer containing respectively 0.1 M, 0.2 M yielding fraction B and 0.5 M sodium chloride yielding fraction C.

F.1.2 Component A.

Component A of the soluble MMO was purified as described by Woodland and Dalton (1984b). Any particulate material was removed from fraction A by centrifugation in a microcentrifuge for 10-15 minutes and filtration through a 0.2 micrometer filter. Chromatography was carried out at room temperature on an Ultra-Pac TSK-G 3000 SWG (21.5 by 600 mm) gel permeation column preceded by a guard column,

Ultra-Pac TSK-GSWPG (21.5 by 75 mm). Degassed and filtered buffer (20 mM sodium phosphate pH 7.0) was supplied to the column at a flow rate of 3 ml/minute by an LKB 2150 pump. Aliquots of the prepared fraction A were loaded onto the column via a 2 ml sample loop incorporated in a Rheodyne 7125 valve. The eluate was collected by means of a Multirac 2111 fraction collector and monitored for protein by UV absorbance at 280 nm with a 2138 Uvicord S linked to a single channel 2210 potentiometric recorder (all the column and fractionation equipment was supplied by LKB, Bromma, Sweden). Pure component A was stored in 50% glycerol at -20^{0}C.

F.1.3 Component B.

Component B of the soluble MMO was purified by Dr. J. Green, as described by Green and Dalton (1985). Fraction B was concentrated by ultrafiltration over a YM-5 ultrafiltration membrane (Amicon Ltd, Woking, Surrey, U.K). Concentrated fractions (3-5 ml) were applied to a Sephadex G-100 column (2.5 by 80 cm) equilibrated with 10 mM sodium phosphate buffer pH 7.0, and the column eluted with the same buffer at a flow rate of 20 ml/h, collecting 4 ml fractions using a RediRac 2112 fraction collector (LKB). Pooled active fractions were applied to a DEAE-Sepharose CL-6B column equilibrated with 10 mM sodium phosphate buffer pH 7.0. The column was eluted with a linear gradient of 10-300 mM sodium chloride in buffer at 20 ml/h, after the column had been washed with three column volumes of buffer containing 10 mM sodium chloride, fractions being collected

as above. Pooled active fractions after ultrafiltration and dilution were applied to a column of hydroxyapatite (2 by 7 cm). The column was developed with a linear gradient of 10-150 mM sodium phosphate pH 7.0 and fractions collected as above.

F.1.4 Component C

Component C of the soluble MMO was purified as described by Lund (1983). Approximately 30 ml of fraction C were pumped at a rate of 60 ml/h onto a 1.5 by 11 cm column of fresh 5'-AMP sepharose 4B (Pharmacia Ltd) equilibrated with buffer (20 mM sodium phosphate buffer pH 7.0 containing 5 mM sodium thioglycollate). The column was washed with about one half column volume of buffer containing 0.5 M sodium chloride to reduce protein protein interactions, followed by one column volume of buffer and the protein C on the column was chased off with 1 mM NADH (ethanol free) in buffer.

F.2 Methylosinus trichosporium (OB3b).

F.2.1 DEAE cellulose ion exchange chromatography.

Soluble cell extracts of Methylosinus trichosporium (OB3b) were concentrated by dialysis against dry polyethylene glycol (PEG) 20000. Concentrated soluble extract was mixed with pre-swollen DEAE cellulose (10 ml extract plus 20 ml DEAE cellulose) equilibrated with 20 mM sodium phosphate buffer pH 7.0 containing 1 mM dithiothreitol (DTT), poured into a column (2.5 by 6.0 cm)

and allowed to settle for 10-15 minutes. Unbound protein was eluted with two column volumes of buffer at a flow rate of 1 ml/minute, yielding fraction A. The column was then eluted with buffer containing 0.3 M sodium chloride which chased off the bound protein, yielding fraction C. Protein concentration of fractions was monitored throughout by UV absorbance at 280 nm using an LKB 2138 Uvicord S linked to an LKB single channel 2210 potentiometric recorder, fractions were collected by means of a LKB RediRac 2112 fraction collector.

F.2.2 Component A.

Component A of the soluble MMO was purified using the same techniques and apparatus as described above for the purification of component A of the soluble MMO of Methylococcus capsulatus (Bath).

F.2.3 Component C.

Component C of the soluble MMO was further purified. Fraction C was concentrated up by ultrafiltration over a PM-10 ultrafiltration membrane (Amicon). Chromatography was carried out on a Reactive Red 120 agarose 3000 CC (Sigma) column (10 by 60 mm, 5 ml volume), equilibrated with buffer (20 mM sodium phosphate pH 7.0, 1 mM DTT). Fraction C was pumped onto the column and followed by 1 ml of buffer and allowed to equilibrate for 10 minutes. The column was eluted with two column volumes of buffer and the component C chased off the column with 2 mM NADH (ethanol free) in buffer. Fractions were collected by use of a LKB RediRac

fraction collector.

F.3 Associated purification procedures.

F.3.1 5'-AMP Sepharose chromatography.

5'-AMP Sepharose chromatography of fraction C of Methylosinus trichosporium (OB3b) was carried out using the procedure described above for the purification of component C of the soluble MMO of Methylococcus capsulatus (Bath) but using a small 2ml volume column and chasing bound protein C with 2 mM NADH. Buffers contained 1 mM DTT rather than 5 mM sodium thioglycollate.

F.3.2 DyeMatrex screening kit.

A 'Dymatrex' screening kit was used to assess the ability of a number of dye affinity chromatography materials to bind component C. The kit contains six 2 ml columns, one each of dye affinity chromatography materials: Blue A, Blue B, Red, Orange, Green and a control column containing support material.

Procedures were those laid down in 'Operating instructions for DyeMatrex screening kit' (Amicon). Columns were washed with six column volumes of 8 M urea, followed by six column volumes of buffer (20 mM tris/HCl pH 7.5). A 0.5 ml sample of fraction C was added to each column followed by 0.1 ml of buffer and allowed to equilibrate for 30 minutes. Columns were eluted with five column volumes of buffer followed by five column volumes of 2 mM NADH in buffer and finally five column volumes of 1.5 M potassium chloride in

buffer. A single fraction was collected for each elution and assayed for protein and component C activity.

F.3.3 Molecular weight determination by gel filtration.

The molecular weight of component A of the soluble MMO of Methylosinus trichosporium (OB3b) was estimated by gel filtration on Sephacryl S-300 superfine (Pharmacia). The following standards were used to calibrate the column: thyroglobin (M_r 669000), ferritin (M_r 440000), catalase (M_r 232000), aldolase (M_r 150000) and bovine serum albumin (M_r 67000). The void volume was calculated by the use of blue dextran. To estimate the molecular weight, K_{av} (the fraction of the stationary gel volume which is available for a given solute species) was plotted against log molecular weight.

Where $$K_{av} = \frac{(V_o - V_e)}{(V_t - V_o)}$$

Where V_o is the void volume, V_e is the elution volume and V_t the total column volume.

G. Gas chromatography procedures.

Measurement of epoxypropane, acetate and acetone were done using a Pye Unicam series 104 gas chromatograph (Pye Unicam, Cambridge, U.K.) fitted with a flame ionisation detector and a "Poropack Q" (Waters Associates, Milford, Massachusetts, U.S.A) column (1 m x 4 mm internal diameter). Nitrogen was used as a carrier gas at a flow rate of 30

ml/min and an oven temperature of 195 °C. This system was linked to a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, Pennsylvania, U.S.A.) for the measurement of peak areas. For epoxypropane and acetone the system was calibrated with a standard 2 mM solution from a sealed reaction flask (7 ml volume) containing 1 mM of the standard, incubated at 30°C or 45 °C in order that equilibration of the epoxypropane or acetone between the liquid and gas phases of the reaction vessel could occur. For the measurement of acetate, the column was pretreated with a series of injections of 10 mM acetate to reduce tailing and the consequent variability in the peak areas (Leek and Dalton 1983), and then a 5 mM acetate standard was used.

H. Spectrophotometric procedures.

Routine spectrophotometry e.g., optical density readings of liquid cultures at 540 nm, optical density readings of protein, nitrite, hydroxylamine and PHB assays, were carried out on a Pye Unicam SP 1800 U.V. recording spectrophotometer. All other spectrophotometry including spectra of enzyme preparations and enzyme assays measuring the rate of oxidation/reduction of NADH, DCPIP, ferricyanide or cytochrome c were carried out using a Pye Unicam SP8-200 U.V./VIS recording spectrophotometer. This instrument incorporates a water jacketed cuvette carridge that can be heated to the required temperature for enzyme assays.

I. Analytical determinations.

Linear regression analysis was carried out on each standard curve produced for the analytical determination of compounds.

I.1. Protein.

Protein was assayed using commercially available Bio-Rad reagent (Bio-Rad Ltd, Watford, Herts, U.K.) using bovine serum albumin as a protein standard.

I.2. Hydroxylamine.

Hydroxylamine was assayed using a modified version of the method described by Magee and Burris (1954). A test solution , volume 0.1-0.2 ml was acidified with 1 M HCl and the volume made up to 1 ml in a test tube. 1 ml of 1% (w/v) 8-hydroxyquinoline in ethanol was added, followed by 1 ml of 1 M sodium carbonate solution with shaking. A cap was placed on the test tube which was incubated for 1 hour at 45 ^{o}C and the optical density read at 680 nm. A standard curve was constructed by addition of 10-200 nmol hydroxylamine to the assay. No interference with ammonia, nitrite or nitrate was observed, however PMS interfered with the assay. With PMS the response to the concentration of hydroxylamine was still linear but lower than that of assays lacking PMS. Separate standard curves were therefore constructed for samples containing PMS.

I.3. Nitrite.

The concentration of nitrite was determined using the method described by Nicholas and Nason (1957). To a test sample, made up to a volume of 1 ml, 0.5 ml of 1% sulfanilamide in 3 M HCl was added followed by 0.5 ml of 0.02% N-(1-napthyl)ethylenediamine hydrochloride with shaking. As both the above solutions are light sensitive they were kept in an amber or foil covered bottle. The colour was left to develop at room temperature for ten minutes and the sample was then centrifuged for 2 minutes in a Microcentrifuge (Quickfit Instrumentation, U.K.) to remove any precipitate formed in the assay, the optical density was then read at 540 nm. A standard curve was constructed by addition of 2-35 nmol nitrite to the assay. Standard solutions of nitrite were prepared in dilute sodium hydroxide solution (25 mg NaOH per 100 ml) to prevent the liberation of nitrous oxide from the reaction of nitrite with carbon dioxide.

I.4. Nitrate.

Nitrate was assayed using a modification of the method described by Hooper et al., (1977). A sample was made up to a volume of 1 ml and an ammonium hydroxide concentration of 1 M, 25 mg of metallic zinc powder was added with vigorous shaking on a Griffin flask shaker. Aliquots of 0.2 ml were withdrawn at four two minute intervals and centrifuged for thirty seconds in a microcentrifuge to remove the zinc powder. 0.1 ml aliquots were then assayed for nitrite as described above. The maximum nitrite value obtained for

each sample was used and adjusted for recovery by comparison with the value of nitrite obtained from standard nitrite solutions containing 0-200 nmol/ml nitrite and treated in the same way. Recovery of both nitrite and nitrate from this assay was consistent at 92% as compared with the nitrite assays.

I.5. Poly β hydroxybutyrate.

Poly β hydroxybutyrate (PHB) was analysed by the method of Law and Slepecky (1961). Samples of cells (25 mg dry weight) were pelleted by centrifugation and resuspended in sodium hypochlorite solution and incubated at 37^{0}C for 60 minutes. The lipid granules were washed with acetone and ethanol and disolved in three volumes of boiling chloroform, filtered and the filtrate used for PHB assay. The chloroform was evaporated and 10 ml of concentrated sulphuric acid was added and heated for 10 minutes at 100^{0}C. The solution is cooled and mixed and the absorbance at 235 nm measured against a sulphuric acid blank.

I.6. Iron.

Iron content of the protein was determined by the method described by Woodland and Dalton (1984a). The protein was wet ashed in concentrated sulphuric, nitric and perchloric acids. The acid digest were transferred to volumetric flasks (5 ml) containing saturated sodium acetate (2.0 ml), 20% ascorbic acid (0.45 ml) and 10 mM

bathophenanthroline sulphonate (0.15 ml) and the volume adjusted to 5 ml with distilled water. The absorbance at 535 nm was measured and compared with a standard curve prepared from solutions of ferrous ammonium sulphate to estimate the iron content.

J. Enzyme assays.

All figures for activity of enzymes are the average of at least three assays.

J.1. Methane monooxygenase (MMO).

The MMO of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) are composed of more than one protein and when their specific activity is plotted as a function of the concentration of extract in the assay a non-linear relationship is obtained (Colby and Dalton 1976, this thesis). Maximum specific activity was obtained when a concentration of 5 mg/ml protein for the soluble MMO of Methylosinus trichosporium (OB3b) and 7 mg/ml protein for the soluble MMO of Methylococcus capsulatus (Bath) was present in the assay. This was therefore the amount of extract present in routine MMO assays of soluble cell free extracts.

J.1.1. Propene oxidation.

Cell extracts and combinations of the components of the MMO complex were assayed as described by Colby et al., (1977).

Assays were done in conical flasks (7 ml internal

volume) containing 1 ml of the reaction mixture and sealed with a Suba-Seal (N037). Propene (3 ml) was added by injection through the Suba-Seal, replacing the same volume of the gas phase of the reaction flask. The reaction mixture contained 20 micromol sodium phosphate buffer pH 7.0, the extract or whole cells plus any inhibitors. The flask was pre-incubated for two minutes in a Gyratory water bath shaker model G76 (New Brunswick Scientific Co Ltd, Edison, New Jersey, U.S.A.) at 90 oscillations/min. The reaction was started by the addition of 5 micromol NADH (ethanol-free). A sample of the liquid phase (5 micro l) was removed after three minutes for analysis of epoxypropane concentration as described above.

J.1.2. Ammonia oxidation.

Assays of ammonia oxidation by the MMO were carried out as above replacing propene with the required amount of ammonium chloride in the reaction mixture. Samples of the liquid phase (0.1-0.2 ml) were withdrawn at intervals over a fifteen minute period for analysis of the concentration of nitrite, nitrate or hydroxylamine.

J.2. Independent NADH:acceptor reductase activity of component C of the soluble MMO.

NADH:acceptor reductase assays were performed as described by Colby and Dalton (1979) and Lund (1983). 2 ml of 20 mM sodium phosphate buffer pH 7.0 containing 0.9 micromoles ferricyanide or 0.19 micromoles of dichloroindophenol (DCPIP) or 0.05 micromoles of horse heart

cytochrome _c_ was placed in a 3 ml cuvette, sealed with a Suba Seal and sparged with helium for 10 minutes to make anoxic. After the addition of the enzyme by injection through the Suba Seal, the reaction was initiated by the addition of 0.23 micromols of NADH and the reaction monitored at the appropriate wavelength for the electron acceptor employed.

J.3. Methanol dehydrogenase.

Methanol dehydrogenase was assayed by the method described by Anthony and Zatman (1967). Assays in a cuvette sealed with a Suba Seal, contained 33 mM pyrophosphate buffer pH 9,
1 mM potassium cyanide, 1.6 mM ammonium chloride and 125 micrograms DCPIP, which was sparged with nitrogen for ten minutes to produce anoxic conditions. 1 mg PMS, extract and methanol to 6.7 mM added, and after each addition the rate of DCPIP oxidation followed at 600 nm. Assays were of 3 ml total volume and carried out at 30^{0}C.

J.4. Hydroxylamine oxidoreductase (HAO).

Assays were done in conical flasks (7 ml internal volume) containing 1 ml of the reaction mixture and sealed with a Suba-Seal. The reaction mixture contained 20 micromol sodium phosphate buffer, pH 7.0, extract (as indicated in the text) and any electron acceptors, electron donors or inhibitors used as detailed in the text. Flasks

were pre-incubated for two minutes at 300C in a Gyratory water bath at 90 oscillations/minute. The reaction was started by the addition of 5 micromol hydroxylamine. Samples (0.1-0.2 ml) were withdrawn over a period of fifteen minutes from the liquid phase and assayed for either nitrite, nitrate or hydroxylamine.

K Preparation of ethanol-free NADH.

2 g of NADH was dissolved in 20 ml of 20 mM sodium phosphate buffer pH 7.0. In a separating funnel 150 ml of diethyl ether (A.R. grade) was added to the NADH solution and the closed funnel shaken violently for a few minutes. The ether was removed and replaced by fresh ether. This extraction was repeated five times. The NADH solution was then made up to 20 ml and the ether evaporated off under vacuum until the levels of both ethanol and ether are at a sufficiently low levels for MMO assays. Ethanol and ether were monitored by gas chromatography as previously described. The solution of NADH was diluted down to 100 mM and stored at -20^{0}C.

L Dry weight determinations of cell suspensions.

Cells were washed in CO_2-free water. The total carbon content of the cell suspension was measured using a Beckman Model 915-B total carbon analyser (Beckman Instruments Inc. Fullerton, California, U.S.A). The cell carbon content was

assumed to be 47% of the dry weight (Dijken and Harder 1975) and thus the dry weight of the cell suspension was estimated. A curve of OD_{540} vs dry weight was therefore prepared giving a figure of 0.429 mg/ml dry weight per unit OD_{540}.

M. Polyacrylamide gel electrophoresis.

Analytical polyacrylamide gel electrophoresis (PAGE) was carried out on vertical 5-20% linear gradient gels (1.5 by 150 mm by 180 mm) with a 4% stacking gel. A discontinuous buffer system was used consisting of 0.375 M tris/HCl pH 8.8 resolving gel buffer, 0.125 M tris/HCl pH 6.8 stacking gel buffer and 0.025 M tris/glycine pH 8.3 reservoir buffer. For PAGE under dissociating conditions all buffers were supplemented with 0.1% sodium dodecyl sulfate (SDS) and protein samples were treated with β-mercaptoethanol and 0.2% SDS for 2 minutes at 100^{o}C prior tp loading on to the gel. Non-dissociating gels were run at 150 mV constant voltage for at least 2000 Vh, dissociating gels were run at 50 mA constant current. On the completion of electrophoresis, dissociating gels were soaked in methanol:acetic acid:water (3:1:6) to remove SDS and then gels were stained in Coomassie brilliant blue R-250 (0.1% w/v) dissolved in the above solvent and destained with the same solvent.

N. Extinction coefficients.

The molar extinction coefficients of the chemicals used were as follows;

Chemical	Wavelength (nm)	Molar extinction coefficient ($M^{-1}cm^{-1}$)
DCPIP	600	21000
Cytochrome c	550	19000
ferricyanide	410	1020
NADH	340	6220

O. Chemicals.

Compounds, inhibitors, substrates and media components were obtained from the following manufacturers: Sigma (London) Chemical Co Ltd, Poole, Dorset, U.K.; Fisons Scientific Apparatus, Loughborough, Leics, U.K.; Aldrich Chemical Co Ltd, Gillingham, Dorset, U.K.

P. Gases.

Methane (technical grade), oxygen, hydrogen, propene and nitrous oxide were obtained from the British Oxygen Co, London, U.K.

Section 3. Results and Discussion.

A. Growth harvesting and the preparation of soluble cell extracts of Methylosinus trichosporium (OB3b) for the purification of the soluble methane monooxygenase.

The methods for the growth, harvesting and preparation of soluble cell free extracts from the cell of Methylosinus trichosporium (OB3b) were employed to maximize the yield of soluble MMO. There are a number of general considerations that need to be taken into account in order to achieve this objective: 1. Growth conditions may alter the level of enzyme produced by the cells, so conditions conducive to the production of the soluble MMO need to be optimised. 2. The stability of the soluble MMO in vitro requires investigation, so that by the addition of stabilising agents the enzyme is sufficiently stable to yield active enzyme at the end of a purification procedure. 3. A cell free extract needs to be produced quickly and efficiently, from which the soluble MMO is easy to purify. These three points are interrelated, but will be dealt with separately referring to the others where appropriate.

A.1. Growth of Methylosinus trichosporium (OB3b).

Both a soluble and a particulate MMO is produced by Methylosinus trichosporium (OB3b) (Scott et al., 1981.

Stanley et al., 1983. Burrows et al., 1984). In the chemostat there are two parameters that affect the availability of copper to cells, the biomass concentration and the concentration of copper in the feedstock.

There are two methods for measuring the type and position of the MMO in cells of Methylosinus trichosporium (OB3b). A crude extract can be separated into soluble and particulate fractions by centrifugation and the specific activity of the MMO in each fraction measured. Alternatively, crude extracts can be analysed by SDS polyacrylamide gel electrophoresis (PAGE) and the distinct bands associated with the soluble MMO observed (see introduction A.4.1.2a). Both these methods were used in conjuction to assess the effect, if any, of a range of culture conditions on the location and activity of the MMO in cell extracts.

For the investigation of the effect of growth conditions on the location and activity of the MMO, Methylosinus trichosporium (OB3b) was grown in a 2l working volume, LH1000 fermenter on ammonia minimal salts (AMS) at a dilution rate of 0.05 h^{-1}, as detailed in Materials and Methods. Under conditions of oxygen limitation, Methylosinus trichosporium (OB3b) attained a biomass concentration of 4.05 mg dry weight/ ml (OD_{540} = 8.3). AMS contains 0.2 mg/l copper sulphate and cells grown under these conditions contained high activities of the soluble MMO (70-90 nmol/min/mg). On the addition of a further 1.0 mg/l copper sulphate to the growth medium, soluble MMO activity drops to between 15-25 nmol/min/mg with a concomitant rise in the particulate MMO activity. The

measurement of the particulate MMO activity was very irreproducible due to its inherent instability (Scott *et al.*, 1981. Cornish *et al.*, 1985). However under conditions of excess copper particulate MMO activities were usually between 10-20 nmol/min/mg, whereas under conditions of copper stress particulate MMO activity was often not detectable and never exceeded 6 nmol/min/mg. Recently it has been shown in our laboratory that the addition of copper ions *in vitro*., to particulate cell extracts of *Methylococcus capsulatus* (Bath) can stimulate the activity of the particulate MMO (Prior and Dalton 1985) and inhibit the activity of the soluble MMO (Green *et al.*, 1985). Assays of particulate MMO activity from *Methylosinus trichosporium* (OB3b) here were carried out without the addition of copper to the assays and so they may not have measured the maximum particulate MMO activity of particulate extracts.

Due to mechanical failure a culture "batched up" in the fermenter to an OD_{540} of 16 on AMS, these cells were extremely copper stressed and had a high soluble MMO activity (107 nmol/min/mg) and no detectable particulate MMO activity. Subsequently cultures of *Methylosinus trichosporium* (OB3b) were grown extremely copper stressed with no copper added to the medium. Under these conditions the biomass concentration was similar to that achieved on media with 0.2 mg/l copper sulphate but the soluble MMO activity was consistently high (85-105 nmol/min/mg) and no particulate MMO activity was detected.

An alternative carbon source for *Methylosinus trichosporium* (OB3b) is methanol. The substitution of

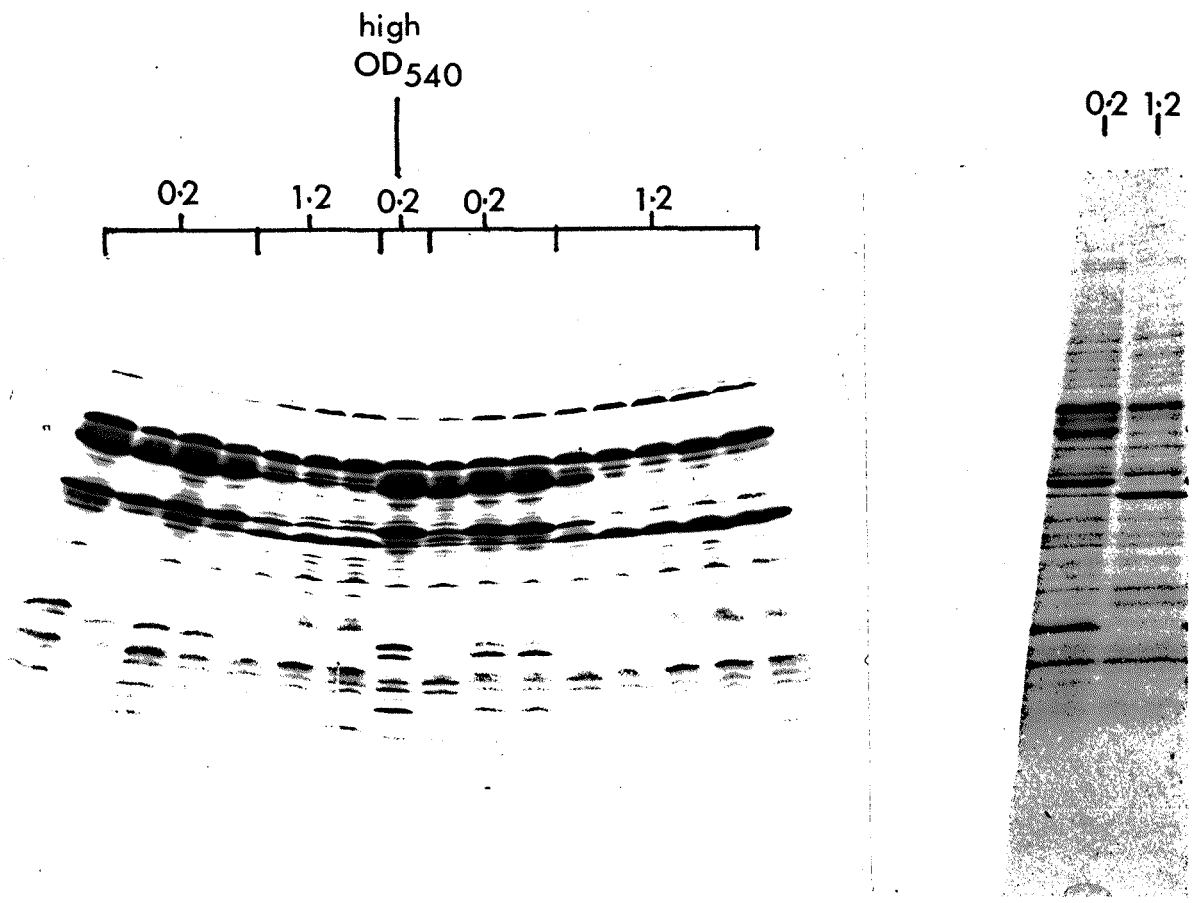
methanol for methane as a carbon source had no obvious effect on the level of soluble MMO of soluble extracts from a chemostat culture grown under copper stress. This agrees with the work of Best and Higgins (1981) and Cornish et al., (1984), who studied methanol-grown Methylosinus trichosporium (OB3b). However, work carried out in our laboratory has failed to demonstrate the production of a soluble MMO by Methylococcus capsulatus (Bath) when grown in a chemostat on methanol at a copper concentration of 0-2 mg/l copper sulphate (Prior and Dalton 1985).

The above results were paralleled in the SDS PAGE protein band patterns of crude cell extracts. Under conditions of copper stress the bands associated with the soluble MMO were prominant whereas under conditions of copper in excess these bands were diminished or had disappeared and a single band (MW 42000) become more prominant. On closer inspection, there were a number of other changes in band pattern not reported by Burrows et al., (1984). An additional band not present in cells with a particulate MMO, was present in soluble MMO-containing cells, and a band not present in cells containing soluble MMO was present in cells with a particulate MMO (FIG 3.A.1).

Whole cells for the preparation of large quantities of soluble cell free extract required for the purification of the soluble MMO were grown in a 100 l batch culture on AMS with no copper added to the medium on methane as detailed in Materials and Methods. The fermenter was inoculated with 5 l from the waste pot of a 2 l chemostat culture. Two such batch fermentations were performed, sequentially using 5 l from the first batch culture as an inoculum for the second.

Figure 3.A.1. Electrophoresis of crude extracts of Methylosinus trichosporium (OB3b).

Concentration of copper sulphate in the growth medium. (mg/l)



Electrophoresis: 5-20% gradient SDS PAGE, 50 micrograms protein per track.

Cells grown in 2 l chemostat on AMS with methane as the carbon and energy source, dilution rate 0.05 h^{-1}.

Arrows indicate protein bands associated with the presence of the soluble MMO.

Each culture grew slowly, achieving a doubling time of only 17 hours. Cultures were harvested when the OD_{540} had reached 8.0 and were still in the exponential phase of growth. Cells were centrifuged down, washed and drop frozen into liquid nitrogen and stored at $-80^{0}C$. These cells contained soluble MMO activity of 40-70 nmol/min/mg with no particulate MMO activity being detected.

In summary, the availability of copper to the cells of Methylosinus trichosporium (OB3b) grown in a chemostat culture affects the type and location of the MMO produced. High copper stimulates the production of a particulate MMO, whereas low copper levels stimulate the production of a soluble MMO. The substitution of methanol for methane as carbon and energy source has little or no effect on this relationship.

A.2. Stability of the soluble MMO.

The stability of a multicomponent enzyme system such as the soluble MMO of Methylococcus capsulatus (Bath) is dependent on the individual stabilities of the enzyme's component proteins. Each component may require different conditions or the addition of different stabilising agents to retain activity. In practice however, one of the components of the enzyme complex will be appreciably less stable than the other(s) and therefore in crude cell preparations, will always appear to be or will quickly become the rate limiting component of the enzyme complex. It is therefore for this component that the initial efforts

at stabilisation are directed in crude cell free extracts.

Colby and Dalton (1976) in their initial report of cell free soluble MMO activity of Methylococcus capsulatus (Bath), reported a loss of 25% activity over 24 hours at $4^{0}C$. On the resolution of the soluble MMO from Methylococcus capsulatus (Bath) into its three components, DEAE- fractions A and B were found to be stable over a period of 24 hours at $0^{0}C$. The instability of the soluble MMO was due to a loss of 60-90% of activity of the reductase component in DEAE- fraction C over a 20 hour period at $0^{0}C$ (Colby and Dalton 1978). All three fractions were stable when frozen in liquid nitrogen and stored at $-80^{0}C$. A number of stabilising agents were tested for their effect on the stability of component C. Sodium thioglycollate (5 mM) and DTT (5 mM), were shown to stabilise as was NADH (5 mM) with no loss of activity in 22 hours at $0^{0}C$.

On the subsequent purification of the components of the soluble MMO of Methylococcus capsulatus (Bath) information has been aquired on the stability of the individual components. Woodland and Dalton (1984), reported a loss of 40% in activity over 72 hours at $4^{0}C$ for DEAE- fraction A. A wide range of stabilising agents failed to prevent this loss of activity. Once purified, component A was not stable to freezing but could be stored in 50% glycerol at $-20^{0}C$ for several weeks without loss of activity. This instability was attributed to the loss of iron from the protein, as activity could be restored by incubation with iron and DTT, though this process was unreliable and variable. Component B of the soluble MMO of Methylococcus capsulatus (Bath) was only stable after treatment with phenyl methyl sulphonyl

fluoride (PMSF), a serine protease inhibitor (Green and Dalton 1985). In the presence of 5 mM sodium thioglycollate, crude preparations of component C lost activity at a rate of 5% an hour at 4°C. Pure component C was more unstable, losing 30% of its activity per hour under the same conditions. It was stable to freezing and could be stored at -70°C indefinitely without loss of activity (Lund 1983). In crude cell free extracts, made in either the presence or absence of 5 mM sodium thioglycollate, component C was shown to be the limiting factor in soluble MMO activity (Pilkington 1983).

The soluble MMO of Methylosinus trichosporium (OB3b) in crude cell free extracts is unstable, losing all activity after 24 hours at 4°C (Stirling and Dalton 1979a. Scott et al., 1981a). Soluble extracts were less stable, losing all activity after only two hours at 4°C. However activity could be restored to these extracts by the addition of DEAE-fractions B and C from the soluble MMO of Methylococcus capsulatus (Bath). DEAE- fraction 1 of the soluble MMO of Methylosinus trichosporium (OB3b) lost all activity in 1-3 hours at 4°C and also when frozen in liquid nitrogen and stored at -80°C. A number of stabilising agents including PMSF (0.05-0.15 mM), DTT (10 mM), and sodium thioglycolate (10 mM) had no effect on the stability of DEAE- fraction 1 (Stirling and Dalton 1979a). Scott et al., (1981), found the soluble MMO of Methylosinus trichosporium (OB3b) was stable at liquid nitrogen temperatures and that the stability of the soluble MMO in soluble extracts was enhanced by a number of stabilising agents particularly PMSF (1 mM) and DTT (1 mM). Storage of extracts under

anaerobic conditions also inhibited the decay in soluble MMO activity (Table 3.A.1).

Initial short-term studies of the stability of the soluble MMO in soluble extracts of Methylosinus trichosporium (OB3b) showed that 25-30% of activity was lost per hour at $0^{0}C$, no activity remaining after 24 hours. Scott et al., (1981a) reported partial stabilisation of the soluble MMO by a number of compounds (Table 3.A.1). An investigation of the effect of a number of compounds on the stability of the soluble MMO was therefore carried out. 1 mM PMSF proved to be the most effective, extracts retaining 40% of the initial activity over 24 hours at $0^{0}C$, a result in agreement with Scott et al., (1981a). However, other agents specifically DTT, sodium thioglycollate and NADH, were found to be less effective than reported by Scott et al., (1981a), as was the incubation of extract under an anaerobic environment (Table 3.A.1).

PMSF is a serine protease inhibitor (Fahrney and Gold 1963) and so two other such inhibitors, aminophenylboronic acid (APBA) and procane were also tested for their ability to stabilise the soluble MMO. Both showed some ability to stabilise the soluble MMO, though they were not as effective as PMSF (Table 3.A.1).

The ability of PMSF to stabilise the soluble MMO was presumably due to its action as a serine protease inhibitor. Enzymic digestion is just one mechanism by which an enzyme may become denatured. Other compounds that enhance the stability of the soluble MMO act to inhibit other de-stabilising processes secondary to enzyme digestion: eg.,

Samples (1ml) of soluble extract were kept at 0°C in sealed polythene tubes in the presence of the test compound for 24 hours. The original MMO specific activity for Scott et al., 1981 was 21 nmol/min/mg and for S.J.Pilkington was 52 nmol/min/mg

MMO assays: 3 minute assays, 30°C, following the oxidation of propene to epoxypropane. 5mg soluble extract per assay.

Table 3.A.1. Stability of the soluble MMO: Effect of a number of stabilising agents.

Test compound	Conc (mM)	MMO activity (% of original)	
		A	B
No additions	-	0-5	0-5
PMSF	1	44	40
	5	32	27
	10	0	nd
Dithiothreitol	1	35	8
	5	11	15
	10	12	9
Na thioglycollate	1	16	4
	5	12	3
	10	13	4
NADH	1	24	nd
	5	0	nd
Anaerobic conditions	-	41	0
Procane	1	nd	10
	5	nd	4
	10	nd	0
APBA	1	nd	14
	5	nd	25
	10	nd	0

A- Scott et al., 1981

B- S.J. Pilkington

thiol reagents such as DTT and thioglycollate, protect active site thiol groups (Cleland 1964). The stabilising effects of such agents that inhibit secondary denaturing processes, may, when used in isolation, be masked by the primary denaturation process that proceeds at a higher rate. Their effect, if any, will only become fully apparent when used in combination with an inhibitor of the primary denaturation process. This may be particularily true of the stabilisation of complex multicomponent enzymes such as the soluble MMO's. Soluble extracts of Methylosinus trichosporium (OB3b) prepared with 1 mM PMSF were incubated with various concentrations of DTT overnight at 0^{0}C (Table 3.A.2). Both 1 mM and 5 mM DTT were found to significantly enhance the stability of the soluble MMO up to a maximum of 79% of the original activity retained over 24 hours at 0^{0}C.

Short term stability studies carried out on a soluble cell free extract of Methylosinus trichosporium (OB3b) showed dramatically the effect of DTT. An extract was divided into three parts and 0, 1, and 5 mM DTT added, specific activities measured over a period of six hours (FIG 3.A.2). After only three hours at room temperature all activity was lost from the extract with no DTT. Extracts with additions of 1 and 5 mM DTT lost 25% of their activity over a six hour period at room temperature.

Extracts were therefore routinely prepared using buffers that contained freshly dissolved PMSF (1 mM) and DTT (1 mM). This allowed the production of crude extracts with soluble MMO activities as high as 105 nmol/min/mg. However, normally the specific activity of soluble extracts was in the range of 50-70 nmol/min/mg. This compares with

Table 3.A.2. Stability of the soluble MMO: Effect of dithiothreitol.

Dithiothreitol (mM)	Specific activity (nmol/min/mg)	% Original activity
0	23	39
1	47	79
5	44	74
10	28	47

Soluble extract was made with 1 mM PMSF was incubated for 24 hours at 4^{0}C in the presence of 0, 1, 5 and 10 mM DTT and assayed for soluble MMO activity. Original specific activity of the soluble extract: 60 nmol/min/mg.
MMO assay: 3 min, 30^{0}C, propene to epoxypropane.

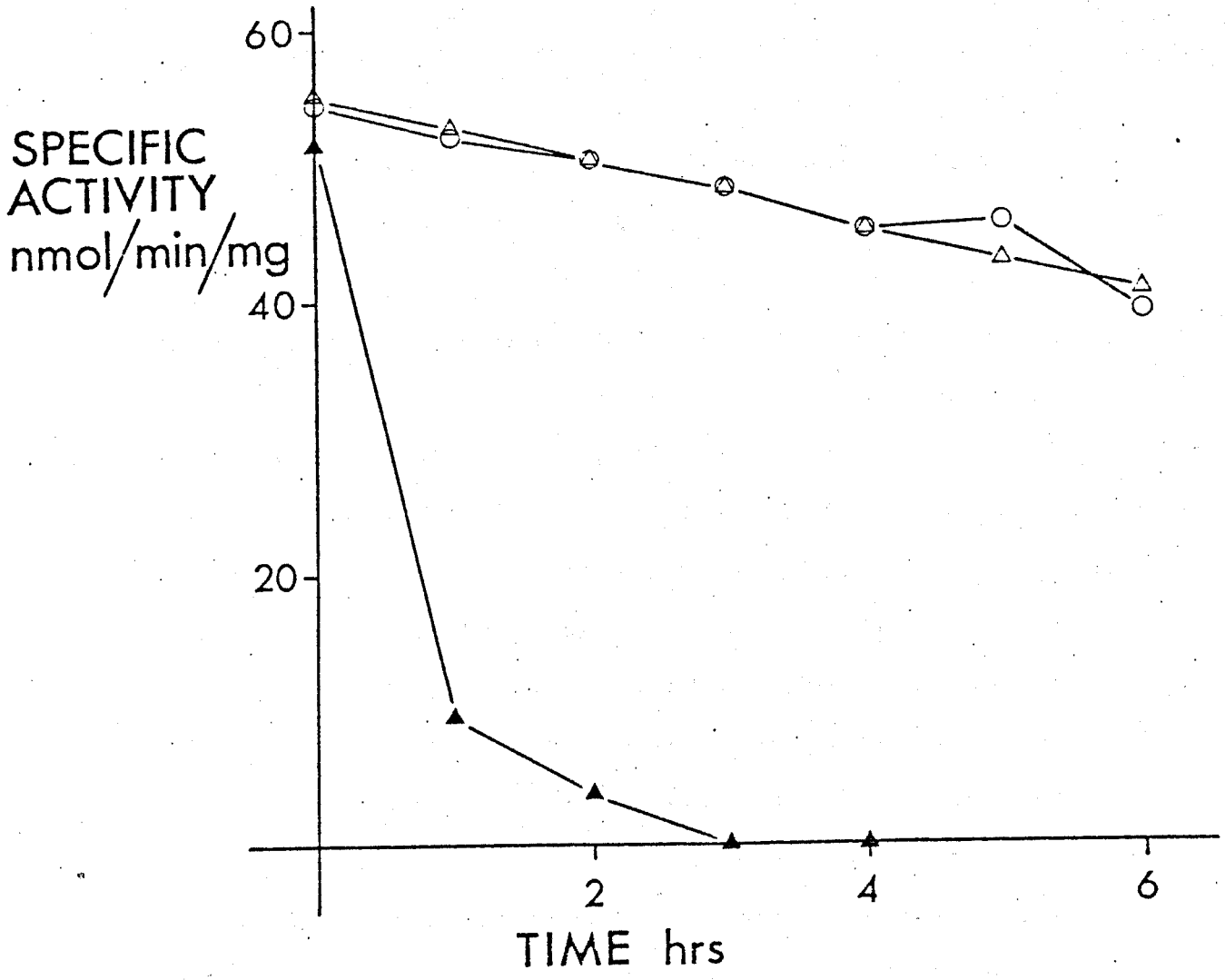


Figure 3.A.2. Stability of the soluble MMO of Methylosinus trichosporium (OB3b): The effect of dithiothreitol.

Soluble extract was incubated with 0, 1 and 5 mM dithiothreitol at room temperature and assayed for MMO activity hourly.

Dithiothreitol ▲ 0 mM. △ 1 mM. O 5 mM.

MMO assay: 3 minute assay, 30^{0}C, 5 mg extract per assay, following the oxidation of propene to epoxypropane.

21-26 nmol/min/mg activity reported for soluble MMO activity in soluble extracts by Scott et al., (1981a) and up to 146 nmol/min/mg reported by Stirling and Dalton (1979a).

The preparation of soluble cell free extracts did not provide constant soluble MMO activities, even when extracts were prepared from the same batch of drop frozen cells. Of the seventeen extracts made from the first 100 l batch culture of Methylosinus trichosporium (OB3b), three had soluble MMO activities below 10 nmol/min/mg, with the lowest having no detectable activity. The majority of extracts had a soluble MMO activity of over 40 nmol/min/mg with the highest activity measured at 68 nmol/min/mg. So despite the addition of both PMSF and DTT the soluble MMO could still be highly unstable during the preparation of soluble cell extracts, yielding little or no activity. Extracts as far as possible were prepared in an identical fashion, with buffers made up directly prior to use, and the process taking 2-2.5 hours in total. So no obvious reason for the variation in the specific activity of the soluble MMO of analogous cell free extracts can be put forward.

Soluble extracts were drop frozen in liquid nitrogen and stored at -80^{0}C until required. No loss of activity was measured when extracts were stored in this manner for up to six months, a result that contradicts Stirling and Dalton (1979a) who, although finding crude cell free extracts stable under these conditions, measured a 25% loss of activity in soluble extracts over ten day period.

Stirling and Dalton (1979a) found that soluble extracts that had lost activity could be restored to full activity by the addition of DEAE- fractions B and C from the soluble MMO

of Methylococcus capsulatus (Bath). Similar findings were observed with extracts prepared in this study (Table 3.A.3). Addition of 1 mg of each of Methylococcus capsulatus (Bath) soluble MMO DEAE fractions B and C increased the specific activity from 13.8 to 69 nmol/min/mg. The addition of greater amounts of either B or C failed to further increase the specific activity and so a component(s) present in the soluble cell free extract of Methylosinus trichosporium (OB3b) must have been limiting. The addition of Methylococcus capsulatus (Bath) soluble MMO DEAE- fraction B to soluble cell extracts of Methylosinus trichosporium (OB3b) failed to increase and in fact slightly lowered the specific activity. Addition of DEAE- fraction C of the soluble MMO of Methylococcus capsulatus (Bath) raised the specific activity to 52.3 nmol/min/mg a significant rise but not as high as on the addition of both components B and C (Table 3.A.3).

These results suggest that it was the hydroxylase component of the soluble MMO of Methylosinus trichosporium (OB3b) that was the most stable: ie that component equivalent to component A of the soluble MMO of Methylococcus capsulatus (Bath), confirming the view of Stirling and Dalton (1979a). This component was also the most stable in the soluble MMO of Methylococcus capsulatus (Bath) when in soluble cell free extracts (Colby and Dalton 1978).

Neither Methylococcus capsulatus (Bath) soluble MMO DEAE fractions B cr C alone would restore full activity to soluble extracts of Methylosinus trichosporium (OB3b) which may suggest that both were required by the hydroxylase

Table 3.A.3. Stability of the soluble MMO: Restoration of activity by the addition of components of the soluble MMO of Methylococcus capsulatus (Bath).

Methylococcus capsulatus soluble MMO components (mg)			Epoxypropane (nmol)	Specific activity (nmol/min/mg)
A	B	C		
0	0	0	206	14
0	1	1	1039	69
0	1	2	1019	68
0	2	1	1028	68
1	0	0	204	14
0	1	0	140	9
0	0	1	785	52
0	0	0	407	27
0	0	1	537	36
0	0	2	637	42
0	0	1*	408	27

Assays contained: 5 mg soluble extract of Methylosinus trichosporium (OB3b) plus DEAE frations of the soluble MMO of Methylococcus capsulatus (Bath) as specified.

* - Denotes use of pure component C.

MMO assay: 3 minutes, 30^{0}C, propene to epoxypropane.

component of the soluble MMO of Methylosinus trichosporium (OB3b) for full activity. However, it is not known whether component B, which couples the flow of electrons through the soluble MMO of Methylococcus capsulatus (Bath) to the oxidation of substrate, acts on component A, component C or a complex of the two. So component B may well act on component C and not the hydroxylase component of the soluble MMO of Methylosinus trichosporium (OB3b). The addition of Methylococcus capsulatus (Bath) soluble MMO DEAE- component C to soluble cell free extracts of Methylosinus trichosporium (OB3b) raised the specific activity, however the addition of pure component C of the soluble MMO of Methylococcus capsulatus (Bath) failed to increase the specific activity (Table 3.A.3). Methylococcus capsulatus (Bath) soluble MMO DEAE component C was known to be contaminated with component B (see results section E), and accounts for this result, showing that both components B and C were required to restore full activity to soluble cell extracts of Methylosinus trichosporium (OB3b). Unfortunately pure component B of the soluble MMO of Methylococcus capsulatus (Bath) was unavailable for use in this work. The addition of pure component A of the soluble MMO of Methylococcus capsulatus (Bath) to soluble cell extracts of Methylosinus trichosporium (OB3b) had little or no effect on the activity of the soluble MMO.

The activities measured for the hydroxylase component of the soluble MMO of Methylosinus trichosporium (OB3b) by the addition of the components of the soluble MMO of Methylococcus capsulatus (Bath) were not expected to be the maximum attainable, since it would not be expected that

the components of the soluble MMO of Methylococcus capsulatus (Bath) would operate efficiently at 30^{0}C or as efficiently as the equivalent components of the soluble MMO of Methylosinus trichosporium (OB3b) in combination with the hydroxylase component of this enzyme.

A.3. Preparation of cell free extracts.

Upon disruption of bacterial cells a large amount of nucleic acid is released. Cell disruption can also release cell wall material that can either be finely dispersed to give a turbid extract or partially solubilised, so that as well as protein and nucleic acid, there is a large amount of viscous polysaccharide in solution causing high viscosity (Scopes 1982).

Cell extracts of Methylosinus trichosporium (OB3b) are extremely viscous, this has been prevously reported by Stirling and Dalton (1979a) and Scott (personal communication). It was found that soluble extracts greater than 20 mg/ml protein prepared for this work, obtained either by sonication or decompression in the French press had a sufficiently high viscosity to prevent significant separation into soluble and particulate fractions by centrifugation at 150000 g for 60 minutes. This compares with the adequate separation achieved in this laboratory with extracts of Methylococcus capsulatus (Bath) of a protein concentration of 40 mg/ml by centrifugation at 38000 g for 30 minutes (Stanley and Dalton 1982).

Two groups, that of Higgins's and Dalton's have prepared soluble cell extracts of Methylosinus trichosporium (OB3b), each used a centrifugation of 150000 g for 20-90 minutes, to give a soluble cell extract (S_{150}). From the data available, though in many papers figures are not supplied, it can be seen that soluble cell extracts of Methylosinus trichosporium (OB3b) were prepared at low protein concentrations (less than 20 mg/ml). This was presumably necessary to enable efficient pelleting of particulate material at 150000 g.

High viscosity extracts can cause a number of problems in the purification and characterisation of enzymes from such extracts:

1. High viscosity soluble extracts can only be prepared at low protein concentration to enable an efficient and reproducible separation of particulate material. At such low protein concentrations enzymes are often less stable than at high protein concentrations. Low protein concentrations also restrict the amount of protein that can be prepared at one time due to the large volumes involved.

2. Viscosity can interfere with a number of purification procedures such as: precipitation, ultrafiltration, running of chromatography columns, etc.

3. All processes such as centrifugation or concentration, are prolonged by viscosity, causing problems in the purification particularily if the enzyme is unstable.

4. Viscosity causes problems in the general handling of the extract, especially when trying to measure out small volumes accurately for assays etc.

To the author's knowledge only two proteins have been

purified from the soluble fraction of cell free extracts of Methylosinus trichosporium (OB3b). These were cytochrome c (Tonge et al., 1977) and methanol dehydrogenase (Higgins et al., 1984). No details were published of the purification of methanol dehydrogenase from Methylosinus trichosporium (OB3b) however it was purified in an analogous way to the methanol dehydrogenase from Methylophilus methylotrophus by the use of an aqueous two-phase partition system consisting of polyethylene glycol (PEG) and potassium phosphate.

Cytochrome c was purified from a 150000 g supernatant using a protamine sulphate precipitation (equal volume of 1% weight/volume) which brought the protein concentration down from 20.0 to 9.0 mg/ml. Further steps including ion exchange and gel-filtration chromatography were employed to give pure cytochrome c. This protein, though totally denatured by one cycle of freezing and thawing, was very stable when stored at 0-4^{0}C, and so would present little problem in purification unlike the highly unstable soluble MMO. During the early stages of purification protein concentrations were kept low presumably due to high viscosity. So the problems caused by high viscosity in cell extracts of Methylosinus trichosporium (OB3b) have not been overcome. No progress had been made on the problem of viscosity by members of Higgins's group over the last few years (D. Scott personal communication).

It is therefore obvious that the problem of very high viscosity of cell extracts of Methylosinus trichosporium (OB3b) needed to be addressed before any purification of the soluble MMO was undertaken. There are a number of strategies available to alleviate the problems caused by

high viscosity extracts. These are:

1. Variation in the growth conditions of Methylosinus trichosporium (OB3b) may reduce the production of viscous components of the cell free extract.

2. Cells can be treated directly prior to disruption to either stimulate the breakdown of viscous components or extract viscous components.

3. Use of a more gentle method of cell disruption than sonication or French pressing to reduce the dispersion and solublisation of 'gum'-like polysaccharides of the cell wall.

4. The treatment of extracts either to digest or to separate viscous components from enzyme(s) of interest.

Attempts were therefore made to prepare manageable extracts using each of these strategies.

A.3.1. Variation of growth conditions.

Methylosinus trichosporium (OB3b) was grown in a chemostat under a variety of different conditions to examine whether the viscosity of cell extracts was affected. Cells were harvested, disrupted using the French pressure cell and cell extracts prepared as detailed for small scale extract preparations in Materials and Methods. The growth conditions investigated were: using methane as a carbon and energy source; oxygen limitation; methane limitation; with either ammonia, nitrate or gaseous nitrogen as nitrogen source in each case, variation in the dilution rate (D = 0.025-0.1 h^{-1}) and therby variation in the cell density in the chemostat. None of these growth conditions appeared to

affect the viscosity of cell extracts. Substituting methane for methanol, the only other known carbon and energy source for obligate methanotrophs also had no effect on the viscosity of the extracts. Because Methylosinus trichosporium (OB3b) was sensitive to methanol (Best and Higgins 1981), all cultures using methanol were grown under methanol limiting conditions.

A.3.2. Treatment of cells prior to disruption.

Under certain growth conditions Methylosinus trichosporium (OB3b) is known to form large quantities of the storage polysaccharide poly-β-hydroxybutyrate (PHB), particularly when it is grown under conditions of carbon excess, when up to 30% of the dry weight of the organism may consist of PHB (Best and Higgins 1981). Such large quantities of PHB may, when released on cell disruption, contribute to the viscosity of cell free extracts.

Thomson et al., (1976) showed that acetate and higher carboxylate ions stimulated the breakdown of PHB in Methylosinus trichosporium (OB3b). Under these conditions 3-hydroxybutyrate is converted to acetone and carbon dioxide by the action of 3-hydroxybutyrate dehydrogenase and acetoacetate decarboxylase, which are excreted from the cell.

Cells of Methylosinus trichosporium (OB3b) from a methane-limited chemostat culture were centrifuged and resuspended in 20 ml of 20 mM phosphate buffer pH 7.0 to an OD_{540} of 100 (43 mg/ml dry weight) in a conical flask. Sodium acetate to a concentration of 5 mM was added, the

flask sealed with a 'Suba Seal' and placed in a gently rotating gyratory water bath set to 30^{0}C. 5 microlitres samples were withdrawn at approximately 15 minute intervals and analysed for acetone and acetate using gas chromatography as described in Materials and Methods.

The concentration of acetate fell to a level of 1 mM after 105 minutes (FIG 3.A.3). The level of acetone rose to reach a maximum at 75 minutes of 3.7 mM from which it slowly declined. At 105 minutes the cells were centrifuged, washed twice and resuspended in buffer. A sample was taken for PHB analysis. The rest of the cells were broken and a soluble cell extract prepared (without the use of protamine sulphate).

Before acetone treatment cells contained 5% of their dry weight as PHB whereas cells after acetate treatment contained no measurable PHB. However the soluble extract prepared from acetate treated cells was still as viscous as that prepared from untreated cells.

This result shows that PHB content of the cells is not a major factor in the viscosity of cell extracts. This confirms the results obtained from experiments where cells were grown under different limitations that greatly affect the levels of PHB in the cell. Cells grown under conditions where carbon was in excess (oxygen limitation) are known to contain 30% of their dry weight as PHB whereas cells grown under carbon limited conditions have less than 5% of their dry weight as PHB (Best and Higgins 1981) yet each yields highly viscous extracts.

Capsular polysaccharide that is tightly bound to bacterial cells can be removed from cells and extracted

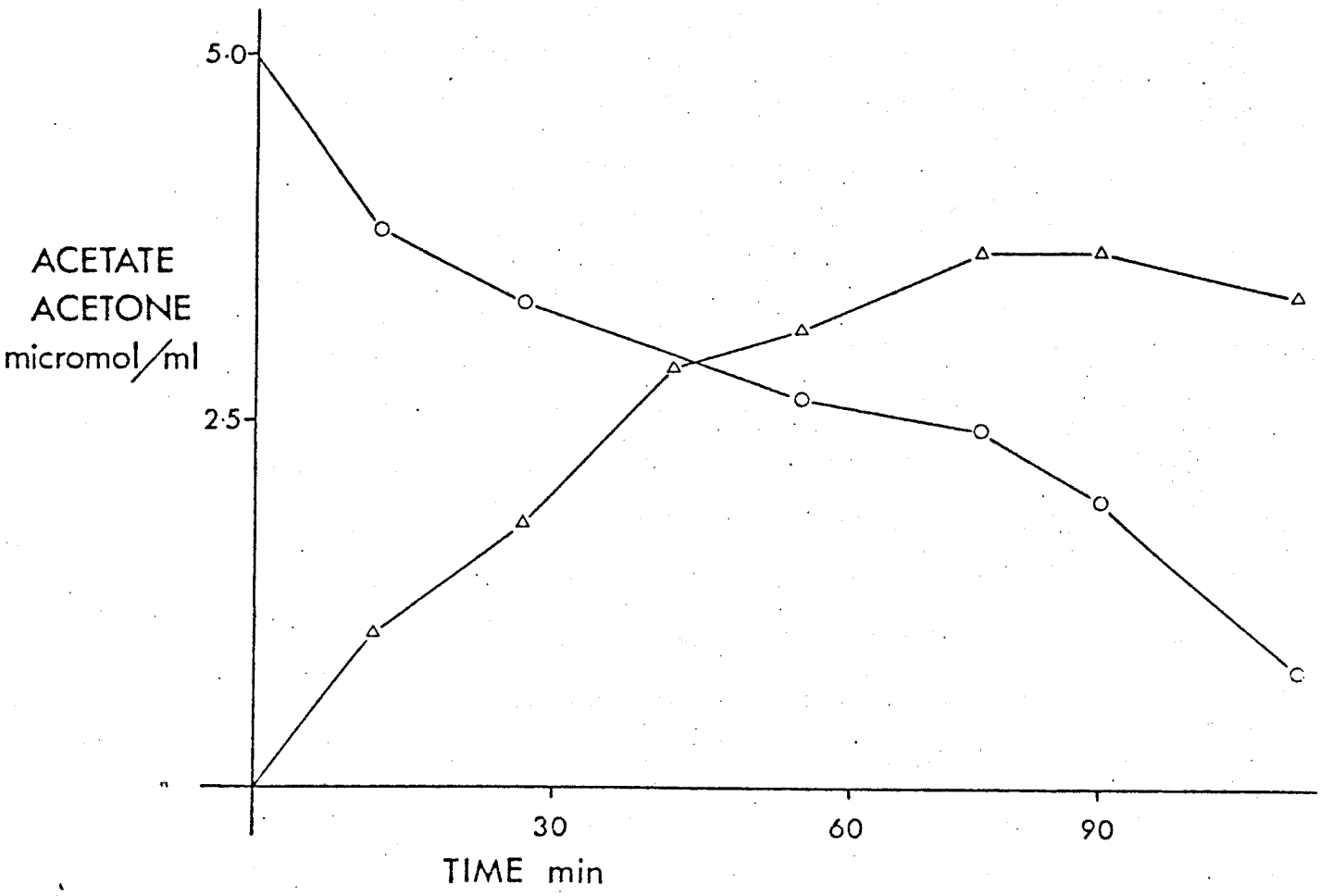


Figure 3.A.3. The assimilation of acetate and the production of acetone by whole cell of Methylosinus trichosporium (OB3b).

Cells (20 ml OD_{540} = 100) were incubated with 5 mM acetate at 30°C in a sealed conical flask, samples were withdrawn over
2 h and assayed for acetate and acetone by gas chromatography.
Acetate O Acetone △

using a number of techniques (Sutherland and Wilkinson 1971). However such techniques are normally designed for the isolation of capsular polysaccharide or nucleic acids, the treatment of cells is often harsh and leads to the denaturation of any enzymes present. Hot aqueous, hot phenol, alkali and acetic acid extractions of capsular polysaccharide obviously fall into this category. A number of less harsh methods were tested for their ability to reduce the viscosity of extracts while preserving high soluble MMO activity. These included , phenol extraction (Sutherland and Wilkinson 1971), cold acetone extraction (Gansalus 1955) and lysozyme digestion. None of these methods proved satisfactory. In all cases where cells were exposed to organic solvents no soluble MMO activity was recoverable from cells.

A.3.3. Methods of cell disruption

Cells of Methylosinus trichosporium (OB3b) were routinely disrupted by two passages through a French pressure cell. This is a violent mechanical method of cell disruption, relying on hydrodynamic shear. Sonication also relies on similar forces to disrupt cells. Both these methods of cell disruption yielded equally viscous extracts, a result in agreement with that observed by Stirling and Dalton (1979a). Methods such as these are the methods of choice when disrupting cells in the preparation of cell extracts for protein purification, because they work efficiently at the high cell densities required. The major disadvantage of these methods is that they can release and

disperse large quantities of cell wall material producing turbid viscous extracts. Gentle chemical methods of cell disruption have been developed, mainly for the isolation of DNA. These methods release the minimum amount of cell wall polymers but are designed to work at low cell densities.

A method using combinations of detergent washes, lysozyme treatment and osmotic shock as described by Schwinghamer (1980) was employed in an attempt to disrupt gently cells of Methylosinus trichosporium (OB3b). Experiments carried out using low cell densities similar to that used by Schwinghamer (OD_{540} 1-5), showed that cells of Methylosinus trichosporium (OB3b) were indeed sensitive to this treatment being efficiently broken as observed by microscopic examination though no soluble MMO activity was measured in extracts prepared using this method. However at the high cell densities required for the preparation of cell extracts for protein purification (OD_{540} = 150) the method proved totally inadequate at disrupting cells. Variation in the type, concentration and length of exposure to, the detergent and exposure to lysozyme as detailed in Materials and Methods had little or no effect on this efficiency.

A.3.4. Treatment of extracts.

As, previously stated, the viscosity of extracts can render the fractionation of proteins by precipitation difficult and irreproducible. However precipitation can be employed as an early step to selectively separate protein from other cellular components, in the hope that viscous components of the extract remain in solution. Protein can

then be pelleted, redissolved and fractionation proceeded with.

Two precipitation methods were tested using ammonium sulphate and acetone. Solid ammonium sulphate was added slowly to a concentration of 80% saturation, to a crude cell extract, a concentration at which all protein should precipitate (Scopes 1982). The extract was then incubated with gentle stirring at $4^{0}C$ for 10 minutes to allow precipitation to take place and centrifuged at 38000 g for 10 minutes to remove precipitate. The resultant pellet was resuspended and redissolved in breakage buffer. It was then necessary to dialyse the protein solution against breakage buffer to remove traces of ammonium sulphate. A similar process using 40% acetone as the precipitant was also undertaken.

The protein solutions obtained were of reduced viscosity however no soluble MMO activity was detected in either the ammonium sulphate- or acetone-prepared protein solutions. This may have been due to a number of reasons but was most likely to be due to the inherent instability of the soluble MMO (even in the presence of DTT and PMSF), combined with the length of time the above procedure takes, particularily dialysis

The acidification of extracts to pH 5-6 can promote the aggregation of particulate material, enabling the more efficient and reproducible separation of the particulate material from soluble extracts by centrifugation. It is not a method that will reduce the viscosity of extracts (Scopes 1982). This method is only useful when the enzyme of interest does not itself precipitate, absorb to the

precipitate and is stable at the reduced pH.

Molar acetic acid was added to cell free extracts reducing the pH value to 5.5. The extract was then gently stirred for 10 minutes, centrifuged at 150000 g for 60 minutes and the pH of the resulting supernatant returned to pH 7.0 with molar sodium hydroxide. Acidification was an aid to the removal of particulate material from cell free extracts, however the treatment resulted in a large drop in the activity of the soluble MMO, from 47.7 to 17.2 nmol/min/mg.

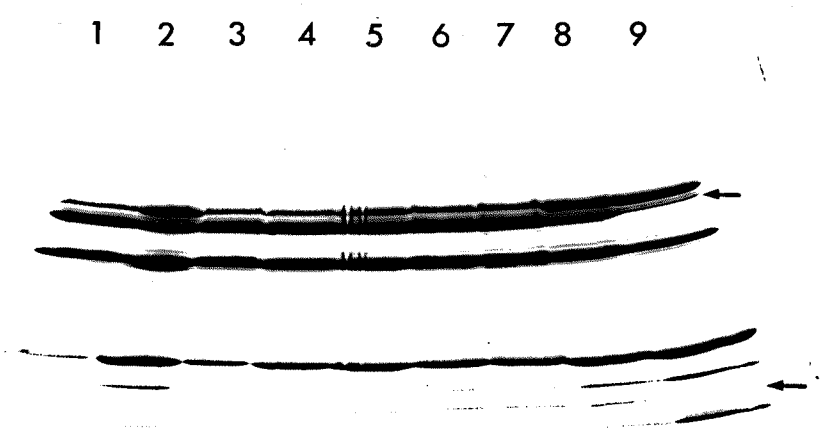
Bacterial extracts contain large quantities of nucleic acid in the form of DNA and ribosomal RNA which may cause high viscosities. All nucleic acid can be precipitated using a poly-cationic macromolecule such as protamine from salmon milt. DNA, all forms of RNA and other forms of nucleic acid are precipitated as well as some proteins which may absorb to the precipitate, in fact protamine precipitate absorption has been used as a step in the purification of certain enzymes (Dalton et al., 1971. Welch and Scopes 1981).

The addition, prior to cell disruption, of deoxyribonuclease 1 (0.01 mg/ml) and/or ribonuclease A (0.1 mg/ml) to cell extracts of Methylosinus trichosporium (OB3b) failed to reduce the viscosity of extracts significantly. However the addition of protamine sulphate to cell extracts of Methylosinus trichosporium (OB3b) was investigated as a method of precipitating nucleic acid and thereby reducing the viscosity of extracts. A cell extract was prepared and split into 5 ml aliquots and to each was added a known percentage of protamine sulphate (0.25- 2.0%) as a 10%

solution and each volume made up to the same final volume with water so that each had been diluted to the same degree. The extracts were then incubated at 4^{0}C for 20 minutes with shaking. Samples were then centrifuged at 150000 g for 60 minutes to remove the precipitate and protein and MMO assays performed (Table 3.A.4). In addition all samples were analysed on SDS PAGE (FIG 3.A.4). The addition of 0.25 - 1.0% protamine sulphate precipitated up to 17% of the protein without affecting the soluble MMO activity of the extract (Table 3.A.4). The addition of 2% protamine sulphate caused the precipitation of 65% of the protein with the accompanying loss of all soluble MMO activity from the supernatant. The addition of 1% and 2% protamine sulphate reduced the viscosity and promoted the precipitation of particulate material to a degree where, after centrifugation at 150000 g for 60 minutes, a consistently clear soluble extract was obtained from extracts with a protein concentration at, or around 20 mg/ml protein.

As analysed on SDS PAGE no major differences can be seen in the protein band pattern between crude extracts, soluble extracts and soluble extracts produced after treatment with 0.25 -1.0% protamine sulphate. However the extract treated with 2% protamine sulphate has one major and one minor protein band greatly reduced in intensity as compared to soluble extracts (FIG 3.A.4). The major band is one of those associated with the presence of the soluble MMO, and was subseqently identified as the large (α) subunit of component A of the soluble MMO (Results section C). Its precipitation may explain the loss of soluble MMO activity from soluble extracts prepared by the addition of

Figure 3.A.4. Electrophoresis of cell extracts of Methylosinus trichosporium (OB3b): Treatment with Protamine sulphate.



Percentage protamine sulphate treatment.

1. 0% crude extract
2. 1% soluble extract
3. 0% soluble extract
4. 1% soluble extract
5. 1% soluble extract
6. 0.5% soluble extract
7. 0.25% soluble extract
8. 1% soluble extract
9. 2% soluble extract

Electrophoresis: 5-20% gradient SDS PAGE, 20 micrograms protein per track.

Arrows denote bands dimminishing after treatment with 2% protamine sulphate.

Table 3.A.4. Protamine sulphate treatment of cell extracts.

Sample	Protamine sulphate	Protein* (mg/ml)	MMO activity (nmol/min/mg)
Crude extract	___	18.7	60.9
Soluble extract	0.25%	15.6	51.2
Soluble extract	0.50%	17.3	56.8
Soluble extract	1.00%	15.9	59.6
Soluble extract	2.00%	6.9	0

* - Protein concentration of the supernatant.

MMO assays: 3 minute assay, 30^{0}C, following the oxidation of propene to epoxypropane. 5 mg extract per assay.

2% protamine sulphate.

In conclusion, of all the many methods employed in an attempt to reduce the viscosity of cell extracts none enabled the production of concentrated cell free extracts containing high soluble MMO activity. The addition of protamine sulphate (1%) to crude extracts, prior to centrifugation at 150000 g enabled the production of clear soluble cell free extracts of protein concentration of around 20 mg/ml, by some reduction of the viscosity and promotion of the aggregation of particulate material. The method was subsequently used to routinely produce soluble cell free extracts of Methylosinus trichosporium (OB3b) for the purification of the soluble MMO.

A.4. Basic characterisation of the soluble methane monooxygenase.

Both reports of the soluble MMO of Methylosinus trichosporium (OB3b) state that NADH or NADPH were required for activity (Stirling and Dalton 1979a, Scott et al., 1981), activity with NADPH as electron donor supporting 60-100% of that with NADH depending on the substrate used in the assay. This was confirmed by assays on extracts prepared for this study. Soluble MMO activity was measured only in the presence of either NADH or NADPH using propene as a substrate. Assays of soluble extracts carried out under anaerobic conditions failed to have soluble MMO activity showing a requirement for oxygen by the soluble MMO. The soluble MMO has been exposed to a wide range of

inhibitors of which only 8-hydroxyquinoline and ethyne (acetylene) were found to be effective.

The pH optimum for the soluble MMO of Methylosinus trichosporium (OB3b) was measured in soluble extracts using a range of phosphate buffers pH 6.2 - 8.5 (FIG 3.A.5). The soluble MMO was found to have an activity optimum (using propene as a substrate) between pH 7.4 - 7.7, higher than that reported for the soluble MMOs of Methylococcus capsulatus (Bath) (pH 6.5 -7.0) (Colby and Dalton 1976) and Methylobacterium CRL-26 (pH 7.0) (Patel et al., 1982), for the oxidation of propene.

When the specific activity of the soluble MMO was measured as a function of the enzyme concentration a non-linear relationship was obtained (FIG 3.A.6). As the protein concentration was increased the specific activity increased up to a maximum at 4 mg/ml. This is indicative of the enzyme consisting of two or more proteins, inactive singly but active as a complex. A rise in the specific activity with enzyme concentration may also be due to the presence of a dissociable activator or co-enzyme in the extract or the presence of a toxic impurity (possibly a metal ion) present in one of the other components of the reaction mixture (Dixon et al., 1979). However it is already known that the soluble MMO from Methylosinus trichosporium (OB3b) is a multicomponent enzyme, consisting of at least two proteins (Stirling and Dalton 1979a) and so the increase in specific activity with enzyme concentration is probably due to the multicomponent nature of the enzyme.

The temperature optimum of the soluble MMO of

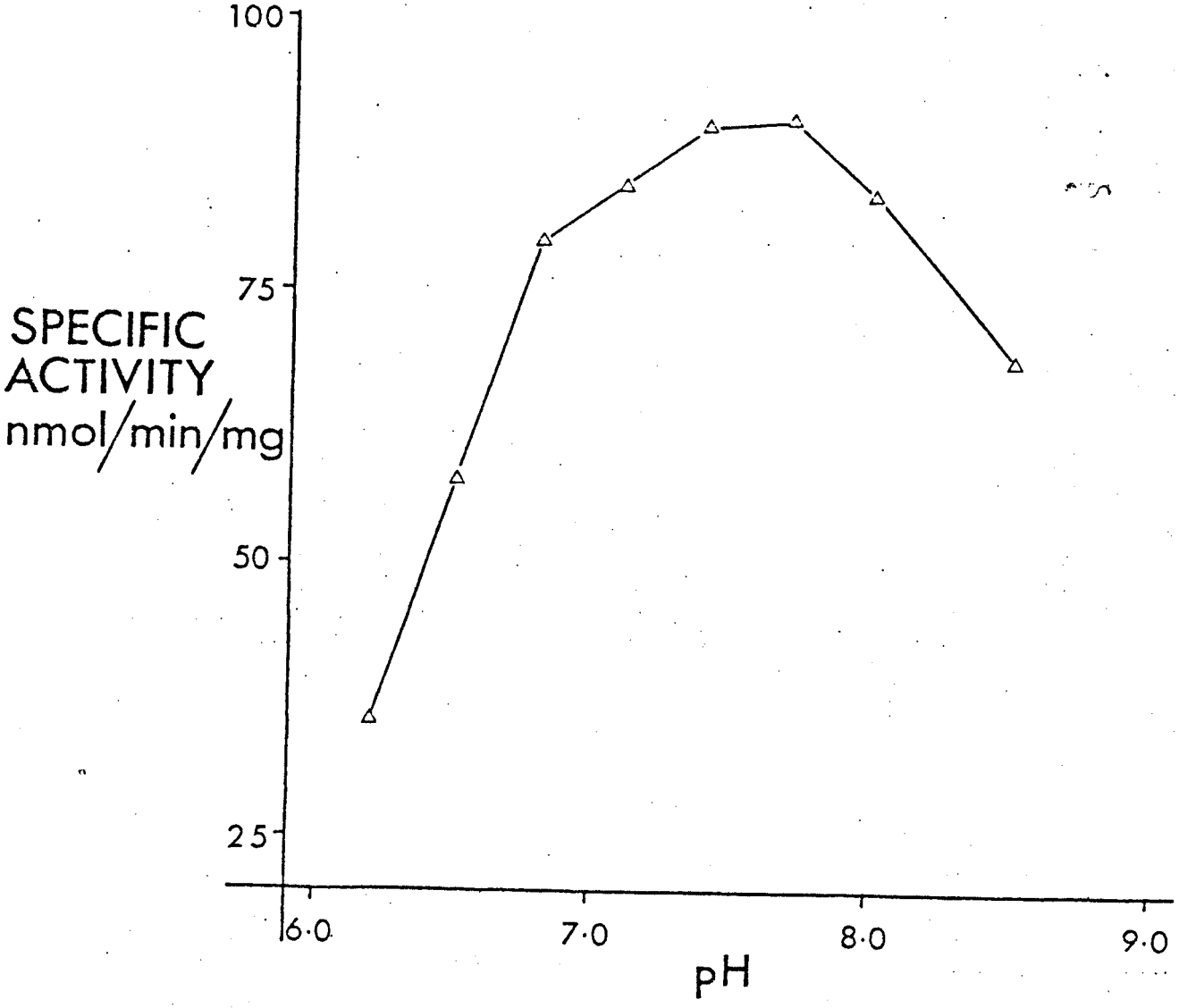


Figure 3.A.5. pH optimum of the soluble MMO of Methylosinus trichosporium (OB3b).

MMO assay: 3 minute assay, 30^{0}C, following the oxidation of propene to epoxypropane, 5 mg soluble extract per assay. pH 6.2 - 8.5 (20 mM sodium phosphate buffer).

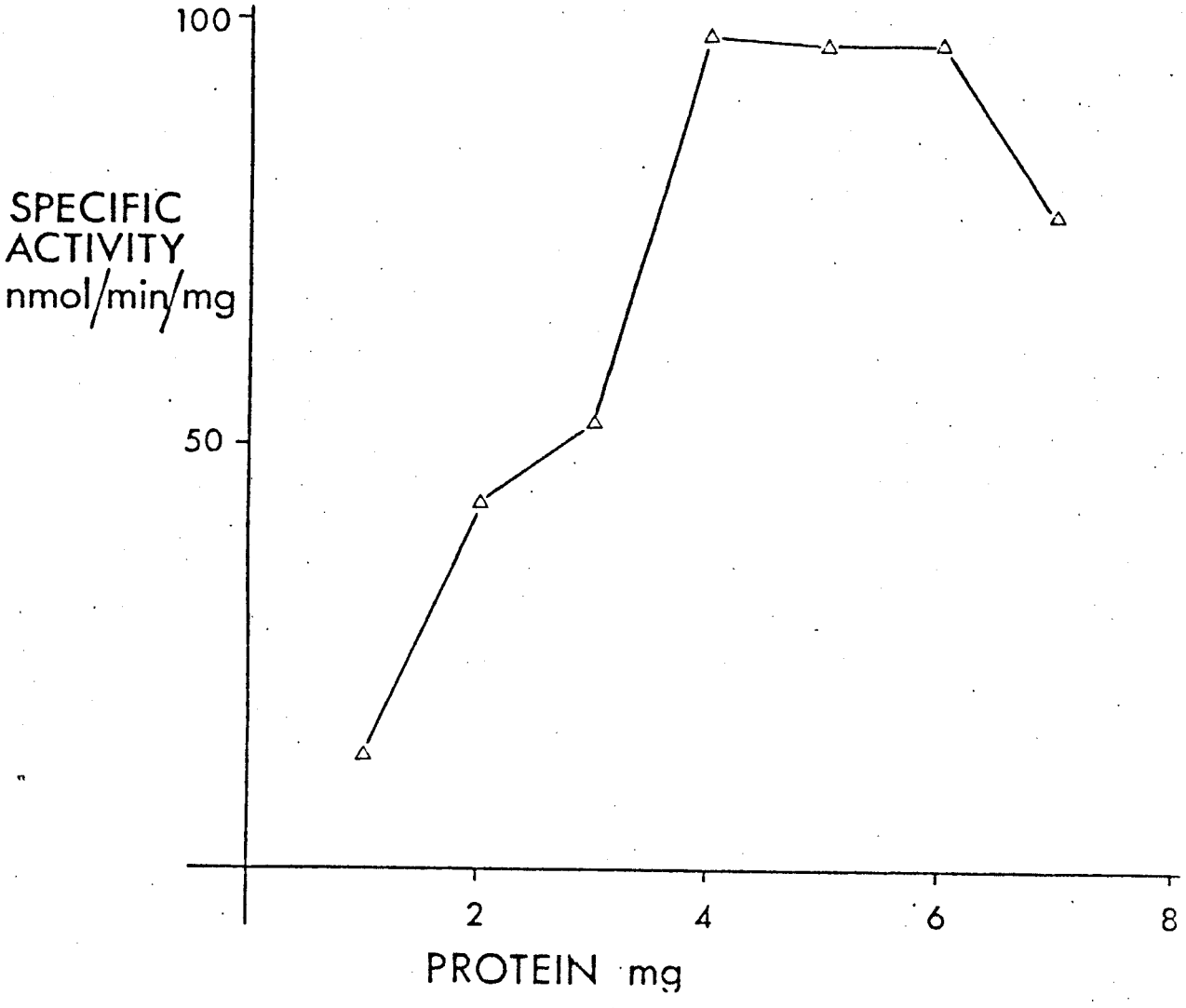


Figure 3.A.6. Effect of protein concentration on the activity of the soluble MMO of Methylosinus trichosporium (OB3b).

MMO assay: 3 minute assay, 30^{0}C, 1 - 7 mg soluble extract per assay, following the oxidation of propene to epoxypropane.

30-35^{0}C, though it was active at 80% of its full activity at 45^{0}C (FIG 3.A.7). The decrease in specific activity at higher temperatures may be due to the instability of the soluble MMO, as extracts incubated at 45^{0}C for three minutes prior to soluble MMO assay lost 70% of their activity. However on the addition of soluble MMO DEAE components B and C Methylococcus capsulatus (Bath), full activity was restored showing the hydroxylase component to be the most stable component of the soluble MMO. Further investigation of the activity of components of the soluble MMO at 30^{0}C and 45^{0}C is reported in results section E.

A.5. Discussion.

The nature and position of the MMO in Methylosinus trichosporium (OB3b) was in dispute for a number of years, until Scott et al., (1981ab) discovered that Methylosinus trichosporium (OB3b) had the ability to produce two MMOs whose subcellular location and properties were significantly different. However it was not until Stanley et al., (1983) showed that the controlling factor in the production of one or the other of the two MMOs in methanotrophs was found to be due to the availability of copper to the organism that any progress could be made on the elucidation of the role of each of the two forms of the soluble MMO in the metabolism of methanotrophs. Subsequently, work by Burrows et al., (1984) and the work contained in this thesis has shown that the regulation of the production of one or the other of the

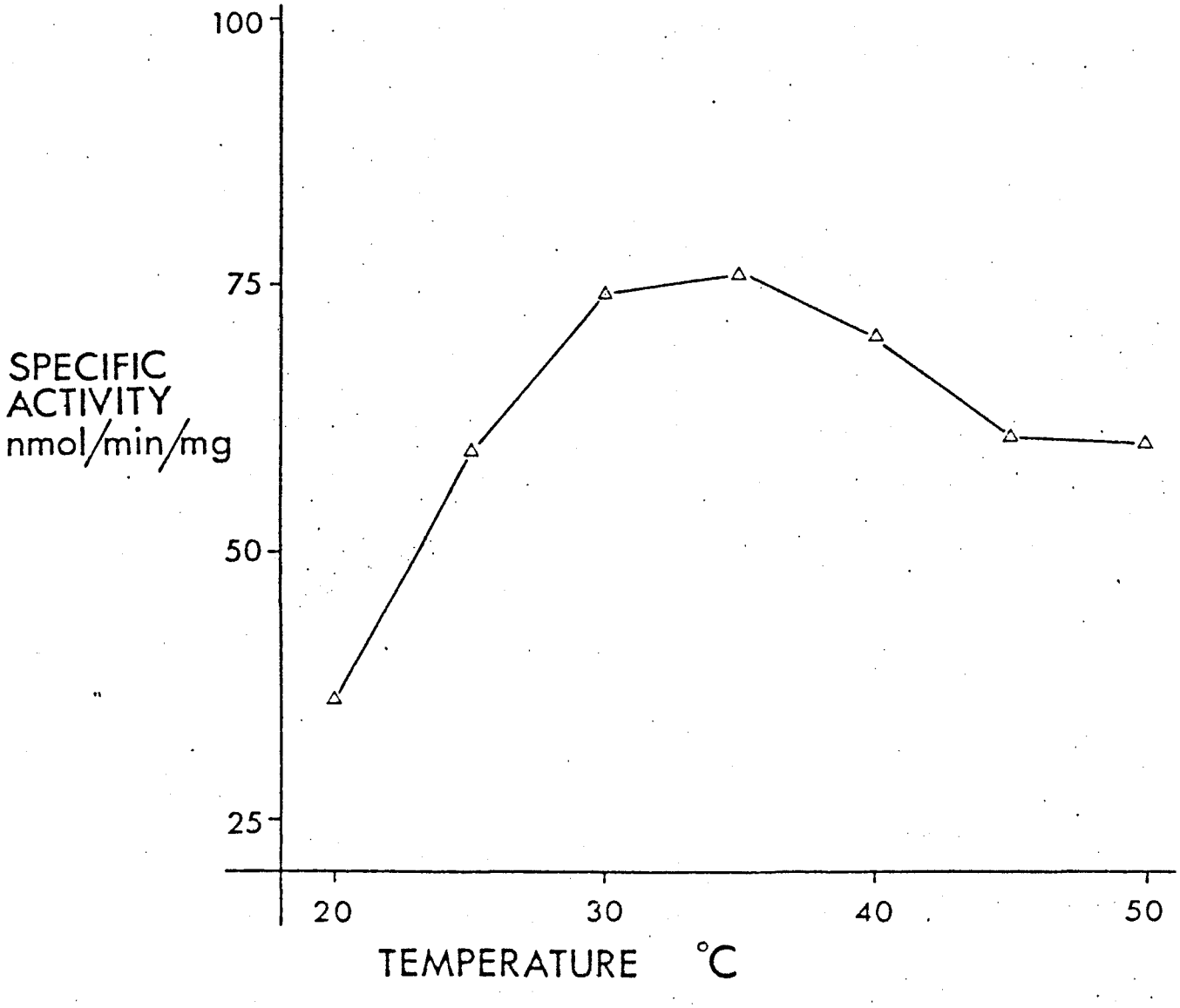


Figure 3.A.7. Temperature optimum for the soluble MMO of Methylosinus trichosporium (OB3b).

MMO assay: 3 minute assay, 30°C, 5 mg soluble extract per assay, following the oxidation of propene to epoxypropane.

definitely controlled by the availability of copper to the organism, a particulate MMO being produced by cells grown with copper in excess and a soluble MMO by cells grown under conditions of copper stress. There is evidence that other organisms (Methylobacterium CRL-26 Patel 1984) , can produce two forms of the MMO and/or have a similar response to copper as cells of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) (Takada and Tanaka 1980. Patel 1984). All organisms so far described appear to either produce both a soluble and a particulate MMO or just a particulate MMO, to the the authors knowledge no organism has been reported to produce only a soluble MMO.

There is however a difference between Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) in the regulation of the MMOs when these organisms are grown on methanol. Methylococcus capsulatus (Bath) grown on methanol can only produce a particulate MMO no matter what the copper concentration of the media. Methylosinus trichosporium (OB3b) grown on methanol can produce either soluble or a particulate MMO (Best and Higgins 1981, Prior and Dalton 1985). Prior and Dalton (1985) suggest that the method of cell disruption employed by Best and Higgins (1981), sonication, may act to release a particulate MMO from the membrane and this was the reason why an MMO activity was observed in soluble extracts of Methylosinus trichosporium (OB3b) grown on methanol. Work contained in this thesis demonstrates that Methylosinus trichosporium (OB3b) grown on methanol responds to the level of copper in the media as occurs during growth on methane,

There is no obvious reason why it would be an advantage for an organism to only produce a particulate MMO when grown on methanol. Paradoxically, it would appear that the production of a soluble MMO may be advantageous to an organism growing on methanol, if as Cornish et al.,(1984) suggest, during growth on methanol, its oxidation by the MMO acts as a sink for NADH producing NAD^+ required for the further oxidation of methanol through to carbon dioxide. The soluble MMO has an absolute requirement for NADH, whereas the particulate MMO can obtain its reducing equivalents from non-NADH linked alcohol and aldehyde dehydrogenases (Leak and Dalton 1983) and so a lower demand for NADH by a particulate MMO would more quickly result in the depletion of NAD^+ and accumulation of formaldehyde.

The three protein bands prominent on SDS PAGE of cell free extracts of Methylosinus trichosporium (OB3b) as described by Burrows et al., (1984) were only present when soluble MMO activity was detected and a further band was also identified to be associated with the presence of the soluble MMO.

The soluble MMO of Methylosinus trichosporium (OB3b) was highly unstable but could be stabilized by the addition of DTT, a thiol protective agent, and PMSF, a serine protease inhibitor, which enhanced the stability of the soluble MMO up to a maximum of 79% activity retained over a period of 24 hours at 0-4^{0}C. This is a similar stabilizing cocktail to that used for the stabilisation of the soluble MMO of Methylococcus capsulatus (Bath), component C requiring the addition of thiol protective agent , sodium thiolglycollate, and component B requiring the addition of

the serine protease inhibitor, PMSF. Although component B of the soluble MMO of Methylococcus capsulatus (Bath) appears to be far more stable than the serine protease requiring component of the soluble MMO of Methylosinus trichosporium (OB3b).

The similarity between the soluble MMOs of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) again was demonstrated by the ability of components B and C of the soluble MMO of Methylococcus capsulatus (Bath) to restore full MMO activity to soluble cell free extracts of Methylosinus trichosporium (OB3b) that had lost activity due to the unstable nature of the enzyme, as first shown by Stirling and Dalton (1979). This also demonstrates that the hydroxylase component of the soluble MMO of Methylosinus trichosporium (OB3b) is the most stable, as it is in soluble extracts of Methylococcus capsulatus (Bath). This work fails to give any indication of the number of components that go to make up the soluble MMO of Methylosinus trichosporium (OB3b), beyond the fact that it may have two or more. Its similarity to the soluble MMO of Methylococcus capsulatus (Bath) would suggest that it has three components but no real indication can be given at this point (see Results section E).

B. Separation of the soluble MMO into its component proteins.

B.1. Introduction.

Stirling and Dalton (1979a) separated the soluble MMO of *Methylosinus trichosporium* (OB3b) into two components using batch DEAE ion exchange chromatography. Of the soluble MMO's presently described, only that from *Methylococcus capsulatus* (Bath) is fully characterised and has three distinct protein components. Other partially characterised soluble MMO's, that of *Methylobacterium* CRL-26 (Patel 1984) and Organism SB1 (Allen *et al.*, 1984) have at least two components. Initially then, concentrated soluble extracts of *Methylosinus trichosporium* (OB3b) were run on DEAE ion exchange chromatography columns in an attempt to separate it into its component proteins.

B.2. Results.

To optimise the separation of the soluble MMO of *Methylosinus trichosporium* (OB3b) into its component proteins by the use of DEAE ion exchange chromatography, column rather than batch chromatography was employed with the use of a number of eluting methods.

Soluble cell free extracts, containing 1 mM PMSF and 1 mM DTT, and prepared with the use of a 1% protamine sulphate precipitation, were concentrated by dialysis against dry PEG

20000. Dialysis was used as a method of concentration because of the inherent viscocity of soluble cell extracts of Methylosinus trichosporium (OB3b) which prevents the concentration of extracts by ultrafiltration. A small column of DEAE cellulose of 20 ml volume was used (60 mm by 20 mm). Typically 10 ml of concentrated soluble extract of 70-100 mg/ml protein concentration, was loaded onto the column. 30 ml of buffer was used to wash the unbound protein from the column at a flow rate of 1.0 ml/min, followed by one of a number of gradients of sodium chloride, fractions of 3.0 ml being collected and the OD_{280} of the eluate being monitored throughout.

Approximately 85% of the protein loaded onto the column as a deep red concentrated protein solution failed to bind and was eluted with the buffer wash (FIG 3.B.1). This fraction is equivalent to fraction 1 as described by Stirling and Dalton (1979a) but henceforth will be known as DEAE OB3b A. DEAE OB3b A had no soluble MMO activity when assayed alone, however when assayed in the presence of Methylococcus capsulatus (Bath) soluble MMO DEAE fraction(s) C and/or B as described in the materials and methods, high soluble MMO activities were measured confirming the findings of Stirling and Dalton (1979a) (TABLE 3.B.1). Activity was also observed when DEAE OB3b A was assayed in combination with pure component C from the soluble MMO of Methylococcus capsulatus (Bath). No activity was measured when DEAE OB3b A was assayed in combination with Methylococcus capsulatus (Bath) DEAE fraction(s) A and/or B.

The specific activities of DEAE OB3b A fractions

Table 3.B.1. MMO assays of combinations of DEAE fractions of the soluble MMOs of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath).

Soluble MMO DEAE fraction (mg)					Epoxypropane	Specific
OB3b		MC			(nmol)	Activity
A	C	A	B	C		(nmol/min/mg)
5	0	7	0	0	0	—
5	0	0	1	0	0	—
5	0	0	0	1	581	39
5	0	0	1	1	829	55
5	0	0	0	1*	110	7
0	1	7	0	0	262	87
0	1	0	1	0	0	—
0	1	0	0	1	0	—
0	1	0	1	1	0	—
0	1	1*	0	0	63	10

DEAE fractions prepared as described in Materials and Methods.

Methylosinus trichosporium (OB3b): A buffer elution.
C 0.3 M NaCl elution.

MMO assay: 3 min, 30^{0}C, propene to epoxypropane.

OB3b- Methylosinus trichosporium (OB3b).

MC- Methylococcus capsulatus (Bath).

* denotes use of a pure component.

on average being 61.1 nmol/min/mg with a standard deviation of 6.5 (19 samples). DEAE OB3b A was drop frozen into liquid nitrogen and stored at -80^{0}C without any loss in activity, at 0-4^{0}C it was very stable losing up to 5% activity over a 24 hour period. Its stability was not dependent on the presence of either PMSF or DTT. PMSF irreversably inhibits the action of serine proteases by sulphonylation at or near the active site (Fahrney and Gold 1963) and so once an extract has been exposed to PMSF it would not be expected that its presence would be further required.

The soluble cell extracts used had been concentrated up to five times their original protein concentration and so their viscosity was greatly increased. This posed a number of problems in the running of DEAE cellulose columns, notably it led to the compacting of the column material when extracts were pumped onto the top of columns, leading to very slow flow rates and often to blocked columns. This was overcome by the mixing of concentrated soluble extracts with the column packing material prior to the pouring of the column. The column was then poured, allowed to settle and run in the normal way. This method had a number of advantages, in that it alleviated the problems caused by viscosity, was quick and only led to the minimum dilution of protein.

The viscous components of the soluble cell free extracts of Methylosinus trichosporium (OB3b) failed to bind to DEAE cellulose columns and were washed through with the initial buffer wash and were therefore present in DEAE OB3b A.

After the buffer wash the columns were eluted with gradients of buffer containing 0-0.2 M to 0-0.5 M sodium chloride. Typically a single broad yellow protein peak was eluted, sometimes with a shoulder on its trailing edge (FIG 3.B.1). Variation in the steepness of the gradient failed to resolve this single peak further, which eluted at a sodium chloride concentration of 0.1 M, the shoulder if present appearing at 0.17 M. Fractions from the protein peak had no soluble MMO activity when assayed alone, however when assayed in the presence of DEAE OB3b A soluble MMO activity was observed (Table 3.B.2). Combinations of early and late fractions from the 0.1 M peak assayed with DEAE OB3b A gave similar activities as when assayed individually. Fractions from this peak are therefore equivalent to fraction 2 as defined by Stirling and Dalton (1979a) which also restored activity to DEAE OB3b A, this fraction will henceforth be known as DEAE OB3b C. When DEAE OB3b C was assayed in combination with Methylococcus capsulatus (Bath) DEAE fraction A, soluble MMO activity was observed, however no activity was measured when it was assayed in combination with Methylococcus capsulatus (Bath) DEAE fraction(s) B and/or C (TABLE 3.B.1). This is in contrast to the findings of Stirling and Dalton (1979a) who found that none of the components of the soluble MMO of Methylococcus capsulatus (Bath) restored activity to DEAE OB3b C. Soluble MMO activity was also restored to DEAE OB3b C when it was assayed in combination with pure component A of the soluble MMO of Methylococcus capsulatus (Bath) (Table 3.B.2).

No enhancement or stimulation of soluble MMO activity was ever observed on the addition of any late gradient

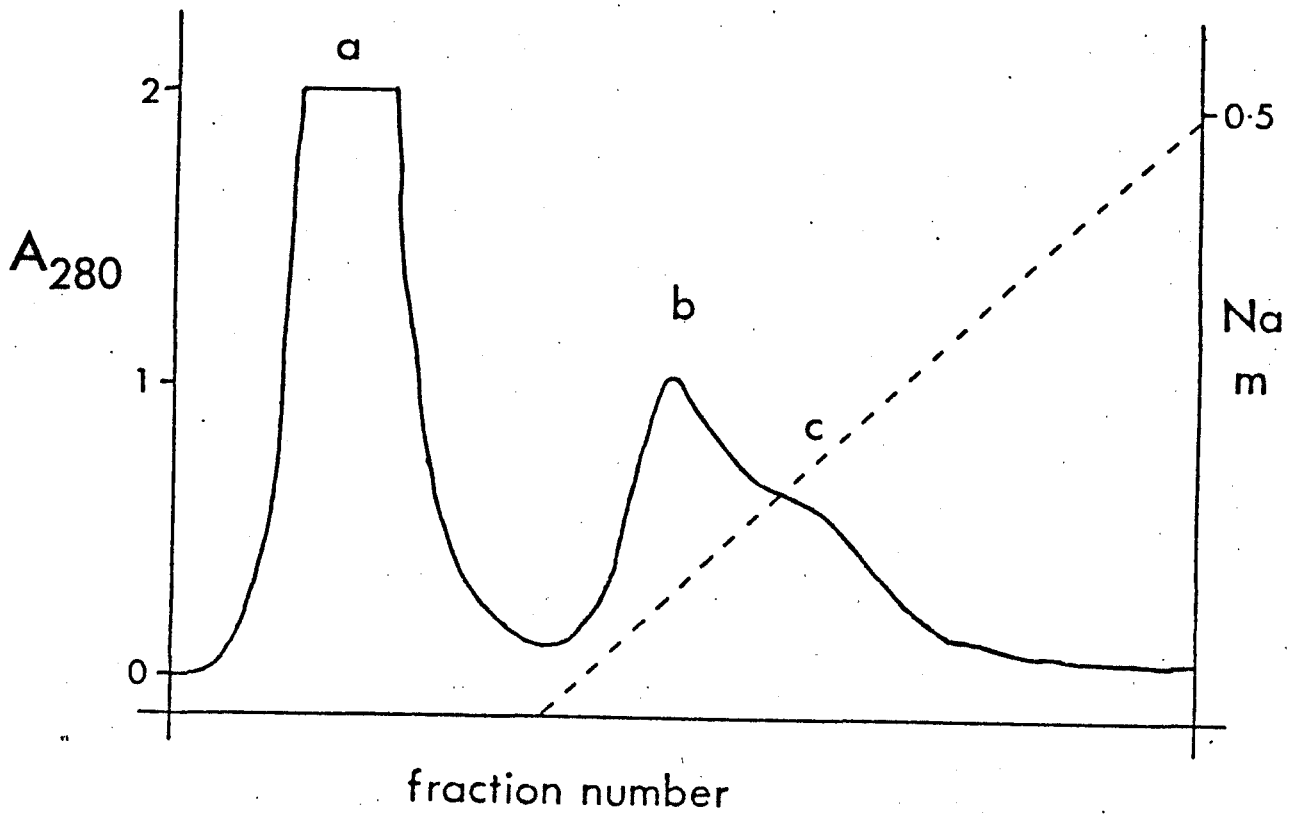


Figure 3.B.1. DEAE ion exchange chromatography of soluble extracts of Methylosinus trichosporium (OB3b).

Protein concentration of fractions was monitored by UV absorbance at 280 nm.

DEAE cellulose ion exchange chromatography: 20 ml volume column eluted with buffer (20 mM sodium phosphate, 1mM DTT, pH 7.0), followed by a linear gradient of buffer plus 0-0.5 M sodium chloride at a flow rate of 1 ml/minute.

a DEAE OB3b A.

b DEAE OB3b C early fractions 0-0.15 M NaCl (C_e).

c DEAE OB3b C late fractions 0.15-0.27 M NaCl (C_l).

Table 3.B.2. MMO assays of combinations of DEAE fractions of the soluble MMO of Methylosinus trichosporium (OB3b).

Soluble MMO DEAE fraction (mg)			Epoxypropane (nmol)	Specific activity (nmol/min/mg)
A	C_e	C_1		
5	0	0	0	—
0	1	0	0	—
0	0	1	0	—
5	1	0	332	22
5	0	1	292	19
5	1	1	324	21

DEAE fractions prepared as described in Materials and Methods using a linear (0 - 0.5 M) gradient of sodium chloride (FIG 3.B.1).

Soluble MMO assays: 3 min, 30^{0}C, propene to epoxypropane.

A - Buffer elution

C_e - early fractions of sodium chloride gradient (0 - 0.15 M).

C_1 - late fractions of sodium chloride gradient (0.15 - 0.27 M).

fractions (of very low protein concentration), eluting after the main single 0.1 M protein peak, to assays containing DEAE OB3b A, and/or C.

The specific activity of DEAE OB3b C varied with its concentration in the assay. In assays containing 5 mg/ml DEAE OB3b A the maximum specific activity for DEAE OB3b C occurs at 0.1 mg/ml DEAE OB3b C, with activity rapidly decreasing as the concentration approached 1.0 mg/ml (FIG 3.B.2). So the specific activity for DEAE OB3b C was measured at this low level where the specific activity is constant.

DEAE OB3b C was drop frozen into liquid nitrogen and stored at -80^{0}C without any loss in activity for up to six months. At 4^{0}C on average a 3.5% loss of activity was measured over a 24 hour period in the presence of DTT (FIG 3.B.3). At room temperature (20^{0}C) although initially stable, 73% of activity was lost over 24 hours in the presence of DTT. DEAE OB3b C was prepared with 1 mM DTT which was present in all the buffers during DEAE cellulose chromatography. On the removal of DTT by dialysis all activity was lost.

The specific activity of DEAE OB3b C prepared by column chromatography from the same batch of whole cells was quite variable, ranging from 97 to 1580 nmol/min/mg, even though it was prepared in the presence of 1 mM DTT. However the greatest influence on the specific activity was due to the variable nature of the soluble MMO activity of the soluble cell extracts which varied from 0 to 79 nmol/min/mg, unlike DEAE OB3b A where the specifc activity was constant and independant of the starting MMO activity. This therefore

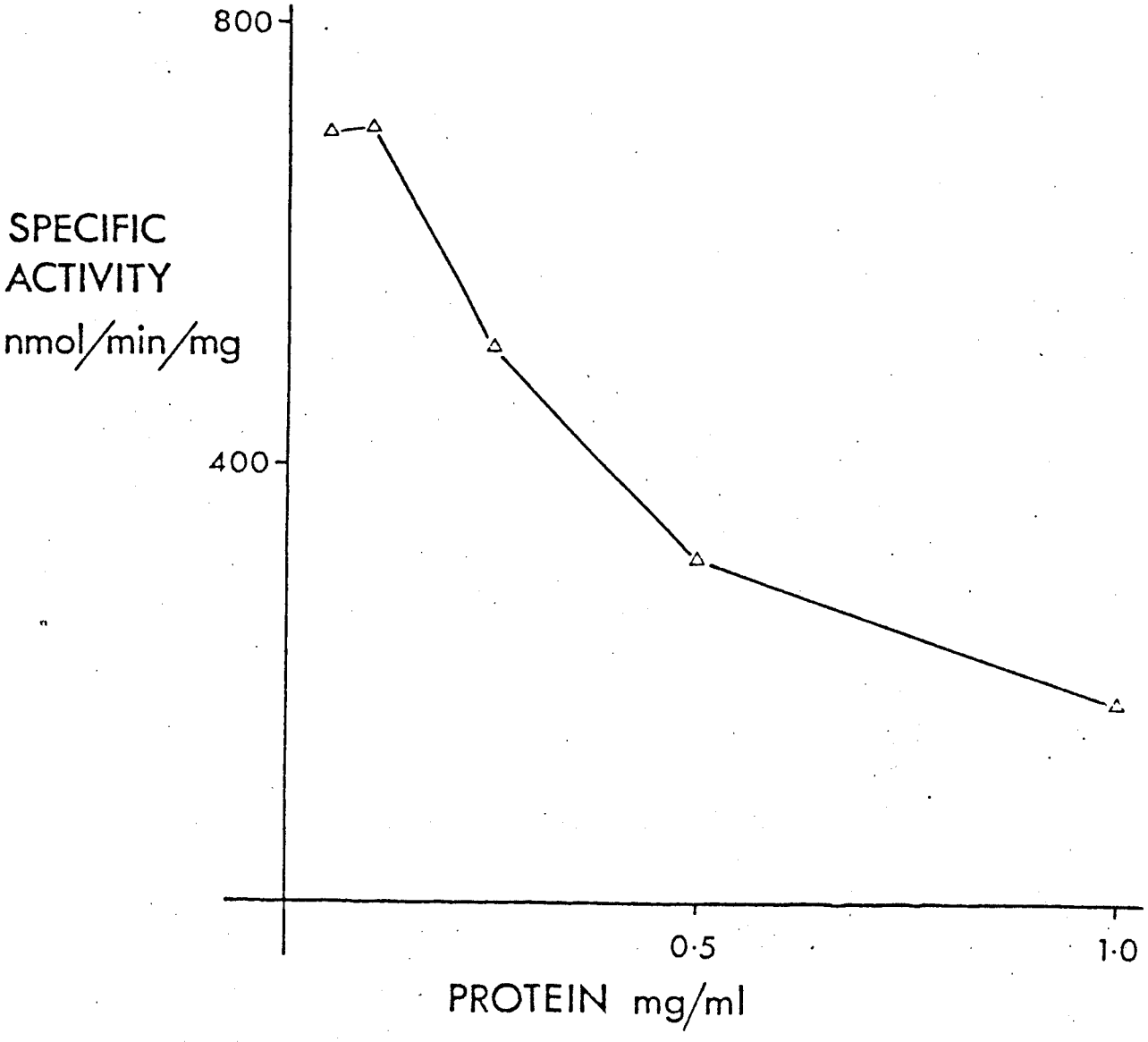


Figure 3.B.2. Effect of the concentration of DEAE OB3b C on its specific activity.

MMO assays: 3 minute assays, at 30^{0}C, containing 5 mg DEAE OB3b A plus 0.05 - 1.0 mg DEAE OB3b C, following the oxidation of propene to epoxypropane.

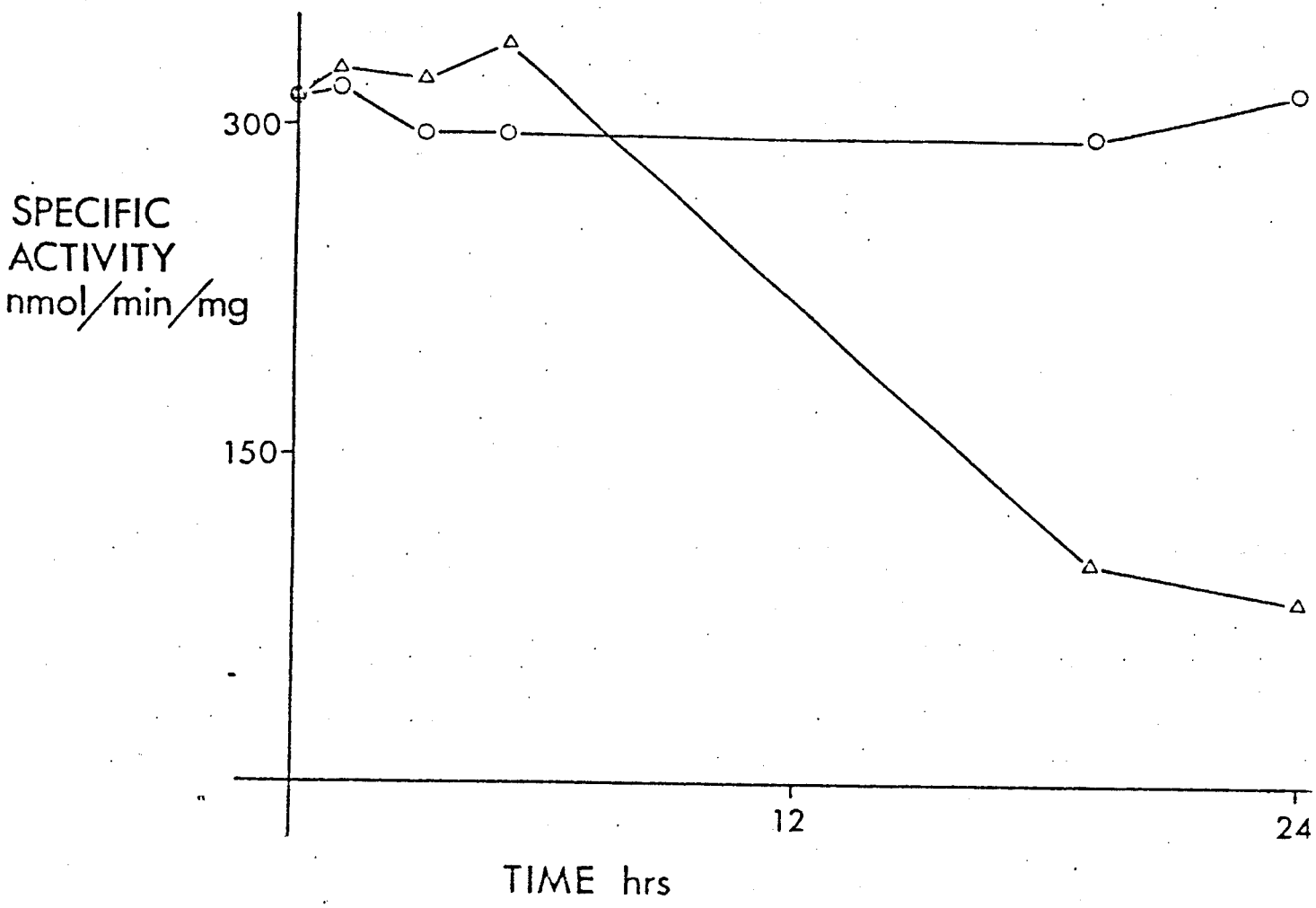


Figure 3.B.3. Stability of DEAE OB3b C.

DEAE OB3b C was incubated at 4°C and 20°C. Samples were withdrawn and assayed for MMO activity.

MMO assay. 3 minute assay, at 30°C, containing 5 mg DEAE OB3b A and 0.2 mg DEAE OB3b C, following the oxidation of propene to epoxypropane.

4°C O . 20°C Δ .

demonstrates that the component(s) of the soluble MMO present in DEAE OB3b C are limiting in soluble MMO assays of soluble cell extracts.

To maximise the yield of DEAE OB3b C from a DEAE cellulose column elution other methods for this fraction were investigated. All fractions containing any soluble MMO activity were eluted in a single protein peak at 0.1 M sodium chloride, this peak containing over 95% of the protein bound to the column. So to elute this peak quickly and efficiently after the buffer wash the column was washed with 0.3 M sodium chloride which brought off the DEAE OB3b C band as a tight concentrated yellow/brown band. This method of elution proved far more reliable in terms of yield than gradient elution where, DEAE OB3b C was eluted as a much broader less concentrated band.

DEAE OB3b C had no viscosity associated with it, the viscosity inherent in soluble extracts of Methylosinus trichosporium (OB3b) does not bind to DEAE cellulose.

B.3. Discussion.

In conclusion the soluble MMO of Methylosinus trichosporium (OB3b) was separated into two fractions by DEAE cellulose chromatography, each of which was essential for soluble MMO activity. DEAE OB3b A failed to bind to DEAE cellulose and was equivalent to component A of the soluble MMO of Methylococcus capsulatus (Bath) in that, in combination with both Methylococcus capsulatus (Bath) soluble MMO components B and C, it had soluble MMO activity. One soluble MMO component contained in DEAE OB3b A was

therefore by analogy the hydroxylase component of the soluble MMO of Methylosinus trichosporium (OB3b). This fraction, when analysed using SDS PAGE, contained the three prominant protein bands associated with the soluble MMO of Methylosinus trichosporium (OB3b) and that roughly corresponded in size to the three subunits of component A of the soluble MMO of Methylococcus capsulatus (Bath) (FIG 3.C.2).

The protein bound to the DEAE cellulose column was eluted as a single yellow protein band at a sodium chloride concentration of 0.1 M and contained a component equivalent to component C of the soluble MMO of Methylococcus capsulatus (Bath) in that, in combination with Methylococcus capsulatus (Bath) soluble MMO component A had MMO activity. One component found in this fraction is therefore presumably the NADH-acceptor reductase component, accepting electrons from NADH and passing them on to the hydroxylase component of the soluble MMO.

The soluble MMO of Methylococcus capsulatus (Bath) has a third component, component B. This acts to mediate the flow of electrons from NADH to substrate via components C and A. It is not known whether component B acts on component A, component C or a complex of the two. The similarity between the soluble MMO's from Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath), in that both their hydroxylases and NADH acceptor reductase components can be interchanged, may suggest that the soluble MMO of Methylosinus trichosporium (OB3b) also has a third component (and possibly others) that is equivalent in action to component B of the soluble MMO of Methylococcus

capsulatus (Bath). However at this stage of the purification of the components of the soluble MMO of Methylosinus trichosporium (OB3b) there is little evidence for the existence of such a component. Component B of the soluble MMO of Methylococcus capsulatus (Bath) was required for maximum activity in assays of DEAE OB3b A, but this may be due to its action on component C of the soluble MMO of Methylococcus capsulatus (Bath) and not any requirement by OB3b A for a B component. Component B of the soluble MMO of Methylococcus capsulatus (Bath) is a small protein of high specific activity (Green and Dalton 1985), that elutes from DEAE cellulose columns at 0.1 M sodium chloride. It does however contaminate both DEAE fractions A and C. There is no evidence for the existence of a third component of the soluble MMO of Methylosinus trichosporium (OB3b) from DEAE cellulose ion exchange chromatography results. The reductase component of the soluble MMO of Methylosinus trichosporium (OB3b) elutes earlier than its equivalent from Methylococcus capsulatus (Bath) (0.1 and 0.5 M sodium chloride respectively) and so may elute at the same stage as any third B component, along with 95% of the bound protein. Alternatively, it may be equally split between DEAE OB3b A and C or absent from the soluble MMO of Methylosinus trichosporium (OB3b). Only further purification of the components of the soluble MMO of Methylosinus trichosporium (OB3b) will reveal the presence or otherwise of a B component (Results section C, D and E).

C. Purification of component A of the soluble methane monooxygenase of Methylosinus trichosporium (OB3b).

C.1. Introduction.

As previously stated the hydroxylase components of the soluble MMOs of both Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) appear to be similar, both in their structure and activity (Stirling and Dalton 1979a, Burrows et al., 1984). The purification of the component A of the soluble MMO of Methylosinus trichosporium (OB3b) was therefore carried out using the same HPLC apparatus as Woodland and Dalton (1984b).

C.2. Results.

The fraction DEAE OB3b A was concentrated by dialysis against dry PEG 20000 to a protein concentration of 50-100

mg/ml. Any residual particulate material was removed by centrifugation and filtration through a 2 micrometre filter.

2 ml aliquots of concentrated DEAE OB3b A containing up to 200 mg of protein were loaded onto the HPGPC column and eluted at a rate of 3 ml/min with degassed filtered buffer. The use of HPGPC resolved DEAE OB3b A into three major peaks as monitored by UV absorbance at 280 nm (FIG 3.C.1). The first peak consisted of high molecular weight lipid-containing material that was cloudy in appearance and was totally excluded from the column packing. The size of the first peak was variable and dependent on the efficiency of the removal of particulate material from concentrated DEAE OB3b A. The second peak contained component A soluble MMO activity, in that it had MMO activity when assayed in combination with DEAE OB3b C or components B and C of the soluble MMO of Methylococcus capsulatus (Bath). The third peak consisted of very pure methanol dehydrogenase as monitored both by enzyme assay and PAGE (FIG 3.C.2). All of the three major peaks had eluted in 35 minutes, however after a further 15 minutes one or two minor peaks were observed. These had a very low protein concentration (< 0.5 mg/ml), but were highly coloured and were thought to contain cytochromes. The whole process was carried out at room temperature and was completed within an hour. None of the fractions listed above had any viscosity associated with them.

The second peak containing component A of the soluble MMO activity was colourless and eluted at a protein concentration of 2-4 mg/ml. Fractions from this peak were pooled and concentrated by ultrafiltration over a PM10

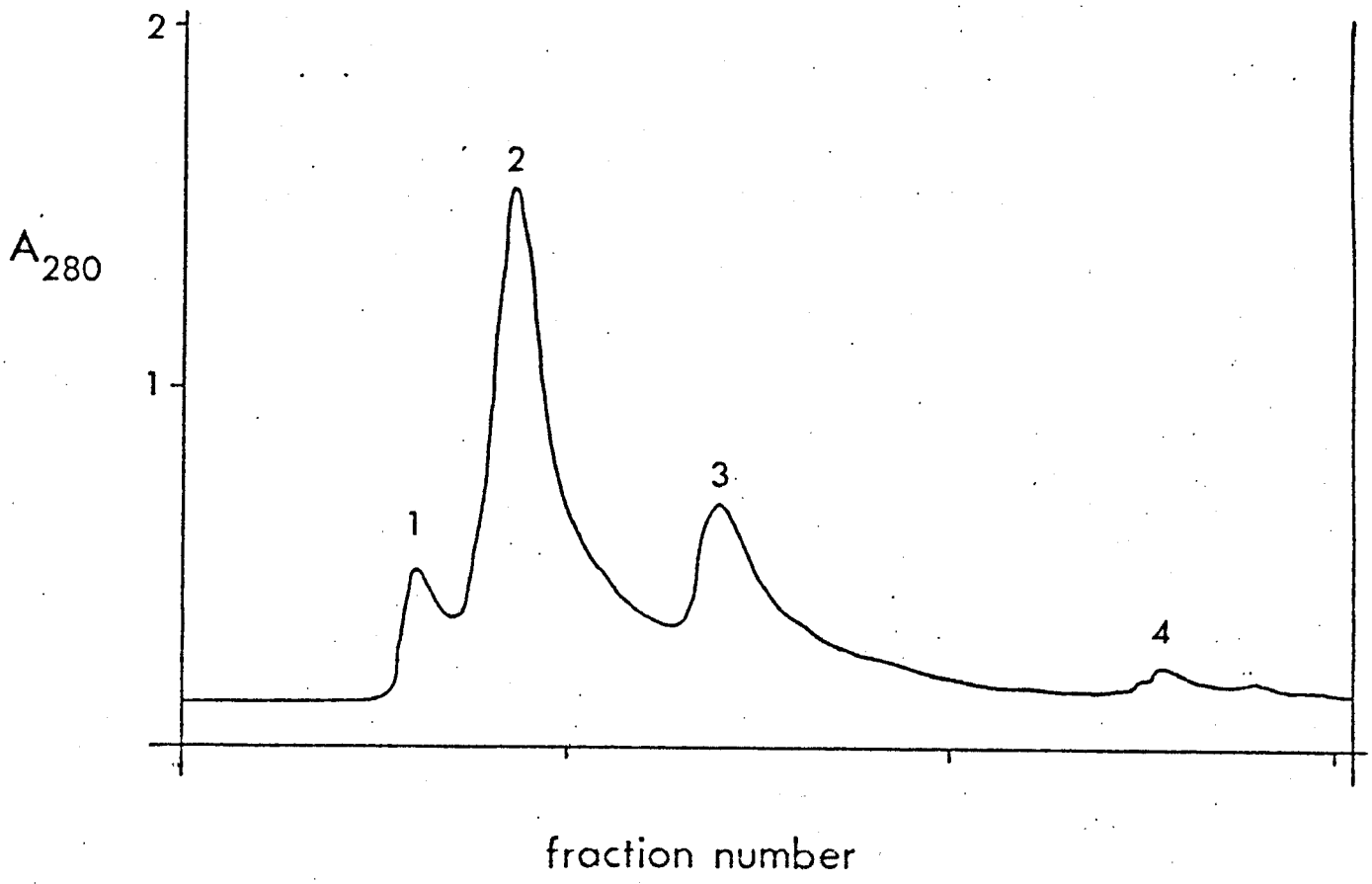


Figure 3.C.1. Purification of component A: HPGPC of DEAE OB3b A.

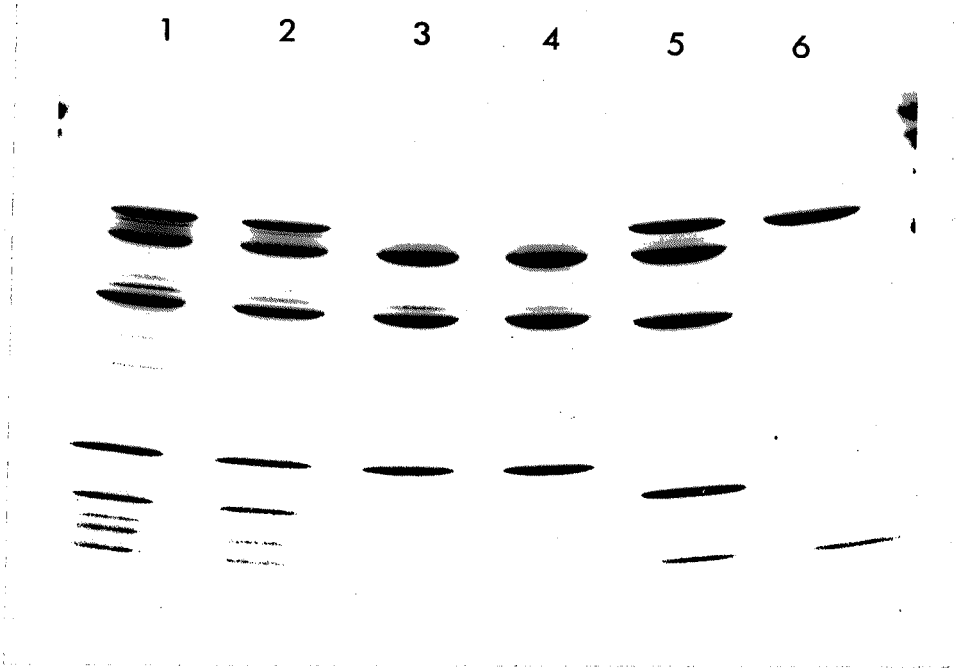
Protein concentration of fractions monitored by UV absorbance at 280 nm.
HPGPC on an 'Ultra-pack' TSK-GSWPG column (21.5 by 600 mm). 115 mg DEAE OB3b A loaded and eluted with de-gassed and filtered buffer (20 mM sodium phosphate pH 7.0) at a flow rate of 3 ml/minute.
1. Material totally excluded from the column. 2. HPLC OB3b A 3. Methanol Dehydrogenase. 4. Cytochrome.

ultrafiltration membrane to a protein concentration of 20-50 mg/ml, at which concentration it was still colourless. This fraction will henceforth be referred to as HPLC OB3b A.

The specific activity of HPLC OB3b A was variable. In many cases the specific activity of HPLC OB3b A was similar to that of the concentrated DEAE OB3b A fraction loaded onto the column. However in approximately 50% of column runs an increase in specific activity of up to 2.5 fold was observed up to a maximum of 159 nmol/min/mg. This apparent loss in activity during the process of purification of component A was also observed during the purification of component A of the soluble MMO from Methylococcus capsulatus (Bath) and was tentatively ascribed to the loss of iron from the protein (Woodland and Dalton 1984ab).

Analysis of HPLC OB3b A by SDS PAGE (FIG 3.C.2) revealed the presence of three major bands, the same three bands that were associated with the presence of the soluble MMO activity in cell free extracts. With HPLC OB3b A loaded onto polyacrylamide gels at high protein concentrations (50 micrograms per track) three or four minor bands were observed though they constituted only approximately 5% of the total protein (FIG 3.C.2). In an attempt to reduce further this already low level of contaminating protein, fractions of HPLC OB3b A were pooled, concentrated by ultrafiltration over a PM30 ultrafiltration membrane and loaded onto the HPGPC column as previously described. As was expected a single major symmetrical peak of protein was observed, which sometimes had preceeding it a minor peak which was probably comprised of material totally excluded from the column consisting of aggregated denatured protein

Figure 3.C.2. Purification of component A: SDS PAGE of the stages of purification.



1. Soluble extract. 2. DEAE OB3b A. 3. HPLC OB3b A.
4. Pure OB3b A. 5. DEAE MC A.
6. OB3b methanol dehydrogenase.
Electrophoresis: 5-20% gradient SDS PAGE 30 micrograms of protein per track.

(FIG 3.C.3). From the single major peak two types of fraction were collected. From the centre of the peak, fractions of very pure component A and from the edges of the peak fractions of impure component A which could be added back to subsequent purifications. The very pure peak fractions will henceforth be referred to as pure OB3b A.

When pure component A of the soluble MMO of Methylosinus trichosporium (OB3b) was analysed using non-denaturing PAGE no protein bands were observed using the standard discontinuous buffer system described in materials and methods (FIG 3.C.4). Samples of soluble cell extracts of Methylosinus trichosporium (OB3b) and DEAE OB3b A were analysed using this system and had a single prominent band corresponding to purified methanol dehydrogenase with no prominent band as would be expected for component A. Samples of soluble cell extracts and DEAE component A from Methylococcus capsulatus (Bath) when analysed on non-denaturing PAGE had two prominent bands corresponding to methanol dehydrogenase and component A of the soluble MMO (FIG 3.C.4 and 3.C.5). Component A of the soluble MMO of Methylosinus trichosporium (OB3b) was the only protein which failed to form a band using this gel system including standard proteins added to gels (thyroglobin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin). Component A gave a long continuous streak of protein extending almost the entire length of the gel with no concentration of material at any point. There was no material at the top of the stacking gel, at the bottom of the well or at the stacking gel / resolving gel interface.

The smearing of protein on non-denaturing gels is

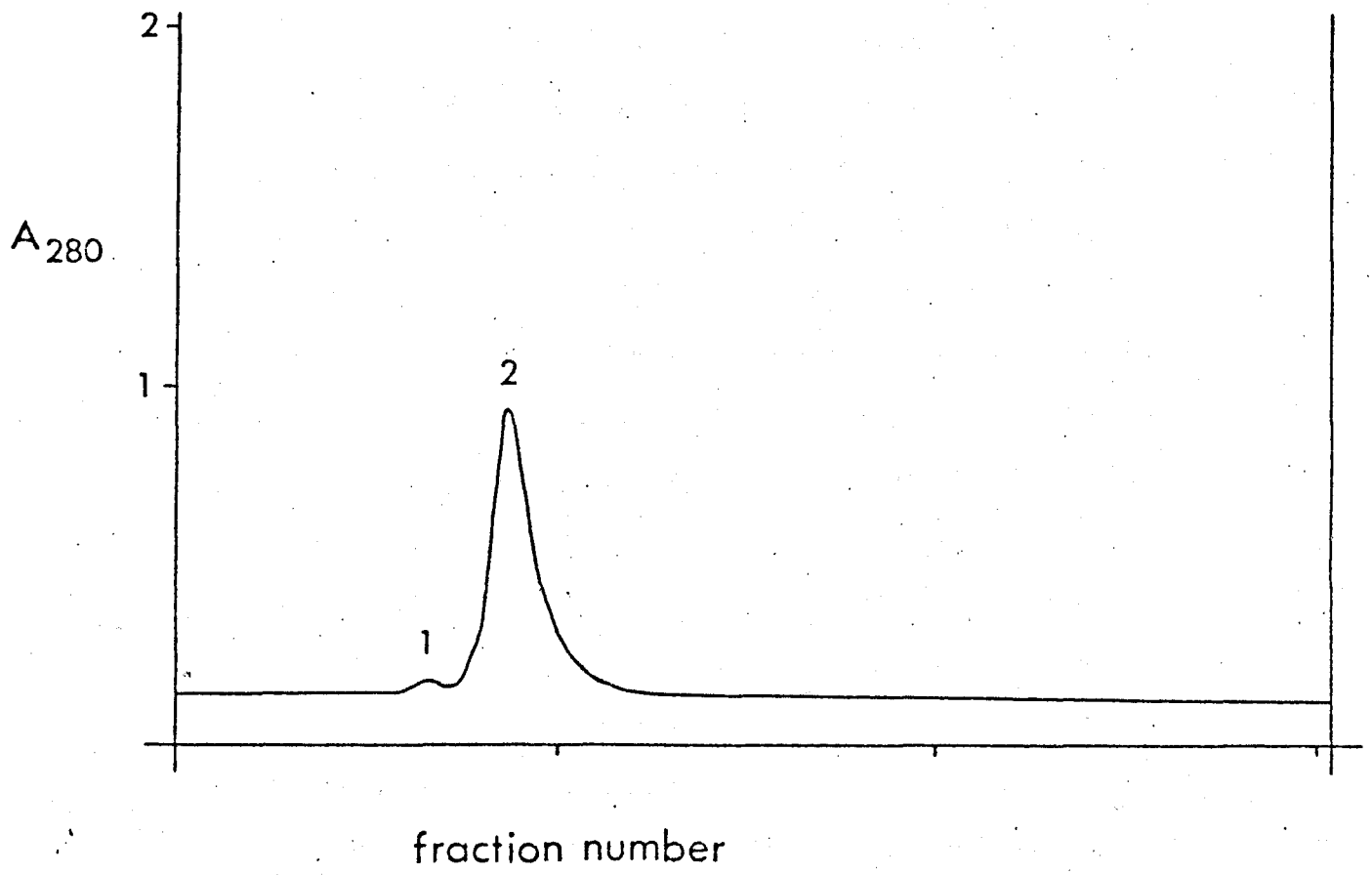


Figure 3.C.3. Purification of component A: HPGPC of HPLC OB3b A.

Protein concentration of fractions monitored by UV absorbance at 280 nm.

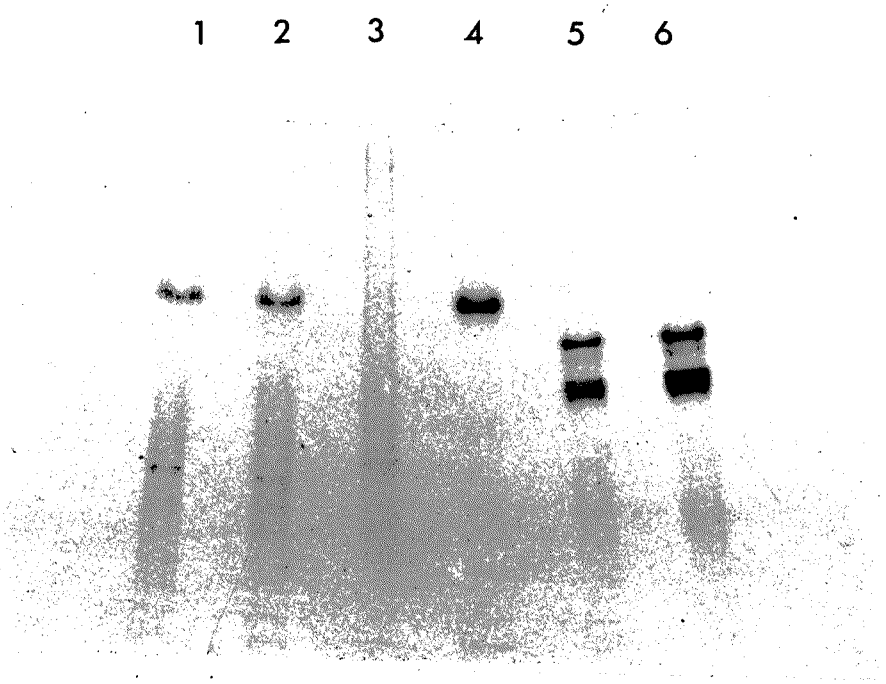
HPGPC on an 'Ultra-pack' TSK-GSWPG column (21.5 by 600 mm). 40 mg DEAE OB3b A loaded and eluted with de-gassed and filtered buffer (20 mM sodium phosphate pH 7.0) at a flow rate of 3 ml/minute.

1. Material totally excluded from the column. 2. Pure OB3b A

normally due to the precipitation of the protein, followed by the dissolution of the protein during electrophoresis (Hames and Rickwood 1984). Precipitation of protein can be caused in two ways in non-denaturing PAGE. The ionic strength of the sample preparation buffer used, if too low, can cause the aggregation and precipitation of protein. Variation in the concentration of the sample preparation buffer used (0.05- 0.5 M) failed to have any effect on the resolution of component A on non-denaturing gels. The second possible cause of precipitation is the addition of an excess of protein, which during the process of stacking forms such a concentrated band of protein that it comes out of solution within the stacking gel. Variation in the level of pure OB3b A loaded onto each track (5-60 micro grams) failed to produce a band or concentrated area of protein. A continuous gel system was also investigated that lacked a stacking gel. Despite giving excellent tight banding for methanol dehydrogenase from both Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) and good banding for component A of the soluble MMO of Methylococcus capsulatus (Bath), no bands were observed for component A of the soluble MMO of Methylosinus trichosporium (OB3b) (FIG 3.C.5). Variation in the pH of the running buffer (pH 6.3-9.0) also failed, and often made worse, the banding of protein on non-denaturing gels.

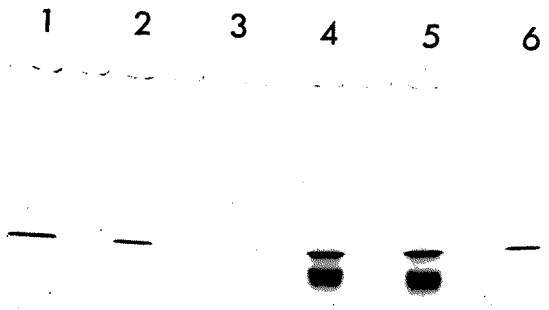
The purification sequence described above yielded component A of approximately 95% homogeneity as judged by the following criteria. A single symmetrical peak was observed for pure OB3b A eluted from gel-filtration columns (HPGPC and Sephacryl S300) (FIG 3.C.3). The specific

Figure 3.C.4. Non-denaturing PAGE of component A: Discontinuous system.



1. OB3b extract. 2. DEAE OB3b A. 3. Pure OB3b A.
4. OB3b methanol dehydrogenase.
5. Methylocuccus capsulatus (Bath) extract. 6. DEAE MC A.
Electrophoresis: 5-20% gradient PAGE, discontinuous buffer system, 20 micrograms protein per track.

Figure 3.C.5. Non-denaturing PAGE of component A: Continuous system.



1. OB3b extract. 2. DEAE OB3b A. 3. Pure OB3b A.
4. Methylocuccus capsulatus (Bath) extract. 5. DEAE MC A.
6. OB3b methanol dehydrogenase.
Electrophoresis: 5-20% gradient PAGE (no stacking gel), continuous buffer system, 20 micrograms protein per track.

activity of each fraction from this peak was the same, a further indication of the purity of the protein. Unfortunatly it was not posible to analyse component A on non-dissociating PAGE as it failed to form a band under these conditions. Dissociating PAGE revealed the presence of three bands of approximately equal intensity corresponding to the three subunits of component A and at high loadings (50 micrograms protein), three contaminating bands. Pure OB3b A therefore contained low levels of contaminating proteins which may have consisted of specific degredation products of component A. However, for most practical purposes this protein can be regarded as sufficiently pure for the analysis of its properties to be carried out.

The molecular weight of component A was estimated to be 230000 as measured by gel filtration on sephacryl S-300 (FIG 3.C.6). The molecular weight of the subunits of the pure component A as determined by SDS PAGE was 54000, 40000 and 18500, in broad, though not close, agreement with the molecular weights reported by Burrows et al., (1984). These subunits will in future be refered to as α, β and γ in descending molecular weight following the terminology adopted by Woodland and Dalton for the subunits of the component A of the soluble MMO of Methylococcus capsulatus (Bath) (Woodland and Dalton 1984a). The subunits appear to be present in stoichiometric amounts suggesting a $\alpha_2\beta_2\gamma_2$ arrangement in the native protein.

Component A of the soluble MMO of Methylococcus capsulatus (Bath) was shown to contain 2.3 mol Fe per mol protein (Woodland and Dalton 1984a). The iron content of

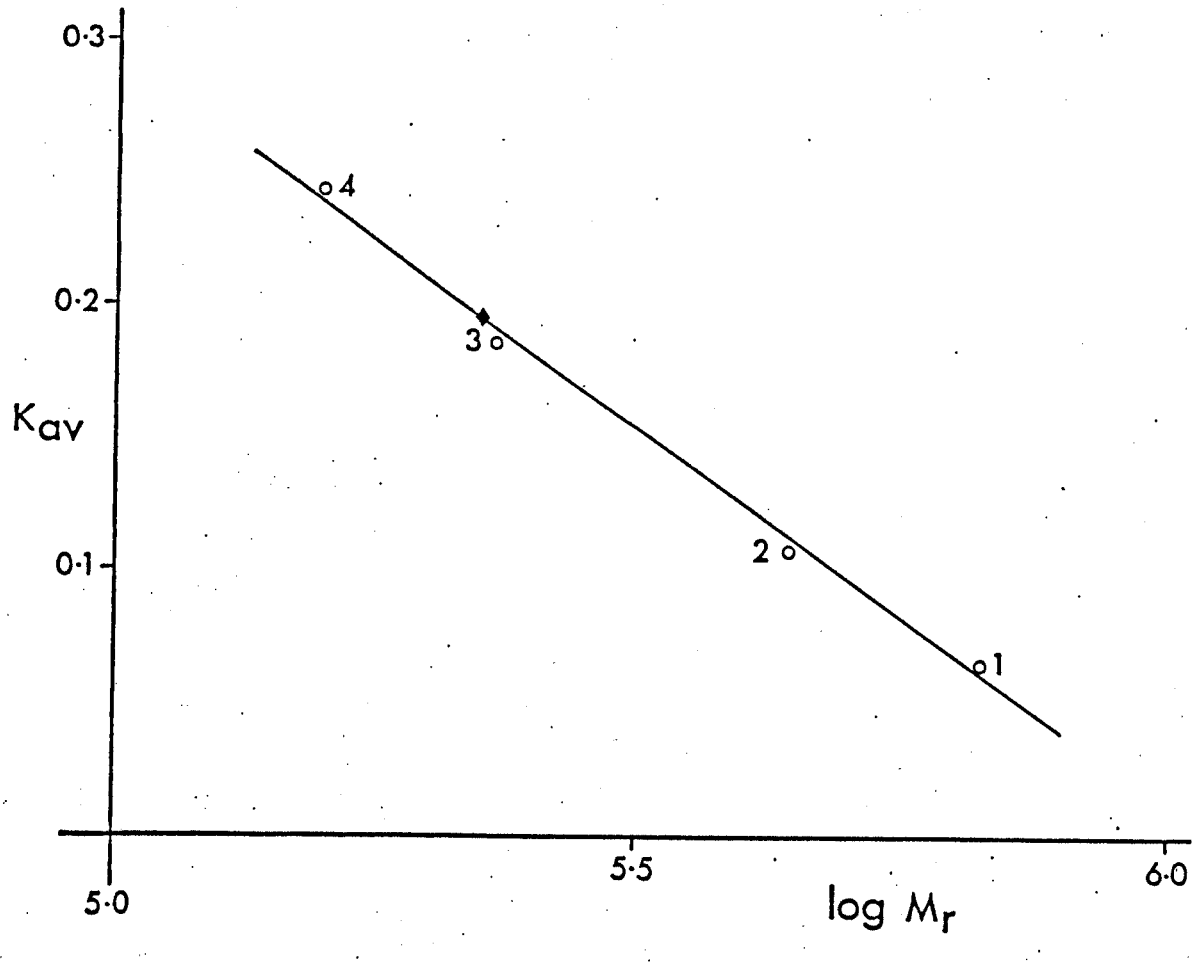


Figure 3.C.6. Estimation of the molecular weight of component A by gel filtration.

The molecular wieght of component A was estimated by gel filtration on Sephacryl S-300 as described in Materials and Methods. Molecular weight standards were: 1. Thyroglobulin (M_r = 669000), 2. Ferritin (M_r = 440000), 3. Catalase (M_r = 232000) 4. Aldolase (M_r = 158000). The plot was calculated by linear regression analysis.

◆ Component A.

component A of the soluble MMO of Methylosinus trichosporium (OB3b) was analysed by colorimetry as described by Woodland and Dalton (1984a). Component A contained 1.9 - 2.4 mol of Fe/mol protein.

Pure component A of the soluble MMO of Methylococcus capsulatus (Bath) was found to be sensitive to freezing (Woodland and Dalton 1984a). Pure component A of the soluble MMO of Methylosinus trichosporium (OB3b) was also found to be sensitive to freezing, losing 40-50% of its activity in one cycle of freeze/thawing and all activity in three cycles of freeze/thawing. However as with pure component A of the soluble MMO of Methylococcus capsulatus (Bath), it was found that pure component A of the soluble MMO of Methylosinus trichosporium (OB3b) could be stored for extended periods of time at $-20^{0}C$ in 50% glycerol without the loss of activity. Pure component A was relatively stable losing up to 10% of its activity over 24 hours at $4^{0}C$. The loss of activity from component A of the soluble MMO of Methylococcus capsulatus (Bath) was ascribed to loss of iron from the protein (Woodland and Dalton 1984a). Preliminary experiments were reported which described how the activity of component A could be stimulated by its incubation with DTT and iron. Subsequently in our laboratory it has been shown that the incubation of pure component A with DTT (10 mM), iron-EDTA (100 micro molar) and material from the first peak that is eluted from the HPGPC step in purification (containing very high molecular weight membranous material) restored activity to component A (Green personal communication). This process was

investigated using pure component A of the soluble MMO of Methylosinus trichosporium (OB3b). Pure component A was inactivated by three cycles of freeze/thawing and then incubated for 20 minutes at 30°C with DTT (10 mM) and iron-EDTA (100 micromolar) with and without high molecular weight material from the first peak of protein from the HPGPC step in purification. No stimulation in the activity of component A was observed, under these conditions.

C.3. Discussion.

The use of HPGPC in the purification of component A of the soluble MMO of Methylosinus trichosporium (OB3b) was a quick, efficient and easy step that could be carried out at room temperature. The whole process was completed within an hour and so a number of runs could be carried out in a single working day producing up to 200 mg of pure component A. One of the reasons why this process was so efficient is because component A already comprises a very large percentage of the protein added to the column in the form of concentrated DEAE OB3b A. The HPGPC column yielded only three major peaks, the first being made up of very high molecular weight membranous material of a cloudy appearance. The second peak was comprised of pure component A and the third peak pure methanol dehydrogenase. This process therefore not only provided a method for the purification of component A but also an alternative method for the purification of methanol dehydrogenase to that published by Higgins et al., (1984). For cell extracts that had lost

soluble MMO activity (component C activity) yet retained component A soluble MMO activity, it was shown that the DEAE step in the purification could be omitted without significant loss in the purification of component A by HPGPC, an effective single stage purification.

One of the major problems in purifying proteins from soluble cell extracts of Methylosinus trichosporium (OB3b) was the very high viscosity of such extracts. Another of the major advantages of HPGPC was that the use of highly viscous extracts had no obvious effect on the running or the efficiency of the purification achieved by the column. Viscous components were separated from the proteins of interest and must have emerged from the column in a highly diluted form as no obvious viscous fractions were observed.

Pure component A has soluble MMO activity when assayed in combination with either DEAE OB3b C or Methylococcus capsulatus (Bath) soluble MMO components B plus C. Component A is therefore the hydroxylase component of the soluble MMO of Methylosinus trichosporium (OB3b). DEAE OB3b C therefore contains not only the NADH: acceptor reductase component of the the soluble MMO of Methylosinus trichosporium (OB3b) but also any other components essential for soluble MMO activity. This does not however exclude the possibility of the presence of such components in DEAE OB3b A, further components may be spread between DEAE fractions. No stimulation of activity was measured by the addition of other HPGPC fractions to assays of component A.

The purification of component A of the soluble MMO of Methylosinus trichosporium (OB3b) was accompanied by an at most 2.5 fold increase in specific activity equating

Table 3.C.1. Purification scheme of component A of the soluble MMO.

Fraction	Volume (ml)	Protein conc (mg/ml)	Total protein (mg)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Recovery (%)	Purification factor
Soluble extract	10	82.3	823	64.7	53248	100	1.00
DEAE OB3b A	9	57.6	518	61.7	31985	60	0.95
HPLC OB3b A	5	23	115	148	17020	32	2.28
pure OB3b A	4.2	19.8	83.2	150.7	12529	23	2.30

directly to the purification factor. This is very low, however when the very high levels of this protein within the cell are taken into account, at best a purification factor of approximately three would be expected, so although the purification factor achieved is low this is not necessarily unexpected.

The purification of component A was accompanied with a 2-2.5 fold increase in specific activity only in approximately 50% of cases investigated. In other purification procedures no increase in specific activity was observed. The variability in specific activity and apparent loss in activity was also observed in component A of the soluble MMO of Methylococcus capsulatus (Bath) where it was tentatively ascribed to the loss of iron from the protein (Woodland and Dalton 1984a).

Until this final purification step component A of the soluble MMO of Methylosinus trichosporium (OB3b) was very stable and lost no activity on freezing, this last stage in the purification therefore removed a stabilising factor from the environment of component A. This factor may be specific, as is thought to be the case in Methylococcus capsulatus (Bath) where the factor present in the first peak of the HPGPC purification is sensitive to protease and heat treatment (Green personal communication). It may also be nonspecific, where association of component A with protein and/or membranous material stabilises the protein. No restoration in activity of component A of the soluble MMO of Methylosinus trichosporium (OB3b) was observed when this factor was incubated with DTT, iron-EDTA and material from the first HPGPC peak. However this section of work was not

comprehensively investigated and therefore no absolute conclusions can be drawn.

The failure of component A of the soluble MMO of Methylosinus trichosporium (OB3b) to form discrete bands on non-denaturing gels was unexplained. Component A merely produced a smear of protein thoughout the length of the gel. This would normally be attributed to the precipitation of the protein either in the gels well or within the gel. However there was no evidence for this precipitation to have taken place, with no concentration of protein either at the top of the stacking gel or at the stacking gel / resolving gel interface. Smearing was not caused by nonspecific interference from another molecule either from contamination of component A with a protease or a small molecule such as an organic solvent, as crude extracts of Methylosinus trichosporium (OB3b) failed to produce a prominent protein band that could be ascribed to component A but did have a single prominent band representing methanol dehydrogenase and a number of minor bands. Methanol dehydrogenase co-purified with component A also formed discrete bands on non-denaturing gels. No explanation can be put forward as to why component A failed to band on non-denaturing gels and only with further chemical analysis of this protein may an answer be forthcoming.

The purification of component A of the soluble MMO has further demonstrated its similarity both functionally and physicochemically to component A of the soluble MMO of Methylococcus capsulatus (Bath). Both are large proteins of M_r 230000 and 220000 respectively, have three different subunits of similar M_r, which are present in $\alpha_2\beta_2\gamma_2$ subunit

structure. Each contain approximately 2 mol Fe per mol protein and in their pure state are inactivated by freezing.

D. Purification of the NADH:acceptor reductase, component C of the soluble MMO.

D.1. Introduction.

DEAE OB3b C contains at least one component of the soluble MMO, which is equivalent to component C of the soluble MMO of Methylococcus capsulatus (Bath), the NADH:acceptor reductase. This component will hence-forth be referred to as component C of the soluble MMO of Methylosinus trichosporium (OB3b). However DEAE OB3b C may also contain at least one other component of the soluble MMO that is essential for activity. There is little evidence for the existence of such a component apart from the observation that a third component exists (component B) in the soluble MMO of Methylococcus capsulatus (Bath), a soluble MMO that resembles that from Methylosinus trichosporium (OB3b). The soluble MMO of Methylobacterium CRL-26 also appears to be a similar enzyme to that from Methylococcus capsulatus (Bath) and was initially reported to have three components, however subsequently only two components were reported to be required for maximum activity (Patel et al., 1982, Patel 1984).

In this chapter the purification of the NADH:acceptor reductase component of the soluble MMO of Methylosinus trichosporium (OB3b) is reported. The NADH:acceptor reductase component must, by definition, bind NADH. There are a number of affinity chromatography materials available specifically designed to bind NADH binding proteins and thereby achieving a high level of purification in a single

step. Affinity chromatography was used as the major purification step in the purification of component C from the soluble MMO of Methylococcus capsulatus (Bath) and so it was decided to expose DEAE OB3b C to affinity chromatography to effect the purification of the NADH:acceptor reductase component.

D.2. Results.

Component C of the soluble MMO of Methylococcus capsulatus (Bath) was originally purified using a combination of ion-exchange and gel filtration chromatography. However subsequently 5'-AMP Sepharose affinity chromatography was used to effect a more efficient purification (Colby and Dalton 1978, 1979, Lund 1983). Initially then, 5'-AMP Sepharose was used in an attempt to purify component C from the soluble MMO of Methylosinus trichosporium (OB3b).

Throughout this section, except where indicated, the activity of component C of the soluble MMO of Methylosinus trichosporium (OB3b) was measured by assay in combination with DEAE OB3b A (5 mg) at 30^{0}C.

The fraction DEAE OB3b C was concentrated by ultrafiltration over a PM10 ultrafiltration membrane to a protein concentration of 5-15 mg/ml. A small test column of 5'-AMP Sepharose 4B (2 ml volume) was prepared within a 2 ml syringe. 1 ml of concentrated DEAE OB3b C was added to the column and allowed to equilibrate for 10 minutes and then the column was eluted with 10 ml of buffer (20 mM sodium phosphate, 1 mM DTT, pH 7.0), followed by 10 ml of buffer

containing 2 mM NADH. 2 ml fractions were collected and assayed for component C activity in combination with DEAE OB3b A. On two occasions no component C activity was recovered from the column, as measured by assay of individual fractions or combined fractions from the buffer wash and buffer plus NADH wash. Component C activity was recovered from other 5'-AMP Sepharose column runs. Activity was measured thoughout the fractions collected with 30% of the activity present in fractions eluted with 2 mM NADH. Component C therefore only bound weakly to 5'-AMP Sepharose. The column was tested for its efficiency of binding by the substitution of DEAE OB3b C by DEAE fraction C of the soluble MMO of Methylococcus capsulatus (Bath), the conditions of the elution of the column being identical to those listed above. Component C of the soluble MMO of Methylococcus capsulatus (Bath) bound efficiently to the 2 ml test column, 80% of the component C activity eluting with 2 mM NADH.

The inadequacy of the purification of component C of the soluble MMO of Methylosinus trichosporium (OB3b) by 5'-AMP Sepharose affinity chromatography led to the testing of a wide range of dye affinity materials, which are known to bind proteins that bind NADH, for the purification of component C. Using a 'DyeMatrex' test kit (Amicon) a range of five affinity dyes were tested for their ability to bind component C. The test columns were of 2 ml volume and were run in an analogous way to the 5'-AMP Sepharose column described above, though instead of collecting 2 ml fractions, a single fraction was collected for each elution i e., elution with buffer, buffer plus 2 mM NADH and buffer

plus 1.5 M potassium chloride as described in the 'Operating instructions for Dyematrex screening kit' and Materials and Methods.

Five dye affinity materials were tested. Blue B, Green, Orange and the control column failed to bind component C, though the Green gel bound 67% of the protein recovered without binding component C (Table 3.D.1). Two gels, Red and Blue A bound component C that was eluted with 2 mM NADH. From the Red gel column 70% of the component C activity was recovered in the 2 mM NADH elution. From the Blue A column 40% of the component C activity was recovered in the 2 mM NADH elution (Table 3.D.1). Repeated runs of the 'Dymatrex' test columns produced similar results, with on average the Red gel binding 60% of the component C activity which was eluted with 2 mM NADH. The overall recovery of component C activity from all of the 'DyeMatrex' columns was excellent ranging from 102 to 113% of the activity added to the column. On the basis of these results the red dye affinity material was chosen for the further purification of component C, where it should have been possible to achieve a purification factor of 20 in a single step using figures based on the the recovery of activity from the test columns (Table 3.D.1).

Reactive Red 120 Agarose (3000 CC) was purchased from Sigma and a test column of 2 ml volume made up and the material tested for its ability to bind component C as described for the 'DyeMatrex' test columns. Reactive red from Sigma had similar properties binding 61% of the component C activity recovered from this column, eluting with 2 mM NADH in 2% of the protein added to the column.

Table 3.D.1. Purification of component C of the soluble MMO: 'Dyematrex' test kit affinity chromatography.

	Blue A	Blue B	Red	Green	Orange	Control
Buffer elution.						
% protein	55	88	43	33	100	100
% C activity	61	100	29	100	100	100
2 mM NADH elution.						
% protein	9	12	5	19	0	0
% C activity	39	0	71	0	0	0
KCl elution.						
% protein	36	0	52	48	0	0
% C activity	0	0	0	0	0	0

'Dymatrex' columns run as described in the text. 0.5 ml of DEAE OB3b C (4.2 mg/ml protein) loaded onto the columns, with a specific activity for the oxidation of propene of 737 nmol/min/mg.

% protein - % of the protein recovered from the column.

% C activity - % of the component C activity recovered from the column.

However, when this process was scaled up with the use of a 40 ml volume column, little or no component C activity was recovered in the buffer elution, buffer plus NADH elution or in desalted fractions of a 1.5 M potassium chloride elution. This failure to recover component C from the 40 ml column was repeated on three separate occasions and could not be attributed to the instability of component C, as the running of the large 40 ml column did not take significantly longer than the 2 ml test columns. The failure to recover any component C activity from this large column and the experiments investigating this failure led to a severe reduction in the amount of active DEAE OB3b C available for the rest of the experimentation. Consequently, and to limit the losses from the failure to recover component C activity, further use of Red dye affinity chromatography was confined to a 5 ml column loading samples containing only, at most, 15 mg of DEAE OB3b C. Of the six purifications carried out using this 5 ml column all but one yielded purified component C of a specific activity ranging from 818 to 6615 nmol/min/mg. These active fractions were pooled and concentrated up by ultrafiltration over a PM10 ultrafiltration membrane yielding 0.5 mg of protein (2.2 ml at 0.229 mg/ml protein) with a specific activity of 2829 nmol/min/mg (Table 3.D.2). With such a small amount of protein any comprehensive characterisation or further purification of this component of the soluble MMO was impossible, however a limited analysis was performed and its activity studied in the presence of purified components of the soluble MMOs of both Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b).

Table 3.D.2. Purification scheme of component C of the soluble MMO.

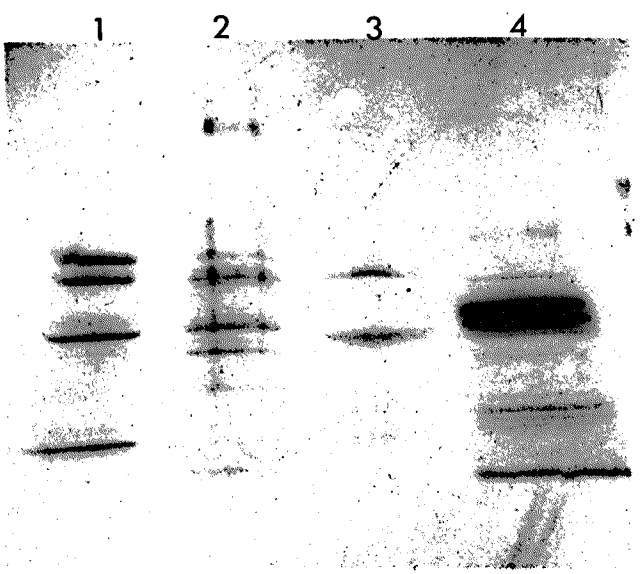
Fraction	Volume (ml)	Protein conc (mg/ml)	Total protein (mg)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Recovery (%)	Purification factor
Soluble extract	13	93.8	1219	19	23161	100	1.00
DEAE OB3b C	5.2	9.6	50	241	12050	52	12.7
pure OB3b C	2.2	0.23	0.5	2829	1414	6	149

Analysis of pure OB3b C using SDS PAGE revealed the presence of two major bands of approximately equal intensity of M_r 58000 and 38000 (FIG 3.D.1). This gel also revealed the degree of purification required in the purification of component C as compared to component A. The subunits of component A are always prominent on SDS PAGE gels of soluble extracts because this protein constitutes such a large proportion of the total protein of the cell. The two proteins present in pure OB3b C are not visible until the final stages of purification and are therefore present in much lower relative amounts than those of component A. FIG 3.D.1 also shows a track that contains component C of the soluble MMO of Methylococcus capsulatus (Bath), with a molecular weight of 37000. The similarity of the soluble MMOs of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath), tempts one to speculate on the possibility that the 38000 MW protein in pure OB3b C is infact component C and the 58000 MW protein a contaminant. However this cannot be proved until further and final purification of this component takes place (see Discussion).

D.3. Independent NADH:acceptor reductase activities of component C.

Component C of the soluble MMO of Methylococcus capsulatus (Bath) has been shown to have an independent NADH:acceptor reductase activity, passing electrons from NADH to a number of electron acceptors such as potassium ferricyanide, dichlorophenol indophenol (DCPIP) and cytochrome c (Colby and Dalton 1979, Lund 1983). Steady

Figure 3.D.1. Purification of component C: SDS PAGE of the stages of purification.



1. Soluble extract. 2. DEAE OB3b C. 3. Purified OB3b C. 4. Pure MC C.

Electrophoresis: 5-20% gradient SDS PAGE 30 micrograms of protein per track, except 3. 10 micrograms protein..

state kinetics were employed to investigate the acceptor reductase activities of component C of the soluble MMO of Methylococcus capsulatus (Bath) and to study the interaction of component C with NADH. The similarity between the soluble MMO's of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) led to the similar investigation of NADH:acceptor reductase activities of the component C of the soluble MMO of Methylosinus trichosporium (OB3b).

NADH: acceptor reductase activities were investigated using spectophotometry under anoxic conditions following the reduction of the electron acceptors ferricyanide, DCPIP and cytochrome$_c$ at 410, 600 and 550 nm respectively (see Colby and Dalton 1979, Lund 1983 and Materials and Methods for details). Both DEAE OB3b C and 'pure' dye affinity generated component C were used for these experiments. NADH:acceptor reductase activities were linearlly related to the concentration of DEAE OB3b C in the assay. This activity was also linearlly related to the soluble MMO activity of component C. DEAE OB3b C with a soluble MMO activity of 654 nmol/min/mg had an NADH:acceptor reductase activity of 2938 nmol/min/mg (with DCPIP as electron acceptor), 4.5 times the soluble MMO activity. The ratio of approximately 4.5:1 for NADH acceptor reductase activity : soluble MMO activity was maintained during the purification of component C.

The maximum initial rates measured at an NADH concentration of 1 mM were low as compared to the activities of component C of the soluble MMO of Methylococcus

capsulatus (Bath), however when the soluble MMO activities of these two proteins were considered the acceptor reductase activities are comparable for the electron acceptors ferricyanide and DCPIP (Table 3.D.3). Activities of cytochrome c reduction by component C of the soluble MMO of Methylosinus trichosporium (OB3b) were very low as compared to those reported for component C of the soluble MMO of Methylococcus capsulatus (Bath). Unfortunately this activity was not measured using 'pure' C.

For further assays of the NADH:acceptor reductase activity of component C, DCPIP was used as the electron acceptor, assays carried out using this acceptor being of a convenient velocity and reproducibility. However a small amount of endogenous activity is measured with DCPIP and NADH which has to be taken into account in the calculation of initial rates.

The km for NADH and the Vmax for the reduction of DCPIP were found by measuring the initial rates of DCPIP (20 micromolar) reduction with an NADH concentration range of 0.135-1.62 mM and 25 micrograms of component C. The reaction was initiated by the addition of NADH and the rate corrected for endogenous activity at the highest concentration of NADH. A Lineweaver-Burke plot gave a linear slope for NADH concentration 0.27-1.62 mM, however at or below an NADH concentration of 0.135 mM points varied from the straight line (FIG 3.D.2). The linear plot indicates that this reaction obeys the behavior predicted by the Michaelis-Menten equation for enzyme catalysis/kinetics. The km was calculated to be 0.685 mM and the Vmax 4.29 micromoles/min/mg.

Table 3.D.3. NADH:acceptor reductase activity of component C: Comparison of the rates of reduction of component C from Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b).

Electron acceptor	NADH:acceptor reductase activity. (micromol/min/mg)	
	OB3b	MC
DCPIP	2.9	30-60
K ferricyanide	12.3	230-260
Cytochrome c	0.05	130-180

Assays contained:

Methylococcus capsulatus (Bath): 50 nmolar component C in 2 ml of 20 mM sodium phosphate buffer pH 7.0. 200 micromolar NADH at 45^{0}C. Component C MMO activity 6000 nmol/min/mg. Figures obtained fron Lund (1983).

Methylosinus trichosporium (OB3b): 5-25 micrograms DEAE OB3b C in 2 ml of 20 mM sodium phosphate buffer pH 7.0, 1 mM NADH at 30^{0}C. Component C MMO activity 645 nmol/min/mg.

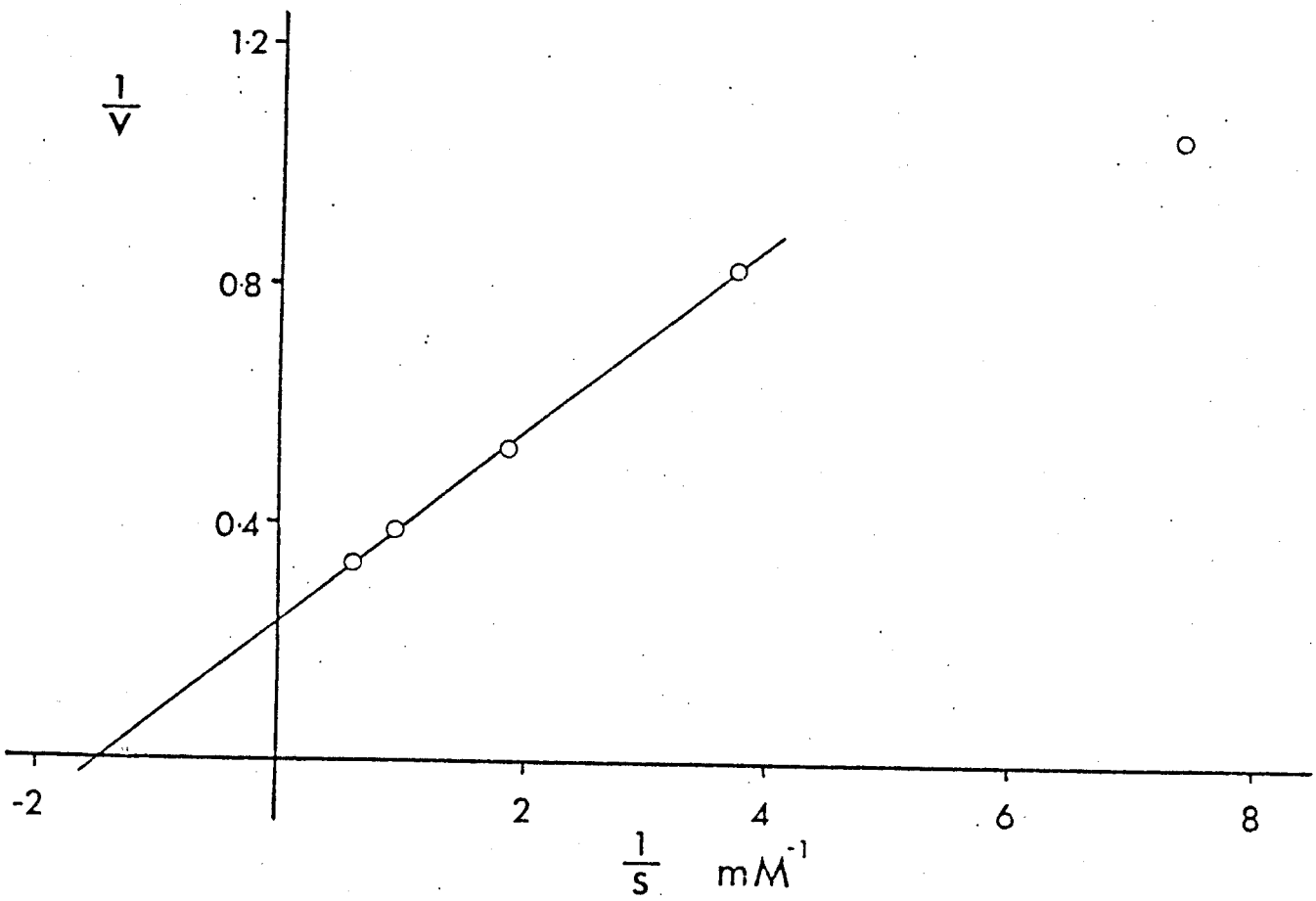


Figure 3.D.2. NADH:acceptor reductase activity of component C: Lineweaver Burke plot for the calculation of the Km with respect to NADH.

Assays contained: 2 ml 20 mM sodium phosphate buffer pH 7.0, containing 0.19 micromoles DCPIP in a sealed cuvette and sparged with helium for 10 minutes to make anoxic. Component C added (10 micrograms DEAE OB3b C) and the assay started by the addition of 2 micromols NADH.

D.4. Discussion.

Component C of the soluble MMO of Methylosinus trichosporium (OB3b) is the NADH:acceptor reductase component of the enzyme. The equivalent component of the soluble MMO of Methylococcus capsulatus (Bath) was purified by 5'-AMP Sepharose affinity chromatography. This technique when applied to extracts of Methylosinus trichosporium (OB3b) comtaining component C activity failed to significantly purify this protein. Recovery of component C activity was erratic, often no activity being recovered from the column. When activity was recovered it was spread thoughout the column fractions, with 30% of the activity being recovered with elution with NADH. The testing of a number of dye affinity materials for their ability to bind component C showed that two, Red and Blue A bound component C, with Red binding most strongly with 60-67% of the component C activity being eluted by 2 mM NADH. All these chromatography procedures were carried out in small (2 ml volume) test columns. On scaling up of Red dye affinity chromatography to a column of 40 ml volume, a complete failure to recover activity was observed. Returning to columns of more moderate size (5 ml) component C was once again recovered, however on multiple runs the specific activities of the component C fractions varied greatly from 818 to 6615 nmol/min/mg with one run of six failing to yield any activity. The overall component C recovery was 12% from this column.

Throughout the purification of component C the recovery

of this activity whether it be from cell free extracts, DEAE cellulose or dye affinity chromatography, has been extremely variable. Component C was shown to be the limiting component of the soluble MMO in cell extracts. Extracts that had no soluble MMO activity retained component A activity but lacked component C activity. The recovery of component C activity from DEAE column chromatography was extremely variable, the specific activity varying between 97 and 1580 nmol/min/mg. In experiments on the stability of component C, its stability was shown to be dependent on the presence of DTT, it losing only 3.5% activity over a 24 h period at 4^{0}C in the presence of 1 mM DTT, whereas no activity was measured when DTT was removed. From these results it would be expected that little or no component C activity would be lost during purification procedures carried out at 4^{0}C and in the presence of DTT. The variability of the yield of component C activity from all purification procedures cannot therefore be attributed to the observed stability of the protein or to its separation from stablising factors present in extracts of Methylosinus trichosporium (OB3b) during the purification of component C.

The variable yield of component C from purification procedures and particularily the failure to recover activity from large scale Red dye affinity chromatography led to a shortage of purified component C for further experimentation. Consequently the purification of component C was curtailed at the Red dye affinity chromatography stage. At this stage fractions containing component C activity had two polypeptides present in significant amounts. These proteins were of significantly different

molecular weights to effect their separation by gel filtration chromatography. So it is suggested that the next stage in the purification of component C be gel filtration on Sephadex G-100.

The soluble MMO activity of component C is discussed in the following chapter, however its independent NADH acceptor reductase activity is dealt with here. Purified component C of the soluble MMO of Methylococcus capsulatus (Bath) has been shown to have NADH:acceptor reductase activity, passing electrons from NADH to a number of electron acceptors (Colby and Dalton 1979, Lund 1983). Component C of the soluble MMO of Methylosinus trichosporium (OB3b) was also shown to possess this activity passing electrons to ferricyanide, DCPIP and cytochrome c. An estimation of the Km for NADH was made for component C passing electrons to DCPIP and was found to be 0.685 mM an order of magnitude higher than that measured for component C of the soluble MMO of Methylococcus capsulatus (Bath) at 0.05 mM (Colby and Dalton 1979).

E. The interaction of the purified components of the soluble methane monooxygenases of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b).

E.1. Introduction

Over the last few years purification schemes have been published for all of the three components of the soluble MMO of Methylococcus capsulatus (Bath). Within the work contained in this thesis are protocols for the purification of component A and partial purification of component C of the soluble MMO of Methylosinus trichosporium (OB3b). The aim of this section of the thesis is therefore to investigate further the interaction of the purified components of the soluble MMOs of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b). This work was hampered by lack of material, specifically component B of the soluble MMO of Methylococcus capsulatus (Bath), and component C of the soluble MMO of Methylosinus trichosporium (OB3b).

E.2. Results

To simplify this section abbreviations have been adopted for the soluble MMO fractions of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) used in this work, some of which were introduced earlier. There follows a table listing the abbreviations adopted for and the characteristics of the soluble MMO components used in this work (Table 3.E.1).

Table 3.E.1. Abreviations adopted for fractions containing components of the soluble MMOs of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b).

Abreviation.	Description.
DEAE MC A DEAE MC B DEAE MC C	DEAE fractions of the soluble MMO of Methylococcus capsulatus (Bath) contaminated by other soluble MMO components.
Pure MC A Pure MC B Pure MC C	Pure components of the soluble MMO of Methylococcus capsulatus (Bath).
DEAE OB3b A DEAE OB3b C	DEAE fractions of the soluble MMO of Methylosinus trichosporium (OB3b) contaminated with other components of the soluble MMO.
Pure OB3b A Pure OB3b C	'Pure' components of the soluble MMO of Methylosinus trichosporium (OB3b) purified as defined in the text.

Table 3.E.2 gives the specific activities of the components of the soluble MMOs of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) used in this work. Each was assayed in the presence of excess amounts of the other components of the soluble MMO from the same organism in their partially purified state (DEAE fractions). Assay of the components of the soluble MMO of Methylococcus capsulatus (Bath) were carried out at 45°C and 30°C. When assayed at 30°C components of the soluble MMO of Methylococcus capsulatus (Bath) had 32-47% of their specific activity at 45°C. DEAE fractions of the soluble MMO of Methylococcus capsulatus (Bath) all had contaminating components present, DEAE MC A contained some component B, DEAE MC B contained some component C and DEAE MC C contained some component B (Table 3.E.3).

The results in this chapter can be separated into three parts; 1. the interaction of pure OB3b A with components of the soluble MMO of Methylococcus capsulatus (Bath). 2. the interaction of pure OB3b C with components of the soluble MMO of Methylococcus capsulatus (Bath), and 3. the interaction of pure OB3b A+C with components of the soluble MMO of Methylococcus capsulatus (Bath).

As previously stated pure OB3b A had activity when assayed in combination with DEAE MC B+C and DEAE MC C at 30°C. No activity was measured if pure OB3b A was assayed in combination with pure MC C or pure MC B+C at 30°C, whereas at 45°C activity was present with pure MC B+C (Table 3.E.4.). This therefore demonstrates that all three components of the soluble MMO were required for activity when Methylosinus trichosporium (OB3b) was the source of

Table 3.E.2. Specific activities of the fractions containing components of the soluble MMO.

Fraction	Specific activity (nmol/min/mg)	
	30°C	45°C
DEAE MC A	33.3	88.1
DEAE MC B	119	253
DEAE MC C	514	1613
Pure MC A	40	132
Pure MC B	nd	17000
Pure MC C	254	836
DEAE OB3b A	35.5	nd
DEAE OB3b C	645	nd
Pure OB3b A	35	nd
Pure OB3b C	2829	nd

Soluble MMO assays: as described in Materials and Methods. Each component being assayed with excess amounts of partially pure complementary soluble MMO components (DEAE fractions) from the same organism. 3 minute assays, propene to epoxypropane.

Table 3.E.3. Soluble MMO assays of combinations of DEAE soluble MMO fractions from Methylococcus capsulatus (Bath).

DEAE fraction (mg)			Epoxypropane
A	B	C	(nmol/3min)
7	1	1	2518
7	1	0	290
7	0	1	1539
0	1	1	0
7	0	0	0
0	1	0	0
0	0	1	0

Soluble MMO assays: 3 min assays, 45^{0}C measuring the oxidation of propene to epoxypropane containing specified soluble MMO fractions.

Soluble MMO assays: 3 minute assays, at both 30^{0}C and 45^{0}C following the oxidation of propene to epoxypropane. Containing:7mg DEAE MC A, 1 mg DEAE MC B, 1mg DEAE MC C, 1 mg Pure MC A, 0.1 mg Pure MC B, 1 mg Pure MC C, 1 mg Pure OB3b A and 0.01 mg Pure OB3b C.

Table 3.E.4. Soluble MMO assays of combinations of the components of the soluble MMOs of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b).

Soluble MMO fraction		Temperature	Epoxypropane
OB3b	MC	(°C)	(nmol)
Pure OB3b A	DEAE MC B + C	30	52
Pure OB3b A	DEAE MC B + C	45	53
Pure OB3b A	DEAE MC B	30	0
Pure OB3b A	DEAE MC C	30	48
Pure OB3b A	Pure MC C	30	0
Pure OB3b A	Pure MC B + C	30	0
Pure OB3b A	Pure MC B + C	45	43
Pure OB3b A	Pure MC B	45	0
Pure OB3b A	Pure MC C	30	0
Pure OB3b C	DEAE MC A	30	60
Pure OB3b C	DEAE MC B	30	0
Pure OB3b C	DEAE MC C	30	0
Pure OB3b C	DEAE MC B + C	30	0
Pure OB3b C	Pure MC A	30	0
Pure OB3b C	Pure MC B	30	0
Pure OB3b C	Pure MC C	30	0
Pure OB3b C	Pure MC A + DEAE MC B	30	45
Pure OB3b C	Pure MC A + B	30	0
Pure OB3b C	Pure MC A + B	45	32
Pure OB3b A + C		30	0
Pure OB3b A + C		45	0
Pure OB3b A + C	DEAE MC B	30	24
Pure OB3b A + C	DEAE MC B	45	26
Pure OB3b A + C	Pure MC B	30	0
Pure OB3b A + C	Pure MC B	45	29

component A and Methylococcus capsulatus (Bath) component C.

The failure to measure MMO activity at 30°C with pure OB3b A in the presence of pure MC B+C was not due to either or both of the Methylococcus capsulatus (Bath) components being inactive at this temperature, as the partially pure components stimulated activity at this temperature. That the activity was either absent totally at 30°C or at a greatly reduced rate at the lower temperature demonstrated that components B and C of the soluble MMO of Methylococcus capsulatus (Bath) were more compatible with and therefore had a higher activity at 45°C than at 30°C.

Assays of pure OB3b A at either 30°C or 45°C with DEAE MC B+C where component A was limiting had very similar specific activities of approximately 17.5 nmol/min/mg, half that measured for pure OB3b A assayed with DEAE OB3b C (35.3 nmol/min/mg). This demonstrates that the activity of component A of the soluble MMO of Methylosinus trichosporium (OB3b) was unaffected by temperature within the range 30-45°C and that the complementary components of the soluble MMO from Methylococcus capsulatus (Bath) were less compatible with component A of the soluble MMO of Methylosinus trichosporium (OB3b) than the equivilent soluble MMO component(s) from the same organism.

Pure OB3b C had soluble MMO activity when assayed in combination with DEAE MC A though not DEAE MC B and/or DEAE MC C. No activity was measured in assays of pure OB3b C in combination with pure MC A, pure MC B, pure MC C or pure MC A+B at 30°C, though activity was measured with pure MC A plus DEAE MC B at 30°C. At 45°C activity was also measured

with pure MC A+B, though not with either component separately. Once again combinations of pure components from both Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) were only active at 45°C and not at 30°C, whereas pure OB3b C assayed in combination with partially pure components from Methylococcus capsulatus (Bath) had activity at both 45°C and 30°C. This demonstrates that this combination with Methylosinus trichosporium (OB3b) being the source of component C was more active at 45°C than at 30°C. All three known components of the soluble MMO of Methylococcus capsulatus (Bath) or their equivalents from Methylosinus trichosporium (OB3b) were shown to be essential for activity when Methylosinus trichosporium (OB3b) is the source of component C and Methylococcus capsulatus (Bath) component A, and as previously discussed it cannot be presumed from these results that component C from Methylosinus trichosporium (OB3b) required a B component for activity, B may well act on component A or on a complex of components A+C.

As described previously pure OB3b A, when assayed in combination with DEAE OB3b C, had activity as does pure OB3b C assayed in combination with DEAE OB3b A. However assays of pure OB3b A plus pure OB3b C failed to yield activity at either 30°C or 45°C. Activity could be restored to a combination of pure OB3b A+C by the addition of DEAE MC B at 30°C and 45°C, or by the addition of pure MC B at 45°C though not at 30°C. Assays where all three pure soluble MMO components from Methylococcus capsulatus (Bath) were assayed in combination had activity at 45°C though not at 30°C.

E.3. Discussion.

The presentation of the above results is necessarily complicated because of the number of combinations of the five components of the soluble MMO identified from Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) and the additional complication of the effect of temperature. To further simplify the presentation, discussion of some of the control experiments has been edited out. The results of these assays appear in the tables accompanying this chapter and are hopefully self-explanatory.

The major conclusion drawn from these assays is that in purifying two components of the soluble MMO of Methylosinus trichosporium (OB3b), component A the hydroxylase and component C the NADH:acceptor reductase, a component of the soluble MMO that is essential for activity of the enzyme is lost. The lost component could be replaced by component B of the soluble MMO of Methylococcus capsulatus (Bath), restoring activity to the Methylosinus trichosporium (OB3b) components. All the purified components from the two organisms were shown to be fully interchangeable so that component A from Methylosinus trichosporium (OB3b) could be combined with components B and C from Methylococcus capsulatus (Bath) and and component C from Methylosinus trichosporium (OB3b) with components A and B from Methylococcus capsulatus (Bath) to produce a complex with MMO activity. No activity was ever measured when one or more of the components of the soluble MMO was absent. The soluble MMO of Methylosinus trichosporium (OB3b) therefore

not only had a hydroxylase and NADH:acceptor reductase component in common with the soluble MMO from Methylococcus capsulatus (Bath) but also a third component, a regulatory component B which must act as a coupling effector in a similar manner to the component B from the soluble MMO from Methylococcus capsulatus (Bath).

All of the assays of only pure soluble MMO components failed to have any measurable activity at 30^{0}C only having activity if all three components of the soluble MMO were present at 45^{0}C. Component B of the soluble MMO of Methylococcus capsulatus (Bath) was the only component common to all these assays, pure components A and C being available from both organisms. A temperature of 45^{0}C has the effect of, if not enabling component B to act then enhancing its action on the other components of the soluble MMO from both Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b). None of the work presented here sheds any light on the precise action of component B, whether it acts on component A, component C or a complex of both A and C.

F. The oxidation of ammonia by Methylosinus trichosporium (OB3b).

F.1. Introduction.

The oxidation of ammonia has been demonstrated in all methanotrophs tested, most reports deal with whole cell oxidations and only in Methylococcus capsulatus (Bath) has ammonia oxidation been comprehensively studied in cell extracts. O'Neil and Wilkinson (1977) used whole cells of Methylosinus trichosporium (OB3b) to study ammonia oxidation. This work was carried out prior to the realisation of the effect of growth conditions on the nature and location of the MMO in Methylosinus trichosporium (OB3b). From the data available it would seem reasonable to assume that the cells used by O'Neil and Wilkinson (1977) possessed a particulate MMO. Cells used for whole cell assays or for the preparation of cell free extracts during this investigation contained a soluble MMO, unless otherwise stated.

F.2. Whole cell assays.

Whole cell assays were carried out using a 1 ml suspension of washed whole cells in 20 mM sodium/potassium phosphate buffer pH 7.0 at an OD_{540} of 50, in a 7 ml volume conical flask at $30^{0}C$ in an analogous way to cell free MMO assays (See Materials and Methods). However, with gaseous propene as a substrate, assays could not be initiated by the addition of substrate due to the delay in its oxidation caused by the time required for its dissolution in the liquid phase. Consequently a zero point reading was

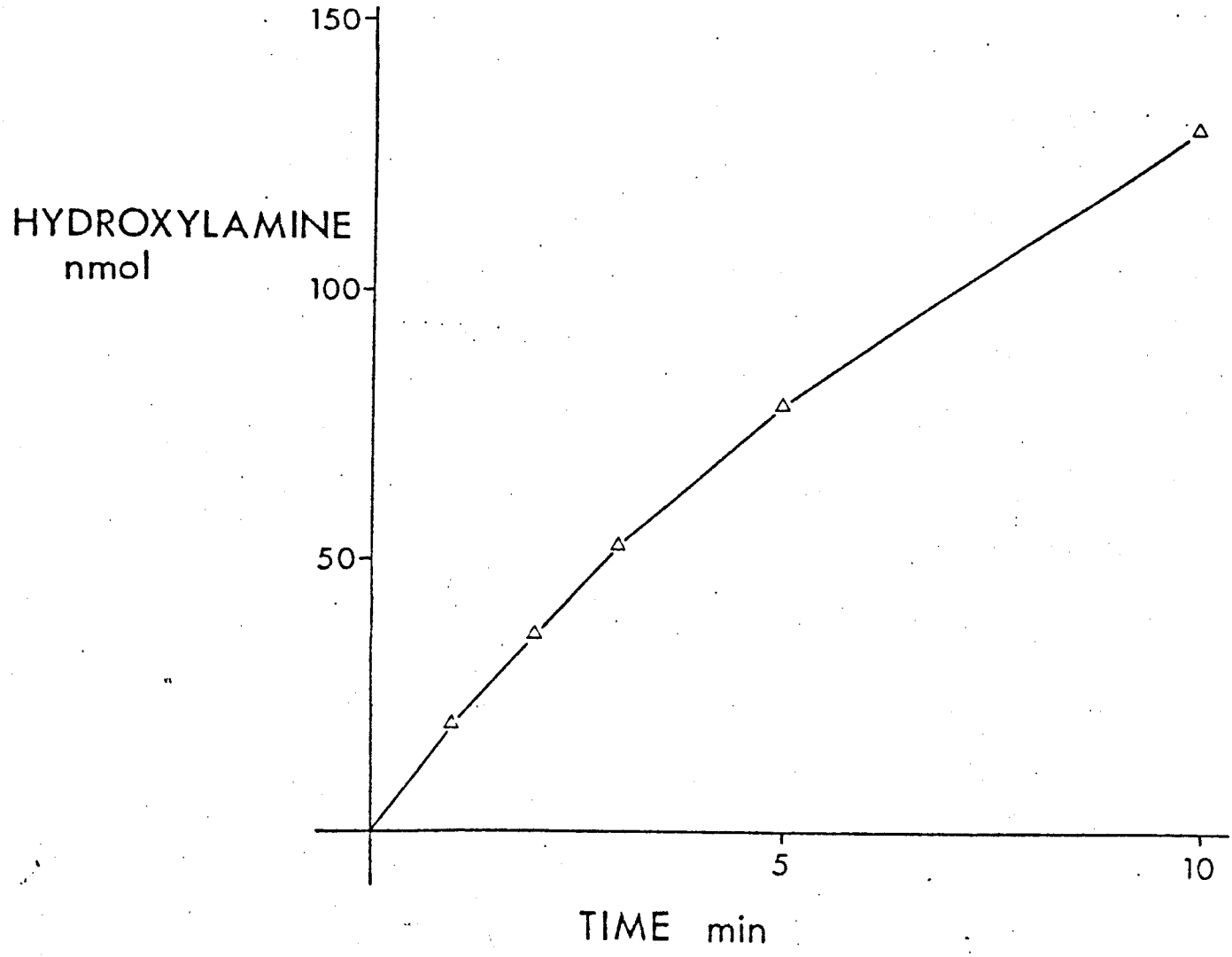


Figure 3.F.1. Ammonia oxidation to hydroxylamine by whole cells of Methylosinus trichosporium (OB3b).

Ammonia oxidation assay as described in Materials and Methods. Assays containing 21 mg dry weight whole cells.

necessary, which was subtracted from subsequent readings. The cells were taken from an oxygen-limited, low copper chemostat culture and therefore possessed a soluble MMO and high levels of the storage polymer PHB.

Whole cells oxidised propene to epoxypropane at a rate of 414 nmol/min/OD_{540} 50, this activity being stimulated by 10% by the addition of 5 mM formate and inhibited 59% and 40% by the addition of 5 mM hydroxylamine and 5 mM ammonium sulphate respectively. Whole cells oxidised ammonia to hydroxylamine at a rate of 17 nmol/min/OD_{540} 50, 4% of the propene rate, and was linear for up to ten minutes (FIG 3.F.1) and was unaffected by the presence of 5 mM formate. No nitrite was detected as a product of ammonia oxidation by whole cells even after incubation for 25 minutes. This is in direct opposition to the findings of O'Neil and Wilkinson (1977) who measured the rate of ammonia oxidation by the accumulation of nitrite. Formate was also shown to stimulate oxidation of ammonia by O'Neil and Wilkinson (1977). Both of these anomalies may well be explained by the difference in the physiological state of the cells used.

Even though no nitrite was detected as a product of the oxidation of ammonia by whole cells of Methylosinus trichosporium (OB3b), whole cells did oxidise hydroxylamine to nitrite at a rate of 20 nmol/min/OD_{540} 50.

F.3. Cell free assays.

F.3.1. Confirmation of the role of the soluble methane monooxygenase in the oxidation of ammonia.

Dalton (1977) listed four points that led him to the conclusion that the soluble MMO of Methylococcus capsulatus (Bath) was responsible for the oxidation of ammonia to hydroxylamine in cell extracts. These were based on the requirement for a reduced pyridine nucleotide and an identical inhibitor spectrum for the oxidation of ammonia and propene. Later Pilkington (1983) using purified components of the soluble MMO of Methylococcus capsulatus (Bath) showed unequivocally that ammonia oxidation was catalysed by the soluble MMO and also presented evidence that suggested that the particulate MMO also catalysed this reaction.

The similarity between the soluble MMOs of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) would lead one to assume that the soluble MMO of Methylosinus trichosporium (OB3b) was responsible for ammonia oxidation in this organism, however a number of experiments were caried out to test this hypothesis.

Oxidation of ammonia required the presence of NADH for activity and was completely inactive in the presence of acetylene (5% in air) or 8-hydroxyquinoline (1mM), potent inhibitors of the soluble MMO (Table 3.F.1). Ammonia oxidation required the presence of both soluble MMO DEAE fractions A and C for activity. Furthermore pure component C of the soluble MMO of Methylococcus capsulatus (Bath)

Table 3.F.1. Confirmation of the role of the soluble MMO in the oxidation of ammonia.

Assay contained.	Ammonia oxidation (nmol/min/mg)	Propene oxidation (nmol/min/mg)
Extract	9.5	68.7
Extract - NADH	0	0
Extract + Ethyne (1%)	0	0
Extract + 8-hydroxyquinoline (1 mM)	0	0
DEAE OB3b A	0	0
DEAE OB3b C	0	0
DEAE OB3b A + C	4.1	24.2
DEAE OB3b A + Pure MC C	8.3	61.3

Soluble MMO assays: As described in Materials and Methods. Propene oxidation; 3 minute assays. Ammonia oxidation; 1 minute assays. Containing: 5 mg extract, 5 mg DEAE OB3b A, 1 mg DEAE OB3b C and 1 mg Pure MC C.

restored ammonia oxidising activity to DEAE OB3b A. Ammonia oxidation activity was never present in extracts that had lost all soluble MMO activity due to the lack of stabilising agents or after being left at room temperature overnight. In addition to these assays ammonia oxidation activity was lost from soluble cell extracts along with soluble MMO activity when extracts were prepared from cells grown in a high copper medium which acts to suppress the production of the soluble MMO and induces the particulate MMO. No ammonia-oxidising activity was ever associated with particulate extracts from such cells, though this may be attributed to the highly unstable nature of the particulate MMO and the consequent low activities measured (<20 nmol/min/mg propene to epoxypropane activity), rather than the particulate MMO lacking the ability to oxidise ammonia. The particulate MMO may indeed oxidise ammonia as demonstrated by O'Neil and Wilkinson (1977) in their studies of whole cell oxidations.

F.3.2. Time course for ammonia oxidation.

Measurement of the rate of hydroxylamine formation from the oxidation of ammonia over a period of ten minutes by soluble cell free extracts of Methylosinus trichosporium (OB3b) revealed that the highest rate of ammonia oxidation occurred in the first minute of the assay, this being independent of the ammonia concentration in the assay (FIG 3.F.2). The concentration of hydroxylamine rose to a peak between 2 and 5 minutes from whence there was an initial sharp drop followed by a steady decline. This decline was

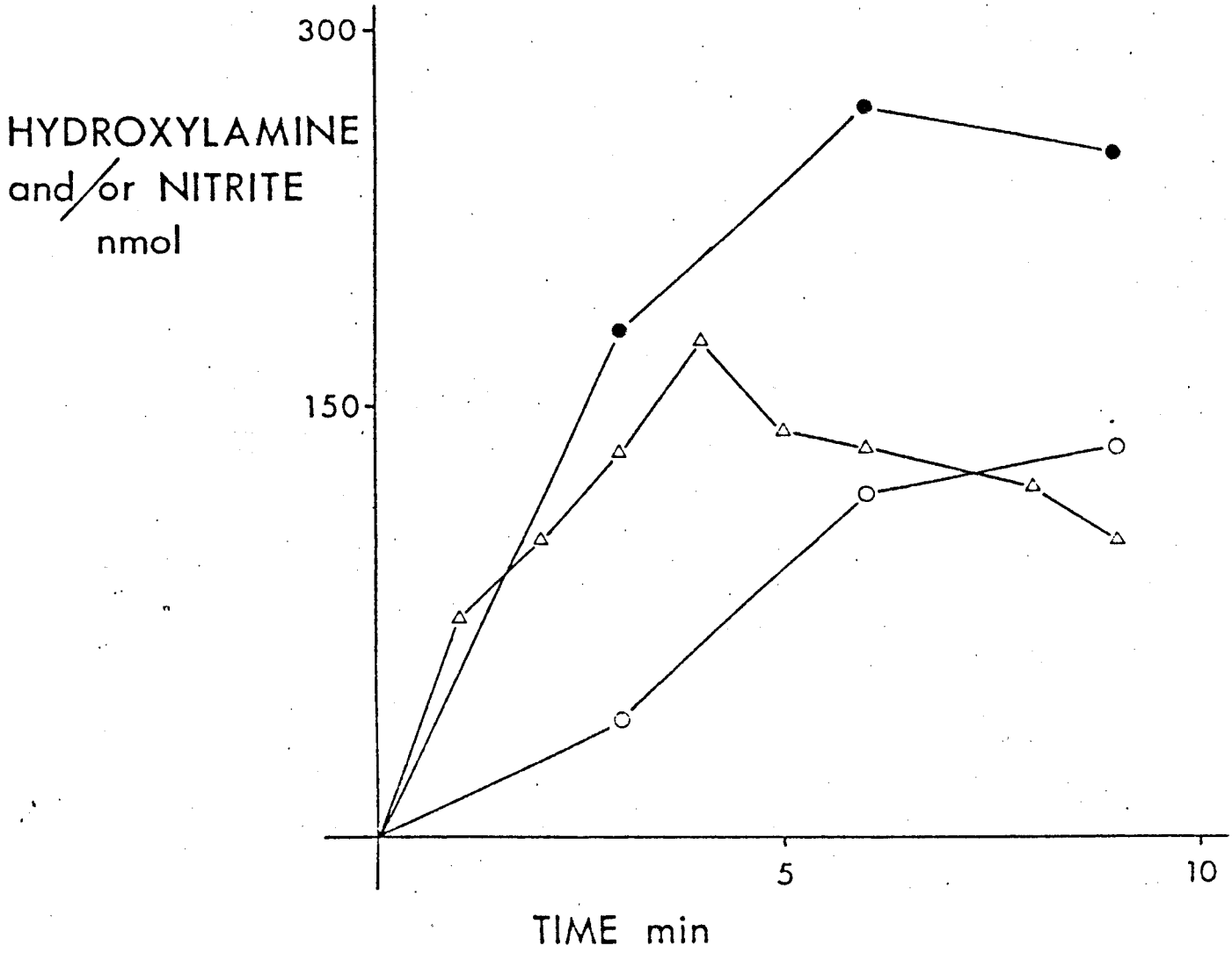


Figure 3.F.2. Ammonia oxidation by crude extracts of Methylosinus trichosporium (OB3b): Production of hydroxylamine and nitrite.

Ammonia oxidation assays as described in Materials and Methods. Containing 5 mg crude extract, 5 mM ammonium chloride.

△ Hydroxylamine. ○ Nitrite.

● Hydroxylamine + Nitrite.

mainly accounted for by the action of hydroxylamine oxidoreductase (HAO) converting hydroxylamine to nitrite. However this did not wholly account for the decline in hydroxylamine concentration. There are two possibilities that may explain this further fall in hydroxylamine concentration. Nitrite may be further converted to an as yet unidentified compound (such as nitrate) and so the measurement of nitrite concentration yields a false low activity for HAO. There is no evidence for the further oxidation of nitrite by cell extracts of Methylosinus trichosporium (OB3b) under these conditions. No disappearance of nitrite was observed when incubated with cell free extracts in the presence or absence of NADH. Alternatively, hydroxylamine may be converted to another compound other than nitrite by the HAO as occurs in Nitrosomonas where the HAO converts hydroxylamine into equal amounts of both nitrite and nitrate (Hooper et al., 1977), or by an as yet unidentified enzyme. However no production of nitrate was detected in assays of ammonia oxidation.

The cessation of hydroxylamine formation after the first few minutes of the assay may be the result of two factors. These being the depletion of a substrate for the oxidation of the ammonia (ammonia, NADH or oxygen) or the inhibition of the soluble MMO by a product of the oxidation (hydroxylamine or NAD^+).

The NADH levels in an MMO assay can be maintained by the use of the formate/formate dehydrogenase NADH-regeneration system (Pilkington 1983). Assays of the soluble MMO activity of soluble cell extracts of Methylosinus trichosporium (OB3b), by the measurement of

propene to epoxypropane oxidation, maintained a linear maximum rate of epoxypropane production for at least ten minutes, at a far higher rate than that observed for the oxidation of ammonia. However the oxidation of ammonia was unaffected by the presence of the NADH-regeneration system in assays, maximum activity still occurring in the first minute of the assay.

To test the ability of hydroxylamine to inhibit the soluble MMO, assays measuring the oxidation of propene to epoxypropane were carried out in the presence of hydroxylamine (0.01 - 1.0 mM) (Table 3.F.2). Propene oxidation activity was inhibited by 50% at 1 mM and 29% at 0.1 mM hydroxylamine.

F.3.3. The oxidation of hydroxylamine to nitrite.

Methanotrophs not only share with Nitrosomonas the ability to oxidise ammonia to hydroxylamine but can also further oxidise hydroxylamine to nitrite via the action of HAO (Dalton 1977, Sokolov et al., 1980). However some methanotrophs have been reported that are not able to carry out this further oxidation (Sokolov et al., 1980). The oxidation of hydroxylamine has been most comprehensively studied in Methylococcus thermophilus, where it was shown that ATP and NADH could be generated from the oxidation of hydroxylamine (Sokolov et al., 1980, 1981).

Whole cells of Methylosinus trichosporium (OB3b) have been shown to be able to oxidise ammonia to nitrite (O'Neil and Wilkinson 1977) and in this study hydroxylamine to nitrite. For the assay of maximum HAO activity in

Table 3.F.2. Inhibition of the soluble MMO by hydroxylamine.

Hydroxylamine concentration (mM)	Soluble MMO Specific activity (nmol/min/mg)
0	52.4
0.01	51.9
0.1	37.4
1.0	26.2

Soluble MMO assays: As described in Materials and Methods, following the oxidation of propene to epoxypropane. Containing 5 mg soluble extract, 3 minute assays at 30^{0}C.

Methylococcus capsulatus (Bath) and Nitrosomonas PMS an electron acceptor was included in the assay. Assays of HAO in crude cell extracts of Methylosinus trichosporium (OB3b) gave very low activities whether assayed in the presence or absence of PMS (2.5 mM) of 0.3-0.5 nmol nitrite formed/min/mg (Table 3.F.3). The possibility that the enzyme was very unstable was investigated by the preparation and very rapid assay of crude cell free extracts, prepared with or without DTT and/or PMSF, however no higher activities were measured than those listed above. A number of other electron acceptors such as DCPIP, cytochrome c and NAD^+ were tested for their ability to stimulate HAO activity in crude extracts without success (Table 3.F.3). No discernible difference in HAO activity was measured in assays of crude extracts prepared from cells that were grown under conditions which induced the production of a particulate MMO. The only co-factor that was found to stimulate the HAO activity was NADH (5 mM) an electron donor, which induced a six fold rise in activity to 3.23 nmol/min/mg, still very low activity by comparison to HAO activity in crude extracts of Methylococcus capsulatus (Bath) which had an HAO activity of 152 nmol/min/mg using PMS as an electron acceptor (Pilkington 1983). The stimulation of HAO activity was however ephemeral. Time course assays measuring nitrite formation over a 9 minute period with various levels of NADH (0-10 mM) showed that the period of maximum nitrite production occured in the first two minutes of the assay. Once again the inclusion of an NADH regeneration system in assays of nitrite production failed to prolong the period of maximum activity. If

Table 3.F.3. Hydroxylamine oxidoreductase in crude extracts.

Electron donor/ acceptor	(mM)	Specific activity (nmol/min/mg)
PMS	2.5	0.50
NAD^+	5.0	0.63
DCPIP	2.0	0.65
Cytochrome c	0.075	0.59
NADH	5.0	3.23

Assay contained: 1 ml 20 mM sodium phosphate buffer pH 7.0 containing, 5 mg crude extract, 5 mM hydroxylamine plus any electron acceptors/donors as detailed above. Assays were of 3 minute duration, at 30^0C, following the production of nitrite from hydroxylamine.

hydroxylamine disappearance rather than nitrite accumulation was measured similar results were obtained, with NADH and not PMS stimulating the disappearance of hydroxylamine.

HAO activity was found to be present in the fraction DEAE OB3b A and was not inhibited by acetylene.

F.4. Discussion

The oxidation of ammonia by the soluble MMO's of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) was a similar short lived activity, the maximum rate of hydroxylamine formation occuring in the first minute of the assay. Various aspects of the assay of ammonia oxidation in Methylosinus trichosporium (OB3b) were further investigated, following procedures established during the investigation of the activity in Methylococcus capsulatus (Bath) (Pilkington 1983). The level of NADH in the assay was maintained by the use of an NADH-regeneration system with no effect on the duration of the period of maximum activity. Propene oxidation under these conditions was constant over a period of 10 minutes at a far higher rate than that observed for the oxidation of ammonia. The level of ammonia in assays also had little or no effect on the duration of maximum activity. So it can be concluded that no substrate for the oxidation of ammonia by the soluble MMO i e., oxygen, ammonia and NADH, was limiting in the assay.

Hydroxylamine is an inhibitor of the soluble MMO's of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) as well as the ammonia monooxygenase of

Nitrosomonas (Suzuki et al., 1976, Pilkington 1983). Hydroxylamine inhibits the soluble MMO of Methylosinus trichosporium (OB3b) by 50% at 1 mM and 29% at 0.1 mM. The concentration of hydroxylamine in assays of ammonia oxidation reached at least 0.2 mM and will presumably inhibited the soluble MMO considerably. This must have then been, if not the only, then a major cause of the rapid cessation of the maximum ammonia oxidation activity. The same conclusion was reached for the oxidation of ammonia by the soluble MMO of Methylococcus capsulatus (Bath) and so the same observations apply.

To extend the oxidation of ammonia by the soluble MMO the product, hydroxylamine, must be rapidly removed from the environment of the enzyme, to prevent its concentration building up to a point where it significantly inhibits the enzyme. This could either be by physical removal of hydroxylamine (technically difficult) or by the conversion of hydroxylamine to a compound that was inert in relation to the oxidation of ammonia but was readily assayable. An obvious candidate for the conversion of hydroxylamine is HAO which converts it to nitrite. The natural levels of HAO in extracts of Methylosinus trichosporium (OB3b) were too low to prevent the rise in hydroxylamine concentration. HAO from other sources (Methylococcus capsulatus (Bath) and Nitrosomonas) require the presence of an electron acceptor such as PMS which inhibited the soluble MMO (Dalton 1977. Pilkington 1983). The HAO of Nitrosomonas can use cytochrome c as an electron acceptor and may well be a possibility for the removal of hydroxylamine from ammonia oxidation assays. Glutamine synthetase has an activity

involving the conversion of hydroxylamine to glutamyl hydroxamate however the products also include ammonia and so the exploitation of glutamine synthetase in experiments on the stoichiometry of the ammonia oxidation reaction would be difficult to interpret.

From the similarities that have been demonstrated between the soluble MMOs of Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) it could be assumed that the soluble MMO from Methylosinus trichosporium (OB3b) was also responsible for the oxidation of ammonia. This does indeed appear to be the case, for a number of reasons: 1. Ammonia and methane oxidation required the presence of a reduced pyridine nucleotide for activity. 2. Ammonia oxidation was inhibited by acetylene and 8-hydroxyquinoline, potent inhibitors of the soluble MMO. 3. Ammonia oxidation required the presence of both of the soluble MMO DEAE fractions for activity. 4. Ammonia oxidation was always found to co-exist with soluble MMO activity and was never found in extracts lacking soluble MMO activity, whether that activity was lost due to the instability of the enzyme or by the repression of the enzyme by the conditions under which Methylosinus trichosporium (OB3b) was grown. However, only when the soluble MMO from Methylosinus trichosporium (OB3b) is purified and thoroughly characterised will its ability to oxidise ammonia be finally proven.

The HAO of Nitrosomonas is a soluble enzyme that is located in the periplasm and is loosely associated with membrane (Suzuki and Kwok 1981b, Olson an Hooper 1983). HAO purified from Methylococcus thermophilus was reported to be a soluble enzyme (Sokolov et al., 1980). The HAO from

Methylococcus capsulatus (Bath) was initially reported to be a soluble enzyme but was subsequently shown to be associated with the membrane fraction of the cell extract (Dalton 1977, Pilkington 1983). In other respects however the HAO from Methylococcus capsulatus (Bath) resembled the highly characterised enzyme from Nitrosomonas. It was stable, required the presence of an electron acceptor (PMS) for activity and had hydroxylamine-dependent cytochrome c reductase activity associated with it.

The HAO activity in extracts of Methylosinus trichosporium (OB3b) was very low when assayed without the addition of the electron acceptor PMS as it is in extracts of Methylococcus capsulatus (Bath). However the inclusion of PMS failed to stimulate the HAO in extracts of Methylosinus trichosporium (OB3b) whereas it stimulated the HAO activity in extracts of Methylococcus capsulatus (Bath) 30 fold. NADH an electron donor was found to stimulate HAO activity in Methylosinus trichosporium (OB3b) whereas it had no effect on the activity of the enzyme from Methylococcus capsulatus (Bath) (Pilkington 1983). These findings may indicate that the nature of the oxidation of hydroxylamine to nitrite in Methylosinus trichosporium (OB3b) is fundamentally different from this activity in Nitrosomonas and Methylococcus capsulatus (Bath). However Methylosinus trichosporium (OB3b) may have an HAO which may either not have or lose during the process of extract preparation, the ability to use PMS as an electron acceptor. It therefore may only be the failure to identify a suitable electron acceptor that prevents the detection of high HAO activities in Methylosinus trichosporium (OB3b).

This however does not explain the stimulation of hydroxylamine oxidation by NADH an electron donor. This stimulation was ephemeral and only raised the level of activity to 3.2 nmol/min/mg as compared to 152 nmol/min/mg measured in extracts of Methylococcys capsulatus (Bath) using PMS as an electron acceptor. The oxidation of hydroxylamine may be carried out by another enzyme which utilizes NADH in the oxidation of hydroxylamine. A good candidate would have been the soluble MMO which oxidises a wide range of compounds including methanol the product of its oxidation of methane (Introduction A.4.1.2d). However acetylene a potent inhibitor of the soluble MMO failed to inhibit the oxidation of hydroxylamine and so the soluble MMO could not be responcible for this activity.

In conclusion, the oxidation of hydroxylamine to nitrite could not necessarily be attributed to HAO as described in Nitrosomonas. This oxidation may be carried out by an as yet unidentified enzyme which utilizes NADH.

Section 4. Conclusions and general discussion.

The results presented in this thesis have been discussed at the end of each results section. This section therefore has a number of aims: 1. To briefly summarise the findings of this research, 2. To compare these findings in Methylosinus trichosporium (OB3b) with those already published for methanotrophs, particularly Methylococcus capsulatus (Bath), 3. To discuss the wider aspects of these results, and 4. To indicate the research required to further characterise the soluble MMO of Methylosinus trichosporium (OB3b) and elucidate the importance (if any) of ammonia oxidation in the metabolism of methanotrophs.

A. Functional and physicochemical properties of the soluble MMO.

The nature and location of the MMO in Methylosinus trichosporium (OB3b) was shown to be dependent on the availability of copper to the cells, a particulate MMO being produced under conditions of excess copper and a soluble MMO under conditions of copper stress. This response to the availability of copper was independent of the carbon source used for growth (methane or methanol), which confirms the findings of Best and Higgins (1981) that Methylosinus trichosporium (OB3b) can produce both soluble and particulate MMO when grown on methanol.

The purification of component A and the partial purification of component C of the soluble MMO of

Methylosinus trichosporium (OB3b) and their characterisation has demonstrated further the similarity between the soluble MMOs from Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) (Table 4.A.1).

With the purification of components A and C it became evident that the soluble MMO of Methylosinus trichosporium (OB3b) had a third component. Purified components A and C assayed for MMO activity in combination failed to show activity. Partially purified component A plus purified component C and partially purified component C plus pure component A had soluble MMO activity. So there was a component that was lost in the purification of components A and C that was essential for MMO activity and that was present in both partially pure (DEAE fractions) A and C.

The purification of component A of the soluble MMO of Methylosinus trichosporium (OB3b) not only confirmed its functional similarity to component A of the soluble MMO of Methylococcus capsulatus (Bath) but also the similarity between these two components in their physicochemical charateristics (Table 4.A.1). Both A components from Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) are large proteins of M_r 230000 and 220000 respectively, have three different subunits of similar molecular weight which are present in an $\alpha_2\beta_2\gamma_2$ subunit structure. They each contain approximately 2 mol iron per mol protein and in their pure state are inactivated by freezing. The component A from Methylobacterium CRL-26 purified by Patel (1984) also shares many of these characteristics though that purified from Organism SB1 has a different subunit structure (α_4) though is similar in

Table 4.A.1. Characteristics of purified Methane Monooxygenases.

Organism	Methylococcus capsulatus (Bath)	Methylobacterium CRL-26	Organism SB1	Methylosinus trichosporium (OB3b)	
			Type 1	Type 2	
Classification	Type 1	Type 2			
Location	Soluble	Soluble	Soluble	Soluble	Particulate
Number of component proteins	Three A Hydroxylase B Regulatory C Acceptor reductase	Two or three A Hydroxylase B ? C Acceptor reductase	At least two A Hydroxylase C Acceptor reductase	Three A Hydroxylase B Regulatory C Acceptor reductase	Two or three 1 2 Cytochrome c_{co}
Component A M_r	220000	220000	260000	230000	1 47000
Number of subunits	Three	Three	One	Three	
Subunit M_r	α54000 β42000 γ17000	α60000 β40000 γ20000	α60000	α53000 β45000 γ23000	
Structure	$\alpha_2\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	α_4	$\alpha_2\beta_2\gamma_2$	
Metal content (mol/mol protein)	Fe 2.3	Fe 2.8	Fe 2.0	Fe 2.2	Cu 1.0
Component B M_r	15000				2. 9400
Number of subunits	One				
Stucture	α_1				
Metal content	none				
Component C M_r	38000	40000	42000	38000 and/or 58000	Cytochrome c_{co} 13000
Number of subunits	One	One	One		
Structure	α_1	α_1	α_1		
Prosthetic groups	1 FAD + 1 Fe_2S_2	1 FAD + 1 Fe_2S_2	FAD		
Catalytic activity	NADH:acceptor reductase	NADH:acceptor reductase	NADH:acceptor reductase	NADH:acceptor reductase	
Inhibitors	Ethyne 8-hydroxyquinoline	Iodoacetamide 5,5'-Dithio-2--nitrobenzoate		Ethyne 8-hydroxyquinoline	Ethyne 8-Hydroxyquinoline Imidazole, Cyanide Thiourea
Electron donors	NADH, NADPH	NADH, NADPH	NADH	NADH, NADPH	NADH, NADPH Non NADH-linked dehydrogenases
References	Colby & Dalton 1978 Colby & Dalton 1979 Woodland & Dalton 1984a Green & Dalton 1985	Patel et al., 1982 Patel 1984	Allen et al., 1984	Stirling & Dalton 1979 Scott et al., 1981a This thesis	Tonge et al., 1975 Tonge et al., 1977 Scott et al., 1981a

molecular weight (M_r 260000). In work presented here component A of the soluble MMO of Methylosinus trichosporium (OB3b) in a pure form was shown to be freely interchangeable with component A of the soluble MMO of Methylococcus capsulatus (Bath); ie in assays of pure components of the soluble MMO of Methylococcus capsulatus (Bath), component A from Methylosinus trichosporium (OB3b) could replace component A from Methylococcus capsulatus (Bath).

Component C of the soluble MMO of Methylosinus trichosporium (OB3b) was not purified to a homogeneous state due to its instability during purification, however it was purified to a point where certain characteristics could be investigated. In a partially pure (DEAE fractions) and purified state component C was shown to be able to replace component C of the soluble MMO of Methylococcus capsulatus (Bath) in assays of pure and partially pure components of the soluble MMO from this organism. The physicochemical charateristics of component C of the soluble MMO of Methylosinus trichosporium (OB3b) were not investigated in detail due to the lack of purified preparations. However purified component C contained two polypeptides of M_r 58000 and 38000. Component C of the soluble MMO's of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) do share other characteristics, they are highly unstable proteins that are stabilised by thiol protective agents (dithiothreitol and thioglycollate), and have an independent NADH:acceptor reductase activity associated with them. The NADH:acceptor reductase components of Methylobacterium CRL-26 and Organism SB1 also share a number of characteristics with those from Methylosinus trichosporium

(OB3b) and Methylococcus capsulatus (Bath) (Table 4.A.1).

Component B of the soluble MMO of Methylococcus capsulatus (Bath) has only recently been purified and characterised (Green and Dalton 1985). The presence of an equivalent component in the soluble MMO of Methylosinus trichosporium (OB3b) was tentatively demonstrated. This component was not purified or even separated from the other components of the soluble MMO but a requirement for the activity of a third component was demonstrated as previously discussed. Further, pure component B of the soluble MMO of Methylococcus capsulatus (Bath) was able to replace the putative component B of the soluble MMO of Methylosinus trichosporium (OB3b) in assays of purified components A and C of the soluble MMO of Methylosinus trichosporium (OB3b). Partially purified components A and C of the soluble MMO of Methylosinus trichosporium (OB3b), apparently both containing component B, restore activity to pure components C and A respectively of the soluble MMO of Methylococcus capsulatus (Bath). Recently in our laboratory it has been demonstrated that soluble extracts of Methylosinus trichosporium (OB3b) contain a protein that cross reacts with antibodies raised against pure component B of the soluble MMO of Methylococcus capsulatus (Bath) (Green, personal communication). Obviously the confirmation of the similarity of components B from Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) awaits the separation of this component from other components of the soluble MMO and its purification from Methylosinus trichosporium (OB3b), however functionally these components can be said to be similar. In Methylobacterium CRL-26 it

was initially reported that three components were required for soluble MMO activity, subsequently it was reported that only components A and C were required (Patel et al., 1982, Patel 1984). The soluble MMO of Organism SB1 has at least two components, no evidence for the existence of a third component was presented but the purification of component C from this organism has not been undertaken and so the presence of a third component cannot be ruled out (Allen et al., 1984).

Methylosinus trichosporium (OB3b) is a type 2 methanotroph as is Methylobacterium CRL-26. Methylococcus capsulatus (Bath) is a type 1 methanotroph but has certain characteristics that it shares with type 2 methanotrophs (Figure 1.A.1). The soluble MMO from Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) (and to a certain extent Methylobacterium CRL-26) are similar both functionally, physicochemically and in the regulation of their expression. This is evidence for this enzyme being highly conserved within methanotrophs. Though the purification and characterisation of other soluble MMOs must be completed before this can be confirmed. To the authors knowledge only one paper on any aspect of the biochemistry of Organism SB1 has been published (Allen et al., 1984). Its soluble MMO appears to be significantly different to others described and so further information on the biochemistry of this organism would be of great interest.

The first MMO to be purified and characterised was the particulate MMO from Methylosinus trichosporium (OB3b) (Tonge et al., 1977) though subsequently this purification

procedure has failed to produce active enzyme (Higgins *et al.*, 1981b). The soluble MMO from *Methylosinus trichosporium* (OB3b) bears no resemblance to the particulate MMO from this organism (Table 4.A.1).

From what we know of the functions of the components of the soluble MMO it may be predicted that a particulate MMO may not have a component equivalent to component C of the soluble MMO. There is evidence that the particulate MMO can accept electrons from NAD^+ independent dehydrogenases via an electron transport chain (Introduction section A.4.1.2c), and therefore may have no requirement for a component that directly interacts with NADH (component C). The particulate MMO must however have a hydroxylase component and it may have been expected that this hydroxylase component would be similar to that found in the soluble MMO. However in *Methylosinus trichosporium* (OB3b) this does not appear to be the case and so it would appear that the two forms of the MMO found in *Methylosinus trichosporium* (OB3b) have evolved independently.

No other particulate MMO has been purified and characterised and as previously discussed, the procedure for the purification of the particulate MMO from *Methylosinus trichosporium* (OB3b) now fails to yield active enzyme (Higgins *et al.*, 1981b). Not until a procedure is established which yields active particulate MMO will a thorough comparison between the soluble and particulate MMOs be possible. However from the studies already completed the soluble and particulate MMOs from *Methylosinus trichosporium* (OB3b) appear to be distinct and unrelated enzymes.

B. Ammonia oxidation.

The oxidation of ammonia to hydroxylamine in Methylosinus trichosporium (OB3b) was shown to be carried out by the soluble MMO. This was demonstrated by: 1. ammonia oxidation required the presence of NAD(P)H for activity as does the soluble MMO, 2. ammonia oxidation was inhibited by acetylene and 8-hydroxyquinoline, specific inhibitors of the soluble MMO, 3. ammonia oxidation required the presence of both DEAE fractions of the soluble MMO for activity, 4. ammonia oxidising activity was always found to co-exist with soluble MMO activity and was never found in extracts lacking soluble MMO activity, whether that activity was lost due to the instability of the soluble MMO or by the repression of the production of the enzyme by the conditions under which Methylosinus trichosporium (OB3b) was grown.

It has been demonstrated unequivocally that the soluble MMO from Methylococcus capsulatus (Bath) oxidises ammonia to hydroxylamine (Dalton 1977, Pilkington 1983). The similarity between the soluble MMOs of Methylosinus trichosporium (OB3b) and Methylococcus capsulatus (Bath) demonstrated by Stirling and Dalton (1979a) and by work contained in this thesis, leads one to predict that ammonia oxidation is also carried out by the soluble MMO of Methylosinus trichosporium (OB3b) and this does appear to be the case. However not until the soluble MMO of Methylosinus trichosporium (OB3b) is purified can this be demonstrated unequivocally.

The product of the oxidation of ammonia by the soluble MMO, hydroxylamine, was shown to inhibit the soluble MMO

(50% at 1 mM, 29% at 0.1 mM). This was identified as the probable major cause of the cessation of ammonia oxidising activity after one minute in assays. Other posible causes such as the depletion of of a substrate for the oxidation of ammonia (ammonia, NADH or oxygen) or the inhibition of the soluble MMO by another product of the reaction (NAD^+) were investigated by parallel studies following the oxidation of propene and found not to affect the duration of the oxidation carried out by the soluble MMO.

HAO has been purified and characterised from Nitrosomonas (Hooper and Nason 1965) and also studied in the methanotrophs Methylococcus capsulatus (Bath) and Methylococcus thermophilus (Dalton 1977, Pilkington 1983, Sokolov et al., 1980; 1981). In each case the presence of an electron acceptor (PMS) is required for maximum activity in vitro. In extracts of Methylococcus capsulatus (Bath), PMS stimulates the HAO activity 30 fold.

Whole cells of Methylosinus trichosporium (OB3b) have been shown to oxidise ammonia to nitrite (O'Neill and Wilkinson 1977) and hydroxylamine to nitrite (this study). The activity of HAO in extracts of Methylosinus trichosporium (OB3b), measured in the presence or absence of PMS was low. PMS had no affect on this activity as did a number of other electron acceptors (NAD^+, DCPIP and cytochrome c). Only NADH an electron donor was found to stimulate HAO activity, six fold from 0.5 nmol/min/mg to 3.2 nmol/min/mg. This is still very low activity in comparison to HAO in extracts of Methylococcus capsulatus (Bath) of 152 nmol/min/mg using PMS as an electron acceptor. There was no

indication that the HAO from Methylosinus trichosporium (OB3b) was highly unstable and being lost during the preparation of extracts. The soluble MMO was shown not to be responsible for the oxidation of hydroxylamine to nitrite.

C. Comparison of the autotrophic ammonia-oxidising and methane-oxidising bacteria.

The close morphological and biochemical relationship between the obligate methanotrophs and the autotrophic ammonia-oxidising bacteria has often been stressed (Quayle and Ferenci, 1978; Drozd, 1980; Higgins et al., 1981; Suzuki 1984). Both groups of organisms possess a complex series of internal membranes, a characteristic shared by only one other group of bacteria, the photosynthetics. They both utilize a very restricted range of substrates for energy production and many methanotrophs (the type 1's) and all the autotrophic ammonia-oxidisers possess an incomplete TCA cycle, which further restricts their metabolic diversity. They both oxidise their energy sources by the action of a monooxygenase which exibits an unusual lack of substrate specificity. The particulate MMO and the ammonia monooxygenase appear to be similar unstable, membrane bound, non-specific copper containing monooxygenases that appear to receive their reducing power indirectly from electrons derived from dehydrogenases. The methane monooxygenases are capable of oxidising ammonia and the ammonia monooxygenases, methane.

Type 1 methanotrophs utilize the ribulose monophosphate (RMP) cycle as their main carbon assimilation

pathway whereas the ammonia oxidisers use the ribulose bisphosphate (RuBP) cycle. Quayle and Ferenci (1978), have suggested possible evolutionary sequences by which these two cycles may be linked. It is interesting to note that Methylococcus capsulatus (Bath) has been shown to possess both RuBP carboxylase and phosphoribulokinase, the two key enzymes in the RuBP cycle. However attempts to grow Methylococcus with ammonia as an energy source for the fixation of carbon dioxide have failed (Drozd, 1980), as have attempts to grow ammonia oxidisers on methane as sole carbon and energy source even in the presence of 1.0 ppm ammonia (Jones and Morita, 1983).

The data presented above has led to speculation on the evolutionary relationship between methane and ammonia oxidisers (Quayle and Ferenci 1978). Whether it is by divergent or convergent evolution that these two groups of bacteria share so many characteristics, it is certain that observations made about one group may, with care, give us an insight into the characteristics of the other.

The significance of the lack of substrate specificity of the MMO in obligate methanotrophs, has been an area of contention for some time (Higgins et al., 1980, 1981; Stirling and Dalton, 1981). With the discovery of a similar lack of specificity in the oxidation catalysed by the ammonia monooxygenase from Nitrosomonas, these arguments may be applied to this system.

The two positions are: 1) That the wide substrate specificity shown by the MMO has evolved because of its (potential) competitive and survival value to the organism which outweighs any harmful effects incurred (Higgins et

al., 1980).

2) that the wide substrate specificity is a consequence of the nature of the active site chemistry of the MMO and that the oxidations carried out are purely fortuitous (Stirling and Dalton, 1979b).

Evidence that methanotrophs are able to benefit from some of the oxidations carried out by the MMO (Patel et al., 1978a; Wadzinski and Ribbons, 1975b; Sokolov et al., 1981) or that the autotrophic ammonia-oxidisers can benefit from the oxidation of methane (Jones and Morita 1983), is not necessarily evidence for evolutionary pressure for a non-specific MMO / ammonia monooxygenase. The organism may well be trying to make the most of a situation where, in order to exploit methane / ammonia as an energy source and in the case of methane, a carbon source, it has to possess an enzyme which by its very nature lacks substrate specificity. An example of such a system occurs with the enzyme RuBP carboxylase which has both carboxylase and oxygenase activities. The latter leading to the formation of phosphoglycollate which many organisms excrete as glycollate. This apparantly wasteful activity has not been eliminated over a long period of biochemical evolution and therefore appears to be a constitutive element of the enzyme's active site, whose primary function is that of a carboxylase (Dalton and Stirling, 1982). However some organisms have evolved a mechanism for the utilization of the glycollate so formed, thereby reducing the deleterious effect of the oxygenase activity.

The resolution of this argument will rest on the discovery of an MMO / ammonia monooxygenase which is more

specific than those already characterised. In a number of methanotrophs where a particulate MMO is present (Stirling et al., 1979; Burrows et al., 1984) a more restricted range of oxidations is carried out as compared to the soluble MMO. Whether this is due to increased specificity or the inhibition of other related processes by these compounds awaits the (re)isolation and characterisation of a stable particulate MMO.

The discussion of this area has primarily been concerned with oxidation of hydrocarbons other than methane by methanotrophs with little or no discussion of the possible advantage gained by methanotrophs through the oxidation of ammonia.

Ammonia is oxidised by all methanotrophs tested, however in only one case has it been demonstrated that the oxidation leads to the production of ATP or reducing power (via ATP-dependent reverse electon flow) (Malashenko et al., 1979). This ability would confer a selective advantage over other methanotrophs lacking this characteristic, either by supplementing the normal metabolic processes or to facilitate growth and survival under conditions of methane limitation. Obviously the recently discovered oxidation of methane to carbon dioxide by Nitrosomonas, which may provide it with a significant source of energy under conditions of ammonia limitation, is analogous.

It is significant that at least in one environment, that of freshwater lakes, the highest rates of both methane and ammonia oxidation always occur at the same location, namely at the oxic-anoxic interfaces of sediments and stratified water columns or throughout the water column

during periods of overturn (Rudd and Taylor, 1980). In situ, measurments of the rates of ammonia oxidation attributable to methane-oxidisers or that of methane oxidation by ammonia-oxidisers have not been attempted. However, Griffiths et al., (1982) reported that in a study of naturally occurring methane oxidation of the Bering Sea the proportion of methane incorporated into biomass was very low at 2%, indicating that it was being used as an energy source. This is suggestive of a role for ammonia oxidisers in the oxidation of methane in the environment. This low methane incorporation rate in marine environments is contrary to reports from freshwater environments of 30-60% methane incorporation (Rudd and Hamilton, 1975; Panganiban et al., 1979), though this discrepancy may be due in part to the relative carbonate concentrations in marine and freshwater environments. However it does appear to further demonstrate the distinct differences between marine and freshwater ammonia oxidisers as regards methane carbon incorporation reported by Jones and Morita (1983). Recently efforts have been made to distinguish between ammonia oxidation carried out by methanotrophs and that carried out by autotrophic ammonia-oxidisers using pyridine derivatives as inhibitors of ammonia oxidation. Only picolinic acid showed any specificity, inhibiting ammonia oxidation by methanotrophs but not by the autotrophic ammonia-oxidisers (Salvas and Taylor 1984). The contribution of methane and ammonia oxidisers to the cycling of ammonia and methane respectively, and the interactions of these two groups of bacteria is an area of research worthy of further study.

Section 5. References.

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