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The Effect of Copper Ions on Methane Oxidation  
by the Obligate Methylotroph Methylococcus capsulatus (Bath)

by

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This thesis is presented for the degree of Doctor of Philosophy  
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Dedication

To Mum, Dad, Sue and Toby

Dedication

To Mum, Dad, Sue and Toby

## List of Contents

	<u>Page no.</u>
Acknowledgements	vii
Summary	viii
List of Figures	ix
List of Tables	xii

## Chapter 1      General Introduction

1.	The Concept of Methylotrophy	1
2.	Occurrence and Isolation of Methane-utilizing Bacteria	1
3.	Grouping and Classification of Methanotrophs	7
4.	Physiology and Biochemistry of obligate methanotrophs	11
4.1	Basic growth requirements	11
4.2	The basis for obligate methylotrophy	12
4.3	Methane oxidation	14
	4.3.1 Introduction	14
	4.3.2 The oxidation of methane to methanol	14
	4.3.3 The particulate methane monooxygenase from <u>M. trichosporium</u> UB3b	17
	4.3.4 The soluble methane monooxygenase from <u>M. capsulatus</u> (Bath)	20
	4.3.5 Methane monooxygenases from other organisms	22
	4.3.6 Final comments on methane oxidation by methylotrophs	24
4.4	Methanol oxidation	25
4.5	Formaldehyde oxidation	26

	<u>Page no.</u>
4.6 Formate oxidation	27
4.7 Carbon assimilation by methylotrophs	27
5. The Industrial Applications of Methylotrophs	28
5.1 Production of single cell protein (SCP)	28
5.2 Overproduction of metabolites	29
5.3 Production of intracellular/extracellular polymers	30
5.4 The use of methylotrophs and their enzymes as biocatalysts	31

## Chapter 2      Materials and Methods

2.1 Organism	34
2.2 Media	34
2.3 Maintenance and growth	34
2.4 Whole cell respiration studies	37
2.5 Preparation of cell extracts	38
2.6 Methane monooxygenase assay	38
2.7 Whole cell MMO activity	40
2.8 Dry weight estimation	40
2.9 Electron microscopy	42
2.10 SDS-polyacrylamide gel electrophoresis	42
2.11 Copper uptake experiments	43
2.12 Antibody studies	45
2.13 <sup>35</sup> S-methionine uptake/incorporation studies	45
2.14 Studies using <sup>14</sup> C-labelled acetylene	47

	<u>Page no.</u>
2.15 Fluorography	48
2.16 Substrate specificity of the particulate MMO	48
2.17 Protein determinations	49
2.18 Gases	50
2.19 Chemicals	50

Chapter 3      The Role of Copper in the Regulation of  
the intracellular location of methane  
monooxygenase activity in Methylococcus  
capsulatus (Bath) grown on Methane

3.1 Introduction	51
3.2 Batch growth of <u>Methylococcus capsulatus</u> (Bath)	52
3.3 Continuous culture of <u>M. capsulatus</u> (Bath)	60
3.4 Studies with other organisms	65
3.5 The effect of addition of copper to copper-limited chemostat cultures of <u>M. capsulatus</u> (Bath)	68
3.6 Is the change in intracellular location of the MMO due to expression of new proteins?	73
3.7 Examination of membrane fractions expressing particulate MMO activity using antibody raised to components of the soluble MMO	78
3.8 The use of cell suspensions rather than chemostat cultures to study the transition from soluble to particulate MMO activity	79

	<u>Page no.</u>
3.9 Pulse-labelling studies to determine whether soluble MMO associates with cell membranes during the transition from soluble to particulate MMO activity	80
3.10 Thin-section electron microscopy of <u>M. capsulatus</u> (Bath) grown under different conditions in chemostat culture	83
3.11 Final comments	88
3.12 Further examples of regulation of expression of proteins by metal ions	89

Chapter 4                      The Effect of Copper Ions on Methanol Grown Cells of M. capsulatus (Bath)

4.1 Introduction	93
4.2 Presence of MMO and intracytoplasmic membranes in methanol-grown cultures	94
4.3 Growth of <u>Methylococcus capsulatus</u> (Bath) on methanol in continuous culture	96
4.4 The effect of copper on MMO activity in <u>M. capsulatus</u> (Bath) grown on methanol	96
4.5 The effect of copper on <u>in vitro</u> MMO activity of methanol-grown <u>M. capsulatus</u> (Bath)	103

Page no.

4.6	The effect of addition of copper on whole cell oxidation rates of methanol-grown <u>M. capsulatus</u> (Bath)	105
4.7	The intracellular location of MMO activity in organisms grown on methanol	107
4.8	Characteristics of the particulate MMO from <u>M. capsulatus</u> (Bath) grown on methanol	108
4.9	Thin-section electron microscopy of methanol- grown cells of <u>M. capsulatus</u>	109
4.10	Final Comments	114

Chapter 5                      Characteristics and Properties of the  
Particulate Methane Monooxygenase from  
Methylococcus capsulatus (Bath)

5.1	Introduction	116
5.2	Growth of <u>M. capsulatus</u> and preparation of cell extracts	117
5.3	Validity of results	117
5.4	The effect of various inhibitors on particulate MMO activity in cell extracts of <u>M. capsulatus</u>	118
5.5	Electron donor specificity of the particulate MMO in <u>M. capsulatus</u>	123
5.6	Stability of the particulate MMO from <u>M. capsulatus</u>	126

Page no.

4.6	The effect of addition of copper on whole cell oxidation rates of methanol-grown <u>M. capsulatus</u> (Bath)	105
4.7	The intracellular location of MMO activity in organisms grown on methanol	107
4.8	Characteristics of the particulate MMO from <u>M. capsulatus</u> (Bath) grown on methanol	108
4.9	Thin-section electron microscopy of methanol- grown cells of <u>M. capsulatus</u>	109
4.10	Final Comments	114

Chapter 5                      Characteristics and Properties of the  
Particulate Methane Monooxygenase from  
Methylococcus capsulatus (Bath)

5.1	Introduction	116
5.2	Growth of <u>M. capsulatus</u> and preparation of cell extracts	117
5.3	Validity of results	117
5.4	The effect of various inhibitors on particulate MMO activity in cell extracts of <u>M. capsulatus</u>	118
5.5	Electron donor specificity of the particulate MMO in <u>M. capsulatus</u>	123
5.6	Stability of the particulate MMO from <u>M. capsulatus</u>	126

	<u>Page no.</u>
5.7 Effect of temperaure on the particulate MMO from <u>M. capsulatus</u>	128
5.8 Effect of metal ions on particulate MMO activity	129
5.9 The effect of pH on particulate MMO activity	139
5.10 The use of phosphate buffer in NMS medium	144
5.11 Does the particulate MMO from <u>M. capsulatus</u> (Bath) contain copper?	145
5.12 Substrate specificity of the particulate MMO	149
 <u>Chapter 6</u> <u>Further characteristics and properties of the Methane Monooxygenase enzymes from M. capsulatus (Bath)</u>	
6.1 Partial purification of the particulate MMO from <u>M. capsulatus</u> (Bath)	156
6.2 The use of radiolabelled acetylene to identify the active site of the particulate and soluble MMO from <u>M. capsulatus</u> (Bath)	164
6.3 Copper uptake and inhibition of soluble MMO activity by <u>M. capsulatus</u> (Bath)	173
6.4 Inhibition of soluble MMO activity by copper ions	180
 <u>Chapter 7</u> <u>Coda</u>	189
 <u>References</u>	192

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### Summary

The work presented in this thesis provided the first report of membrane-bound methane monooxygenase (MMO) activity in the obligate methylotroph Methylococcus capsulatus (Bath). Experiments in batch and continuous culture showed that the intracellular location of the MMO enzyme was regulated by the copper:biomass ratio. Membrane-bound (particulate) MMO activity was associated with a high copper:biomass ratio, whereas, at low copper:biomass ratios the soluble form of the enzyme predominates. Growth of the organism on methanol showed that the expression of the particulate form of the enzyme was constitutive and that expression of the enzyme increased with increasing concentrations of copper in the growth medium.

Thin-section electron microscopy of M. capsulatus (Bath) grown on methane and methanol showed that the cell exhibited changes in intracellular morphology which could be correlated with the presence or absence of particulate MMO activity, although a direct connection between particulate MMO activity and intracytoplasmic membranes was not demonstrated. The change in intracellular location of the MMO was accompanied by changes in the protein banding pattern on SDS-polyacrylamide gels. Particulate MMO activity was characterized by an increase in at least 3 major proteins in the membrane fractions of cell extracts.

Addition of copper ions to chemostat cultures of Methylococcus capsulatus expressing wholly soluble MMO activity led to a rapid "switch-off" of soluble MMO activity and an equally rapid increase in particulate MMO activity. Studies on in vitro MMO activity demonstrated that particulate MMO activity was stimulated by the addition of copper ions to the assay system; however, addition of copper ions to fractions expressing soluble MMO activity led to a rapid inhibition of this form of the enzyme by irreversible inactivation of the NADH acceptor:reductase activity of the protein complex. The stimulation of particulate MMO activity by copper ions was found to be pH dependent due to the binding of the copper ions to the sodium/potassium phosphate buffer used in the assay system.

The particulate MMO was compared with the soluble enzyme from M. capsulatus (Bath) and was shown to differ in several respects including: inhibitor sensitivity, substrate specificity and activity in the presence of various metal ions. Studies using radiolabelled acetylene demonstrated that this compound appeared to act as a suicide-substrate for both forms of the enzyme by irreversibly binding to the active site of the proteins. A method for the partial purification of the particulate enzyme was developed which provided some stabilization of the protein. Studies on copper uptake by the organism demonstrated that this process was energy-dependent and occurred very rapidly when copper was added to copper-limited cultures of the organism.

List of FiguresPage no.

2.1	Relationship between optical density and dry weight of cells for <u>M. capsulatus</u> (Bath)	41
3.1	Batch growth of <u>M. capsulatus</u> (Bath) on low copper medium	55
3.2	Batch growth of <u>M. capsulatus</u> (Bath) on high copper medium	58
3.3	Effect of copper on optical density and intracellular location of the MMO activity in <u>M. capsulatus</u>	64
3.4	Effect of addition of copper sulphate to a chemostat culture of <u>M. capsulatus</u> expressing wholly soluble MMO activity (1)	71
3.5	Effect of addition of copper sulphate to a chemostat culture of <u>M. capsulatus</u> expressing wholly soluble MMO activity (2)	72
3.6	SDS-polyacrylamide gel electrophoresis of cell extracts of <u>M. capsulatus</u> (Bath)	74
3.7	SDS-polyacrylamide gel electrophoresis of membrane fractions of <u>M. capsulatus</u> (Bath)	76
3.8	SDS-polyacrylamide gel electrophoresis of soluble fractions of <u>M. capsulatus</u> (Bath)	77
3.9	<sup>35</sup> S-methionine pulse-labelling of cells during a switch from soluble to particulate MMO activity	82
3.10	Electron photomicrographs of thin-sections of <u>M. capsulatus</u> grown on methane in a low copper medium	85
3.11	Electron photomicrographs of thin-sections of <u>M. capsulatus</u> grown on methane in a high copper medium	86

	<u>Page no.</u>
4.1 SDS-polyacrylamide gel electrophoresis of cell extracts of <u>M. capsulatus</u> (Bath) grown on methanol	100
4.2 Thin-section electron micrographs of <u>M. capsulatus</u> (Bath) grown on methanol - no copper in the growth medium	110
4.3 Thin-section electron micrographs of <u>M. capsulatus</u> (Bath) grown on methanol - effect of copper on intracytoplasmic membrane content	112
4.4 Thin-section electron micrographs of <u>M. capsulatus</u> (Bath) grown on methanol - effect of high levels of copper in the growth medium on intracytoplasmic membranes	113
5.1 The effect of copper ions on <u>in vivo</u> particulate MMO activity from <u>M. capsulatus</u> (Bath)	136
5.2 The effect of the pH of the sodium/potassium phosphate buffer in the assay medium on the <u>in vitro</u> particulate MMO activity	141
6.1 Procedure for the partial purification of the particulate MMO from <u>M. capsulatus</u> (Bath)	159
6.2 The effect of copper ions on partially purified particulate MMO activity	162
6.3 The binding of radiolabelled acetylene to proteins in cell extracts of <u>M. capsulatus</u> (Bath)	168
6.4 Copper uptake by cell suspensions of <u>M. capsulatus</u> (Bath)	175

	<u>Page no.</u>
6.5 Copper uptake by cell suspensions of <u>M. capsulatus</u> (Bath) - the effect of temperature on the intracellular accumulation of copper ions	179
6.6 Inhibition of whole-cell soluble MMO by copper in the presence and absence of formate	184
6.7 Energy requirement for whole cell soluble MMO inhibition	186

List of Tables

	<u>Page no.</u>
1.1 Sources of Atmospheric Methane	3
1.2 C-1 compounds of enviromental significance	4
1.3 Classification of obligate methane-utilizing bacteria	10
2.1 Composition of trace element solution used in nitrate mineral salts (NMS) medium	35
3.1 Location of MMO in nutrient-limited chemostat cultures of <u>Methylosinus trichosporium</u> UB3b	53
3.2 Continuous culture of <u>M. capsulatus</u> (Bath)	61
3.3 Summary of continuous culture experiments	63
3.4 Efficiency of Biomass Production from Methane	66
4.1 The effect of the level of copper in the growth medium on MMO activity in cell extracts of <u>M. capsulatus</u> grown on methanol	99
4.2 Effect of copper ions on MMO activity in cell-free extracts of <u>M. capsulatus</u> (Bath) grown on methanol with various levels of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in the growth medium	104
4.3 Effect of adding $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to the growth medium on whole cell oxidation rates of <u>M. capsulatus</u> (Bath) grown on methanol	106
5.1 Effect of various potential inhibitors on <u>in vitro</u> MMO activity in <u>M. capsulatus</u> (Bath)	120
5.2 The effect of various electron donors on <u>in vitro</u> particulate MMO activity in <u>M. capsulatus</u> (Bath)	124

	<u>Page no.</u>
5.3 The effect of temperature on the stability of <u>in vitro</u> particulate MMO activity	127
5.4 Effect of temperature on particulate MMO activity from <u>M. capsulatus</u>	130
5.5 The effect of various metal ions on <u>in vivo</u> particulate MMO activity in <u>M. capsulatus</u> (Bath)	132
5.6 The effect of various metal ions on <u>in vitro</u> particulate MMO activity in <u>M. capsulatus</u> (Bath)	134
5.7 Effect of a variety of copper compounds on particulate MMO activity	138
5.8 Effect of pH and buffer on copper-stimulated <u>in vitro</u> particulate MMO activity	143
5.9 Functional classes of copper proteins	148
5.10 Substrate specificity of the particulate MMO from <u>M. capsulatus</u>	151
6.1 Particulate MMO activity in partially purified fractions of <u>M. capsulatus</u> (Bath)	161
6.2 The effect of copper ions on particulate MMO activity in the P100 fraction of cell extracts	161
6.3 Inhibition of methane monooxygenase activity by acetylene	166
6.4 Inhibition of soluble MMO by a variety of metal ions	181

## Chapter 1

### General Introduction

## 1. The Concept of Methylo-trophy

The generally accepted definition of methylo-trophic organisms was provided by Colby and Zatman (1972). They described methylo-trophs as organisms which possess the ability to grow non-autotrophically at the expense of carbon compounds containing one or more carbon atoms, but containing no carbon-carbon bonds. This group of organisms could be subdivided into obligate and facultative methylo-trophs, the latter class having the additional ability to grow and replicate on a variety of other multicarbon compounds.

Methylo-trophs, as defined above, are thus able to utilise methane, methanol, N-methyl compounds and S-methyl compounds as sole sources of carbon for growth and replication. The methylo-trophs include both prokaryotic and eukaryotic organisms but for the purposes of the introduction to this thesis only the methylo-trophic bacteria will be discussed. The extensive and varied physiology and biochemistry of all the various types of methylo-trophs have been comprehensively covered in an excellent book by Anthony (1982), and the reader is referred to this source for details of the many aspects of methylo-trophic organisms not discussed in the introduction to this thesis.

## 2. Occurrence and Isolation of Methane-utilising Bacteria

Methane is produced naturally in a number of diverse situations; coal and oil deposits, anaerobic sewage digestors, the rumen of ruminant animals and in lakes and ponds. As a source of C-1 compounds methane is

of the greatest quantitative importance since approximately 50% of the carbon in organic material degraded anaerobically is eventually converted to methane. However, only a small fraction of the methane generated finds its way into the atmosphere because most of the gas is oxidized to  $\text{CO}_2$  by aerobic methane-utilising (methanotrophic) bacteria present in soils and natural waters. It has also been shown that some methane is oxidized anaerobically (Keeburgh, 1981). The amount of hydrocarbon actually released into the atmosphere is given by the data in Table 1. As noted in Table 1 most biogenic methane does not reach the atmosphere but is oxidized by both aerobic and anaerobic microorganisms. It is not possible to give even an approximate figure for total biogenic methane production because of the influence of the methane-utilising organisms which prevent release of the gas into the atmosphere. Since the atmospheric methane concentration is fairly constant (approx. 1.4 ppm), and the fact that methanotrophic bacteria are thought not to play a significant role in methane oxidation once it has been released into the atmosphere (Ehhalt, 1976), the hydrocarbon must be continually removed, this occurs by interaction of the gas with ozone and its radicals which produces  $\text{H}_2$ , CO,  $\text{CO}_2$  and HCHO.

The other major sources of C-1 compounds are listed in Table 2. Microorganisms play a significant role in their oxidation and the reader is referred to the review of Schlegel *et al.* (1976) for a detailed discussion of their importance. The vast majority of methanotrophs that have been isolated are obligate, aerobic, Gram-negative bacteria capable of growth only on methane, and in some cases, methanol. They are to be found wherever methane passes into an aerobic environment, e.g. soils, sediments, natural waters, buccal cavities of ruminants. In addition to

Table 1                      Sources of Atmospheric Methane  
(from Ehhalt, 1976)

<u>Source</u>	<u>Global Production in 10<sup>6</sup> tonnes of methane/year</u>
1. Biogenic *	
Enteric fermentation in animals	100-220
Paddy fields	280
Swamps, marshes	130-260
Freshwater lakes	1.3-25
Upland fields	10
Forests	0.4
Tundra	0.8-8
Ocean            a) Open	4-6.7
b) Continental Shelf	<u>0.7-1.4</u>
Total Biogenic	<u>528-812</u>
2. Other Sources	
Coal mining	6.3-22
Lignite mining	1.6-5.7
Industrial Losses	7-21
Automobile exhaust	0.5
Volcanic emissions	0.2
	<hr/>
Total other sources	<u>15.6-49.4</u>
Total ALL sources	<u>544-862</u>

Other relevant figures for comparison:

Output of natural gas wells for consumption (1965)	$520 \times 10^6$ tonnes/yr.
Annual production of dry organic matter	$1.65 \times 10^{11}$ tonnes/yr.
Amount of atmospheric methane (about 1.4 ppm)	$4 \times 10^9$ tonnes

\* These figures do not include biogenic methane oxidized by methanotrophs.

Table 2

C-1 Compounds of Environmental Significance(from Higgins et al., 1985)

<u>Compound</u>	<u>Formula</u>	<u>Comments</u>
Methane	CH <sub>4</sub>	End product of anaerobic fermentation processes. Generated by free-living and rumen-inhabiting methanogens.
Methanol	CH <sub>3</sub> OH	Generated during the breakdown of methyl-esters and ethers (e.g. pectin) and released by methanotrophs.
Formaldehyde	HCHO	Common combustion product, intermediate microbial oxidation of other C-1 compounds and methylated biochemicals (e.g. lignin)
Formate ion	HCOO <sup>-</sup>	Present in plant and animal tissues. Common product of carbohydrate fermentation.
Formamide	HCONH <sub>2</sub>	Natural product formed from plant cyanides.
Carbon dioxide	CO <sub>2</sub>	Combustion, respiration and fermentation end product. A major reservoir of carbon on Earth.
Carbon monoxide	CO	Combustion product, common pollutant. Product of plant, animal and microbial respiration, highly toxic.
Cyanide ion	CN <sup>-</sup>	Generated by plants, fungi and bacteria. Industrial pollutant, highly toxic.

oxidizing C-1 compounds, some methanotrophs can oxidize hydrocarbon and substituted hydrocarbon compounds which are not growth substrates (see Section 5.4), and they may play an important role in the nitrogen cycle since they can oxidize ammonia to nitrite and nitrate (Whittenbury et al., 1970) and fix atmospheric nitrogen (de Bont and Mulder, 1974; Murrell and Dalton, 1983).

Prior to 1970 only three species of methane-utilising bacteria had been successfully isolated and characterised despite the fact that the first isolate had been found at the turn of the century (Söhngen, 1906). The three species were Pseudomonas methanica (Söhngen, 1906; Dworkin and Foster, 1956), Methanomonas methano-oxidans (Brown et al., 1964; Stocks and McClesky, 1964) and Methylococcus capsulatus (Foster and Davis, 1966). The apparent difficulty in isolating pure cultures of methane-utilising bacteria was almost certainly due to the lack of a reliable enrichment and isolation technique for these organisms. This situation was remedied by Whittenbury and his colleagues at Edinburgh University who devised simple, effective techniques for the enrichment of methane-utilising bacteria and who described the isolation of over 100 methane-utilising organisms using their new methods (Whittenbury et al., 1970). Since this important watershed in the study of methane-utilising bacteria a number of reports on obligate methane-utilising bacteria have been published. In 1976 Malashenko described the isolation and characterisation of several new species of thermophilic and thermotolerant methane-oxidisers, and Galchenko (1977) has isolated a number of Methylocystis species. The isolation of Methylococcus mobilis by Hazeu et al. (1980) provided a description of a novel obligate methanotroph that was occasionally motile possessing one or two

flagella. In addition to the description of new strains many reports of isolates similar to those of Whittenbury et al. (1970) have appeared in the literature (Hou et al., 1979b; Galchenko et al., 1977; Galchenko et al., 1975; Heyer et al., 1984).

The first report of the isolation of a facultative methane-utiliser was made in 1974 by Patt et al. who isolated an organism which could utilise not only methane or methanol as a sole source of carbon and energy, but a variety of organic compounds such as acetate, mono- and di-saccharides and amino acids. This organism, Methylobacterium organophilum XX, has since been extensively characterised (Patt et al., 1976; Lynch et al., 1980), and has been joined by other facultative methane utilising bacteria which are described in detail by Patt et al. (1976) and Lynch et al. (1980). The organism Methylobacterium ethanolicum described by Lynch et al. (1980) has since been shown to be a mixed culture (Lidstrom-O'Connor et al., 1983) and indicates the care that needs to be taken when dealing with the facultative methanotrophs. In this case the 'organism' was composed of a 'Methylocystis' sp and a 'Xanthobacter' sp which formed stable associations during growth on methane. This confirms other findings (Whittenbury and Dalton, 1981) that ordinary methods for determining culture purity are not sufficient in the case of methanotrophs and it is worth noting that, in certain cases, results suggesting the existence of plasmid-mediated characteristics, such as have been reported for several facultative methylotrophs, can be explained by stable associations like that of 'Methylobacterium ethanolicum'.

One of the most interesting new isolates that have been reported is the

organism Methylobacter sp Strain 761M (Zhao and Hanson, 1984a) which is a Type I methanotroph (see next Section) that requires methane as an energy source but can derive most of its cellular carbon from complex carbon sources. Variants of this methanotroph have been isolated (Zhao and Hanson, 1984b) that are capable of growth with methanol as the energy source (761AR) and also glucose as the energy source (761H). Both isolates maintained the ability to grow on methane and contained methane monooxygenase, the enzyme responsible for the initial conversion of methane to methanol. The ability to maintain methane monooxygenase whilst the organism is grown on methanol indicates that this organism would be well suited for genetic studies on the enzyme as it should be possible to isolate mutants capable of growth on methanol but not on methane.

### 3. Grouping and Classification of Methanotrophs

At the last Symposium on Microbial Growth on C1 Compounds (Minnesota, USA 1984) it was reported by Galchenko and Andreev that "the present taxonomic status of the methanotrophs is rapidly becoming unsatisfactory". The reason for this is that the classification scheme used by most researchers in this field, which was developed by Whittenbury and his co-workers (Colby et al., 1979; Whittenbury et al., 1970, 1976; Whittenbury and Dalton, 1981), has not yet been officially approved because it was not published according to the rules of the International Code of Nomenclature of Bacteria. It remains, however, the best scheme produced to date and is outlined below.

On the basis of thin-section electron microscopy of numerous methane-utilising bacteria, Whittenbury and his co-workers divided the isolates into two groups based on the internal membrane arrangement (Davies and Whittenbury, 1970). The two different types are designated Type I and Type II. Type I organisms were described as possessing bundles of stacked, disc-shaped membrane vesicles which were randomly distributed throughout the interior of the cell, whereas Type II organisms possessed layers of paired membranes situated around the periphery of the cell. These membrane arrangements were confirmed for a number of species by other workers using similar techniques (Proctor et al., 1969; Smith et al., 1970; Smith and Ribbons, 1970; Weaver and Duggan, 1975).

This major difference in internal membrane morphology plus a number of divergent properties between the two groups, i.e. nature of the resting stage, cell and colony morphology of the organisms, prompted the proposal of a provisional classification of the isolates into two groups each of which could be subdivided into subgroups (Whittenbury et al., 1970). This scheme designated only group and subgroup names, but many workers have subsequently adopted these names as genus/species titles although these names have no formal nomenclature standing in Bergey's Manual (8th Edition, 1974). Subsequent to the publication of Whittenbury et al. in 1970, Lawrence and Quayle (1970) found a correlation between the carbon assimilation pathways (see Section 4.7) and the intracytoplasmic membrane arrangements of a number of methane-utilising bacteria. The Type I membrane system correlated with the ribulose monophosphate pathway, whereas organisms possessing Type II membrane systems used the serine pathway. The classification scheme was finally completed when Davey et al. (1972) examined cell-free extracts

of various methanotrophs and found disparities between Type I and Type II organisms with respect to the activity of a number of key enzymes concerned with intermediary metabolism. The full scheme is shown in Table 3 (Whittenbury et al., 1976).

It should be noted that since the appearance of this classification scheme a number of reports have appeared that suggest that the two groups are not as clearly defined as was first thought. It has been shown that a number of Type I and Type II methylotrophs can possess both hexulose phosphate synthase and hydroxypyruvate reductase activity, which are indicative of the presence of the ribulose monophosphate cycle and the serine pathway respectively (Whittenbury et al., 1976; Malashenko, 1976; Shishkina et al., 1976). This situation is clearly demonstrated in the organism Methylococcus capsulatus (Bath). This organism possesses the key enzymes of the ribulose monophosphate pathway, marking it as a Type I organism, but can also incorporate glycollate (which is formed by the oxygenase function of the enzyme ribulose-1,5-bisphosphate carboxylase), via glyoxylate and the enzymes of the serine pathway (Taylor et al., 1981). The diversity of carbon incorporation pathways in this organism has led to the suggestion that it should be reclassified into a third group, Type X (Whittenbury and Dalton, 1981).

The other major problem with the classification scheme as it stands is that the membrane structures, on which the initial grouping was based, have been shown to be non-static and a particular organism may vary greatly in its intracellular morphology depending on the conditions under which it is grown and the growth phase at which samples are taken

Table 3                      Classification of Obligate Methane-utilising Bacteria

<u>Character</u>	<u>Type I</u>	<u>Type II</u>
Membrane arrangement	Bundles of vesicular discs	Paired membranes in layers around periphery
Resting stage	Cysts (Azotobacter-like)	Exospores or "lipid-cysts"
Carbon Assimilation	Kibulose monophosphate pathway	Serine pathway
TCA cycle	Incomplete (lacks 2-oxoglutarate dehydrogenase)	Complete
Glucose-6-phosphate and 6-phosphogluconate dehydrogenase	Present	Present
<hr/>		
Examples	<u>Methylococcus</u> <u>Methylomonas</u> <u>Methylobacter</u>	<u>Methylosinus</u> <u>Methylocystis</u> <u>Methylobacterium</u>

(Hyder et al., 1979; Ohtomo et al., 1977; Takeda and Tanaka, 1980; Scott et al., 1981; see also Chapters 3 and 4 of this thesis). It would thus seem that this division between the two groups is not as rigid as was first thought and should be regarded with caution.

The latest scheme published is that of Galchenko and Andreev (1984) which considers phospholipid composition, fatty acid composition, antigenic characteristics, DNA homology and protein electrophoretic patterns. The final classification is based on protein electrophoretic patterns and corresponds well with the scheme of Whittenbury, but the reader is referred to Chapters 3 and 4 of this thesis where evidence is provided of changes in protein banding patterns due to differences in the growth conditions of M. capsulatus (Bath), suggesting that this scheme may be flawed.

The present situation of classification of methanotrophs is obviously not satisfactory but the nature of the organisms makes classification extremely difficult and poses an interesting problem for taxonomists to tackle.

#### 4. Physiology and Biochemistry of obligate Methanotrophs

##### 4.1 Basic growth requirements

All methane-oxidising bacteria are strictly aerobic due to their requirement for gaseous oxygen for the initial oxidation of methane (Higgins and Quayle, 1970), and can utilise either methane or methanol as a sole source of carbon and energy. It has been reported that the

growth of methane-utilisers is inhibited by addition of normal heterotrophic metabolites at usual heterotrophic concentrations (Eccleston and Kelly, 1972; 1973). No growth factors are required for growth of the organisms and they are normally grown on a mineral salts medium containing a nitrogen source, divalent cations ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), sulphate, phosphate, iron and trace elements (Dalton and Whittenbury, 1976).

#### 4.2 The basis for obligate methylotrophy

The inability of the obligate methylotrophs to use multicarbon compounds as their sole source of carbon and energy has not yet been fully explained. Obviously the toxicity of multicarbon compounds as noted above may be important in limiting growth but other possible reasons that have been proposed by Anthony (1982) are:

- a) The presence of a lesion in a central metabolic pathway, the most likely being the absence of 2-oxoglutarate dehydrogenase.
- b) The lack of a system for coupling ATP synthesis to NADH oxidation.
- c) The lack of transcriptional control mechanisms.
- d) The lack of suitable transport systems for multicarbon compounds or their products of metabolism.

Of these possibilities the most probable reason is (a) which will result in an incomplete TCA cycle but as explained in Section 3.1 of this introduction, it is only Type I organisms that lack 2-oxoglutarate

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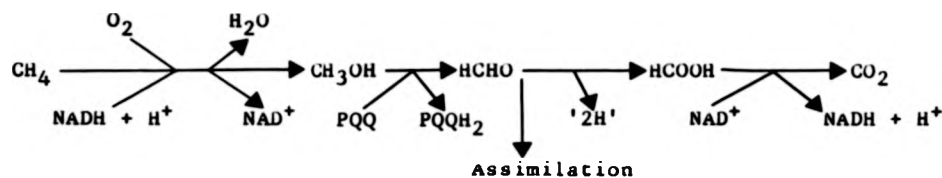
dehydrogenase so the reason for obligate methylotrophy in Type II organisms is not the same as in Type I organisms. It is, however, worth noting that the loss of 2-oxoglutarate dehydrogenase by mutation in Pseudomonas AM1 (a typical facultative methylotroph) is sufficient to confer upon the mutant the property of obligate methylotrophy (Taylor and Anthony, 1976). The Type II organisms are known to possess a complete TCA cycle but the results of experiments on the facultative methylotrophs have shown that growth of these organisms on multicarbon compounds leads to only low levels of activity of the TCA cycle enzymes when compared to the activity of the same enzymes when the organism is grown on methane. It thus remains possible that the failure to grow on multicarbon compounds may sometimes be because such compounds cannot induce the enzymes of the TCA cycle to sufficiently high levels for it to subserve both oxidative and biosynthetic functions (Anthony, 1982). It has also been suggested by Anthony (1982) that the low (or zero) levels of pyruvate dehydrogenase, present in Type II methylotrophs, may play a role in obligate methylotrophy as this enzyme is essential for growth on most compounds with three or more carbon atoms and it is not known whether they have the enzymes for conversion of  $C_4$  compounds to  $C_3$  compounds essential for growth on  $C_4$  compounds such as succinate.

The possibility that obligate methylotrophy is due to the lack of a system for coupling ATP synthesis to NADH oxidation cannot be discounted as respiration-coupled ATP synthesis has not yet been demonstrated in extracts of obligate methylotrophs, but this is probably due to technical reasons rather than a lack of such coupling during growth (Anthony, 1982).

### 4.3 Methane oxidation

#### 4.3.1 Introduction

The pathway for the complete oxidation of methane was first proposed by Dworkin and Foster (1966), it has since been refined by several workers and is now shown below:



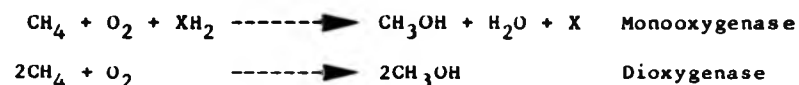
PQQ = Pyrrolo-Quinoline Quinone

The first reaction is hydroxylation catalysed by the enzyme methane monooxygenase (MMO) which in vitro requires NADH as reductant. The second reaction is the conversion of methanol to formaldehyde and is catalysed by a methanol dehydrogenase which contains the novel prosthetic group PQQ. Formaldehyde oxidation is sometimes coupled to reduction of  $\text{NAD}^+$ , but it may be oxidised by the methanol dehydrogenase, or by other dye-linked dehydrogenases. The conversion of formaldehyde to formate and then to carbon dioxide may be catalysed by formaldehyde dehydrogenase and an  $\text{NAD}^+$ -linked formate dehydrogenase or alternatively some bacteria, lacking formate dehydrogenase, can carry out the conversion by a cyclic variant of their carbon assimilation pathway. These reactions are discussed in detail in the following sections.

#### 4.3.2 The oxidation of methane to methanol

The early studies on the oxidation of methane were concerned with the origin of the oxygen atom found in methanol. The work of Leadbetter and

Foster (1959) and Johnson (1967) had led to the suggestion that molecular oxygen was involved in the reaction, but conclusive evidence for this was not obtained until 1970 when Higgins and Quayle (1970) used  $^{18}\text{O}_2$  to demonstrate the incorporation of one atom of dioxygen into methanol. They used a high phosphate concentration (80 mM) to accumulate methanol in whole-cell suspensions of Pseudomonas methanica and Methanomonas methanooxidans which were oxidising methane in the presence of  $^{18}\text{O}_2$ . The results suggested that one of two oxygenase mechanisms catalysed the oxidation of methane to methanol:



The major problem associated with trying to elucidate the mechanism of initial enzymic attack on methane by methylotrophs has been the difficulty in obtaining stable cell-free extracts which exhibit methane-oxidising activity. The first report of such activity was by Ribbons and Michalover (1970) who demonstrated methane-stimulated respiration and methane-stimulated NADH oxidation by cell-free particulate preparations of Methylococcus capsulatus (Texas). Subsequently, similar results were obtained by Ferenci using cell-free particulate preparations of Pseudomonas (Methylomonas) methanica (Ferenci, 1974; Ferenci et al., 1975). Both systems gave tentative stoichiometric relationships which suggested a monooxygenase mechanism for methane oxidation, but as neither substrate disappearance nor product accumulation was demonstrated, the work could not be considered conclusive. This evidence was partially supplied by Ribbons (1975) who

demonstrated simultaneous NADH oxidation, methane and oxygen uptake by particulate extracts of Methylococcus capsulatus (Texas). No initial product of methane oxidation was detected, presumably due to methanol-oxidising activity in the crude, particulate fraction. The first report of methanol accumulation from methane in a cell-free system appeared in 1975 when it was shown that particulate extracts of Methylosinus trichosporium OB3b accumulated methanol from methane in the presence of 150 mM phosphate (Tonge et al., 1975). The stoichiometry of the oxidation, methane utilization:methanol formation:NADH utilization:O<sub>2</sub> consumption, was 1.0:0.9:1.6:1.3 and, as such, would be inconsistent with a monooxygenase-type reaction. However, the authors did suggest that such a stoichiometry would be consistent if methane caused a redirection of existing electron flow to oxygen into the methane oxygenase system (Higgins et al., 1976).

In addition to the above report 1975 also saw the demonstration that crude, particulate fractions of Methylomonas methanica could catalyse the oxygen- and NAD(P)H-dependent disappearance of bromomethane (Colby et al., 1975). The stoichiometric relationship between the specific activities of bromomethane-dependent NADH oxidation and bromomethane disappearance suggested a monooxygenase catalysed reaction. These workers also proposed that in Methylomonas methanica the enzyme that catalysed bromomethane disappearance in vitro was in fact the enzyme that catalysed methane oxidation in vivo, based on the following evidence: similar inhibitor patterns for bromomethane oxidation by extracts of M. methanica and for methane oxidation by Methylosinus trichosporium OB3b (Hubley et al., 1975) plus the inhibition of bromomethane oxidation caused by substrate analogues and acetylene.

In the following year Colby and Dalton (1976) reported that a soluble methane-oxidising system could be reproducibly extracted from Methylococcus capsulatus (Bath) and Higgins et al. (1976) reported the partial purification of a particulate methane-oxidising system from Methylosinus trichosporium OB3b. These two systems remain the best characterised methane-oxidising enzymes and are considered in detail in the following sections.

#### 4.3.3 The particulate methane monooxygenase from M. trichosporium OB3b

Following the initial report of methane-oxidising activity in a particulate preparation from M. trichosporium by Tonge et al. (1975) the Kent group reported a complete purification scheme in which the solubilized protein was resolved into three components by DEAE-cellulose, sephadex gel and hydroxyapatite chromatography (Tonge et al., 1977). The purified components comprised a soluble CO-binding cytochrome of molecular weight 13,000, a copper-containing protein of molecular weight 45,000 and a colourless protein of molecular weight 9,400. The purified system used only ascorbate, or methanol in the presence of partially purified methanol dehydrogenase from the same organism, as the electron donor by directly reducing the CO-binding cytochrome c. NAD(P)H would only serve as the electron donor for the crude or partially purified enzyme. It was indicated by the authors that electrons were recycled in vivo from the further oxidation of methanol in a non-NADH-linked reaction. The inability of NAD(P)H to act as an electron donor in the purified monooxygenase system was said to be due to the lack of electron transport chain proteins (Tonge et al., 1977). It has since been reported that this procedure no longer gives active enzyme (Higgins et al., 1971a), and this coupled with the

findings of Stirling and Dalton (1979), which are discussed below, have cast serious doubts about the reproducibility of these results. It is worth noting at this point that cell-free particulate methane-oxidising activity from M. trichosporium has also been observed by Hou et al. (1979c), but the report was contained in a patent application and no confirmation of such activity has ever been published by the authors in the scientific literature although it was noted by Higgins et al. (1981b).

The methane monooxygenase from M. trichosporium UB3b isolated and purified by Tonge et al. (1977) was found in the particulate fractions of cell extracts. However, work performed by Stirling et al. (1979) failed to substantiate this report and, in addition, found that the only effective electron donor for methane-oxidising activity in crude extracts of M. trichosporium was NAD(P)H. This observation coupled with similar substrate and product specificity of the enzymes from M. trichosporium and Methylococcus capsulatus (Bath), suggested that the organisms had similar methane-oxidizing enzymes. Further studies by Stirling and Dalton (1979) revealed that the crude enzyme systems from M. trichosporium UB3b and M. capsulatus (Bath) were similar in the following respects: both were soluble (i.e. were not sedimented after centrifugation at 150,000 g for 90 minutes); only NADH or NADPH would serve as effective electron donors for in vitro MMO activity; high phosphate concentrations were not necessary for methanol accumulation from methane; and methanol was a very poor electron donor for M. trichosporium UB3b and was completely ineffective for M. capsulatus. Both extracts were unstable at 4°C but were completely stable on freezing at -70°C, in addition 1 mM cyanide had no inhibitory effect on

methane oxidation and the pattern of inhibitors on MMO activity by both enzymes was very similar. They also showed that purified components of the MMO from M. capsulatus would restore activity to DEAE-cellulose fractionated components from M. trichosporium supporting the theory that the two enzyme systems were similar. These characteristics were very different from those described by Tonge et al. (1975, 1977) for the particulate enzyme from M. trichosporium and the situation remained unclear for several years. In 1981 Scott et al. reported that the intracellular location of the MMO in M. trichosporium OB3b was dependent on the growth conditions under which the organism was grown. Particulate MMO, as reported by Tonge et al. (1975, 1977) was present in organisms grown in continuous culture under oxygen-limited, nitrate-excess conditions, whilst soluble MMO, as reported by Stirling et al. (1979), was present in nitrate-depleted cultures. It was suggested by these workers (Scott et al., 1981a, b) that the availability of nitrate might determine the intracellular location of MMO in M. trichosporium OB3b. It has since been demonstrated (this thesis, and Stanley et al., 1983) that this was an incorrect assertion and that the intracellular location of the MMO in M. trichosporium OB3b and M. capsulatus (Bath) is dependent on the availability of copper. The conditions used by Scott et al. (1981a, b, c) masked this copper effect due to the changes in biomass which occurred under the various limiting-nutrient conditions employed in their experiments (see also Chapter 3.3 of this thesis).

Since the publication of the results presented in this thesis (Society for General Microbiology 97th Annual Meeting, Warwick 1983; Stanley et al., 1983; Dalton et al., 1984) the Cranfield research group led by Higgins have reinvestigated the intracellular location of MMO activity

in M. trichosporium and have confirmed the results showing that copper is the determining factor in this organism (Burrows et al., 1984). It is interesting to note that the presence of soluble MMO activity in M. trichosporium is accompanied by the detection of three major polypeptides on SDS-polyacrylamide gels. These polypeptides have Mr values of 53,000, 45,000 and 23,000 and are similar to the polypeptides of Protein A of the soluble MMO enzyme of M. capsulatus (Bath) (Woodland and Dalton, 1984a) suggesting that the observations of Stirling et al. (1979), which were discussed earlier, may be correct and that the two organisms have similar soluble MMO enzymes.

#### 4.3.4 The soluble methane monooxygenase from M. capsulatus (Bath)

This enzyme system remains the best characterized MMO from any of the methanotrophic organisms studied so far and has been purified to near homogeneity. The early work on this system has been extensively reviewed by Dalton (1980) and the reader is referred to this source for details of the purification process from the first report (Colby and Dalton, 1978) to 1980. The advances since 1980 are discussed below.

The soluble MMO was found to be a complex of three proteins which could be resolved by DEAE-cellulose chromatography and purified by chromatographic techniques (Colby and Dalton, 1978; Dalton, 1980; Woodland and Dalton, 1984a; Woodland and Dalton, 1984b). The three components are; protein A, which is a 220,000 molecular weight protein, comprised of three subunits of Mr 54,000, 42,000 and 17,000 which contains non-heme iron (2-3 g atoms per mole) and zinc (0.2-0.5 g atoms per mole) but no acid labile sulphide; protein B, a 20,000 molecular weight protein which is acidic but which contains no prosthetic group

nor metals and protein C, a single polypeptide protein of molecular weight 40,000 containing 1 mol each of FAD and a 2Fe-2S centre per mol of protein (Woodland and Dalton, 1984a; Lund and Dalton, 1985; Lund et al., 1985).

Of the three soluble MMO components only Protein C has any individual catalytic activity. It is an NAD(P)H:acceptor reductase which catalyses the NAD(P)H-driven reduction of cytochrome c, potassium ferricyanide, dichlorophenol-indophenol or, rather poorly, oxygen (Colby and Dalton, 1979). Using purified proteins the NADH-reduced protein C will also transfer electrons to oxidized protein A under anaerobic conditions in the absence of protein B, but there is no substrate oxidation in the absence of protein B. The electron transfer reactions in the soluble methane monooxygenase of M. capsulatus (Bath) have recently been reviewed by Lund et al. (1985). The only component that has not yet been assigned a function is protein B, although it is absolutely necessary for methane oxidation, and this component is currently under investigation in our laboratory and appears to be the regulator of electron flow in the enzyme system (Green and Dalton, 1985).

The soluble MMO from M. capsulatus (Bath) is capable of oxidising a wide variety of compounds including alkanes, alkenes, alicyclic, aromatic and heterocyclic hydrocarbons as well as ethers (Dalton, 1980). The enzyme was not inhibited by metal-binding agents but was very sensitive to the presence of acetylene (Stirling and Dalton, 1979). The only suitable electron donor was NAD(P)H, neither ascorbate, nor electrons derived from the oxidation of methanol could substitute for NAD(P)H (Stirling and Dalton, 1979). The enzyme is thus very different from the

particulate MMO from M. trichosporium OB3b described by Tonge et al. (1975, 1977), but resembled the soluble MMO from that organism (Stirling and Dalton, 1979). It was at this point that the studies outlined in this thesis began in an effort to determine whether M. capsulatus (Bath) could express a particulate MMO and whether such an enzyme, if it could be found, exhibited any similarity to the particulate enzyme from M. trichosporium.

#### 4.3.5 Methane monooxygenases from other organisms

There are only two other reports of the purification of MMO enzymes, one is from the organism SB-1 (Allen et al., 1984) and the other from the facultative methanotroph, Methylobacterium sp. CRL 26 (Patel et al., 1982; Patel, 1984). The enzyme from the obligate methane-oxidizing bacterium SB-1 (a Type II methanotroph) was resolved into two components by DEAE-chromatography. Protein A, which does not absorb to DEAE cellulose, was electrophoretically homogeneous in nondenaturing and SDS gel systems and had a molecular weight of 60,000. It was reported that the protein was a tetramer containing 2 g atoms of iron per mol of tetramer (Allen et al., 1984). Analysis by electron paramagnetic resonance spectroscopy and visible spectroscopy have shown that this component was an iron-sulphur protein. The bound fraction, which was labelled protein C, had NADH:acceptor reductase activity and was capable of reducing cytochrome C and dichlorophenol-indophenol. Spectral analysis indicated the presence of flavin-moieties and the molecular weight of the protein was found to be 48,000. This is analogous to the protein C of the soluble MMO from M. capsulatus (Bath) (see Section 4.3.4 of this general introduction), but unlike the soluble enzyme from M. capsulatus there was no requirement for a third component (Protein B)

for full MMO activity. It was suggested that as the protein C fraction from organism SB-1 is not homogeneous the involvement of a third protein in this system cannot be ruled out (Allen et al., 1984).

The soluble MMO purified from Methylobacterium sp. CRL 26 was originally reported to have been resolved into three components by ion-exchange chromatography and that all three components were required for fully functional MMO activity (Patel et al., 1982). The properties of this enzyme were very similar to those reported for the soluble MMO from M. capsulatus (Bath) and only NAD(P)H was effective as an electron donor. However, a recent report by the same author (Patel, 1984), indicates that component B was not required for MMO activity in the soluble enzyme from Methylobacterium sp. CRL 26, this was in direct contrast to results presented in 1982 when all three components were required. No explanation for this apparent change in the composition of the enzyme was given, by the author in the latter paper and suggests that both reports should be treated with caution. The three components specified in the first paper (Patel et al., 1982) were not purified but the two components in the second paper (Patel, 1984) were purified and are described below. Component A was designated the hydroxylase component, (but no results were presented to substantiate this claim), it had a molecular weight of 220,000 and was composed of three subunits of Mr 60,000, 40,000 and 20,000. The purified protein contained  $2.8 \pm 0.2$  mol of iron per mol of protein and contained no acid labile sulphide. In these respects it was very similar to protein A of the soluble MMO from M. capsulatus. The flavoprotein component, component C, had a molecular weight of 40,000 and was composed of a single polypeptide. The protein contained 1 mol of FAD per mol of protein as well as 2 mol of iron and

2 mol of acid labile sulphide per mol protein. In this respect it was very similar to the protein C of the soluble MMO from M. capsulatus (see Section 4.3.4 - introduction to this thesis). In view of the similarities in the properties of these two components to the components of the soluble MMO from M. capsulatus the assertion that there is no requirement for a third protein (which presumably would be equivalent to protein B of M. capsulatus) seems even more suprising. Further reports on this system will be awaited with interest to discover whether a third component is necessary and whether this component is similar to protein B of the soluble MMO from M. capsulatus (Bath).

#### 4.3.6 Final comments on methane oxidation by methylotrophs

The purification and characterization of the soluble MMO from M. capsulatus (Bath) has been extremely useful when studying soluble MMO activity in other organisms. Preliminary results from the organisms M. trichosporium OB3b, Methylobacterium sp. CRL 26 and organism SB-1 as discussed here suggest that there may be a similarity between the soluble MMOs from a variety of sources but that direct comparisons of properties, must await further purification. The situation with particulate MMO activity is much more difficult to summarise. To date the only particulate MMO to be purified is the enzyme from M. trichosporium OB3b and this work has not been reproduced by any workers since the initial reports. The problem with particulate MMO's remains their instability and the difficulty experienced on trying to solubilize the enzyme to facilitate purification. Until such time as purification can be readily achieved the properties of such systems remain unknown and a direct comparison between the soluble and particulate MMO's remains unresolved.

#### 4.4 Methanol Oxidation

Methanol oxidation to formaldehyde by methylotrophs is catalysed by an  $\text{NAD}^+$ -independent dehydrogenase which was first described in Pseudomonas M27 by Anthony and Zatman (1964a, b). Enzymic activity in cell extracts required the addition of phenazine methosulphate as an electron acceptor, plus ammonia or methylamine as an activator, the in vitro activity had an optimum pH of around 9. Since the original description of the enzyme from Pseudomonas M27 the same enzyme has been isolated from approximately thirty other methylotrophic bacteria (Anthony, 1982). In general, the substrate specificity of the enzyme is restricted to primary alcohols (hence the alternative name of primary alcohol dehydrogenase), with the affinity of the enzyme decreasing with increasing carbon chain length. It has been reported that certain secondary alcohols are substrates for the enzymes isolated from Methylobacterium organophilum (Wolf and Hanson, 1978), Pseudomonas C (Goldberg, 1976), and Diplococcus PAK (Bellion and Wu, 1978). The majority of enzymes are dimers of two identical subunits of molecular weight 60,000, notable exceptions are Methylomonas methanica and Methylosinus sporium which contain monomeric enzymes of molecular weight 60,000 (Patel et al., 1978b; Patel and Felix, 1976). The in vivo electron acceptor from methanol dehydrogenase is thought to be cytochrome C, this conclusion is based on the reduction of cytochrome C by methanol, catalyzed by anaerobically prepared methanol dehydrogenase (Duine et al., 1979; O'Keefe and Anthony, 1980).

Anthony and Zatman (1967) described the in vitro fluorescence characteristics of methanol dehydrogenase and showed that it possessed a novel prosthetic group which has since been characterized and determined

to be a novel ortho-quinone compound named pyrrolo-quinoline quinone (PQQ) (Salisbury *et al.*, 1979; Duine *et al.*, 1980). The catalytic role of PQQ in methanol oxidation by methanol dehydrogenase has been discussed by Forrest *et al.* (1980), DeBeer *et al.* (1983) and Duine *et al.* (1984). PQQ has also been found in other quinoproteins including glucose-, amine-, alcohol- and aldehyde-dehydrogenases (Anthony, 1982).

Recently a novel methanol dehydrogenase which is NAD-dependent has been isolated by Duine *et al.* (1984). This enzyme still contains the prosthetic group PQQ but is tightly bound to NADH dehydrogenase. The enzyme was initially isolated from a facultative methanol-utilizing *Nocardia* sp. (strain 239 - Duine *et al.*, 1984) but has also been shown in the obligate methane-utilizing bacterium *Methylococcus capsulatus* (Bath) (Duine *et al.*, 1984). The implications for this discovery of an NAD<sup>+</sup>-linked dehydrogenase in *M. capsulatus* (Bath) are important as this enzyme could possibly provide reducing power for the MMO systems in the form of NADH. This possibility is currently under investigation and the results may provide the answer to the question of where the reducing equivalents for MMO systems are derived from.

#### 4.5 Formaldehyde oxidation

The complete oxidation of formaldehyde to carbon dioxide in methylotrophic bacteria results from successive dehydrogenase action or from a cyclic series of reactions involving C-1-assimilation enzymes. The formaldehyde dehydrogenases can either be NAD(P)<sup>+</sup>-dependent or NAD(P)<sup>+</sup>-independent, and were discussed in detail by Stirling and Dalton (1978), Zatman (1981) and Anthony (1982). Some bacteria which utilise

the ribulose monophosphate pathway for formaldehyde assimilation (see Anthony, 1982) have little or no formaldehyde or formate dehydrogenase activity and rely entirely on this cyclic route for formaldehyde dissimilation (Anthony, 1982). One obligate methylotroph, Methylococcus capsulatus (Bath) has the enzymic capability to oxidize formaldehyde to carbon dioxide by either successive dehydrogenation steps or via the cyclic route involving the ribulose monophosphate pathway. A detailed discussion of the routes for formaldehyde dissimilation can be found in the book by Anthony (1982).

#### 4.6 Formate oxidation

Oxidation of formate by methylotrophic bacteria can occur by one of two routes both of which involve formate dehydrogenases. The first type is a soluble,  $\text{NAD}^+$ -dependent enzyme which is specific for formate, the second involves a membrane-bound  $\text{NAD}^+$ -independent enzyme which donates electrons to the cytochrome chain at the level of cytochrome b (Dijkhuizen et al., 1978, 1979; Rodinov and Zakharova, 1980). The distribution of these enzymes in a variety of bacteria and their specific activities are detailed by Zatman (1981).

#### 4.7 Carbon Assimilation by Methylotrophs

Methylotrophic bacteria initiate carbon assimilation at the level of formaldehyde utilising one of two pathways either the ribulose monophosphate pathway (RuMP) and/or the serine pathway. The methylotrophic bacteria (other than autotrophs which assimilate carbon in the form of carbon dioxide via the ribulose bisphosphate pathway) can

be split into two groups based on carbon assimilation pathways, i.e. Type I and Type II (Colby et al., 1979). Type I organisms use the RuMP pathway and Type II organisms use the serine pathway (see also Section 3.1 - introduction to this thesis).

The detailed biochemistry of the carbon assimilation pathways of the methylotrophic bacteria is beyond the scope of this introduction and the reader is referred to any of the following reviews for coverage of these pathways: Colby et al., 1979; Quayle, 1980; Anthony, 1982.

## 5. The Industrial Applications of Methylotrophs

In his recent book on the biochemistry of methylotrophs, Anthony (1982) suggested three main areas of commercial exploitation of methylotrophs, namely: production of single cell protein (SCP), overproduction of metabolites and the use of methylotrophs and their enzymes as biocatalysts. A fourth category may be added, namely that of the production of intracellular and extracellular polymers. Some aspects of the biotechnological exploitation of methylotrophs will be discussed very briefly in this section and the reader is referred to the following articles for a more detailed account: Anthony (1982); Best and Higgins, 1983; Whittenbury and Dalton, 1983; Kato et al., 1983).

### 5.1 Production of Single Cell Protein (SCP)

Methylotrophs have been considered excellent candidates for SCP production for some time and the majority of studies have indicated that

methylotrophic bacteria are better candidates for SCP production than methylotrophic yeasts by virtue of their higher growth rates, yields and protein content. Obligate methanotrophs were initially considered by companies like Shell mainly because of the fact that the carbon and energy source, methane, was readily available and was fairly cheap. However, methane utilization has a high oxygen requirement and the growth yields are lower than those of methanol-utilizing bacteria. In addition to these factors, methane has a low solubility and in conjunction with oxygen forms an explosive mixture. The final difficulty with methane-utilising bacteria is that trace levels of higher alkanes such as ethane and propane in the gas mixture can be toxic (Drozd and McCarthy, 1981).

It is therefore to the methanol-utilizing bacteria that industrial concerns have turned. Methanol is inexpensive, available in large amounts, very pure, completely miscible with water, easy to store, transport and handle and any residual methanol after growth can readily be removed from the SCP product (Anthony, 1982). The only commercial continuous process for SCP production using a methylotroph is the ICI "Pruteen" plant at Billingham, England. The organism used is the obligate Type I methanol-utilizing bacterium Methylophilus methylotrophus AS1 and details of the process can be found in articles by Senior and Windass (1980) and Smith (1981).

## 5.2 Overproduction of Metabolites

Several reports have appeared in the literature concerning the accumulation of extracellular metabolites by cultures of methylotrophs.

Most work has been concentrated on overproduction of amino acids and vitamins by methylotrophic bacteria. The first report of production of an amino acid by a methylotroph came in 1972 when Kuono et al. (1972) demonstrated the production of L-glutamate by Methanomonas methylovora. This was followed by several other reports of L-glutamate production by this organism (Oki et al., 1973; Nakayama et al., 1975). Subsequently, several amino acids have been produced by mutants of methylotrophs these include L-valine (Izumi et al., 1977); L-phenylalanine, L-tyrosine and L-tryptophan (Suzuki et al., 1977a, b); and L-serine (Keune et al., 1976). Vitamin B12 has been produced during growth on methanol by a number of pink facultative methylotrophic bacteria but only small quantities have been excreted (Tanaka et al., 1974; Nishio et al., 1978). The use of methanol grown organisms to produce useful metabolites has recently been reviewed by Kato et al. (1983).

### 5.3 Production of intracellular/extracellular polymers

The most common polymer which is synthesized by methylotrophs is poly- $\beta$ -hydroxybutyrate (PHB) it is hoped that this compound can be used as a precursor for chemical polymer synthesis. Extracellular polysaccharide production by methylotrophs has been reported for both methane-utilizing bacteria (Hou et al., 1979) and methanol-utilizing bacteria (Tam and Finn, 1977; Misaka et al., 1979). This area of the biotechnological exploitation is still in its infancy but may become important as the search for new polymers continues.

#### 5.4 The use of methylotrophs and their enzymes as biocatalysts

There is little doubt that it is the use of methylotrophs and/or their enzymes to perform biocatalytic conversions of hydrocarbons that has attracted the greatest interest from industry in the past few years. This is witnessed by the large number of patents which have been taken out by researchers working in this field of study (see Anthony, 1982). The basis of this potential exploitation of methylotrophs is the broad specificity of the methane monooxygenase which allows this group of organisms to effect the partial oxidation of hydrocarbons and derivatives to commercially interesting products (Colby *et al.*, 1977). The use of methane-utilising bacteria as biocatalysts has been extensively discussed by Best and Higgins (1983) and only a few salient points will be considered in this discussion.

One of the major problems with the use of *in vitro* enzyme systems is the inherent instability of the MMO enzyme and preparations with appropriate stability for commercial exploitation have yet to be achieved. In addition, supply of reductant (NADH) for the soluble form of the enzyme may affect the economic feasibility of commercial processes. The use of the particulate form of the enzyme may be more attractive from the point of view that it may be possible to produce reducing equivalents without the expensive addition of NADH (see Section 4.3.2 of the introduction to this thesis and also Chapter 5), but the range of substrates oxidised by this enzyme is much more limited than its soluble counterpart and the homogenous state of the membrane fraction can lead to further oxidation of the products. It is therefore unlikely that the *in vitro* catalysis of compounds by enzymes from methylotrophs will become commercially viable in the near future, but some of the difficulties may be overcome

by using whole organisms, either immobilised or in suspension. However, in some cases, the non-specific oxidative enzymes may effect further metabolism of primary oxidation products (as is the case with the membrane fraction of cell extracts), and it may then be necessary to limit this product drain, for example by employing enzyme inhibitors.

One example of a reaction that is effected by whole cell suspensions is the epoxidation of propylene to propylene oxide (this conversion is used as a convenient assay system for MMO activity - Colby *et al.*, 1977). This conversion is very difficult to effect using chemical reactions as the system requires several steps and is energetically expensive. It has been shown that whole cells of obligate methylotrophs will catalyze the conversion quantitatively and excrete the product (Stirling and Dalton, 1979; Dalton, 1980; Hou *et al.*, 1979b, 1980; Dalton and Stirling, 1982). It should be noted at this point that this process, as with any bioconversion process, may require selection of a suitable strain; Best and Higgins (1983) suggested that the following factors are of importance when selecting the organism:

- i) The kinetics of the oxidative process.
- ii) Product inhibition and product recovery.
- iii) Mechanism of reducing power supply for the oxygenase.
- iv) The stability of the biocatalytic system.

In addition to these factors the results presented in this thesis will show that a fifth factor should be considered, namely, the growth conditions under which the organism is grown, as changes in the environment of the organism can markedly alter the biochemical

properties of the cell, which may influence production of the desired product. The use of the methylotrophs as biocatalysts for industrially important oxidation reactions should become commercially feasible within this decade. The research so far reported has provided important results which have indicated that such reactions might become economically viable, but there are still substantial problems to be overcome. Further investigations on the detailed biochemistry of these organisms may provide the necessary information for commercial exploitation but perhaps more importantly, will allow for a greater understanding of the complexities of the growth of this fascinating group of organisms.

## Chapter 2

### Materials & Methods

## 2.1 Organism

The organism used throughout this study was Methylococcus capsulatus strain Bath which was originally isolated by Whittenbury et al. (1970), and which was maintained as a stock culture in our laboratory.

## 2.2 Media

A basic nitrate mineral salts medium (NMS) which has been described previously (Whittenbury et al., 1970; Whittenbury and Dalton, 1981) was used throughout these studies for the routine growth of the organism. The composition of this medium is given in Table 2.1. In order to prevent precipitation of the phosphates in this medium these were sterilized separately and added aseptically to the sterile medium when the temperature of the medium was less than 60°C.

For solid medium, 15 g litre<sup>-1</sup> Difco bacto-agar was added to the basic nitrate mineral salts medium (minus phosphates) prior to sterilization. As with liquid culture the phosphate solution was added aseptically to the sterile medium when the temperature of the agar was less than 60°C.

## 2.3 Maintenance and Growth

Cultures of the organism were maintained on NMS agar plates as described

Table 2.1

Composition of Trace Element Solution used in Nitrate  
Mineral Salts (NMS) Medium

<u>Compound</u>	<u>mg l<sup>-1</sup></u>
CuSO <sub>4</sub> .5H <sub>2</sub> O	200
FeSO <sub>4</sub> .7H <sub>2</sub> O	500
ZnSO <sub>4</sub> .7H <sub>2</sub> O	400
H <sub>3</sub> BO <sub>4</sub>	15
CoCl <sub>3</sub> .6H <sub>2</sub> O	50
EDTA	250
MnCl <sub>2</sub> .4H <sub>2</sub> O	20
NiCl <sub>2</sub> .6H <sub>2</sub> O	10
NaMoO <sub>4</sub> .2H <sub>2</sub> O	500

N.B. This is the trace element solution for low copper medium. In order to produce high copper medium an additional 1 g l<sup>-1</sup> (1000 mg l<sup>-1</sup>) should be added to the above list.

Nitrate Mineral Salts Medium

KNO <sub>3</sub>	1 g l <sup>-1</sup>
MgSO <sub>4</sub>	1 g l <sup>-1</sup>
CaCl <sub>2</sub>	0.26 g l <sup>-1</sup>
Fe/EDTA	4 mg l <sup>-1</sup>
Trace element soln.	1 ml l <sup>-1</sup>

Separate addition phosphate soln. (10%) 10 ml l<sup>-1</sup>.

10% Phosphate Solution

Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	644.4 g
KH <sub>2</sub> PO <sub>4</sub>	254.0 g
in 9 l of H <sub>2</sub> O	Final pH = 6.8

previously (Whittenbury and Dalton, 1981). Routine growth on liquid medium was achieved by using narrow-necked conical flasks containing sterile NMS medium which were inoculated with a small amount of M. capsulatus, sealed with a suba-seal cap (William Freeman & Co. Ltd., Barnsley, U.K.), and then injected with a methane/air gas mixture (40% (v/v) methane). The flasks were then incubated on a rotary shaker at 45°C until the liquid becomes turbid. These liquid cultures (25 ml) were added aseptically to fermenter systems for large scale cultivation of the organism.

Studies on the batch culture of the organism were performed in a 15 litre fermenter (L.H. Fermentation Ltd., Stoke Poges, Bucks, U.K.) on NMS medium which was supplemented with an additional 1.0 g litre<sup>-1</sup> KNO<sub>3</sub> to prevent the cells becoming nitrate-limited during the latter stages of growth. The sole source of carbon and energy was methane which was added as a methane/air gas mixture (20% (v/v) methane in air).

Studies on continuous culture of the organism were performed in a variety of systems (L.H. Fermentation Ltd., Stoke Poges, Bucks., U.K.; New Brunswick Scientific, Edison, N.J., U.S.A.), with working volumes from 1.3 litre to 3 litre, using a dilution rate of 0.1 hour<sup>-1</sup> in all cases.

Growth of M. capsulatus in continuous culture with methanol as sole carbon and energy source was established by initially growing the culture on a methane/air gas mixture (20% (v/v) methane in air) prior to

addition of 0.5% (v/v) methanol to the medium. The methane flow rate was then gradually reduced to zero over a period of 4 days.

Subsequently, the methanol concentration in the growth medium was raised to 1.0% (v/v). This method was adapted from Hou *et al.* (1979a) and was found to be a much more rapid method than those previously published for the "training" of the organism to growth on methanol as sole carbon and energy source.

#### 2.4 Whole cell respiration studies

For whole cell studies, cells were harvested by centrifugation (10,000 g for 10 min) at 4°C, washed twice in ice-cold 20 mM sodium/potassium phosphate buffer, pH 7.0 and resuspended in the same buffer to an OD<sub>540</sub> of 20 (approximately 4 mg dry wt ml<sup>-1</sup>). Whole cell respiration studies were performed in a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) with a reaction vessel of 3 ml capacity. All assays were performed at 45°C and contained 20 mM sodium/potassium phosphate buffer, pH 7.0, and sufficient whole cell suspension to give a final OD<sub>540</sub> of 2.0 (0.5 mg dry wt cells ml<sup>-1</sup>). Gaseous compounds were added as saturated buffers, the compound concentration being calculated using Henry's Law and the relevant Henry's constant (International Critical Tables, Vol. 3, pp.255-261).

## 2.5 Preparation of cell extracts

cell-free extracts for the soluble and particulate forms of the enzyme were prepared by slightly different methods. Cells were harvested by centrifugation at 10,000 g for 10 min at 4°C, washed in cold 20 mM sodium/potassium phosphate buffer, pH 7.0, and then resuspended in the same buffer. Sodium thioglycollate (5 mM) was added to the breakage buffer for preparations of the soluble MMO; this stabilized protein C of the enzyme (Colby and Dalton, 1978). Cells were broken by two passages through a pre-cooled French pressure cell at 137 mPa. Any whole cells remaining were removed by centrifugation at 10,000 g for 15 min at 4°C. The cell-free extract was then separated into soluble and particulate fractions by centrifugation at 38,000 g for 30 min.

The particulate MMO is inhibited by sodium thioglycollate (Stanley *et al.*, 1983) and so preparations of this form of the enzyme were broken in the absence of thioglycollate. Centrifugation to obtain soluble and particulate fractions were as described for the soluble MMO.

## 2.6 Methane monooxygenase assay

Methane monooxygenase (MMO) activity in cell extracts was determined by gas chromatographic assay of propylene epoxidation with NADH as the electron donor. The assay for the soluble enzyme was determined in a 5 ml conical flask which contained 40 mM sodium/potassium phosphate

buffer, pH 7.0 and sufficient soluble extract to give a final protein concentration of  $7 \text{ mg ml}^{-1}$ . Propylene (2.5 ml) was added by injection through a suba-seal and the flask equilibrated at  $45^{\circ}\text{C}$  for 30 sec prior to addition of NADH (5 mM). The flask was incubated at  $45^{\circ}\text{C}$  on a shaking water bath and gaseous samples removed by use of a gas-tight syringe before determination of the extent of propylene epoxidation.

The quantitative estimation of propylene epoxidation was determined by gas chromatography using a Pye series 104 gas chromatograph (Pye Unicam, Cambridge, U.K.). Liquid (5  $\mu\text{l}$ ) samples were injected into a glass column (2.1 M x 4 mm i.d.) packed with Porapak Q (Waters Associates, Milford, Mass., U.S.A.). The oven temperature was  $190^{\circ}\text{C}$  and the carrier gas ( $\text{N}_2$ ) flow rate  $30 \text{ ml min}^{-1}$ . The quantity of propylene oxide in the sample was measured by flame ionization detection and compared with authenticated propylene oxide using a Hewlett-Packard 3390 reporting integrator (Hewlett-Packard, Avondale, PA., U.S.A.). The particulate enzyme was assayed similarly, but the pH of the sodium/potassium phosphate buffer was raised to pH 7.5 (the particulate enzyme was found to exhibit maximal activity at pH 7.5).

The effect of temperature on particulate MMO activity were determined by altering the temperature of the shaking water bath, and the effect of pH and the buffer system on particulate MMO activity were determined using the assay system described above. The effect of metal ions and a range of potential inhibitors on MMO activity were determined after the cell extracts had been pre-incubated for 1 min in the presence of the test

metal ion or inhibitor prior to addition of propylene and NADH.

Experiments designed to investigate the effect of various potential electron donors on particulate MMO activity were performed by replacing the NADH with a variety of compounds including NADPH, ascorbate, succinate, methanol and ethanol - the assay was then performed as normal.

#### 2.7 Whole cell MMO activity

Whole cell MMO assays were performed in 7 ml conical flasks containing 1 ml of cell suspension and sealed with rubber suba-seal caps. The electron donor for MMO activity was supplied by the addition of 5 mM potassium formate to the assay system (Leak and Dalton, 1983). MMO activity was measured by following the conversion of propylene to propylene oxide using gas chromatography (2.1 M x 4 mm i.d. Porapak Q (Waters Associates, Milford, Mass., U.S.A.)) with flame ionization detection.

#### 2.8 Dry weight estimation

The concentration of biomass was normally determined by measuring the optical density of cell suspensions at 540 nm in a Pye Unicam SP800 spectrophotometer using water as a standard reference. The relationship

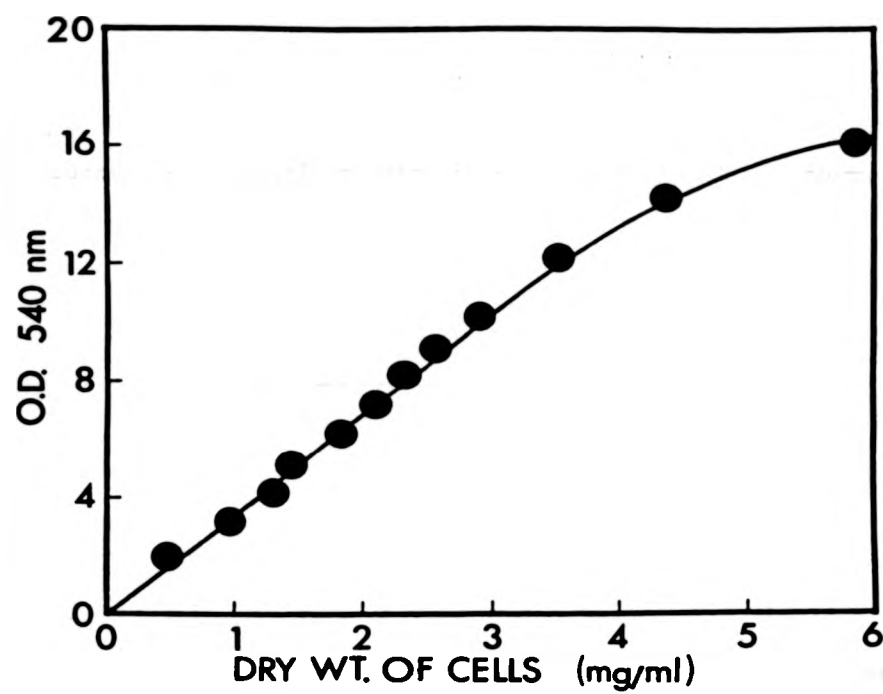


Figure 2.1      Relationship between optical density and dry weight of  
cells for *M. capsulatus* (Bath)

between optical density at 540 nm and dry weight of cells was estimated using the following method: A known volume of culture was filtered through a preweighed Millipore filter (heated at 80°C before weighing). The filter with the cells was then weighed to constant weight at 80°C. The dry weight was then compared to the optical density (540 nm) of the culture prior to filtration. The results are shown in Figure 2.1.

## 2.9 Electron Microscopy

Samples of cells for thin-section electron microscopy were fixed using the standard method of Kellenberger et al. (1958). After fixation, cells were embedded in agar, diced and stained for 2 h in 0.5% (w/v) uranyl acetate in Kellenberger buffer. Stained cells were sequentially dehydrated in a graded ethanol/water series before embedding in Araldite epoxy resin (Luft, 1961). The araldite was polymerized at 60°C for 48 h. Sections were cut on a Reichert OEB U2 ultramicrotome. Post-staining was performed according to the method of Keynolds (1963) and the samples examined in a Jeol 2 electron microscope.

## 2.10 SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed using a linear gradient vertical slab gel and discontinuous buffering system (Laemmli, 1970). Polypeptide bands were visualized by

staining with Coomassie Blue R250 stain (BDH Chemicals, Poole, Dorset, U.K.).

Preparations of membrane fractions from cells expressing particulate MMO activity often exhibited ill-defined polypeptide bands at the lower edge of the gel (low molecular weight proteins). This "smearing" of the bands was thought to be due to interference from the lipids of the intracytoplasmic membranes and was readily removed using the following method. Membrane fractions which were resuspended in buffer (either sodium/potassium phosphate or Pipes) had an equal volume of chloroform added and were then shaken for 30 sec. The two layers were allowed to separate by simple partitioning and the clear chloroform layer was drawn off using a pasteur pipette. The sample was then placed in a boiling water bath for five minutes to remove any residual chloroform. The chloroform-extracted membrane-fraction was then prepared for SDS-polyacrylamide gel electrophoresis in the normal way (Laemli, 1970). Tests on the chloroform layer showed that no proteins were present in this fraction indicating that the method does not alter polypeptide banding patterns, but was effective at removing the "smearing" at the lower edge of the gel.

#### 2.11 Copper uptake experiments

The rate at which copper was removed from cell suspensions and accumulated within cells was determined using atomic absorption

spectrophotometry of cell suspensions and cells digested by nitric acid (Norris and Kelly, 1977).

Cells were removed from chemostat cultures of M. capsulatus grown on low copper medium ( $0.2 \text{ mg l}^{-1} \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and harvested by centrifugation at 10,000 g for 10 min. The cells were washed twice by resuspension in 5 mM Pipes buffer, pH 7.0 (Pipes - piperazine-N,N'-bis(2-ethane sulphonic acid) and resuspended in the same buffer. This buffer was used in preference to sodium/potassium phosphate buffer as it has negligible metal-chelating properties (Good et al., 1966). The cell suspension was then placed in an erlenmeyer flask in an orbital shaker at  $45^\circ\text{C}$ . The flask was sealed using a rubber suba-seal and methane gas injected through the rubber seal to provide a methane/air atmosphere in the flask (this step was omitted when the energy dependence of copper uptake was studied). Samples (12 ml) were removed prior to addition of the copper ions and rapidly filtered through  $0.45 \text{ }\mu\text{M}$  pore size membrane filters (Oxoid Ltd., Basingstoke, U.K.). The filters were then washed immediately with 100 mM EDTA and transferred to 5 ml centrifuge tubes. Copper ions were added by injection through the suba-seal and samples removed at intervals after addition and filtered as described above. The experiments to determine the loss of copper ions from the cell suspension involved the measurement of the filtrate resulting from the rapid filtration method outlined above.

The cells and membrane filters had 1 ml of concentrated nitric acid added to each tube which were then boiled for 30 min in a boiling water

bath to release the cell associated metal ions. Samples were made up to a known volume and centrifuged to remove any undigested material. The supernatant fluids were analysed for copper content using a Varian AA-1275 atomic spectrometer (Varian Ltd., Walton-on-Thames, Surrey, U.K.) according to the standard procedure prescribed in the instrument manual.

#### 2.12 Antibody studies

Experiments to determine whether there was any cross-reactivity between antibody, raised to purified components of the soluble MMO, and the membrane fraction of cells expressing particulate MMO activity were possible due to the generous donation of such antibody from Dr. J. Lund of our laboratory. The procedures involved testing by the standard Ouchterlony double-diffusion technique, and by use of rocket-gel electrophoresis (Laurell, 1966). The latter method involves the migration of antigen and antibody in an electrophoretic field and has the advantage of extreme sensitivity. The procedure used was that of Laurell (1966) using a commercially available apparatus (Shandon Southern Products Ltd., Runcorn, Cheshire, U.K.)

#### 2.13 <sup>35</sup>S-methionine uptake/incorporation studies

This experiment was designed to test whether the protein bands associated with particulate MMO activity were derived from the proteins

of the soluble MMO complex. The procedure involved growing the cells in chemostat culture on low copper medium to produce cells with wholly soluble MMO activity. The cells were then removed from the chemostat and labelled with  $^{35}\text{S}$ -methionine such that the proteins in the cell all contained radiolabelled methionine. (Experiments on uptake of  $^{35}\text{S}$ -methionine showed that the cell contents were fully labelled after incubation with the radiolabel for 60 min). The excess label was then removed by centrifugation and washing of the cells in ice-cold buffer (20 mM sodium/potassium phosphate buffer, pH 7.0) (twice) followed by resuspension in buffer lacking radiolabelled methionine, but containing unlabelled methionine. Copper ions were then added to the cells using the method previously described in the copper uptake experiments (Section 2.11). Samples were removed at intervals after the addition of copper ions, and prepared for SDS-polyacrylamide gel electrophoresis. The samples were run on SDS-PAGE and visualized using coomassie blue R250 stain. This gel was then dried onto filter paper and fluorographed. The resultant fluorographs were then compared to the polypeptide banding pattern on SDS-PAGE.

The theory behind this procedure was as follows: If the particulate MMO was newly synthesized after addition of copper ions the polypeptide banding pattern should change and the corresponding fluorograph should indicate that these new proteins are unlabelled as they were synthesized in the presence of unlabelled methionine. If there was a constitutive level of particulate MMO the fluorograph should show that the level of label in these proteins decreases as newly-formed, unlabelled, proteins

cause a dilution of the  $^{35}\text{S}$ -methionine labelled proteins. The final possibility, for which the experiment was designed, would involve the incorporation of soluble MMO proteins into the membrane, in this case the fluorograph should indicate an increase in the radiolabelling of the proteins as the soluble MMO was fully labelled with  $^{35}\text{S}$ -methionine before the copper was added.

It is important to note that for this method to be effective the amount of material loaded onto the gel must be constant with regard to the level of radiolabel rather than the level of protein which is the normal procedure in order that changes in the relative levels of the radiolabelled proteins can be determined.

#### 2.14 Studies using $^{14}\text{C}$ -labelled acetylene

The inhibition of cell extracts by acetylene was investigated using the methods outlined previously for other inhibitory compounds. In order to determine whether the binding was irreversible or not, the gaseous phase of the assay system was evacuated using a vacuum line. The flasks were evacuated and then flushed with air four times prior to assay for MMO activity using the routine procedure previously described.

Experiments to determine which proteins in the extract bound the acetylene involved incubation of the  $^{14}\text{C}$ -labelled acetylene with the sample in a 5 ml conical flask with a suba-seal cap which prevented the

escape of the radiolabelled material. After a suitable incubation period (10 min) the acetylene which was not bound to the protein was removed by evacuation of the gaseous phase. The samples were then prepared for SDS-PAGE and run as described previously. The resultant gel was stained in the routine manner, dried onto filter paper and fluorographed.

#### 2.15 Fluorography

Gels which had been stained with Coomassie Blue R250 (BDH) were destained using a methanol/acetic acid/water mixture (40:10:50 (v/v)) and then dehydrated by immersion in DMSO (dimethyl sulphoxide) for 1 hr with one change of DMSO. The dehydrated gel was then soaked in a DMSO/PPQ solution (PPQ = 2,5-diphenyloxazide) (DMSO/PPQ solution was 33.5 g PPQ in 100 ml DMSO) for 2 hr prior to recrystallization of the PPQ in the gel. Recrystallization was achieved by immersing the gel in running cold water for 1 hr. The gel was then dried down onto Whatman filter paper and when fully dry was placed in an X-ray cassette with Kodak X-ray film (Kodak Ltd., Hemel Hempstead, Herts., U.K.). The cassette was stored at -70°C for 7 days before developing the film.

#### 2.16 Substrate specificity of the particulate MMO

The ability of cell-free extracts, prepared from cells of M. capsulatus

exhibiting wholly particulate MMO activity, to oxidize a range of substrates was examined using gas chromatography. All assays were performed in 5 ml conical flasks which were sealed with rubber suba-seal caps. The samples contained sufficient of the membrane fraction, resuspended in 40 mM sodium/potassium phosphate buffer (pH 7.5), to give a protein concentration of 2 mg ml<sup>-1</sup>. Gaseous substrates were added as saturated buffers, the compound concentration being calculated as described previously (section 2.4), liquid substrates were diluted in buffer and added as solutions. The electron donor for all assays was 5 mM NADH which was added to the system by injection after the sample and test substrate had been preincubated at 45°C on an orbital shaker for 1 min. Samples were removed after a known interval and the products determined by gas chromatography. All analyses were conducted isothermally at temperatures between 150-190°C and carrier gas (N<sub>2</sub>) flow rates of 30-60 ml min<sup>-1</sup>, the actual conditions depending on the substrate being tested. Compounds were identified by comparing their retention times with those of authentic standards and quantitatively estimated by using a Hewlett-Packard 3390 reporting integrator.

#### 2.17 Protein determinations

Protein determinations were performed using the Bio-Rad protein assay system (Bio-Rad Laboratories Ltd., Watford, Herts., U.K.) with bovine serum albumin standards.

## 2.18 Gases

Methane (technical grade), ethylene and propylene were obtained from British Oxygen Company Ltd., London, U.K. All other gases were obtained from Matheson Gas Products, Croydon, U.K.  $^{14}\text{C}$ -labelled acetylene was obtained from New England Nuclear, Boston, Mass., U.S.A.

## 2.19 Chemicals

Most compounds were obtained from the following manufacturers: BDH Chemicals, Poole, Dorset, U.K.; Aldrich Chemical Co. Ltd., Gillingham, Kent, U.K.; Fisons Scientific Apparatus, Loughborough, Leics., U.K. Electron microscopy materials were obtained from Polaron Equipment Ltd., Watford, Herts., U.K. and Emscope, Ashford, Kent, U.K. Photographic material was obtained from Kodak Ltd., Kirkby, Liverpool, U.K. The detergent Mega-9 was a gift from the British Technology Group, London, U.K.

### Chapter 3

The Role of Copper in the Regulation of the Intracellular  
Location of Methane Monooxygenase activity in Methylococcus  
capsulatus (Bath) grown on Methane

### 3.1 Introduction

In the general introduction to this thesis the section on methane oxidation (Section 4.3) discussed in some detail the different methane monooxygenase (MMO) enzymes which have so far been reported in the literature. It was clear from these reports that there was considerable debate concerning the intracellular location of MMO in some methanotrophs, notably Methylosinus trichosporium OB3b (see Stirling and Dalton, 1979; Dalton, 1981; Higgins et al., 1981b). In addition to this controversy several other areas of methanotrophic biochemistry required thorough investigation in an effort to resolve the long-standing debate regarding the intracellular location of MMO in methanotrophs, these included; i) "was it possible for M. capsulatus (Bath) to express particulate MMO activity in addition to the soluble MMO already isolated by Colby et al. (1977)?"; ii) "if particulate MMO activity is expressed in M. capsulatus (Bath) is it similar to any previously reported MMO?", iii) "if a particulate MMO is present in M. capsulatus (Bath) what are the factors controlling the expression of the soluble/particulate systems?" and iv) "are such factors applicable to the control of expression of soluble/particulate MMO activity in M. trichosporium OB3b and other methanotrophs?" It was with a view to answering these questions that the investigation described in this thesis began.

Several papers (Higgins et al., 1981a; Scott et al., 1981a,b) have attempted to rationalize the differences in localisation of MMO activity

reported for M. trichosporium OB3b on the basis of the nature of the growth-limiting nutrient (oxygen, methane or nitrate). These results are summarised in Table 3.1. One notable feature of the results in Table 3.1 is that the biomass concentration used when the cells were grown under nitrate limitation (and expressing 100% soluble activity) is much higher ( $2-5 \text{ g l}^{-1}$ ) than when cells were grown under oxygen-/methane-limited conditions ( $0.2 \text{ mg l}^{-1}$ ). It was therefore possible that cells exhibiting soluble MMO activity were not only nitrate-limited but were also limited for other nutrients due to the increased biomass concentration used in this part of the experiment. The effect of biomass concentration on MMO activity in M. capsulatus (Bath) was therefore investigated by growing the organism in batch culture as outlined in the next section.

### 3.2 Batch Growth of Methylococcus capsulatus (Bath)

Several workers in our laboratory had noted that the soluble extracts from shake-flask cultures of M. capsulatus (Bath) grown on methane did not possess methane monooxygenase activity, and yet when the cells were transferred and grown to a much higher cell density in a 100 L fermenter, MMO activity was readily detected in the soluble fraction of cell extracts (H. Dalton, personal communication). A similar effect has also been observed by Patel *et al.* (1982) in the facultative methanotroph Methylobacterium sp K6. The possibility that the intracellular location of MMO activity in M. capsulatus (Bath) was

Table 3.1      Location of MMO in nutrient-limited chemostat cultures of  
Methylosinus trichosporium OB3b

<u>MMO</u>	<u>Limiting Nutrient</u>		
	<u>Oxygen</u>	<u>Methane</u>	<u>Nitrate</u>
	Particulate (100%)	Particulate (22%) Soluble (78%)	Soluble (100%)
<u>Internal</u>	Membranes	Membranes	Vesicles
<u>Morphology</u>	(Many)	(Few)	
<u>Biomass</u>			
<u>concentration</u> (g l <sup>-1</sup> )	0.2	0.2	2.0-5.0

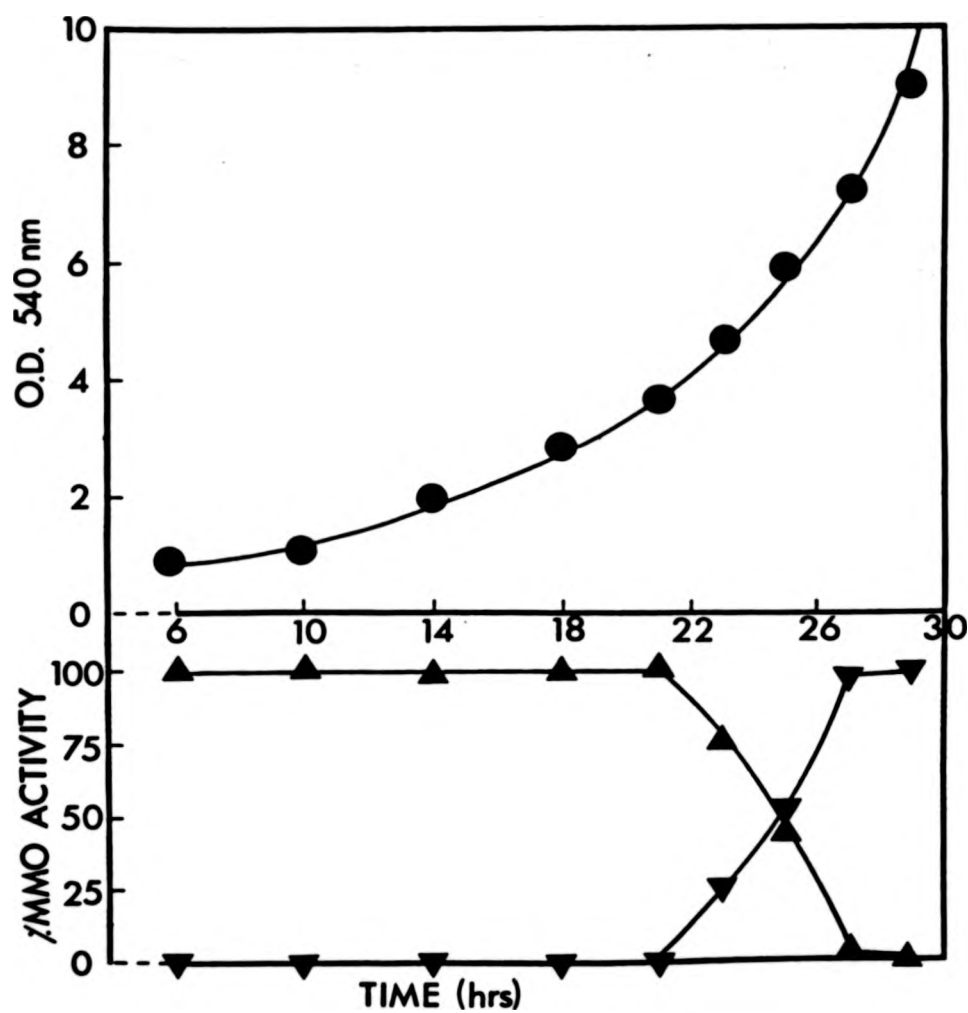
Taken from Scott et al., 1981a and Scott et al., 1981b.

dependent on the biomass concentration was investigated by growing the organism in a 15 L batch fermenter with methane and oxygen flow rates capable of supporting a biomass concentration in excess of 3 g dry wt. cells  $l^{-1}$  ( $OD_{540} = 10$ ). Cell extracts were prepared and assayed for MMO activity by following the epoxidation of propylene on a gas chromatograph (Colby et al., 1977). The results are shown in Figure 3.1.

The experiment showed that at biomass concentrations of less than 1.2 mg dry wt.  $ml^{-1}$  ( $OD_{540} = 4$ ) the MMO activity was found to be associated with a membrane fraction that was sedimented after centrifugation at 38,000 g/30 min. This was contrary to all earlier reports of in vitro MMO activity in M. capsulatus (Bath) (Colby et al., 1977; Colby and Dalton, 1978; Stirling and Dalton, 1979) which had demonstrated that the MMO activity in this organism was found in the soluble fraction of cell extracts (i.e. was not sedimented after centrifugation at 38,000 g/30 min) and was not associated with the membrane fraction of cell extracts. At biomass concentrations higher than 1.2 mg dry wt  $ml^{-1}$  ( $OD_{540} = 4$ ) soluble MMO activity was detected and the ratio of soluble to particulate MMO activity increased with increasing biomass concentrations until at a concentration of 2.3 mg dry wt  $ml^{-1}$  ( $OD_{540} = 8$ ) only the soluble form of the enzyme could be detected.

The switch between particulate and soluble forms of MMO activity appeared therefore to be related to the biomass concentration rather than the type of vessel (shake-flask or chemostat) in which the organism

Figure 3.1      Batch Growth of *M. capsulatus* (Bath) on low copper medium



The experiment was performed as outlined in the text. MMO activity was determined by measuring the rate of propylene epoxidation by gas chromatography. Particulate MMO (▲) and soluble MMO (▼) were assayed as described in Materials and Methods.

was grown. Such a transition is indicative of a change in nutritional status of the organism since all other parameters in the batch culture (temperature, pH, stirring rate) were kept constant. Because of the gas flow rates employed and the media used (see Materials and Methods) the nutrient limitation involved in the "switching" in intracellular location was unlikely to be oxygen, methane or nitrate and suggested that the results of Scott et al., (1981a, b) may be misleading because of the different biomass concentrations employed in their experiments (see Introduction to this section for details).

This experiment provided the first report of particulate or membrane-bound MMO activity in M. capsulatus (Bath) and suggested that it should be possible to determine the parameters involved in the regulation of the intracellular location of MMO activity in this organism. The work might also then be extended to cultures of M. trichosporium UB3b to try and explain the dichotomy between the Kent/Cranfield and Warwick research groups concerning the intracellular location of MMO in this organism.

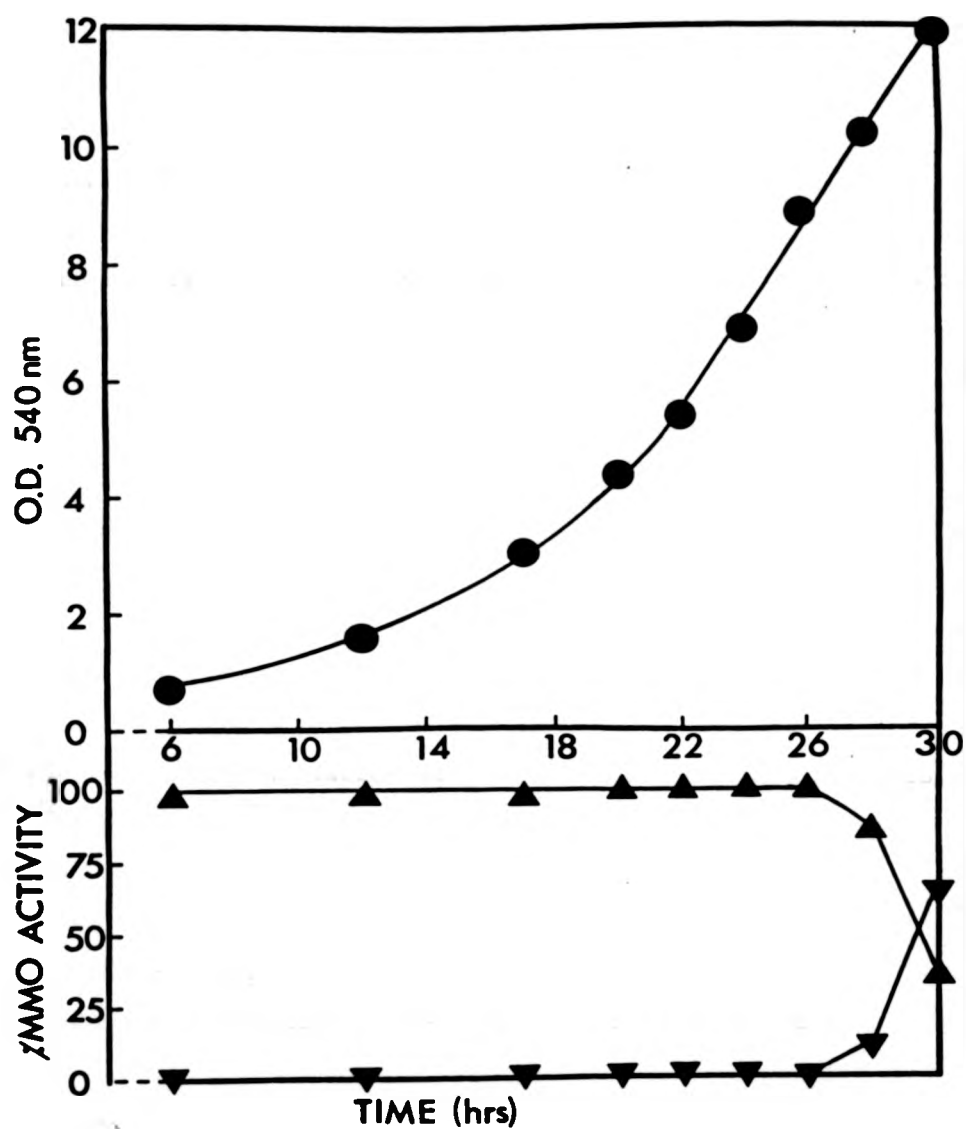
The fact that it was not methane-, oxygen- or nitrate-limitation that was regulating the change in intracellular location of MMO activity in M. capsulatus (Bath) led to an examination of the literature on growth of methanotrophs to determine which nutrient-limitation might be worthy of investigation. Several reports demonstrated a requirement for copper (Sheehan and Johnson, 1971; Vary and Johnson, 1967; Harwood and Pirt, 1972) and there were four reports of enhancement of growth rates of a

variety of methanotrophs when the level of copper ions in the growth medium was increased (Takeda *et al.*, 1976; Ohtomo *et al.*, 1977; Takeda and Tanaka, 1980; Harwood and Pirt, 1972). Furthermore, studies in our laboratory several years ago had shown that the copper requirement for maximum growth of methanotrophs varied between strains (H. Dalton, personal communication). It therefore seemed that copper was a good starting point for studies on nutrient-limitation.

The batch-culture experiment was repeated, but with the copper level in the growth medium raised from 0.2 mg/l to 1.2 mg/l. The results are shown in Figure 3.2.

The initial results were similar to the experiment with only 0.2 mg/l copper in the growth medium and there was no significant change in the doubling time of the culture. The major differences only became apparent during the latter part of the experiment. At 1.2 mg/l copper sulphate in the growth medium the only form of the enzyme detectable until the biomass concentration reached 2.6 mg dry wt ml<sup>-1</sup> (OD<sub>540</sub> = 9) was found to be associated with the membrane fraction of cell extracts. At higher biomass concentrations soluble MMO activity became detectable but at the termination of the experiment at 3.5 mg dry wt ml<sup>-1</sup> (OD<sub>540</sub> = 12) the culture still retained 35% particulate MMO activity. The increased level of copper had thus maintained the particulate form of the enzyme in the culture. It therefore appeared that the level of copper in the growth medium, or more precisely the copper:biomass ratio of the culture, determined the switching of the intracellular location

Figure 3.2      Batch Growth of *M. capsulatus* (Bath) on high copper medium



This experiment was similar to the one shown in Figure 3.1 except that the medium contained  $1.2 \text{ mg l}^{-1}$  copper sulphate (high copper medium). Particulate MMO (▲) and soluble MMO (▼) were assayed as described in Materials and Methods.

of MMO activity in M. capsulatus (Bath) between the particulate and soluble fractions of cell extracts.

The hypothesis that it is the copper:biomass ratio that regulates expression of the two forms of the enzyme would explain the absence of soluble MMO activity in shake-flask culture as under these conditions the maximum cell density attained is only 0.25-0.3 g dry wt  $l^{-1}$  ( $OD_{540} = 1-1.5$ ), such that copper is always in excess of growth requirements, whereas at higher cell densities, such as are attained in chemostat culture, copper was depleted from the growth medium and a switch to the soluble form of the enzyme was effected. Previous experiments with M. capsulatus (Bath) had either been in continuous culture (Colby and Dalton, 1976; Stirling and Dalton, 1977; Stirling, Colby and Dalton, 1979) or in batch culture at a high cell density (Colby, Stirling and Dalton, 1979) such that the low copper:biomass ratio would lead to expression of the soluble form of the enzyme and explains the lack of reports of particulate MMO activity in this organism.

Experiments involving changes in the levels of iron or zinc in the growth medium did not affect the transition from particulate to soluble MMO activity during batch culture, but were examined as both are known to be present in purified soluble MMO proteins (Woodland and Dalton, 1984a). It therefore seemed likely that the transition may be due solely to changes in the copper concentration in the growth medium.

### 3.3 Continuous culture of *M. capsulatus*(Bath)

Experiments performed in batch culture are limited by the fact that the system is always in a transient state due to changes in both the biomass and in the environment in which it is grown. This limitation can, however, be overcome by the use of chemostat culture. *M. capsulatus* (Bath) was therefore grown in chemostat culture under steady-state conditions which simulated the environment at various points during batch growth. Samples were removed from steady-state culture and cell extracts prepared to determine the intracellular location of the MMO activity. The results (Table 3.2) showed that when cells were grown on media which contains  $0.2 \text{ mg l}^{-1}$  copper sulphate the intracellular location of the MMO was dependent on the biomass concentration. Wholly particulate MMO activity was maintained under all conditions until the cell density reached  $1.5 \text{ mg dry wt ml}^{-1}$  ( $\text{OD}_{540} = 5$ ); at biomass concentrations higher than this soluble MMO activity was detected and the ratio of soluble:particulate MMO activity increased as the biomass concentration increased, with 100% soluble activity occurring at a cell density of  $2.0 \text{ mg dry wt ml}^{-1}$  ( $\text{OD}_{540} = 7$ ). This was similar to the results shown in batch culture and reaffirms the suggestion that the change in intracellular location for the MMO activity may be due to nutrient limitation. Supplementation of the growth medium with  $1 \text{ mg l}^{-1}$  copper sulphate (to give a final concentration of  $1.2 \text{ mg l}^{-1}$ ) led to the particulate MMO activity being maintained at much higher biomass concentrations and soluble MMO activity was only detected when the cell density reached  $2.5 \text{ mg dry wt ml}^{-1}$  ( $\text{OD}_{540} = 9.5$ ). The results show that

Table 3.2 Continuous culture of *M. capsulatus* (Bath)

OD <sub>540</sub>	Oxygen limited/0.2 mg l <sup>-1</sup> CuSO <sub>4</sub> ·5H <sub>2</sub> O		Methane limited/0.2 mg l <sup>-1</sup> CuSO <sub>4</sub> ·5H <sub>2</sub> O	
	% Soluble MMO	% Particulate MMO	% Soluble MMO	% Particulate MMO
4	0	100	0	100
5	0	100	0	100
6	41	59	0	100
7	97	3	36	64
8	100	0	81	19

OD <sub>540</sub>	Oxygen limited/1.2 mg l <sup>-1</sup> CuSO <sub>4</sub> ·5H <sub>2</sub> O		Methane limited/1.2 mg l <sup>-1</sup> CuSO <sub>4</sub> ·5H <sub>2</sub> O	
	% Soluble MMO	% Particulate MMO	% Soluble MMO	% Particulate MMO
4	0	100	0	100
6	0	100	0	100
8	0	100	0	100
10	28	72	6	94

The results are the values for soluble/particulate MMO activity in steady-state chemostat cultures of

*M. capsulatus* (Bath).

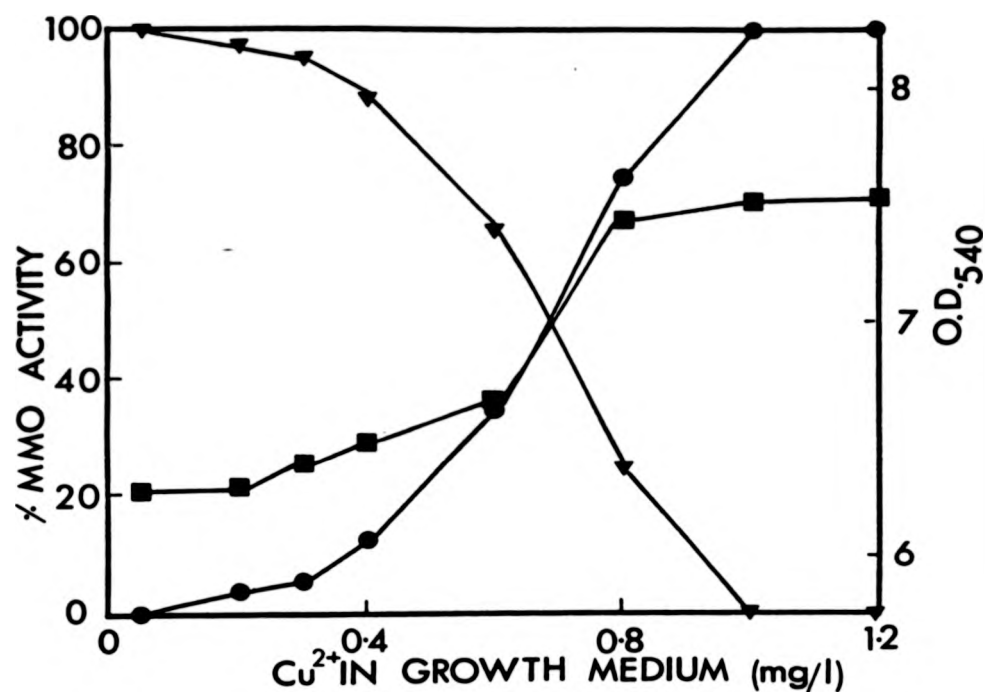
in oxygen-limited chemostat culture the intracellular location of MMO was dependent on the copper:biomass ratio of the growth medium.

The experiment was repeated under methane-limited conditions and the results showed a similar change in location of MMO activity, but the biomass concentration at which the "switch" from particulate to soluble MMO occurred was  $1.8 \text{ mg dry wt ml}^{-1}$  ( $\text{OD}_{540} = 6.5$ ), i.e. slightly higher than the results obtained under oxygen limitation. These results are summarised in Table 3.3. In subsequent studies over a range of biomass concentrations it has been observed that soluble MMO activity was evident at lower biomass concentrations under oxygen limitation than under methane limitation in low copper medium and that the transition from 100% particulate to 100% soluble activity spans a wider range of biomass concentration under methane limitation than under oxygen limitation. The possible explanation for this phenomenon is that under low oxygen tensions there was an additional requirement for copper for a terminal oxidase with a lower  $K_m$  for oxygen than that present at higher oxygen tension. Thus, under oxygen limitation copper limitation occurs at a lower biomass concentration than under methane limitation.

In an effort to determine the copper:biomass ratio at which the transition from particulate to soluble MMO occurs a chemostat culture of M. capsulatus (Bath) was set up and the intracellular location of the enzyme examined at a variety of copper concentrations in the growth medium. The results of this experiment are shown in Figure 3.3. The results confirm those of earlier experiments and show that the level of

Table 3.3Summary of continuous culture experiments

	<u>High Copper</u> (1.2 mg l <sup>-1</sup> )	<u>Low Copper</u> (0.2 mg l <sup>-1</sup> )	
<u>Oxygen-limited</u>	Particulate	Particulate (OD <sub>540</sub> <5) <u>MMO</u> Soluble (OD <sub>540</sub> >7.5)	
<u>Methane-limited</u>	Particulate	Particulate (OD <sub>540</sub> <6) <u>MMU</u> Soluble (OD <sub>540</sub> >9.5)	



**Figure 3.3** Effect of copper on optical density and intracellular location of the MMO activity in *M. capsulatus*

The figure shows the effect of a variety of copper concentrations in the growth medium on the optical density of the culture (■) and on soluble (▼) and particulate (●) MMO activity. All values were determined from steady-state chemostat cultures of *M. capsulatus* (Bath).

copper in the growth medium regulates the intracellular location of MMO activity in M. capsulatus (Bath). The experiment also demonstrates that the cells do not simply express one or other form of the enzyme but can express both forms, i.e. soluble and particulate MMO, in the same cell extract. The implications of this result are discussed in Section 3.5. The other important result in this study was the demonstration that an increase in the copper concentration in the growth medium led to an increase in cell density (as measured by a change in OD<sub>540</sub>) and that this increase occurred concomitantly with the expression of the particulate form of the enzyme. All other parameters remained the same suggesting that growth of M. capsulatus (Bath) under conditions where particulate MMO activity is expressed is more efficient than under conditions where soluble MMO activity is expressed. In a recent publication Leak et al. (1985) studied the efficiency of biomass production from methane by M. capsulatus (Bath) and demonstrated that when grown in chemostat culture with nitrate as a source of nitrogen the carbon conversion efficiency (CCE) of cells exhibiting particulate MMO were higher than when cells were expressing soluble MMO activity, these results are shown in Table 3.4.

### 3.4 Studies with other organisms

A change in the intracellular location of MMO activity that was dependent on the copper:biomass ratio has also been demonstrated in Methylosinus trichosporium OB3b by Stanley et al. (1983). Furthermore,

Table 3.4      Efficiency of Biomass Production from Methane

<u>Limitation</u>	<u>MMO Activity</u>	<u>Carbon conversion efficiency</u> <u>(CCE %)</u>
Oxygen	Soluble	31.3
Oxygen	Particulate	43.3
Methane	Soluble	31.2
Methane	Particulate	41.9

Taken from Leak et al., 1985.

they suggested that it was the level of copper in the growth medium that was regulating the intracellular location of MMO in the studies of Scott et al. (1981a, b) and that the increased biomass concentration present when the cells were grown under nitrate-limitation masked the underlying cause of the change in location of the enzyme. The results of Stanley et al. (1983) have recently been confirmed by Burrows et al. (1984) and it would appear that the long-standing controversy surrounding the intracellular location of MMO activity in M. trichosporium OB3b is close to being resolved (Stirling and Dalton, 1979; Dalton, 1980; Higgins et al., 1981a, b, c). The one remaining problem is the report by Scott et al. (1981a, b) that under methane-limitation at a biomass concentration of 0.2 g dry wt l<sup>-1</sup> the cells exhibited 78% soluble activity and 22% particulate activity. From the results of Stanley et al. (1983) and Burrows et al. (1984) the copper:biomass ratio in this experiment should yield 100% particulate activity. On balance it would seem likely that the report of Scott et al. is erroneous, but there remains the possibility that when M. trichosporium OB3b is grown in methane-limited culture the regulation of the intracellular location of MMO activity is not solely dependent on the copper:biomass ratio. Until this experiment is repeated the situation in M. trichosporium OB3b remains unresolved.

Stanley et al. (1983) also reported that growth of the Type I organism Methylomonas albus Bg8 and the Type II organism Methylocystis parvus OBBP became copper-limited at biomass concentrations similar to those at which soluble MMO activity began to appear in M. capsulatus (Bath) and M. trichosporium OB3b but the intracellular location of MMO activity has

not been demonstrated in these cases. It is therefore possible that some methanotrophs such as M. capsulatus (Bath) and M. trichosporium UB3b can avoid growth limitation due to copper deficiency by switching from a copper-requiring MMO (particulate) to an enzyme that has no discernable copper requirement (soluble). However, the ability to avoid copper-limitation is not universal amongst methanotrophs and some organisms such as M. albus Bg8, M. parvus UBBP and Methanomonas margaritae exhibit copper-limited growth. Whether the failure of these organisms to overcome copper-limitation is due solely to an inability to express a non-copper requiring MMO awaits the demonstration of cell-free MMO preparations from these organisms.

### 3.5 The effect of addition of copper to copper-limited chemostat cultures of M. capsulatus (Bath)

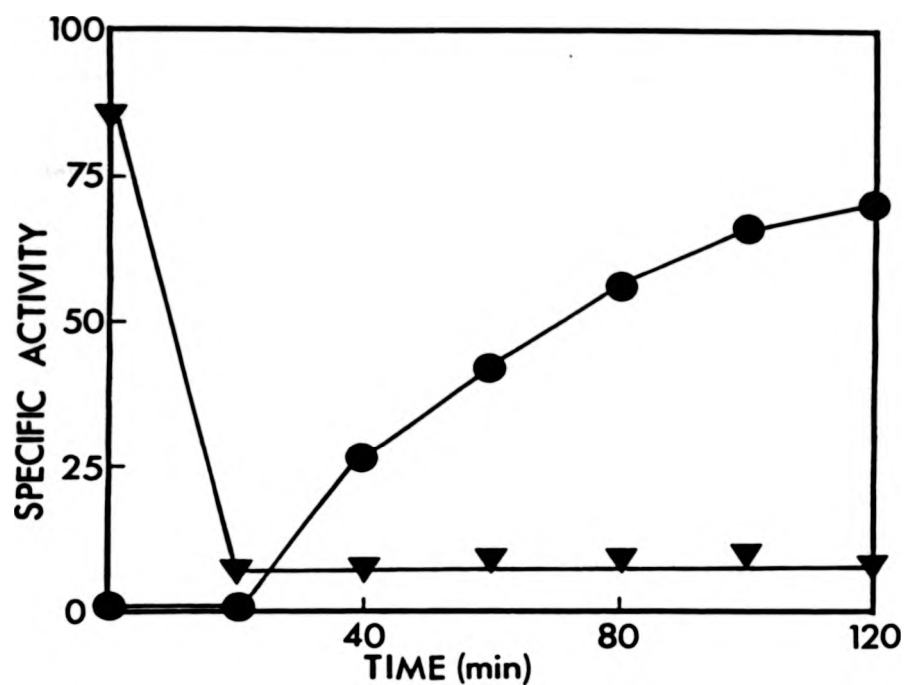
The results of experiments performed on steady-state cultures of M. capsulatus (Bath) have shown that increasing the level of copper in the growth medium permits the cells to express the particulate form of MMO at higher biomass concentrations, and that this leads to suppression of soluble MMO activity that is present when the cells are limited for copper. A series of experiments were set up to examine the affect of adding copper to a copper-limited culture in an effort to determine the rate at which the culture could "switch" from expressing 100% soluble MMO activity to expression of 100% particulate MMO activity.

The level of copper in the growth medium was raised from  $0.2 \text{ mg l}^{-1}$  to  $1.2 \text{ mg l}^{-1}$  by addition of a copper sulphate solution to the incoming medium, and at the same time, by addition to the culture vessel, samples were removed and cell extracts prepared which were then assayed for MMO activity. The results showed that a complete transition from 100% soluble activity to 100% particulate activity was achieved within 48 hr (7 generations), 70% particulate activity being evident after 24 hr (4 generations). The transition was accompanied by an increase in biomass concentration as evidenced by a change in  $\text{OD}_{540}$  from 6.5 to 8.0 (this affect has been discussed in Section 3.3). The transition was reversed by returning to the low copper growth medium, although complete reversal to 100% soluble activity was much slower than the initial transition, taking approximately 80 hr (12 generations). The reason for the much slower transition from wholly particulate to wholly soluble activity is that this transition requires removal of copper from the culture. This can only be achieved by dilution of the higher copper medium by the incoming low copper medium a process which is much slower than the reverse process which is achieved almost immediately by adding copper to the medium and the culture vessel.

The ability of copper to effect a transition from soluble to particulate MMO activity confirms and extends the earlier results of chemostat culture of *M. capsulatus* (Bath). The change in intracellular location was therefore investigated to determine how rapidly the switch from soluble to particulate MMO occurred. The method for preparation of cell extracts was similar to previous experiments except that the samples

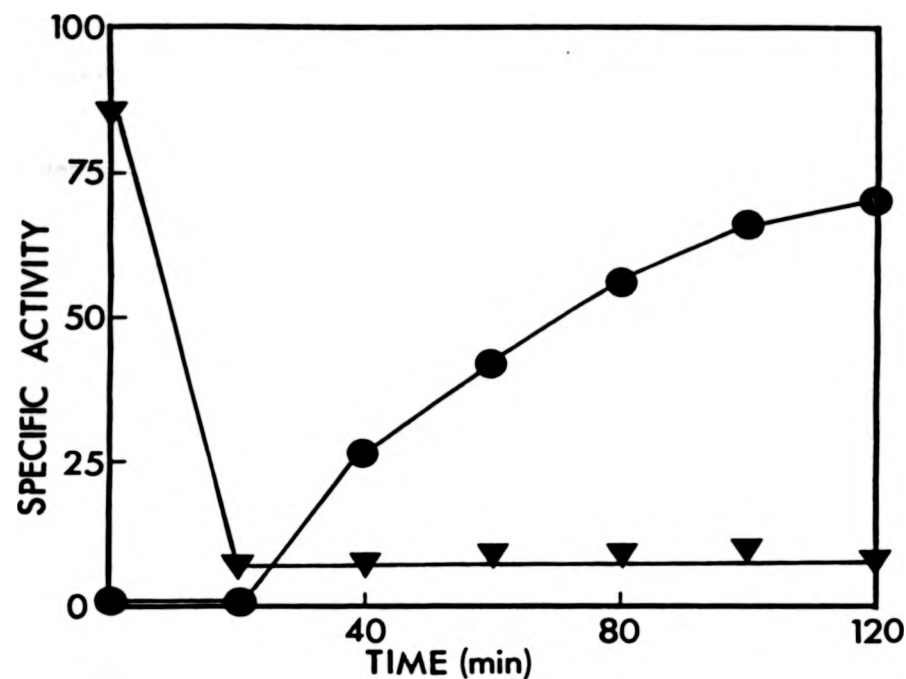
were taken every 20 min. The results shown in Figure 3.4 demonstrate that on addition of copper to the culture, the MMO activity in the soluble fraction decreased very rapidly over the first twenty minutes. Particulate MMO activity was detectable after twenty minutes and was found to increase rapidly over the period of the experiment. In order to determine whether the transition occurs faster than indicated in this experiment the culture was returned to the low copper medium and when a steady-state culture was attained with wholly soluble activity the culture was supplemented with copper sulphate ( $1.0 \text{ mf l}^{-1}$ ) and the culture sampled at 10 min intervals. The results are shown in Figure 3.5 and indicate that the induction of particulate MMO occurs within 10 min after the addition of copper and that in the same period the soluble MMO activity was inhibited by approximately 20%. It was apparent from this study that the addition of copper to the culture had a concerted effect leading to a "switch off" of the soluble MMO activity and the "switch on" of the particulate form of MMO. The mechanism by which soluble MMO activity is "switched off" is discussed in Chapter 6 of this thesis. The observation that in the presence of excess copper it is the particulate form of the enzyme that is preferentially expressed is possibly due to an increased carbon conversion efficiency which occurs in cells containing particulate MMO when compared to cells with soluble MMO (Leak and Dalton, 1985). Thus expression of the particulate MMO provides the cell with a competitive advantage over cells which are limited to expression of the more energetically expensive soluble MMO.

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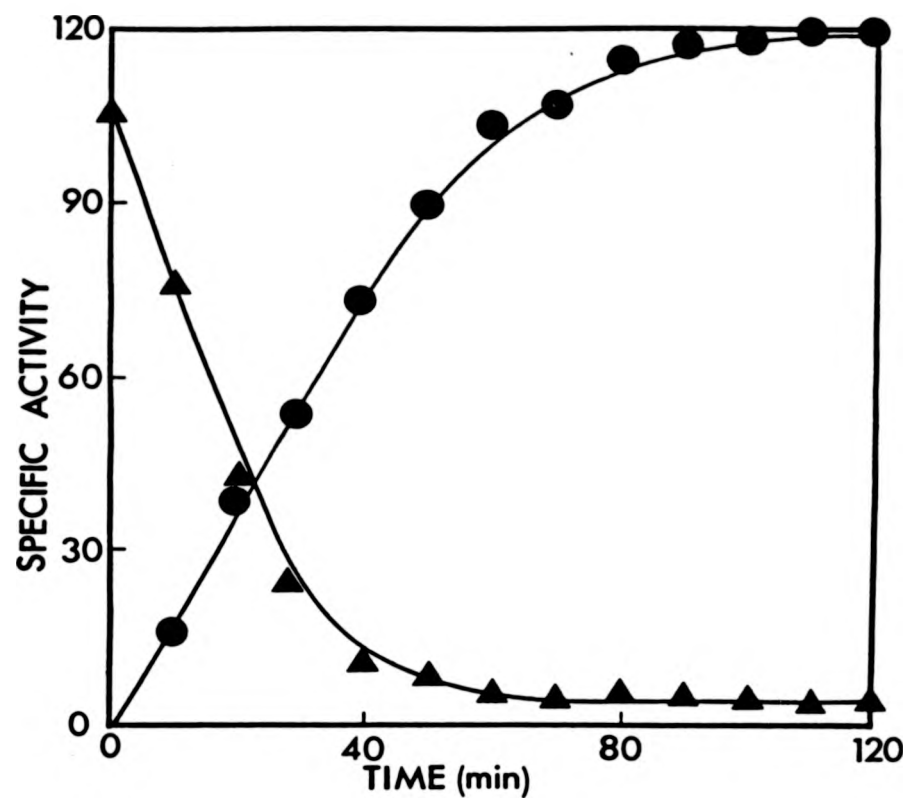
**Figure 3.4** Effect of addition of copper sulphate to a chemostat culture of *M. capsulatus* expressing wholly soluble MMO activity

The figure shows the effect of the addition of copper sulphate ( $1 \text{ mg l}^{-1}$ ) to a culture of *M. capsulatus* expressing wholly soluble MMO activity. Samples were taken at 20 min intervals and the extent of soluble (▼) and particulate (●) MMO activity determined by measuring the epoxidation of propylene. The results are presented in terms of specific activity [ $\text{nmol propylene oxide formed min}^{-1} (\text{mg protein})^{-1}$ ].



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**Figure 3.5** Effect of addition of copper sulphate to a chemostat culture of *M. capsulatus* expressing wholly soluble MMO activity

This figure is similar to Figure 3.4 except that samples were taken at 10 min intervals rather than 20 min intervals. Particulate MMO (●) and soluble MMO (▲) were determined by measuring the epoxidation of propylene and the results are presented in terms of specific activity [nmol propylene oxide formed  $\text{min}^{-1}$  (mg protein) $^{-1}$ ].

### 3.6 Is the change in Intracellular location of the MMO due to expression of new proteins?

It has been clearly demonstrated in the preceding sections of this thesis that copper ions play an important role in determining the intracellular location of MMO activity in M. capsulatus (Bath). A change in the intracellular location of MMO activity can arise either by copper-dependent association of the pre-existing soluble enzyme with the cell membrane or by copper-dependent expression of a novel membrane-bound enzyme. If the cells are producing a new MMO this would be detectable by changes in the protein banding patterns of cell fractions subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Cell extracts from the experiment outlined in Section 3.3 were prepared as described in the Materials and Methods section, and run on a vertical gel system using the discontinuous buffer system of Laemmli (1970). The results are shown in Figure 3.5.

Cells expressing soluble MMO activity were characterised by the presence of three major bands in the soluble fraction of cell extracts (Figure 3.6a). These bands are attributed to the  $\alpha$ ,  $\beta$  and  $\gamma$  subunits of Protein A of soluble MMO in M. capsulatus (Bath) (Woodland and Dalton, 1984a). The soluble fractions from cell extracts which have 100% particulate MMO activity were noted for the decreased levels of these three bands (Figure 3.6b). The membrane fraction of cell extracts with wholly particulate activity were characterised by the increased level in the expression of three bands (Mr 46,000, 35,000, 25,000) (Figure 3.6b)

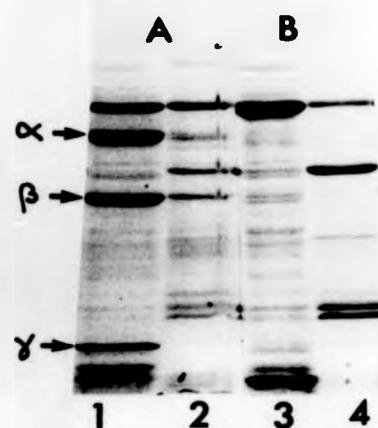


Figure 3.6      SDS-Polyacrylamide gel electrophoresis of cell extracts  
of *M. capsulatus* (Bath)

SDS-polyacrylamide gel of cell extracts of *M. capsulatus* grown under conditions which lead to expression of 100% soluble activity (A) and 100% particulate activity (B). Track 1, soluble fraction of cells exhibiting wholly soluble MMO activity showing the three components of Protein A of the soluble MMO; Track 2, membrane fraction of cells exhibiting wholly soluble activity. Track 3, soluble fraction of cells exhibiting wholly particulate MMO activity and lacking the components of Protein A of the soluble MMO; Track 4, particulate fraction of cells exhibiting wholly particulate MMO and showing an increase in at least 3 proteins lacking in cells exhibiting soluble MMO activity (Track 2).

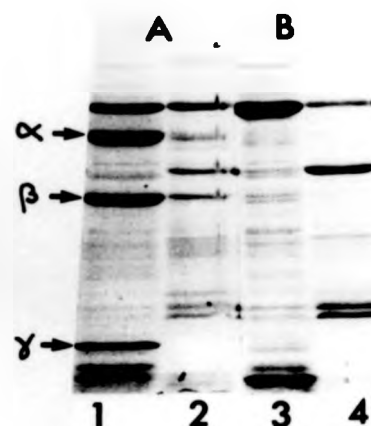


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which were not apparent in the membrane fraction of cell extracts expressing soluble MMO activity (Figure 3.6a). It appeared from these initial results that the change in the intracellular location of MMO activity due to the addition of copper was reflected by corresponding changes in the protein content of cell extracts. The difference in the molecular weights of the proteins associated with each of the MMO's further suggested that the transition from soluble to particulate MMO was not simply an association of existing proteins with the cell membrane.

The possibility that the transition from soluble to particulate MMO activity leads to the synthesis of new proteins in the membrane fraction of cell extracts was investigated using samples from the "switching" experiments outlined in Section 3.5. If particulate MMO activity was dependent on the expression of new proteins then the increased activity detected after addition of copper to the cells should be reflected by increased levels in the proteins visualized by SDS-PAGE. The results shown in Figure 3.7 indicate quite clearly that the increase in particulate MMO activity was paralleled by an increase in the expression of three major proteins (Mr 46,000, 35,000, 25,000). The samples from the soluble fractions of cell extracts from the same experiment (Figure 3.8) demonstrate the persistence of the subunits of Protein A of the soluble MMO despite the rapid loss of MMO activity that occurs when copper was added to the culture (see Figures 3.4 and 3.5). This result is discussed in the context of the inhibition of soluble MMO in section 6.3.

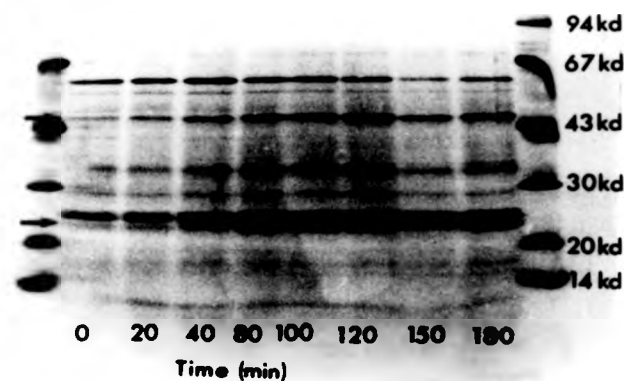


Figure 3.7      SDS-Polyacrylamide gel electrophoresis of membrane  
fractions of *M. capsulatus* (Bath)

SDS-polyacrylamide gel electrophoresis of samples from a "switchover" experiment showing the change in polypeptide banding patterns in membrane fractions over the course of the experiment. An increase in particulate MMO activity is accompanied by an increase in the level of three major proteins which are clearly discernable on the gel. Protein standards phosphorylase B (97,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), and lactalbumin (14,400). Proteins were stained using Coomassie Blue R250.

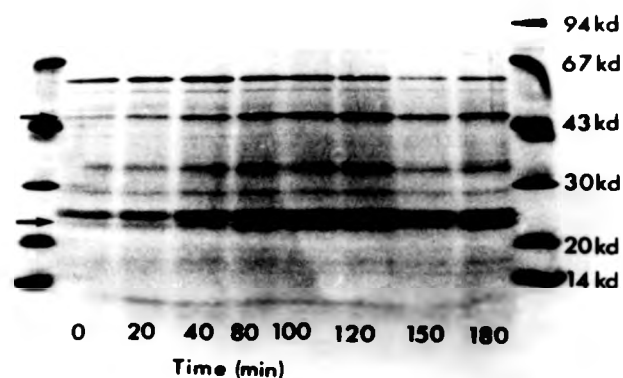


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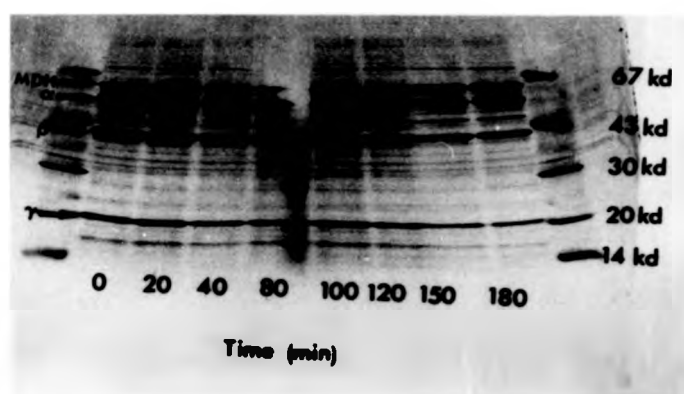


Figure 3.8      SDS-Polyacrylamide gel electrophoresis of soluble  
fractions of *M. capsulatus* (Bath)

SDS-polyacrylamide gel electrophoresis of samples from a "switchover" experiment showing the change in polypeptide banding patterns in soluble fractions over the course of the experiment. The loss in soluble MMO activity which occurs very rapidly (20 min) is not reflected in a loss of the three components of Protein A of the soluble MMO. Protein standards BSA (67,000), Ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and lactalbumin (14,400). Proteins were stained using Coomassie Blue R250.

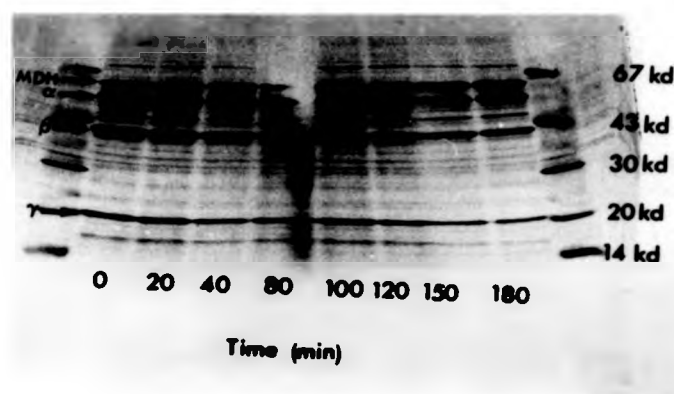


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SDS-polyacrylamide gel electrophoresis of samples from a "switchover" experiment showing the change in polypeptide banding patterns in soluble fractions over the course of the experiment. The loss in soluble MMO activity which occurs very rapidly (20 min) is not reflected in a loss of the three components of Protein A of the soluble MMO. Protein standards BSA (67,000), Ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and lactalbumin (14,400). Proteins were stained using Coomassie Blue R250.

It was clear from these results that the addition of copper to a culture of M. capsulatus (Bath) leads to a change in the intracellular location of MMO activity and that accompanying this change was the expression of at least three new proteins in the membrane fraction of cell extracts. This suggests that the transition from soluble to particulate MMO activity was not due to association of the pre-existing enzyme with the cell membrane, but was due to expression of a new membrane-bound MMO. This possibility was further investigated as outlined in the following sections.

### 3.7 Examination of membrane fractions expressing particulate MMO activity using antibody raised to components of the soluble MMO

The availability of antibody raised to purified components of the soluble MMO from M. capsulatus (Bath) allowed for an examination of membrane fractions containing particulate MMO activity to determine whether there was any cross-reaction between the particulate MMO and antibody raised to the soluble enzyme. The experiments involved the use of anti-Protein A antibody and anti-Protein C antibody which were kindly provided by Dr. J. Lund.

Two methods were used to try and determine if any cross-reactivity occurred; these were Ouchterlony double-diffusion plates and rocket gel electrophoresis (Laurell, 1966). The results showed that the membrane fraction containing the particulate MMO showed no cross-reaction to

antibody raised to either of the soluble MMO components. This lack of cross-reaction could be due to several possibilities including: i) association of Proteins A or C with the membrane may lead to conformational changes such that they no longer react with antibody raised to them when they are not membrane-bound; ii) association of the proteins with the membrane may lead to a masking of the antigenic site within the membrane such that it is not available for reaction with the antibody, or iii) that the membrane fraction does not contain either Protein A or Protein C. These possibilities were further examined by using a detergent, namely sodium deoxycholate, which released the particulate proteins from the membrane. The results were again negative, i.e. no cross-reactivity occurred suggesting that the proteins associated with particulate MMO activity are antigenically distinct from purified proteins of the soluble MMO.

### 3.8 The use of cell suspensions rather than chemostat cultures to study the transition from soluble to particulate MMO activity

The use of chemostat cultures to study the change in the intracellular location of MMO activity due to the addition of copper to the growth medium has been outlined in the preceding sections of this thesis. The results show that the transition from soluble to particulate MMO occurs over a relatively short period of time (approximately 20-40 min), but that the reverse transition took much longer due to the difficulty in removing copper from the growth medium. Therefore, experiments on the

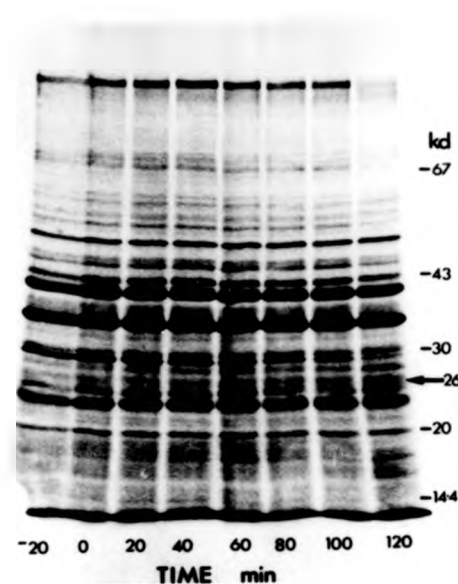
transition from soluble to particulate MMO were preceded by long periods of time which were necessary to return the chemostat to the low copper medium such that cells would exhibit 100% soluble activity. In order to overcome this difficulty an experiment was performed in which cells were removed from the chemostat and maintained with a methane/air atmosphere in a conical flask on a shaking water-bath at 45°C. Copper sulphate was added to the flask to give a final concentration of 1.2 mg l<sup>-1</sup>, samples were then removed to determine the intracellular location of the MMO. The results demonstrated that the transition from soluble to particulate MMO occurred in the same manner as was found in the chemostat (Section 3.5). The ability to study the transition in a flask rather than in the chemostat allowed for maintenance of cells, grown on low copper medium and exhibiting soluble MMO activity, that provided the constant source of cells necessary to detail the switch from soluble to particulate MMO.

3.9 Pulse-labelling studies to determine whether soluble MMO associates with cell membranes during the transition from soluble to particulate MMO activity

The demonstration in section 3.6 of this thesis that the expression of particulate MMO activity was accompanied by the increase in the presence of three proteins in the membrane fraction of cell extracts, coupled with the antibody results presented in Section 3.7, suggests that the two forms of the enzyme are composed of different proteins. It is however possible that the change in intracellular location of the MMO is

due to association of proteins, derived from the soluble MMU, with the cell membrane. This was investigated using pulse-labelling of cells with [ $^{35}\text{S}$ ]-methionine. The experimental details are given in the Materials and Methods section. The experiment involved pulse-radiolabelling of all the proteins in cells exhibiting 100% soluble MMU activity with [ $^{35}\text{S}$ ]-methionine, and then "switching" the cells to expression of particulate MMU activity by the addition of copper sulphate to the suspension. Samples were removed at various times after addition of copper sulphate, run on SDS-PAGE and then fluorographed. The resultant films are shown in Figure 3.9.

The results of the experiments detailed in Section 3.6 have shown that transition from soluble to particulate MMU activity leads to expression of three major proteins in the membrane fraction of cell extracts. If these proteins were derived from the proteins of the soluble MMU then during the course of the pulse-labelling experiment, the bands on the fluorograph associated with these proteins should increase in intensity due to the incorporation of the [ $^{35}\text{S}$ ]-methionine. If, however, the three proteins are newly synthesized (i.e. are not derived from soluble MMU) the intensity of the bands will not increase as the newly-synthesized proteins will incorporate only "cold" (unlabelled) methionine. The results showed that the proteins expressed by cells switched from soluble to particulate MMU activity are the product of de novo protein synthesis. This was further evidence that the transition from soluble to particulate MMU activity is not due to association/dissociation of a single enzyme with the cell membrane, but



**Figure 3.9**  $^{35}\text{S}$ -methionine pulse-labelling of cells during a switch from soluble to particulate MMO activity

Fluorograph of cell extracts of *M. capsulatus* labelled with  $^{35}\text{S}$ -methionine. The cells were "switched" from soluble to particulate MMO activity by the addition of copper sulphate ( $1\text{ mg l}^{-1}$ ) to the cell suspension. The molecular weight standards are marked and the 26 Kd protein is indicated. The figure shows that during the course of the experiment there is no increase in radiolabel in the 26 Kd protein showing that it is not derived from the soluble MMO which is fully labelled.

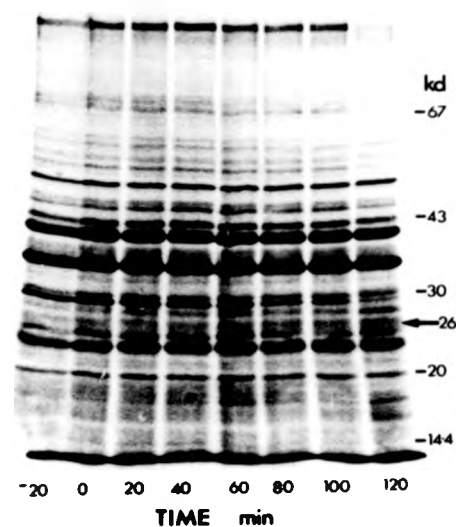


Figure 3.9 <sup>35</sup>S-methionine pulse-labelling of cells during a switch from soluble to particulate MMU activity

Fluorograph of cell extracts of *M. capsulatus* labelled with <sup>35</sup>S-methionine. The cells were "switched" from soluble to particulate MMU activity by the addition of copper sulphate (1 mg l<sup>-1</sup>) to the cell suspension. The molecular weight standards are marked and the 26 Kd protein is indicated. The figure shows that during the course of the experiment there is no increase in radiolabel in the 26 Kd protein showing that it is not derived from the soluble MMU which is fully labelled.

that the two activities are due to the presence of two distinct enzymes in the cell. This hypothesis is further examined in Section 5 of this thesis.

### 3.10 Thin-section electron microscopy of *M. capsulatus* (Bath) grown under different conditions in chemostat culture

#### 3.10.1 Background

As discussed in the general introduction to this thesis, methane-utilizing bacteria are known to possess complex arrays of intracytoplasmic membranes (Smith and Ribbons, 1970; Smith et al., 1970; Whittenbury et al., 1970). The intracellular structure of *M. capsulatus* (Bath) had not been examined prior to this study, but there were reports on the membrane structure of the related species *M. capsulatus* (Texas) (Procter et al., 1969; Smith et al., 1970). These concluded that the organism "possessed elaborate intracellular membranes arranged in stacks of disc-shaped sacs". The membranes were thought to be intimately associated with the oxidation of methane and/or the transduction of energy to or from the enzyme concerned (Ribbons, 1975). In 1979 Hyder et al. investigated the membrane structure of *M. capsulatus* (Texas) grown on different carbon/energy sources (methane vs. methanol), and also during different stages of growth (exponential vs. stationary). They demonstrated that the organism, when grown on methane, exhibits a progressive degeneration of its internal membrane structure and organization with increasing age of the culture. The loss

of membrane organization in older cultures has also been observed in a Methylomonas sp. by De Boer and Hazeu (1972). The results presented in this thesis have shown that during batch growth of M. capsulatus (Bath) the intracellular location of MMO activity changes from a membrane-bound enzyme to a soluble enzyme. It is therefore possible that the changes observed by Hyder et al. (1979) during the batch growth of M. capsulatus (Texas) may be a response to a transition in the intracellular location of the MMO in this organism. A study was therefore undertaken to investigate the membrane structure of M. capsulatus (Bath) grown in batch and continuous culture under conditions which lead to expression of the two forms of MMO activity.

#### 3.10.2 Experimental

The organism was grown in batch and continuous culture as described previously (sections 3.2 and 3.3) with either 100% soluble, 100% particulate or mixed amounts of soluble/particulate MMO activity. Samples were prepared for thin-section electron microscopy as described in Materials and Methods. The results are shown in Figures 3.10 and 3.11. Cells expressing 100% particulate activity had extensive tightly-packed arrays of intracytoplasmic membranes throughout the cell. These sections were very similar to the electron micrographs of M. capsulatus (Texas) during exponential growth phase as reported by Hyder et al. (1979). Cells expressing 100% soluble activity were of similar size and shape to those with particulate MMO activity, but the intracytoplasmic membranes were less abundant and were not as tightly packed as shown previously. These sections correspond to the sections of M. capsulatus

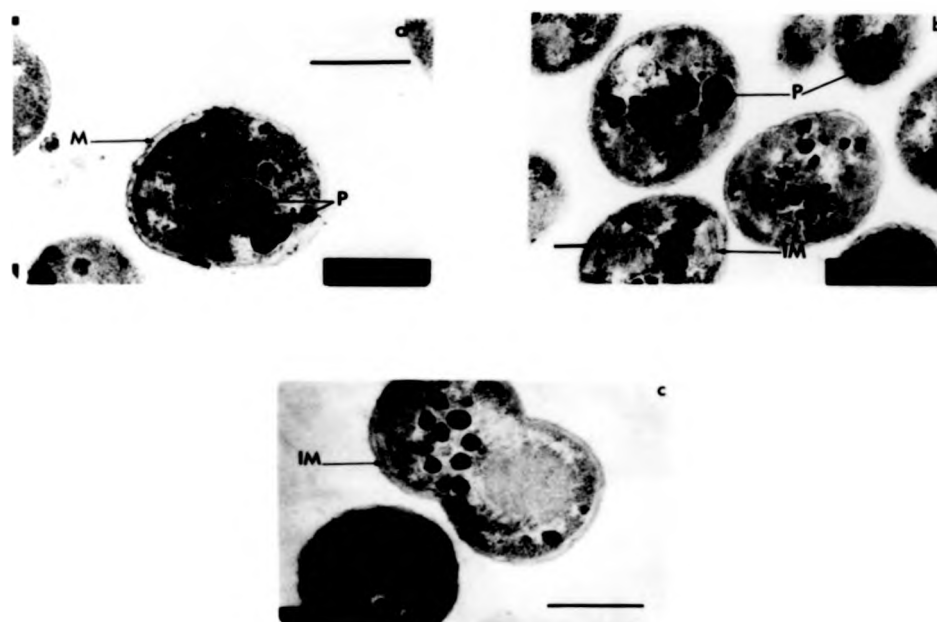


Figure 3.10      Electron photomicrographs of thin-sections of *M. capsulatus* grown on methane in a low copper medium

Electron photomicrographs of thin-sections of *M. capsulatus* grown on methane. Cells expressing soluble MMO activity (a) are characterized by membranes at the periphery (M) and a series of electron-dense particles (P). Samples from cultures expressing both soluble and particulate MMO activity (b) and (c) exhibit a mixed morphology with electron-dense particles (P) and poorly developed intracytoplasmic membranes (IM). Bar markers represent 0.5  $\mu\text{m}$ .

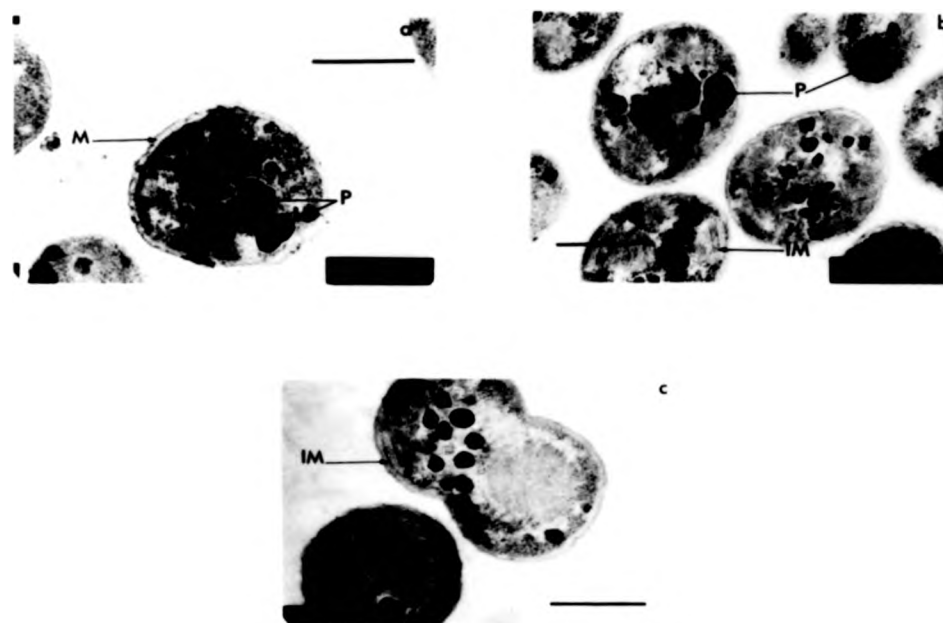


Figure 3.10      Electron photomicrographs of thin-sections of *M. capsulatus* grown on methane in a low copper medium

Electron photomicrographs of thin-sections of *M. capsulatus* grown on methane. Cells expressing soluble MMO activity (a) are characterized by membranes at the periphery (M) and a series of electron-dense particles (P). Samples from cultures expressing both soluble and particulate MMO activity (b) and (c) exhibit a mixed morphology with electron-dense particles (P) and poorly developed intracytoplasmic membranes (IM). Bar markers represent 0.5  $\mu\text{m}$ .

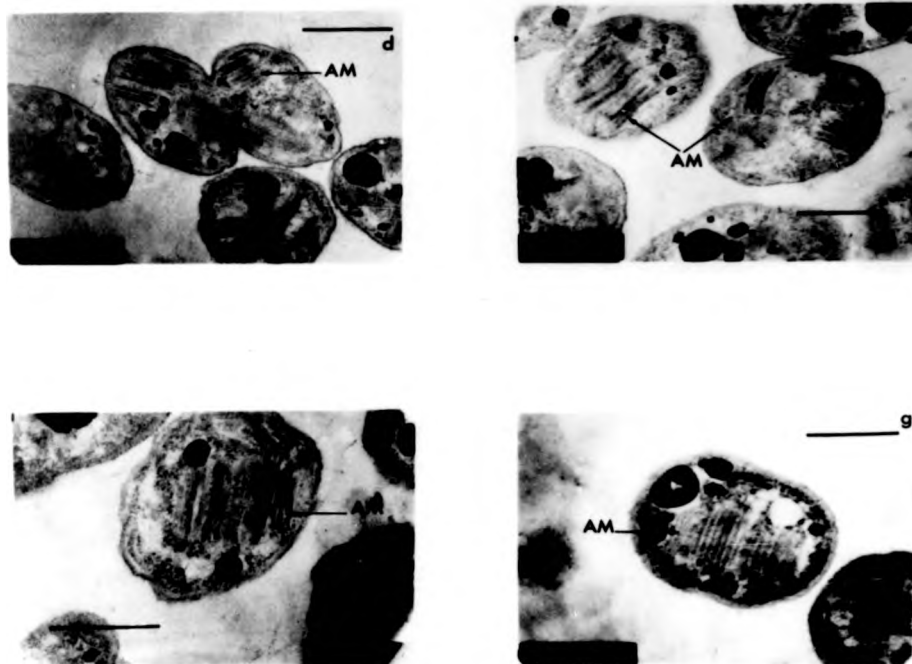


Figure 3.11      Electron photomicrographs of thin sections of *M. capsulatus* grown on methane in a high copper medium

Electron photomicrographs of thin-sections of *M. capsulatus* grown on methane in a higher copper medium. Cells expressing particulate MMO activity are characterized by large numbers of arrays of intracytoplasmic membranes (AM). Bar markers represent 0.5  $\mu\text{m}$ .

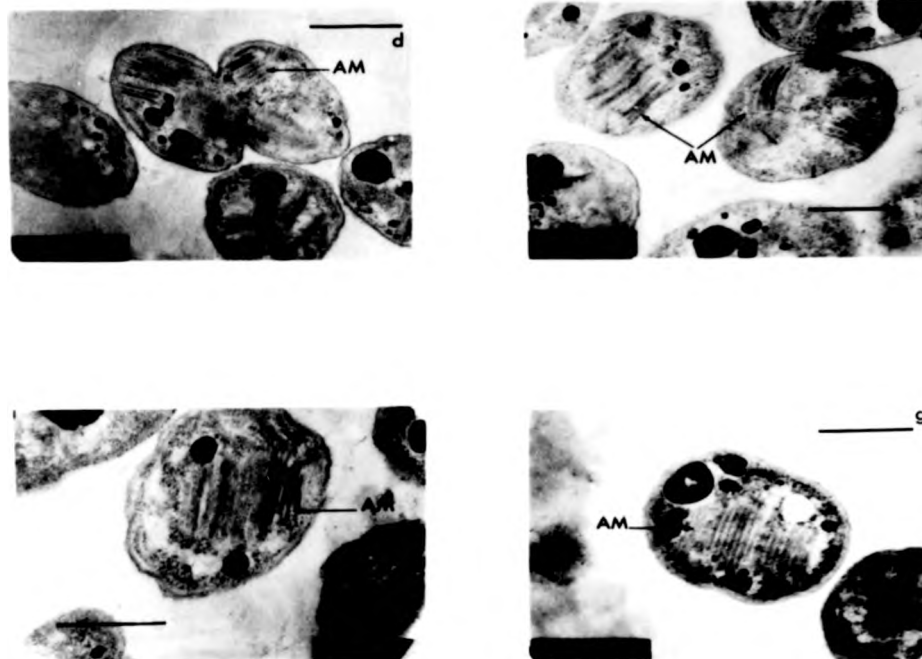


Figure 3.11      Electron photomicrographs of thin sections of *M. capsulatus* grown on methane in a high copper medium

Electron photomicrographs of thin-sections of *M. capsulatus* grown on methane in a higher copper medium. Cells expressing particulate MMU activity are characterized by large numbers of arrays of intracytoplasmic membranes (AM). Bar markers represent 0.5  $\mu\text{m}$ .

(Texas) during late exponential and early stationary phase of growth (Hyder et al., 1979).

These results suggest that the expression of particulate MMO activity was linked to the presence of tightly packed arrays of intracytoplasmic membranes and that a transition to expression of soluble MMO activity was accompanied by a decrease in the organization of these membrane arrays. The fact that the intracellular content of cells changes at different phases of growth and under different growth conditions (see also Section 4), means that assertions that there exists a direct correlation between membrane orientation and the type of intermediary metabolism (Whittenbury et al., 1976) should be treated with caution. The results of these experiments appear to demonstrate an intimate association between expression of particulate MMO activity and the presence of intracytoplasmic membranes, thus supporting the proposals of Ribbons and Michalover (1970), Ribbons et al. (1970) and Ribbons (1975). The results presented here also suggest that the presence of intracytoplasmic membranes may not be essential in cells expressing soluble MMO activity as under these conditions the level of such membranes is decreased. It is not, however, possible at this time to determine the precise role that intracytoplasmic membranes play in the metabolism of methanotrophs.

### 3.11 Final Comments

The work presented in this chapter provides the first report of membrane-bound MMO activity in cell extracts of M. capsulatus (Bath). The organism was found to be capable of expressing two forms of MMO activity, one membrane-bound (particulate MMO) and one not bound to cell membranes (soluble MMO). Experiments showed that during batch growth the relative levels of the two forms of the enzyme fluctuated as a result of a presumed nutrient limitation. Studies performed in batch and continuous culture established that the nutrient concerned was copper and that conditions could be maintained in continuous culture, such that expression of either form of the enzyme was favoured. Conditions were also established under which the organism exhibited either 100% soluble or 100% particulate activity, it was also demonstrated that conditions could be attained such that both forms of the enzyme were expressed in the same culture.

The addition of copper to a copper-limited culture demonstrated that the transition from 100% soluble to 100% particulate activity was dependent solely on the level of copper in the growth medium. The "switching" experiments also demonstrated the concerted effect of copper which caused an increase in expression of particulate MMO activity and inhibited soluble MMO activity.

SDS-polyacrylamide gel electrophoresis provided evidence for a change in the expression of cellular proteins due to the addition of copper and

pulse-labelling experiments and antibody experiments, suggested that the transition from soluble to particulate MMO activity was not due to association of the soluble enzyme with the cell membrane. Thin-section electron microscopy demonstrated that the transition from one form of the enzyme to the other led to a change in the morphology of intracytoplasmic membranes and that the expression of particulate MMO activity was intimately associated with the presence of intracytoplasmic membranes with the cell.

The study of how copper regulates expression of the particulate MMO may be of great value in view of the interest in this organism as a biocatalyst (see Introduction), as the ability to control the expression of particulate MMO by production of mutants may be more convenient than the addition of copper ions to the growth medium. Certainly anyone attempting to determine the genetic systems of the methane-oxidising bacteria should consider the growth conditions very carefully before embarking on experimentation.

### 3.12 Further examples of regulation of expression of proteins by metal ions

The regulation of genes by metal ions is not a new concept and there are many examples of such systems to be found in the literature. The induction of metalloproteins by metal ions has been observed in a wide range of animal tissues and microorganisms. The prototype for such

studies has been the protein Ferritin, a major iron storage protein in liver and other organs (Drysdale and Munro, 1966). Since these studies numerous reports have appeared describing the regulation of non-ferrous metalloproteins and metalloenzymes by specific metal ions (e.g. Evans et al., 1970; Winge and Rajagopalan, 1972; Premakumar et al., 1975; Butt et al., 1984). The majority of metalloproteins discovered thus far function as enzymes which often have limited and quite specific binding capacities for metals and thus may have regulatory mechanisms different from those of metallothioneins which are often induced by a number of different metal ions. The metal ion component may not be required for synthesis of the protein but may be required for final assembly of a protein into a functioning enzyme, an example of this form of metal ion regulation is yeast cytochrome oxidase which requires copper ions for fully functional activity (Keyhani and Keyhani, 1975).

Returning to the specific activation of genes required for synthesis of metalloenzymes, an example of such regulation is the induction, by copper, of lysyl oxidase, a copper metalloenzyme found in aortic tissue (Rayton and Harris, 1979). The synthesis of this enzyme is closely controlled by the availability of copper - a phenomenon which has been demonstrated in vivo and in vitro (Harris, 1976; Rayton and Harris, 1979). A similar control of gene expression by copper has been reported in Saccharomyces cerevisiae by Butt et al. (1984) who demonstrated that the addition of copper to yeast cells led to the induction of a low molecular weight protein that binds copper. They further showed that transcriptional activation of the gene coding for the protein has a

specific requirement for copper indicating that metal ions can be very closely involved in expression of proteins at the earliest stage of synthesis.

Metal ion activation of proteins in bacteria has also been observed and one of the best examples is the induction of the enzyme superoxide dismutase (SOD) in E. coli. The enzyme in E. coli has two forms, one is a manganese-containing enzyme (MnSOD) and the other an iron-containing enzyme (FeSOD). In a recent set of experiments Pugh et al. (1984) demonstrated that in E. coli growth of the organism in media enriched with Mn(II) resulted in the elevation of the levels of MnSOD, whereas growth in medium enriched with Fe(II) caused increased content (Sic) of the FeSOD. Enrichment with Co(II), Cu(II), Mo(IV), Zn(II) or Ni(II) had no effect indicating the specificity of metal ions for inducing metalloenzymes as mentioned earlier. In this case the apoenzyme (i.e. the enzyme without the metal ion inserted) acted as an autogenous repressor so that on addition of the metal ion the apoenzyme was immediately activated and this activation derepressed the gene encoding for production of further enzyme.

Although there is no evidence for a similar mechanism in M. capsulatus (Bath) involving copper, the observation that addition of copper to preparations of cell extracts leads to an immediate increase in in vitro particulate MMU activity (see Chapter 5) suggests that the organism may contain an apoenzyme that requires copper for full activity. It is tempting to extrapolate and suggest that this may be analogous to the

situation in E. coli and that addition of copper to a culture of M. capsulatus permits activation of an apoenzyme which in turn derepresses the gene encoding for particulate MMO, which then leads to synthesis of more of this enzyme. Obviously at this point this suggestion requires experimental verification, but such a system may account for the results so far obtained and presented in this thesis.

Chapter 4

The Effect of Copper ions on Methanol grown  
cells of M. capsulatus (Bath)

#### 4.1 Introduction

Several reports have appeared in the literature concerning the growth of methylotrophs on methanol as their sole source of carbon and energy. Early reports suggested that growth was poor and that methanol was toxic to methylotrophs even at concentrations as low as 0.01% (v/v) (Leadbetter and Foster, 1958; Stocks and McCleskey, 1964; Whittenbury et al., 1970). The reason for this poor growth was not known, but it has been suggested that growth may be inhibited by the accumulation of formaldehyde in the culture supernatant of methanol grown cultures (Ribbons et al., 1970). The accumulation of formaldehyde in the medium of shake-flask grown cultures of Methylococcus NCIB 11083 was demonstrated by Linton and Vokes (1978); they also reported that viability of the organism was lost after six successive transfers in shake flask culture with 0.1% (v/v) methanol as sole source of carbon and energy. Formaldehyde inhibition of whole cells has been reported in Methylomonas sp. BC3 at 0.015% (w/v) (Chen et al., 1977) and in Methylocystis parvus OBBP at 0.012% (w/v) (4 mM) (Hou et al., 1979a). The mechanism of formaldehyde inhibition is not yet clear but can be overcome by two means; growth of methylotrophs in shake-flask culture on methanol can be achieved by using low concentrations of methanol in the starter culture medium and slowly increasing the concentration over successive transfers to fresh medium so that cells become "adapted" to growth on methanol. Using this method it was possible to adapt Methylocystis parvus OBBP to growth on methanol at a concentration of 4% (w/v) (Hou et al., 1979a). Growth on methanol can also be achieved by

growing the organism in chemostat culture under methanol limitation in which the methanol concentration in the in-flowing medium was 0.25% v/v but the concentration in the vessel was ostensibly zero (Linton and Vokes, 1978; Best and Higgins, 1981). It is not clear at this time whether the methods outlined above for growth on methanol are successful due to the production of methanol-tolerant mutants or whether the original cells become tolerant of higher levels of methanol by increasing the concentration of one or more existing enzymes.

#### 4.2 Presence of MMO and intracytoplasmic membranes in Methanol-grown cultures

There have been several conflicting reports in the literature concerning the presence of the MMO enzyme in methanotrophs which have been grown on methanol. The Exxon group have claimed that growth of Methylosinus trichosporium OB3b, Methylococcus capsulatus CML-M1 and Methylobacterium organophilum CML-26 on methanol caused the loss of MMO activity and they concluded that MMO was induced by methane (Hou et al., 1979b). Other reports of the growth of methanotrophs on methanol (Linton and Vokes, 1978; Hyder et al., 1979; Chetina and Trotsenko, 1981; Best and Higgins, 1981) have shown that MMO activity was retained when cells were grown for periods up to nine months on methanol. As outlined in the previous chapter it has recently been demonstrated that the intracellular location of MMO activity in Methylococcus capsulatus (Bath) and Methylosinus trichosporium OB3b was dependent on the

copper:biomass ratio in the growth medium (Stanley et al., 1983).

Thin section electron micrographs of methanotrophs grown on methanol have also been contradictory; several workers have demonstrated that growth on methanol causes a loss of intracytoplasmic membranes (Patt and Hanson, 1978; Hyder et al., 1979), whereas other groups have shown that such structures were maintained during growth on methanol (Linton and Vokes, 1978; Takeda and Tanaka, 1980; Best and Higgins, 1981). It was further suggested by Best and Higgins (1981) that in Methylosinus trichosporium OB3b, because the presence of methane or methanol as growth substrate does not influence the formation of membranes, the intracytoplasmic membranes present in this organism are not induced by methane per se and are not obligatorily linked to the oxidation of methane.

The purpose of this investigation was to determine to what extent MMO is synthesized in methanol grown M. capsulatus (Bath) and how the concentration of copper in the medium affects the activity and intracellular location of MMO. In addition to the above aims an examination of the intracellular morphology of the organism was undertaken to try and determine the relationship, if any, between carbon source, methane-oxidising activity and intracytoplasmic membrane content of the cells.

#### 4.3 Growth of *Methylococcus capsulatus* (Bath) on Methanol in continuous culture

In order to overcome the long periods of time involved with "training" the organism to grow on methanol as sole carbon and energy source (Hou *et al.*, 1979a) a new method was established which was much more rapid, but still resulted in a culture that was able to utilise methanol as sole carbon and energy source.

*Methylococcus capsulatus* (Bath) was initially grown on methane with no methanol present in the growth medium. When a steady-state culture was attained 0.5% (v/v) methanol was added to the growth medium and the methane supply reduced to zero over a period of 4d (40 hrs). Subsequently the methanol concentration in the medium was raised to 1.0% (v/v) (Prior and Dalton, 1985). The culture was maintained at an OD<sub>540</sub> of 10 (approximately 3 mg dry wt ml<sup>-1</sup>) under methanol-limiting conditions for 2 months. Samples of culture supernatant assayed by gas chromatography showed no detectable levels of methanol or formaldehyde.

#### 4.4 The Effect of copper on MMO activity in *M. capsulatus* (Bath) grown on methanol

It has recently been reported (Stanley *et al.*, 1983) that the intracellular location of MMO in *Methylococcus capsulatus* (Bath) grown on methane was dependent on the copper:biomass ratio of the growth

medium. To determine whether there was a copper-dependent change in localization of the MMO activity when cells were grown on methanol the organism was grown at a variety of copper concentrations.

Cells were grown on 1.0% (v/v) methanol as sole carbon and energy source in chemostat culture until steady-state conditions were attained at which time samples were taken for analysis. The copper sulphate concentration in the medium was then increased and a new steady-state reached before further samples were taken. This process was repeated for each of the copper concentrations studied (0, 0.2, 0.6, 1.0, 1.2, 2.0 mg  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O l}^{-1}$ ).

When M. capsulatus was grown on methane as the sole carbon and energy source an increase in copper sulphate concentration in the medium led to an increase in cell density of approximately 25-30% as evidenced by increased  $\text{OD}_{540}$  and dry wt of cells ( $\text{mg ml}^{-1}$ ), (see previous chapter). However, with growth on methanol, the addition of copper to the medium did not lead to an increase in cell density. This phenomenon of an increase in cell density when copper is added to methane-grown cells, but not to methanol-grown cells has also been observed in batch cultures of the obligate methylotroph Methanomonas margaritae (Takeda and Tanaka, 1980). The reasons for the change in cell density were discussed in the previous chapter.

Addition of copper to methanol-grown cells does, however, affect the activity of the MMO located in the particulate fraction of cell

extracts. As the copper concentration in the growth medium was raised there was an increase in the specific activity of the particulate MMU located in the membrane fraction of cell extracts (Table 4.1). This activity was totally inhibited by the addition of 0.1 mM potassium cyanide to the assay system showing that the enzyme activity was due to the particulate form of MMU rather than the soluble MMU, since potassium cyanide is a potent inhibitor of the particulate, but not the soluble, form of the enzyme (Stanley et al., 1983).

The lack of soluble MMU activity in cell extracts from M. capsulatus grown on methanol could have been due to the absence of any one of the components of the soluble enzyme complex, and in order to confirm that this was not the case purified components of the soluble MMU (provided by Dr. J. Lund) were added back to the cell extracts. Neither Protein A, B or C of the MMU complex nor any combination of these components reconstituted MMU activity, but addition of all three components to the soluble fraction gave activity and showed that there was no inhibitor of propylene oxidizing activity present in the extract. The protein banding pattern on SDS-PAGE (Figure 4.1a) confirmed the absence of the three subunits (Mr 54,000, 36,000, 17,000) associated with Protein A of soluble MMU (Woodland and Dalton, 1984a). This further demonstrates the lack of expression of soluble MMU by M. capsulatus grown on methanol.

The increase in particulate MMU activity with increasing copper concentration in the growth medium suggests that copper may be acting as an inducer for the expression of proteins associated with MMU activity.

Table 4.1      Effect of copper sulphate on Particulate MMO activity in  
cell extracts of M. capsulatus (Bath) grown on methanol

<u>CuSO<sub>4</sub> in Growth</u>	<u>Particulate MMO Specific Activity</u>
<u>medium (mg l<sup>-1</sup>)</u>	<u>[nmol propylene oxide formed min<sup>-1</sup> (mg protein)<sup>-1</sup>]</u>
0	0
0.2	3.9 ± 0.5
0.6	10.5 ± 1.6
1.0	44.7 ± 1.4
1.2	110.1 ± 4.7
2.0	58.2 ± 10.1

Values given are the means of three experiments performed on separate days on samples taken from the chemostat. Included is the spread of results from the mean.

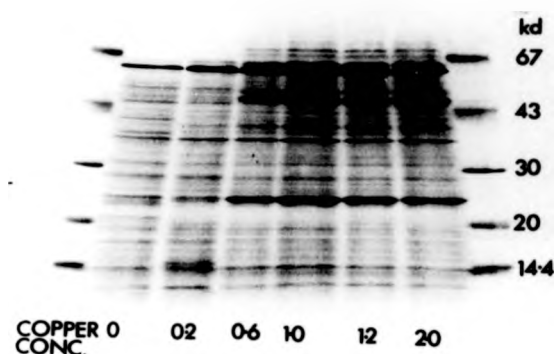


Figure 4.1a      SDS-polyacrylamide gel electrophoresis of cell extracts  
of M. capsulatus (Bath) grown on methanol - Effect of  
copper on the protein content of soluble fractions of  
cell extracts

SDS-PAGE of soluble fractions of cell extracts on methanol with different concentrations of copper sulphate in the growth medium. Standards used were BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and lactalbumin (14,400). Proteins were stained using Coomassie Blue R250.

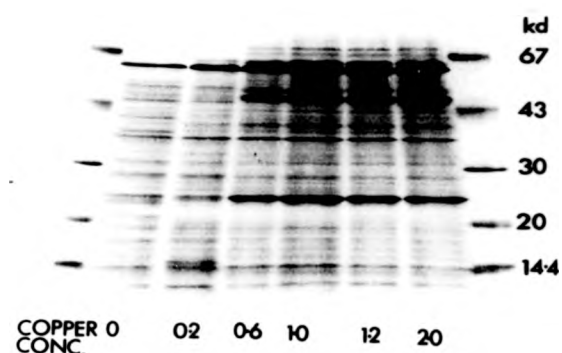


Figure 4.1a      SDS-polyacrylamide gel electrophoresis of cell extracts  
of *M. capsulatus* (Bath) grown on methanol - Effect of  
copper on the protein content of soluble fractions of  
cell extracts

SDS-PAGE of soluble fractions of cell extracts on methanol with different concentrations of copper sulphate in the growth medium. Standards used were BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and lactalbumin (14,400). Proteins were stained using Coomassie Blue R250.

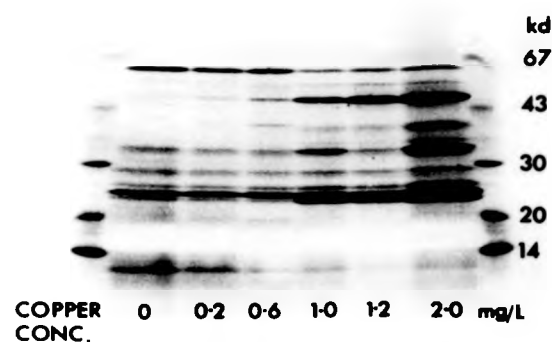


Figure 4.1b

SDS-polyacrylamide gel electrophoresis of cell extracts of *M. capsulatus* (Bath) grown on methanol - Effect of copper on the protein content of soluble fractions of cell extracts

Samples from the same experiment as Figure 4.1a, but showing the effect of different concentrations of copper sulphate in the growth medium on particulate fractions of cell extracts. Protein standards were as in Figure 4.1a. Proteins were stained using Coomassie Blue R250.

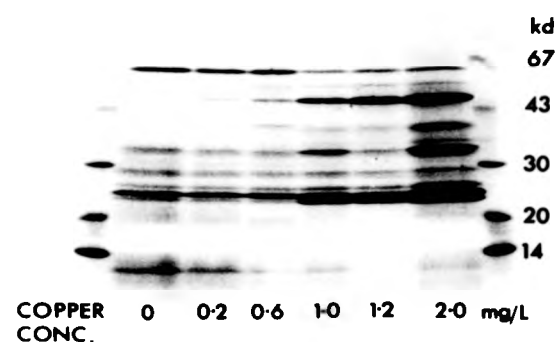


Figure 4.1b

SDS-polyacrylamide gel electrophoresis of cell extracts  
of *M. capsulatus* (Bath) grown on methanol - Effect of  
copper on the protein content of soluble fractions of  
cell extracts

Samples from the same experiment as Figure 4.1a, but showing the effect of different concentrations of copper sulphate in the growth medium on particulate fractions of cell extracts. Protein standards were as in Figure 4.1a. Proteins were stained using Coomassie Blue R250.

This was investigated by examining the protein banding patterns of membrane fractions on SDS-PAGE, grown with different concentrations of copper in the growth medium. The results are shown in Figure 4.1b and demonstrate that in response to an increase in the level of copper in the growth medium, there was an increase in the expression of at least three major proteins, associated with the membrane fraction, which were either absent or present at very low levels in copper-deficient media. These proteins had molecular weights similar to the protein bands that increase when methane-grown cells were switched from conditions under which they exhibited soluble MMO activity to those where they exhibited particulate MMO activity (Mr 46,000, 35,000, 25,000) (Stanley et al., 1983; Dalton et al., 1984).

It was therefore apparent that when M. capsulatus was grown on methanol as sole carbon and energy source expression of particulate MMO activity was regulated by the availability of copper ions, but, unlike the situation where the growth substrate was methane, the cells cannot express the soluble form of the enzyme. The inability to overcome the copper-limitation on the expression of particulate MMO activity does not however lead to a limitation on the growth of the organism (such as is the case with Methylomonas albus Bg8, Methylocystis parvus OBBP and Methanomonas margaritae as discussed in the previous chapter) as growth on methanol was not dependent on expression of MMO activity. When methane was the sole source of carbon and energy the ability of M. capsulatus (and M. trichosporium) to overcome a limitation on growth due to copper involves the expression of a non-copper requiring (soluble)

MMO. When M. capsulatus was grown on methanol the lack of copper in the growth medium does not lead to expression of a soluble MMO due to the fact that the cell does not have an obligate requirement for methane monooxygenase activity as it can derive carbon and energy from methanol via methanol dehydrogenase. These results indicate that in M. capsulatus (Bath) expression of particulate MMO activity was regulated by copper ions rather than methane.

#### 4.5 The Effect of Copper on In Vitro MMO activity of methanol-grown M. capsulatus (Bath)

The particulate MMO exhibited by M. capsulatus grown on methanol was shown to exhibit maximal activity at pH 7.5 and was stimulated by the addition of copper ions to the assay system at pH 7.0. No stimulation of particulate MMO activity was observed when copper ions were added to the assay system at pH 7.5. This phenomenon has also been observed when M. capsulatus was grown on methane (Chapter 5 - this thesis) the reasons for this observation have been discussed in detail in Chapter 5 of this thesis. It is important to note that at very low concentrations of copper in the growth medium (less than  $0.2 \text{ mg l}^{-1}$ ) it was only possible to detect in vitro particulate MMO activity in methanol-grown cells by adding copper to the assay system (Table 4.2). The results demonstrate that when M. capsulatus (Bath) was grown on methanol as sole carbon and energy source, methane-oxidising activity is maintained supporting the results of Linton and Vokes (1978), Takeda and Tanaka (1980), Chetina

Table 4.2      Effect of copper sulphate on the assay system of cell  
extracts of M. capsulatus (Bath) grown on methanol

<u>CuSO<sub>4</sub> in</u>	<u>Particulate MMO Specific Activity</u>	
<u>Growth Medium</u>	<u>[nmol propylene oxide formed min<sup>-1</sup> (mg protein)<sup>-1</sup>]</u>	
<u>(mg l<sup>-1</sup>)</u>	<u>Normal Assay (pH 7.5)</u>	<u>CuSO<sub>4</sub> in Assay (pH 7.0, 0.4mMCu<sup>2+</sup>)</u>
0	0	4.1 ± 1.1
0.2	3.9 ± 0.5	7.3 ± 1.1
0.6	10.5 ± 1.6	18.7 ± 0.9
1.0	44.7 ± 1.4	75.4 ± 2.5
1.2	110.1 ± 4.7	175.6 ± 5.5
2.0	58.2 ± 10.1	92.1 ± 16.1

Values given are the means of three experiments performed on separate days on samples taken from the chemostat. Included is the spread of results from the mean.

and Trotsenko (1981) and Best and Higgins (1981). Furthermore, the results indicate that this activity was solely associated with the particulate MMO, that expression of this form of the enzyme was regulated by copper ions and that the particulate MMO can be expressed in the absence of methane.

#### 4.6 The Effect of addition of copper on whole cell oxidation rates of methanol-grown *M. capsulatus* (Bath)

Most of the work on the retention of methane-oxidising activity when organisms were grown on methanol was performed with whole cells. This was because of the problems of obtaining reproducible in vitro methane-oxidising activity. The results of experiments on whole cell oxidation rates of methanol-grown *M. capsulatus* (Bath) are shown in Table 4.3.

With no copper in the growth medium the rate of oxygen consumption when methane was added to the cell suspension was only 15% of the rate of oxygen consumption when  $1.2 \text{ mg l}^{-1}$  of copper sulphate was present in the growth medium. This is presumably due to the increased levels of particulate MMO which are present at the higher copper concentration (see previous Section 4.5). It should be noted at this point that although the concentration of copper in the growth medium was nominally zero it was possible that traces of copper may have been present in the growth medium, even though all chemicals were of analytical grade. The fact that neither ethylene nor propylene were oxidized by whole cells in

Table 4.3      Effect of adding  $\text{CuSO}_4$  to the growth medium on whole  
cell oxidation rates of *M. capsulatus* (Bath) grown on  
methanol

<u>Oxidation Rates</u>		
<u><math>[\text{nmol O}_2 \text{ consumed min}^{-1} (\text{mg protein})^{-1}]</math></u>		
<u>Substrate</u>	<u>No <math>\text{Cu}^{2+}</math> in</u>	<u><math>\text{Cu}^{2+}</math> (1.2 mg l<sup>-1</sup>)</u>
<u>(concn. mM)</u>	<u>Growth medium</u>	<u>in Growth Medium</u>
Methane (0.1)	61 $\pm$ 5	480 $\pm$ 22
Methanol (1.0)	130 $\pm$ 4	312 $\pm$ 10
Ethanol (1.0)	100 $\pm$ 10	158 $\pm$ 16
Formaldehyde (1.0)	94 $\pm$ 4	117 $\pm$ 4
Formate (1.0)	86 $\pm$ 7	94 $\pm$ 10
Ethylene (1.2)	0	12 $\pm$ 2
Propylene (1.5)	0	6 $\pm$ 3
Acetylene (1.2)	0	0

Values given are the means of two experiments. Included is the spread of results from the mean. Experiments were performed as outlined in Materials and Methods.

the absence of electron donors was due to the fact that unlike the other compounds tested, these gases cannot be further metabolised by the cell and so cannot supply the reducing equivalents necessary for continued MMO activity (Stirling and Dalton, 1979).

The influence of copper ions on the whole-cell oxidation rates of M. capsulatus (Bath) grown on methanol may provide an explanation for the lack of epoxidising and hydroxylating activity in methanol-grown cells which was reported by Hou et al. (1979b). The epoxidation rates of whole cell suspensions grown on methane reported by these workers were very low, presumably due to the small number of cells present in the assay system; when grown on methanol the combined effect of the low number of cells and the low level of copper in the growth medium (5  $\mu\text{g}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per litre) would probably reduce the whole cell oxidation rates to undetectable levels.

#### 4.7 The intracellular location of MMO activity in organisms grown on methanol

As discussed in an earlier section, the majority of studies on retention of methane-oxidising activity when methanotrophs are grown on methanol have been performed on whole cells. Apart from the results presented here there are only two other reports of in vitro MMO activity in cells grown on methanol, these involve the organisms Methylomonas methanica (Chetina and Trotsenko, 1981) and Methylosinus trichosporium UB3b (Best

and Higgins, 1981). The results of the experiments with M. methanica are similar to the results for M. capsulatus presented in this thesis, i.e. the MMO activity which was retained when the cells were grown on methanol occurs exclusively in the particulate fractions of cell extracts (Chetina and Trotsenko, 1981). In contrast to these results Best and Higgins (1981) reported that the MMO activity which was retained when M. trichosporium was grown on methanol occurred in the soluble fraction of cell extracts. It is therefore apparent that M. capsulatus and M. methanica exhibit a different (particulate) form of MMO to M. trichosporium when the organisms are grown on methanol. Whether this difference is due to an as yet unknown factor in the experiments (as was the case with copper when cells were grown on methane - see Chapter 3), or whether the difference is a reflection of a divergence between Type II organisms, such as M. trichosporium, and other methanotrophs has not yet been resolved.

#### 4.8 Characteristics of the Particulate MMO from M. capsulatus (Bath) grown on methanol

The particulate MMO observed when M. capsulatus was grown on methanol was examined with respect to inhibitor profile, electron donor specificity and ability to oxidize aromatic and alicyclic compounds to determine whether it was different from the particulate MMO expressed by cells grown on methane.

The methanol-grown enzyme was found to be totally inhibited by potassium

cyanide, thiourea, 2-mercaptoethanol and acetylene at concentrations similar to the concentrations of the same compounds that caused total inhibition of particulate MMO isolated from methane-grown cells. NAD(P)H was found to be the only electron donor for in vitro activity and membrane fractions could not oxidize benzene, phenol or cyclohexane. These results are the same as those reported for particulate MMO from methane-grown cells (Stanley et al., 1983; Dalton et al., 1984), and show that the enzyme expressed when cells are grown on methanol is similar to the enzyme found in membrane fractions of M. capsulatus grown on methane under conditions which lead to expression of particulate MMO activity.

#### 4.9 Thin-section electron microscopy of methanol-grown cells of M. capsulatus

Thin sections of cells grown on methanol were prepared using the method previously employed for cells grown on methane and which is outlined in the Materials and Methods section of this thesis. Thin-section electron microscopy of M. capsulatus grown on methanol showed that in copper-deficient medium (i.e. copper concentration in growth medium is zero) the cells were smaller than those grown in copper containing medium and that they totally lacked intracytoplasmic membranes (ICM) (Figure 4.2). The cells appeared to have no internal membrane structures but contained inclusions similar to the electron-translucent droplets described by Hyder et al. (1979) who grew M. capsulatus (Texas) on methanol.

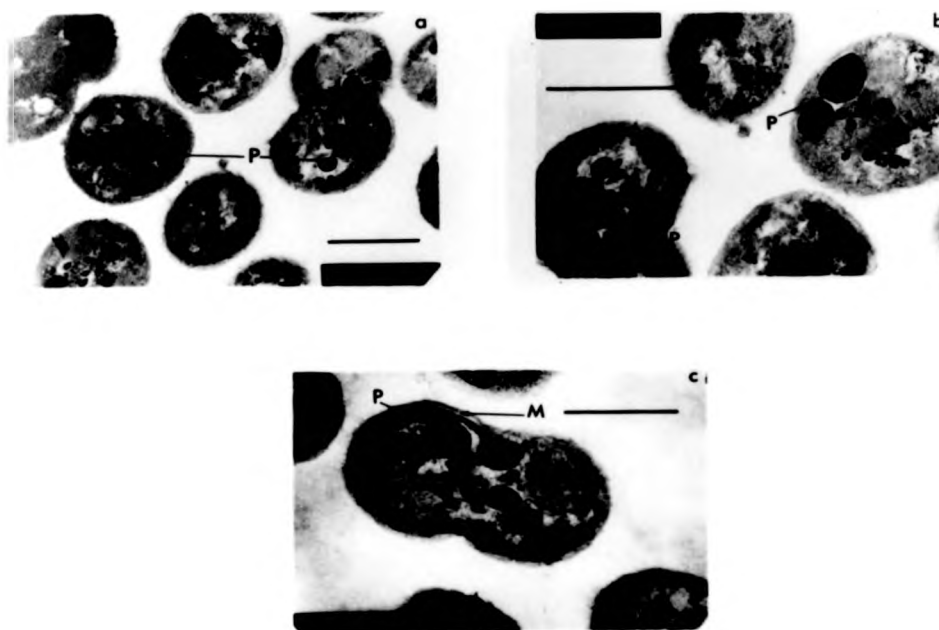


Figure 4.2      Thin section electron micrographs of *M. capsulatus*  
(Bath) grown on methanol - no copper in the growth  
medium

Electron photomicrographs of thin sections of *M. capsulatus* (Bath) grown on methanol with no copper added to the growth medium. (a) Field view of cells displaying electron-dense particles (P) and lacking intracytoplasmic membrane arrays. (b) and (c) Thin-sections of cells exhibiting electron-dense particles (P) and a small number of peripheral membranes (M). Bar markers, 0.5  $\mu\text{m}$ .

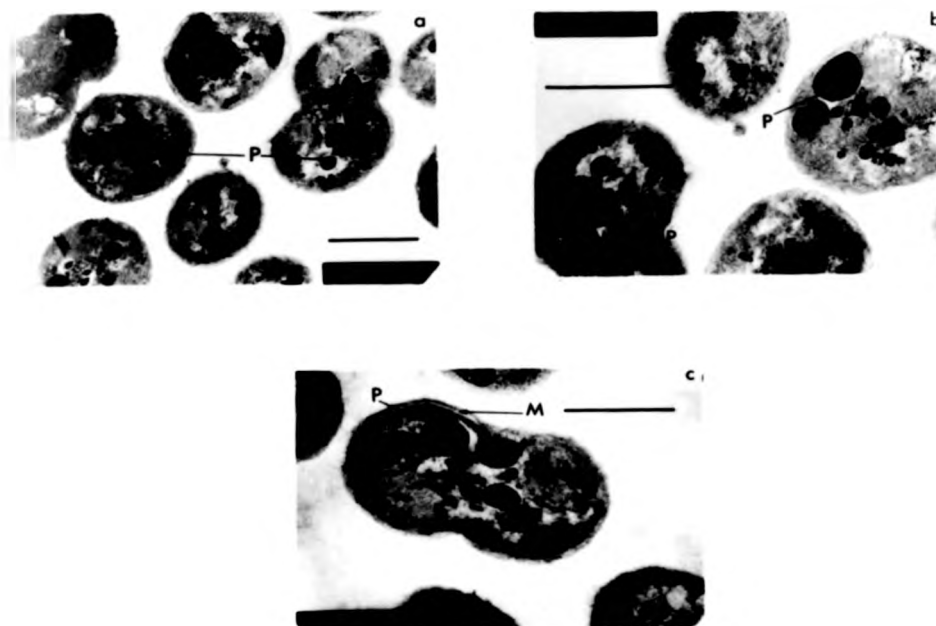


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As the concentration of copper in the growth medium was raised the cells began to exhibit tightly packed arrays of ICM which increased in frequency as the copper concentration increased (Figure 4.3). The number of electron-translucent droplets within the cells decreased as the ICM were formed. It has been suggested that the inclusions observed in cells lacking ICM are composed of lipid material (Procter *et al.*, 1969) and that they are the site of deposition of lipid and other material released from degeneration of the membranes (De Boer and Hazeu, 1972; Hyder *et al.*, 1979). It is therefore possible that new ICM are formed from material deposited in these cell inclusions and that as the number of ICM increases the number of inclusions decreases.

At very high levels of copper in the growth medium ( $2 \text{ mg l}^{-1} \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) the ICM arrays appeared to lose the tight packing and exhibited larger luminal spaces between the membranes (Figure 4.4).

The role of copper in the transition of cells from an intracellular structure lacking membrane arrays to one containing large numbers of stacked arrays of intracytoplasmic membranes is not clear. It is possible that the membranes are necessary for functional particulate MMO activity, either by providing the increased membrane content into which the membrane-bound enzyme can be incorporated or by providing specific electron transport systems necessary for particulate MMO activity. A link between methane oxidation and intracellular membrane content has been postulated (Hyder *et al.*, 1979; Takeda and Tanaka, 1980; Takeda *et al.*, 1976) and the results presented here demonstrate that an

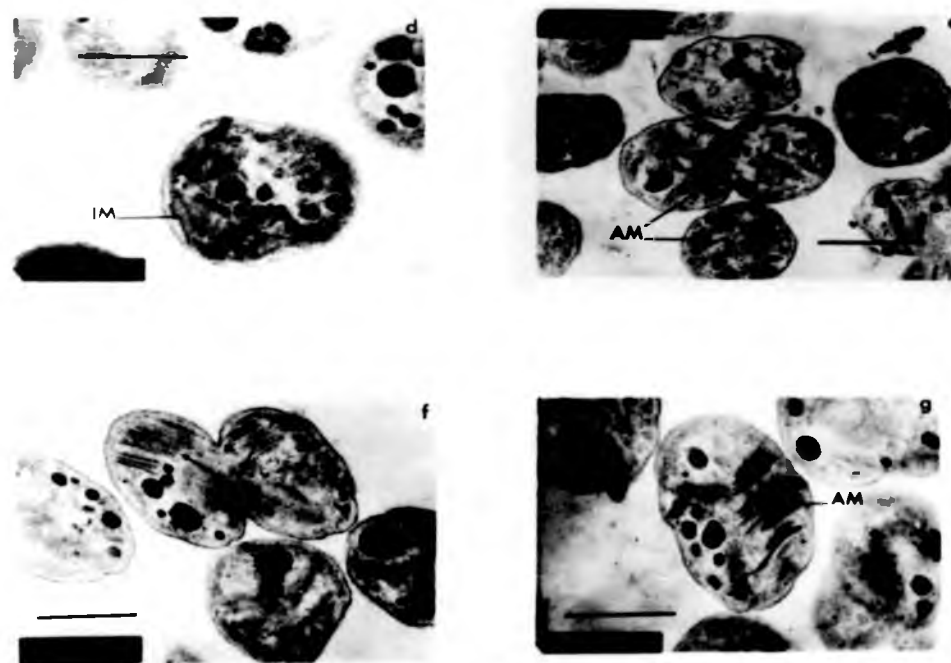


Figure 4.3      Thin-section electron micrographs of *M. capsulatus*  
(Bath) grown on methanol - Effect of copper on  
intracytoplasmic membrane content

Thin-section electron photomicrographs of *M. capsulatus* (Bath). (d) Cells grown on medium containing methanol and  $0.2 \text{ mg l}^{-1}$  copper sulphate. Intracytoplasmic membranes are visible within the cell (IM). (e) and (f) cells grown on medium containing methanol and  $0.6 \text{ mg l}^{-1}$  copper sulphate. Intracytoplasmic membrane arrays (AM) are now clearly discernable within the cell. (g) Cells grown on medium containing methanol and  $1.2 \text{ mg l}^{-1}$  copper sulphate. Intracytoplasmic membrane arrays are clearly visible throughout the interior of the cell and appear to exhibit a lamellar structure. Bar markers,  $0.5 \mu\text{m}$ .

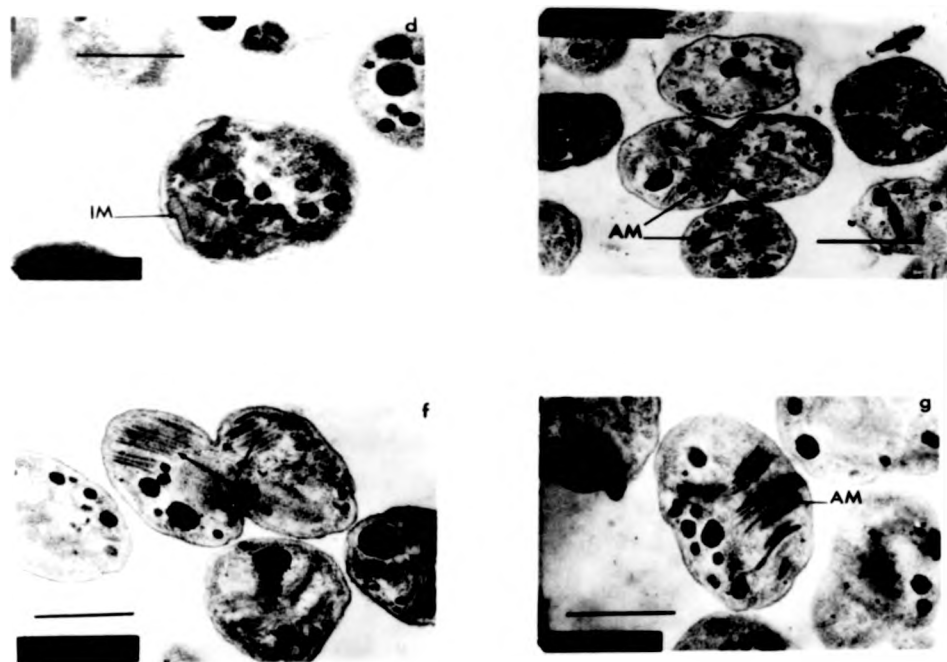


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Figure 4.4      Thin-section electron micrograph of *M. capsulatus* (Bath)  
grown on methanol - Effect of high levels of copper in  
the growth medium on intracytoplasmic membranes

Thin-section electron photomicrograph of cells grown on medium containing  $2.0 \text{ mg l}^{-1}$  copper sulphate. The intracytoplasmic membrane arrays (AM) fill the interior of the cell and are no longer tightly-packed. (This pattern of intracytoplasmic membranes corresponds to a decrease in particulate MMU activity as discussed in Section 4.4).



Figure 4.4      Thin-section electron micrograph of *M. capsulatus* (Bath)  
grown on methanol - Effect of high levels of copper in  
the growth medium on intracytoplasmic membranes

Thin-section electron photomicrograph of cells grown on medium containing  $2.0 \text{ mg l}^{-1}$  copper sulphate. The intracytoplasmic membrane arrays (AM) fill the interior of the cell and are no longer tightly-packed. (This pattern of intracytoplasmic membranes corresponds to a decrease in particulate MMO activity as discussed in Section 4.4).



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increase in methane-oxidising activity was paralleled by an increase in intracytoplasmic membrane content, but a direct correlation between the two events has yet to be shown. One method which was recently published (Tinglu et al., 1984) may provide a way to determine whether the enzyme is attached to the membranes. This involves the use of antibody tagged with colloidal gold which can be visualised using electron microscopy, but its use is precluded until such time as the enzyme can be purified and antibody specific to the particulate MMO can be raised.

#### 4.10 Final Comments

Results of experiments presented here have demonstrated that growth of M. capsulatus (Bath) on methanol can be achieved and that continuous culture of the organism was possible with methanol concentrations as high as 1.0% (v/v). In addition a new method for adaptation of M. capsulatus to growth on methanol was presented and may prove useful in adapting other methanotrophs to growth on methanol, it has the advantage of being much more rapid than previously published methods (Hou et al., 1979a). Methane monooxygenase was found to be constitutively synthesized when the organism was grown on methanol, and was additionally found to be exclusively associated with the membrane-fraction of cell extracts. The particulate MMO observed when M. capsulatus was grown on methanol was similar to the particulate MMO expressed, under certain conditions, when the organism was grown on methane. Soluble MMO was not expressed when the organism was grown on

methanol. The expression of particulate MMO activity was linked to the level of copper in the growth medium and an increase in MMO activity was paralleled by an increase in the intracytoplasmic membrane content of the cell. It was not clear from this study how methane-oxidising activity and the intracytoplasmic membrane content of the cell are linked, but the study clearly demonstrated that, as with methane, the conditions under which methylotrophs are grown can influence both enzyme activity and intracellular morphology, and great care must be taken over interpretation of results which were based on a single set of growth conditions.

## Chapter 5

### Characteristics and Properties of the Particulate Methane

#### Monooxygenase from Methylococcus capsulatus (Bath)

### 5.1 Introduction

In the General Introduction (Chapter 1) the problems associated in obtaining cell-free particulate MMO activity were discussed. The major difficulties encountered during studies on in vitro methane-oxidising activity are the irreproducibility in obtaining active preparations and the labile nature of many of the in vitro systems examined so far (Ribbons, 1975; Colby et al., 1975; Ferenci et al., 1975). To date only two systems have been characterized to any great extent these being the soluble MMO from Methylococcus capsulatus (Bath) by Dalton and his co-workers (for details see Section 4.3.5 of the General Introduction), and the particulate MMO from Methylosinus trichosporium UB3b (Tonge et al., 1975, 1977). The fact that recent attempts to repeat the work of Tonge et al. have resulted in preparations that lack MMO activity (Higgins et al., 1981a; Best and Higgins, 1983) has led to doubts being cast about the validity of the reports of this system and leaves the soluble MMO from M. capsulatus (Bath) as the only reproducible system for experimentation.

The demonstration in this thesis that M. capsulatus (Bath) can also exhibit reproducibly active preparations with particulate MMO activity provides a unique opportunity to compare the properties of the two enzymes in a single organism. It also allows for a comparison between the particulate MMO from M. capsulatus and the system from M. trichosporium reported by Tonge et al. (1975, 1977), furthermore, an examination of the properties of the particulate MMO in M. capsulatus may provide an answer to the question "are soluble and particulate MMO

activities due to two different enzymes or are the two activities due to a single enzyme with properties that are dependent on its intracellular location?"

## 5.2 Growth of *M. capsulatus* and Preparation of Cell Extracts

The organism was grown in continuous culture on NMS medium (Whittenbury et al., 1970) with methane as the sole source of carbon and energy, as described in Materials and Methods. The intracellular location of MMO activity in the cells was regulated by the addition of copper sulphate to the growth medium such that cells exhibited either 100% soluble or 100% particulate MMO activity. Cell extracts were prepared as detailed in Materials and Methods. MMO activity, except when stated, was determined by measuring the epoxidation of propylene to propylene oxide by gas chromatography.

## 5.3 Validity of Results

The particulate MMO from *M. capsulatus* has not been purified to any great extent (see Section 6.1) and, as a consequence, the studies on the enzyme presented in this thesis have been made with fairly crude membrane fractions. Attempts to purify the enzyme by solubilization of the membrane are discussed in Chapter 6. To ensure that the measured methane-oxidising activity was due to particulate MMO, and not contamination of the membrane fraction with soluble MMO, extracts were

subjected to a variety of treatments to provide the necessary controls - details of any such treatments are included with the relevant results.

#### 5.4 The Effect of various Inhibitors on Particulate MMO activity in cell extracts of *M. capsulatus*

##### 5.4.1 Introduction

The presence or absence of inhibition by chelating or metal-binding agents has often been used to provide evidence for or against the involvement of a metal ion in an enzyme. Studies on methane-oxidising organisms have shown that they are sensitive to a whole range of inhibitors, especially metal binding agents (Hubley *et al.*, 1975; Ribbons, 1975; Colby *et al.*, 1975; Patel *et al.*, 1976). These reports suggested that metal ions (and in particular iron and copper) may be involved in methane oxidation. The problem with any such study is that in whole cells or crude membrane fractions the site of inhibition is not always clearly defined. The sensitivity of methane oxidation may be due to inhibition of a number of other proteins which are required for fully functional enzyme activity - these include enzymes that generate reducing equivalents for the enzyme and also electron transport chain components which may play a role in methane oxidation in some methanotrophs (Tonge *et al.*, 1977). The results of inhibition data obtained with homogenous fractions should therefore be treated cautiously. However, the results from studies on whole cells and membrane preparations from a range of methylotrophs are remarkably consistent and show that methane oxidation is sensitive to a variety of

metal chelators, thiol chelators and electron transport inhibitors (Hubley et al., 1975; Ribbons, 1975; Colby et al., 1975; Patel et al., 1976). These results are contrasted the report of Stirling and Dalton (1977) which demonstrated that the soluble MMO in cell extracts of M. capsulatus (Bath) was insensitive to the majority of inhibitors tested and was only inhibited by 8-hydroxyquinoline, and the acetylenic compounds ethylene and propyne. The lack of inhibition of this enzyme by metal chelating agents other than 8-hydroxyquinoline, suggested that if any metal ion complex was directly involved in the soluble MMO system it must be well shielded from attack by the metal binding compounds.

The ability of M. capsulatus (Bath) to express two forms of the MMO enzyme allows for a comparison to be made between the inhibitor profiles of the enzyme when it occurs in the soluble or in the particulate fraction of cell extracts; the results are presented below.

#### 5.4.2 Experimental

All the early studies on the inhibition of methylotrophs used either oxygen uptake in the presence of methane, or methane disappearance as the means of assaying for MMO activity (Hubley et al., 1975; Ribbons, 1975; Tonge et al., 1977; Stirling and Dalton, 1977). In this study the sensitivity of cell extracts to inhibitors was monitored by assaying for the epoxidation of propylene by gas chromatography. This reaction in M. capsulatus is specifically catalysed by MMO; thus all the inhibition data obtained is a measurement of the sensitivity of MMO to the inhibitors and compounds which affect respiration, such as potassium cyanide, will not provide false positive results. The results are shown in Table 5.1.

Table 5.1      Effect of various potential inhibitors on in vitro MMO  
activity in M. capsulatus (Bath)

<u>Inhibitor (0.1 mM)</u>	<u>% Activity</u>	
	<u>Soluble MMO</u>	<u>Particulate MMO</u>
None	100	100
Potassium cyanide	100	0
2-Mercaptoethanol	100	0
2,2-Bipyridyl	99	10
Thiourea	90	0
Dithiothreitol	100	25
Imidazole	81	42
8-hydroxyquinoline	29	0
Neocuproine	100	0
Bathocuproine	100	0
Sodium azide	99	0
Thioglycollate	100*	40
Ethyne (3% in air)	0	0

\*Required as a stabilizing agent for the soluble MMO.

Cell extracts were preincubated for 1 min with inhibitor prior to addition of propylene. MMO activity was determined by measuring the epoxidation of propylene by gas chromatography.

The soluble MMO from M. capsulatus (Bath) was only inhibited by 8-hydroxyquinoline (71%) and acetylene (ethyne) (100%) and was insensitive to the other compounds tested. This result confirms the observations of Stirling and Dalton (1977) and suggests that methane oxidation by this form of the enzyme was either independent of the presence of metal ions or that the metal ions involved are shielded from the effects of metal-ion chelators. The extensive characterization of this enzyme by Dalton and his co-workers has shown that the enzyme is composed of three subunits (see General Introduction) and that one of these subunits (Protein A) contains two antiferromagnetically coupled iron atoms (Woodland and Dalton, 1984a). Presumably these metal ions are not sensitive to the presence of metal ion chelators by virtue of their position within the protein molecule.

The particulate MMO was found to exhibit a totally different inhibitor profile that closely resembles the pattern of inhibition described for the particulate systems from Methylosinus trichosporium UB3b (Tonge et al., 1977), Methylomonas methanica (Colby et al., 1976) and Methylococcus capsulatus (Texas) (Ribbons, 1975; Stirling and Dalton, 1977). It also confirms the results of Scott et al. (1981a) who observed that the inhibition pattern of M. trichosporium UB3b was dependent on the intracellular location of the MMO. The particulate enzyme was potently inhibited by metal chelators, thiol chelators and electron transport inhibitors. This pattern of inhibition suggests a close association of this form of the enzyme with metal ions and also with membrane-bound electron transport proteins.

The inhibition of particulate MMO activity by potassium cyanide and sodium azide suggest that either NAD(P)H was not acting as the direct electron donor or that, under conditions where particulate MMO activity is expressed, an inhibitor-sensitive NAD(P)H acceptor:reductase component for the enzyme is synthesised. The possibility that NAD(P)H is not acting as the direct electron donor for the particulate MMO is further examined in the next section of this chapter. The suggestion that methanotrophs can utilize an electron donor other than NAD(P)H has been made by Tonge *et al.* (1977) who proposed a theory that in *M. trichosporium* UB3b the enzyme methanol dehydrogenase might recycle electrons to the MMO without the involvement of NADH, but the irreproducibility of such activity has remained a problem. Leak and Dalton (1983) observed that ethanol and higher primary alcohols incapable of reducing NAD(P) directly could act as electron donors for the MMO *in vivo* both in organisms incapable of avoiding copper limitation (e.g. *Methylomonas albus* Bg8 and *Methylocystis parvus* UBBP) and in *M. capsulatus* (Bath) when grown on high copper medium. However, this property was lost when the organism was transferred from a high- to a low-copper medium (i.e. a switch from particulate to soluble MMO) (Stanley *et al.*, 1983). On the assumption that primary alcohols are oxidised by the methanol/primary alcohol dehydrogenase in methanotrophs, for which there is substantial evidence, this indicates that methanol can act as an electron donor for further oxidation of methane by the particulate but not the soluble MMO.

### 5.5 Electron Donor specificity of the Particulate MMO in *M. capsulatus*

In the preceding section the inhibition of particulate MMO activity by electron transport inhibitors led to the suggestion that when cells were expressing this form of the enzyme NAD(P)H may not be acting as the direct electron donor in vivo. The possibility that in vitro particulate MMO activity may utilize an electron donor other than NAD(P)H was investigated using *M. capsulatus* grown in high copper medium leading to expression of wholly particulate MMO activity. The experiments with membrane fractions prepared from the organism showed that the only effective donors for in vitro MMO activity were NADH and NADPH (Table 5.2).

These results contrast with studies on cell suspensions of *M. capsulatus* (Bath) which indicated that both methanol and ethanol could act as electron donors for the particulate MMO (Stanley et al., 1983). Ethanol has been shown to act in vivo as an electron donor for the wholly particulate MMO in *Methylobacterium methanica* (Ferenci et al., 1975; Leak and Dalton, 1983), although no NAD-linked alcohol dehydrogenase was detected in cell extracts (Ferenci et al., 1975). From this evidence it was proposed that ethanol (and presumably methanol) could indirectly reduce NAD by reversed electron transport (Ferenci et al., 1975). It is possible, therefore, that NADH may not be the immediate electron donor for the particulate form of the enzyme (even though NADH is effective as a donor for the enzyme in vitro) and that reductant for the MMO from methanol and ethanol was generated via an electron transfer protein

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Table 5.2      The effect of various electron donors on in vitro  
particulate MMO activity in M. casulatus (Bath)

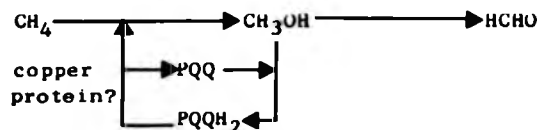
<u>Donor</u>		<u>Specific Activity</u>
		[nmol propylene oxide formed min <sup>-1</sup> (mg protein) <sup>-1</sup> ]
NADH	(5 mM)	114
NADPH	(5 mM)	56
Methanol	(5 mM)	0
Ethanol	(5 mM)	0
Succinate	(4 mM)	0
Ascorbate	(2 mM)	0
Ascorbate/TMPD	(4 mM/1 mM)	0

TMPD-NNN'-tetramethyl-p-phenylenediamine

The assays were performed as outlined in Materials and Methods with the various electron donors replacing NADH in the assay mixture.

without the involvement of NADH. One possible candidate for this electron transfer protein might be a copper-containing protein that was only synthesized when cells are grown under conditions of copper excess and that this protein was capable of donating electrons directly to the particulate MMO without the involvement of NADH. If this protein was unstable in cell extracts then NADH may be necessary to observe in vitro particulate MMO activity.

The possibility that, under conditions when particulate MMO activity was expressed, the cell was capable of methane oxidation without the need for NADH may provide a way for the cell to conserve energy. It has already been noted by Dalton and Leak (1985) that cells expressing particulate MMO activity are energetically more efficient (in terms of g cells/g methane consumed) than cells expressing soluble MMO. If cells expressing particulate MMO activity also produce a copper-containing electron transfer protein such a protein may be able to couple reducing equivalents from methanol dehydrogenase (as PQQH) to the particulate MMO as indicated in the scheme below:



The fact that in M. capsulatus this active has not been observed in vitro may reflect the need for fully activity PQQH and reports of PQQ-linked methanol dehydrogenase activity (Duine et al., 1984a, b) have shown that in vitro activity only occurs under specific conditions.

These conditions include the presence of  $\text{NH}_4^+$  ions; thus, it may require conditions which have not yet been defined in order to demonstrate in vitro particulate MMO activity which can utilize electrons derived from the oxidation of methanol by the PQQ-linked methanol dehydrogenase.

#### 5.6 Stability of the Particulate MMO from *M. capsulatus*

Using the method for preparations of cell extracts from *M. capsulatus* given in Materials and Methods there were no problems encountered with the routine preparation of particulate cell-free MMO activity. The major difficulty with the preparations was the instability of the activity of the membrane fraction. This loss of activity was found to be temperature sensitive as shown in Table 5.3. The results indicated that preparations could be maintained on ice provided that the loss of activity at this temperature during the course of an experiment was taken into account.

The preparations were found to be stable at  $-20^\circ\text{C}$ ; this contrasts with the results of Tonge et al. (1977) who reported that the particulate MMO isolated from *Methylosinus trichosporium* OB3b was totally inactivated on freezing. Extracts from *M. capsulatus* were able to maintain activity when subjected to a single freeze/thaw cycle, but would not withstand repeated cycles of freezing and thawing.

The instability of the membrane-bound MMO from *M. capsulatus* closely resembles the labile nature of membrane preparations from other

Table 5.3      The effect of temperature on the stability of in vitro  
particulate MMO activity

<u>Incubation</u> <u>Temperature</u> °C	<u>Loss of</u> <u>Activity</u> (% Loss h <sup>-1</sup> )
45	60-70
30	45-55
20	30-40
0	15-20
-20*	0

\*Sample incubated at -20°C was frozen, activity was stable to one freeze/thaw cycle but was not stable to repeated freeze/thaw cycles.

organisms (Ferenci et al., 1975; Ribbons, 1975; Colby et al., 1975) and the fact that the preparation is unstable above 0°C was similar to the instability noted by Colby et al. (1975) for the soluble MMO from M. capsulatus (Bath).

During the preparation of soluble MMO from M. capsulatus (Bath) sodium thioglycollate (1-10 mM) is routinely added to the sample to act as a sulphydryl-group protecting reagent for Protein C of the enzyme complex; addition of this compound to particulate preparations led to a reversible inhibition of MMO activity and did not provide any enzyme stabilization. In addition to sodium thioglycollate a number of other compounds with stabilizing properties were tested; these included dithiothreitol and glycerol, but none proved effective at stabilizing the enzyme activity. Extracts prepared in the presence of the protease inhibitor PMSF (phenylmethylsulfonyl fluoride) did not exhibit any increased stability, suggesting that the loss of activity was not due to degradation of the enzyme but was due to the labile nature of the enzyme or proteins necessary for expression of full in vitro activity of the enzyme. It should be noted that some stability was apparent in partially purified preparations of the enzyme which are discussed in Chapter 6.

#### 5.7 Effect of Temperature on the Particulate MMO from M. capsulatus

The optimum temperature for growth of M. capsulatus (Bath) is 45°C although the organism can tolerate temperatures as high as 48°C for

limited periods of time. The soluble MMO from this organism exhibits maximal activity at 45°C and is inactivated at higher temperatures. An investigation was undertaken to determine the optimum temperature for in vitro activity of the particulate MMO from M. capsulatus (Bath). The results are shown in Table 5.4. The temperature at which maximal activity occurs was found to be 45°C with a marked loss in activity at temperatures above or below this level.

## 5.8 Effect of Metal Ions on Particulate MMO activity

### 5.8.1 Introduction

The results so far presented have demonstrated that the availability of copper to the organism M. capsulatus (Bath) plays an important role in determining the intracellular location of MMO activity. Experiments performed on Methanomonas margaritae have shown that the cells grown in the presence of added copper ions exhibited higher methane-oxidizing activity than cells grown in the absence of copper ions (Takeda et al., 1976). In addition, they reported that methane oxidation was inhibited by copper-chelating agents such as neocuproine. The inhibitor studies performed on the particulate MMO from M. capsulatus (Bath) (Section 5.4) demonstrated that methane oxidation in this organism was inhibited by several compounds including many which act as copper-chelating agents (neocuproine, bathocuproine, thiourea). This suggests that copper may play a dual role in methane oxidation by M. capsulatus (Bath), acting as an inducer of particulate MMO activity and also participating directly in the reaction catalysed by the particulate enzyme. An investigation

Table 5.4      Effect of temperature on particulate MMO activity from  
M. capsulatus

<u>Temperature</u>	<u>Specific Activity</u>
<u>of Assay</u>	[nmol propylene oxide min <sup>-1</sup> (mg protein) <sup>-1</sup> ]
(°C)	
30	25
36	53
40	118
45	150
48	108
51	97

The assays were performed as outlined in Materials and Methods. The epoxidation of propylene was measured after a 6 min incubation using NADH (5 mM) as the electron donor.

into the effect of metal ions on methane-oxidising activity in particulate preparations of M. capsulatus was thus undertaken.

#### 5.8.2 Whole cell studies

The organism was grown in chemostat culture under conditions which led to expression of 100% particulate MMO activity. Before experimentation samples of the cells were fractionated to ensure that the activity present was wholly particulate and was totally inhibited by the addition of potassium cyanide (1 mM). Cell suspensions were incubated for 3 mins in the presence of the test metal ion prior to addition of propylene. Samples were removed after 10 min and the extent of propylene epoxidation determined by gas chromatography. The results are shown in Table 5.5.

Addition of some metal ions caused marked inhibition of methane-oxidizing activity notably  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Ag}^{2+}$ . Two other metal ions,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ , caused more than 50% inhibition and several ions showed little or no inhibitory activity ( $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ). It was interesting to note that there was no stimulation of methane-oxidising activity by  $\text{Cu}^{2+}$  in cell suspensions of M. capsulatus in view of the possible role of this metal ion in methane oxidation. The major difficulty with studies on whole cell suspensions was that any inhibition of methane-oxidizing activity which occurs due to the addition of metal ions (or any inhibitory compound) may not be due to inhibition of the MMO per se. Several metal ions including cobalt, copper, nickel, silver and zinc are toxic to organisms because of their ability to denature proteins (Gadd and Griffiths, 1978). Copper has

Table 5.5

The effect of various metal ions on in vivo particulate  
MMO activity in *M. capsulatus* (Bath)

<u>Metal ion</u>	<u>Concentration</u> (mM)	<u>% Activity</u>
No addition	-	100
Cu <sup>2+</sup>	1	96
	0.1	101
Mg <sup>2+</sup>	1	94
	0.1	99
Zn <sup>2+</sup>	1	42
	0.1	49
Mn <sup>2+</sup>	1	34
	0.1	36
Co <sup>2+</sup>	1	11
	0.1	33
Fe <sup>2+</sup>	1	64
	0.1	84
Fe <sup>3+</sup>	1	71
	0.1	87
Ag <sup>2+</sup>	1	16
	0.1	21
Ni <sup>2+</sup>	1	12
	0.1	16

Specific activity of sample with no addition was 122 nmol propylene oxide formed min<sup>-1</sup> (mg dry wt of cells)<sup>-1</sup>.

Samples were prepared in Pipes buffer (5 mM, pH 7.0) to minimise metal ion binding by the buffer system. Samples were preincubated at 45°C in the presence of the test metal ion prior to assaying for MMO activity, with sodium formate (5 mM) supplying reducing equivalents for the reaction.

been shown to exert an inhibitory effect on the respiratory chain of Escherichia coli (Domek et al., 1984), and silver is known to act as an inhibitor of respiration and can also cause uncoupling in bacteria (Tilton and Rosenberg, 1978). It is therefore difficult to draw conclusions from whole cell data as inhibition of respiratory chain components, etc. may well play a role in the expression of in vivo particulate MMO activity (see Section 5.5). Consideration was therefore given to the effect of metal ions on in vitro particulate MMO activity.

#### 5.8.3 Cell-free studies

Cell extracts were prepared from the organism which had been grown in chemostat culture under conditions which led to expression of wholly particulate MMO activity. Particulate fractions were checked for possible contamination with soluble MMO by incubating the samples with KCN (1 mM) prior to assay using the epoxidation of propylene assay system. The results showed that the MMO activity present in the samples was totally inhibited by the addition of KCN indicating that all the activity was due to the particulate form of the enzyme. Assays were performed as outlined in Materials and Methods except that samples were preincubated for 1 min in the presence of the test metal ion prior to addition of NADH and propylene. The samples were assayed for propylene epoxidation after 6 min incubation.

The results shown in Table 5.6 differ slightly from the results obtained with whole cells indicating that the pattern of inhibition observed in whole cells reflects the metal ion sensitivity of several enzymes which are required for particulate MMO activity and was not simply an

Table 5.6

The effect of various metal ions on in vitro particulate  
MMO activity in *M. capsulatus* (Bath)

<u>Metal ion</u>	<u>Concentration</u> (mM)	<u>% Activity</u>
No addition	-	100
Cu <sup>2+</sup>	1	133
	0.1	142
Mg <sup>2+</sup>	1	98
	0.1	80
Zn <sup>2+</sup>	1	72
	0.1	77
Mn <sup>2+</sup>	1	95
	0.1	91
Co <sup>2+</sup>	1	75
	0.1	100
Fe <sup>2+</sup>	1	81
	0.1	92
Fe <sup>3+</sup>	1	74
	0.1	83
Ag <sup>2+</sup>	1	3
	0.1	47
Ni <sup>2+</sup>	1	65
	0.1	69

Specific activity of sample with no addition was 117 nmol propylene oxide formed min<sup>-1</sup> (mg protein)<sup>-1</sup>.

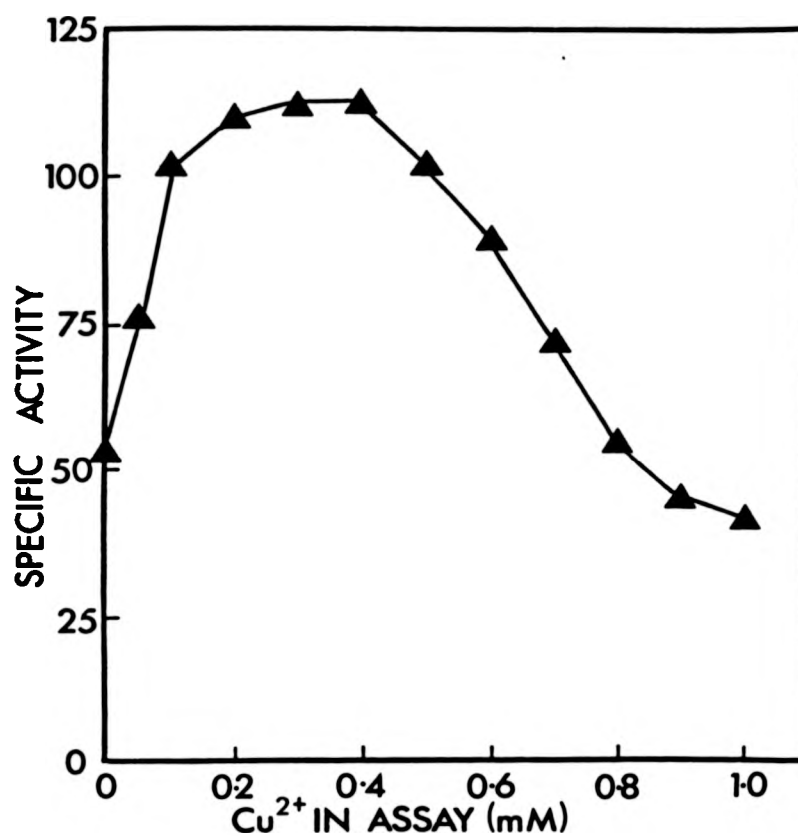
Samples were assayed as indicated in Table 5.5 except that the electron donor in all cases was 5 mM NADH.

indicator of the metal ion sensitivity of the MMO. Of the metal ions tested,  $\text{Ag}^{2+}$  was the most potent inhibitor causing almost total inhibition of MMO activity at a concentration of 1 mM. The only other metal ion which caused notable inhibition was  $\text{Ni}^{2+}$ . In contrast to the whole cell suspensions  $\text{Cu}^{2+}$  caused a stimulation of particulate MMO activity, thus confirming the observations of Patel *et al.* (1979) who also reported enhanced expodizing activity in the presence of added copper ions. The reason for this stimulation by copper ions was not clear but, taken in conjunction with the fact that particulate MMO activity was found to be sensitive to copper-chelating compounds, it suggests that copper may be involved in the catalytic activity of the particulate MMO. The effect of copper on particulate fractions of *M. capsulatus* was investigated further as detailed in the next section.

#### 5.8.4 Stimulation of in vitro Particulate MMO activity by copper ions

The stimulation of activity by the addition of copper ions to the assay system as outlined above, and reported for '*Methylocystis*' sp CRL-15 (Patel *et al.*, 1979) occurred immediately after the copper was added to the assay system and suggests that copper may act as a cofactor or prosthetic group for a protein that was already present in the extract and that the increase in activity does not require *de novo* protein synthesis. The stimulation of particulate MMO activity was investigated further to determine how the enhancement of activity was achieved.

The effect of copper concentration in the assay system was determined and the results presented in Figure 5.1. The results indicate that maximal activity occurred when the copper concentration in the assay



**Figure 5.1**      The effect of copper ions on in vivo particulate MMU activity from *M. capsulatus* (Bath)

Copper was added to the assay system in the form of hydrated copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) at the concentrations indicated in the figure. The particulate MMU activity was measured by assaying for the epoxidation of propylene by gas chromatography and is expressed as specific activity ( $\text{nmol propylene oxide formed min}^{-1} (\text{mg protein})^{-1}$ ).

system was 0.4 mM. This figure is of little intrinsic value in determining the role of copper in particulate MMO activity because of the heterogenous nature of the membrane fraction which does not allow for quantitation of the level of enzyme present in the extract. It is, however, worth noting at this point that in all the assays performed with various preparations of particulate MMO the optimum concentration for stimulation of MMO activity was 0.4 mM  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , the significance of this figure is discussed in section 5.9. The loss of activity which occurs when higher concentrations of copper ions are present in the assay system is possibly due to a change in the enzyme or factors associated with the enzyme, such as denaturation, which are known to occur when metal ions are present in high concentrations.

#### 5.8.5 Effect of a variety of copper compounds on particulate MMO activity

The copper added to the trace element solution used in the growth medium for cultivation of methanotrophs (see Materials and Methods) was in the form of hydrated copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and it was this form of copper which was used in the studies on the effect of metal ions on particulate MMO activity. The effect of other copper compounds was examined to determine whether these were any more or any less effective than copper sulphate in causing an enhancement of particulate MMO activity. The results are shown in Table 5.7 and indicate that all three test compounds caused a stimulation in particulate MMO activity and that the order of "effectiveness" was

$\text{CuCl}_2 > \text{CuSO}_4 \cdot 5\text{H}_2\text{O} > \text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ . As all copper (II) compounds disproportionate in water to form the  $[\text{Cu}(\text{H}_2\text{O})_6]^{2+}$  ion the increased activity

Table 5.7      Effect of a variety of copper compounds on particulate  
MMO activity

<u>Copper compound</u>	<u>Concn.</u>	<u>Specific Activity</u>
		<u>[nmol propylene oxide formed</u> <u>min<sup>-1</sup> (mg protein)<sup>-1</sup>]</u>
No Addition	-	102
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.4 mM	164
CuCl <sub>2</sub>	0.4 mM	176
Cu(NO <sub>3</sub> ) <sub>2</sub> ·3H <sub>2</sub> O	0.4 mM	143
CuCl	0.1 mM	49

Assays were performed as outlined in Materials and Methods using sodium/potassium buffer (40 mM, pH 7.0). Copper compounds were added prior to addition of NADH and propylene. Propylene epoxidation was measured by gas chromatography.

of  $\text{CuCl}_2$  compared to  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$  was presumably a result of increased disproportionation of this compound into a form that was available to the enzyme when compared with the latter two compounds.

An experiment to determine the effect of copper (I) compounds on particulate MMO activity was limited by the insoluble nature of copper (I) compounds, but using  $\text{CuCl}$  a 0.5 mM solution was prepared by adding a drop of 1 mM  $\text{HCl}$  which allowed for solubilization of the compound. The addition of  $\text{Cu}$  (I) (final concentration in assay was 0.1 mM) caused 50% inhibition of the sample when compared to a control assay which had no addition, suggesting that the stimulatory effect of copper compounds was limited to  $\text{Cu}$  (II) ions.

#### 5.9 The Effect of pH on Particulate MMO activity

The influence that the pH of the assay system has on enzymic activity has been established by studies on many enzymes and is known to be extremely complex. A shift in pH can lead to changes in ionizable groups on the enzyme surface, it may cause ionization of either cofactors, substrates or products and can also alter the availability of hydrogen ions which are essential for some enzymic reactions (e.g. dehydrogenase enzymes). All of the above can alter the rate of reaction and it is therefore important to know the "optimum pH", that is the pH at which maximal activity of the enzyme occurs. However, interpretation of the factors involved in pH effects is often difficult and the "optimum pH" often requires careful definition and cautious

interpretation.

Particulate MMO activity in the assay system was determined by measuring the epoxidation of propylene. The optimum pH for particulate MMO activity was determined by using a series of sodium/potassium phosphate buffers which altered the pH of the assay system over the range pH 6.5 to pH 8.0. The results shown in Figure 5.2 indicate that maximal activity in this buffer system occurred at pH 7.5. This contrasts with the soluble MMO from the same organism which exhibits maximal activity in the range pH 6.8-7.0 (Colby and Dalton, 1976).

It had been noted that in studies on metal ions uptake by bacteria and yeasts use was made of the buffer piperazine-N,N'-bis(2-ethane sulphonic acid), which has the trivial name of Pipes, (Norris and Kelly, 1977). This buffer was used because of the buffering capacity in the range pH 6-8, but more importantly, because it had negligible metal binding capacity (Good et al., 1966). The report that Pipes had negligible metal-binding capacity also reported that phosphate buffers tend to precipitate most polyvalent cations. This observation was interesting in view of the results reported in this thesis which suggest that copper (II) ions may be important in the particulate MMO activity. The pH profile of particulate MMO activity in Pipes buffer was determined and the results showed that there was no change in particulate MMO activity in the range pH 6-8 (outside this range Pipes was no longer effective as a buffer). This result suggested that the pH-dependent particulate MMO activity observed when phosphate buffer was used in the assay system might reflect a binding of metal ions to the buffer which would thus

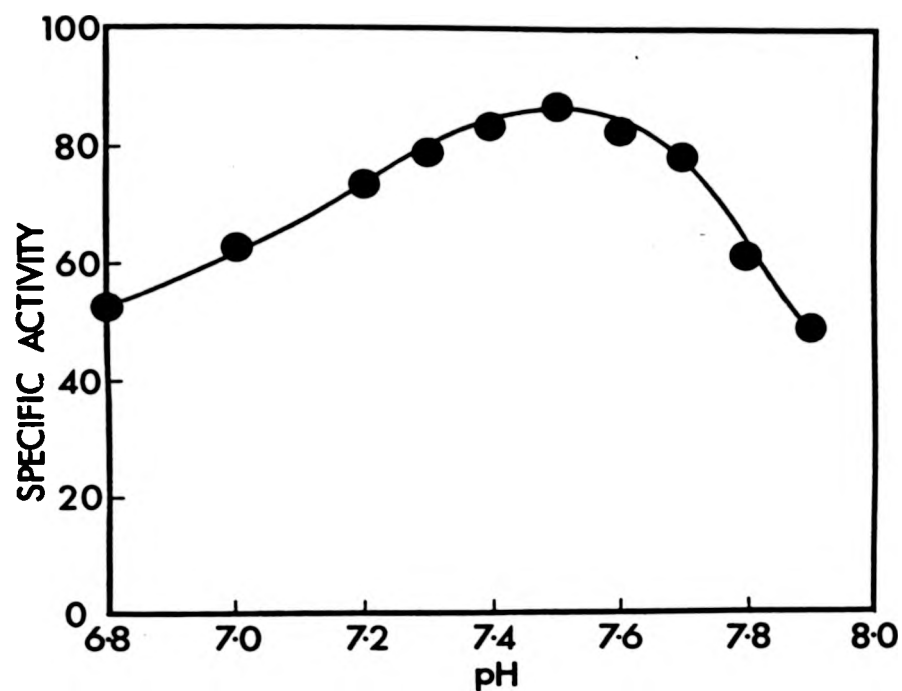


Figure 5.2      The effect of the pH of the sodium/potassium phosphate  
buffer in the assay medium on the in vitro particulate  
MMO activity

The experiment was performed as outlined in the text. All buffers were adjusted to the required pH at a temperature of 45°C to ensure that the pH of the assay system was not affected by any temperature change during the measurement of the particulate MMO activity.

affect the activity of the enzyme. This was confirmed by studying the effect of the addition of copper ions to the assay system in Pipes and sodium/potassium phosphate buffers.

When particulate MMO activity was determined in Pipes buffer with and without added copper ions there was no enhancement of MMO activity at any of the pH values which were tested (pH 6-8). In contrast to this result the addition of copper ions to the assay system with sodium/potassium phosphate buffer showed a pH-dependent stimulation by copper ions (Table 5.8). The pH dependence of particulate MMO activity in the sodium/potassium phosphate buffer was thought to be a reflection of the binding of metal ions to this buffer. The binding capacity of this buffer might change in response to pH changes because of protonation/deprotonation effects on the phosphate moiety. The stimulation of particulate MMO activity by copper ions, which was maximal when the metal ion concentration was 0.4 mM, as discussed in section 5.8, is thus explained by consideration of a pH-dependent exchange of copper ions between the enzyme and the phosphate buffer system. The fact that at pH 7.0 the maximal activity occurs when 0.4 mM copper ions are present in the assay suggests that this was the amount of metal ions which were bound to 20 mM sodium/potassium phosphate buffer. The use of Pipes buffer precludes this effect and the addition of copper ions does not enhance the activity of the enzyme.

It is interesting to note that because of the wider pH range in which the particulate MMO was active (pH 6-8) when compared to the soluble enzyme (pH 6.8-7.0) it would be possible to use Tris buffer in

Table 5.8      Effect of pH and buffer on copper-stimulated in vitro  
particulate MMO activity

<u>Pipes buffer (5 mM)</u>	<u>Specific Activity</u>
<u>+ 0.4 mM CuSO<sub>4</sub>·5H<sub>2</sub>O</u>	<u>[nmol propylene oxide formed min<sup>-1</sup></u>
<u>(pH)</u>	<u>(mg protein)<sup>-1</sup>]</u>
6.5	129
7.0	130
7.5	127
8.0	124

Sodium/Potassium Phosphate Buffer (40mM)

<u>+ 0.4 mM CuSO<sub>4</sub>·5H<sub>2</sub>O</u>	
<u>(pH)</u>	
6.5	69
7.0	111
7.5	136
8.0	102

Assays were performed as outlined in Materials and Methods and contained the appropriate buffer and 0.4 mM CuSO<sub>4</sub>·5H<sub>2</sub>O.

experiments on the particulate enzyme. It should however be pointed out that reports have been made that demonstrate that Tris buffer is capable of binding copper ions (Masi et al., 1984) and that inhibition of the copper-containing enzyme, bovine superoxide dismutase, due to the binding of copper ions to Tris buffer has been demonstrated (McPhail and Goodman, 1984). It is therefore apparent that the choice of buffer can be important as these compounds may influence the results obtained. The best buffer for studies on the particulate MMO from M. capsulatus (Bath) was found to be Pipes as the limited binding of metal ions by this buffer does not alter the activity of the enzyme.

#### 5.10 The Use of Phosphate Buffer in NMS Medium

The results of experiments on pH/buffer effects as detailed in the preceding section have shown that inhibition of the particulate MMO can occur due to the ability of sodium/potassium phosphate buffer to bind copper ions. The NMS medium of Whittenbury et al. (1970) which is routinely used in our laboratory for cultivation of methylotrophs, uses sodium/potassium phosphate buffer to provide buffering capacity for the growth medium. This is also the case with other research groups currently investigating methane oxidation. The results of the experiments outlined in this thesis have demonstrated that in M. capsulatus (Bath) there is a relationship between the intracellular location of MMO activity and the availability of copper ions. This relationship has also been established for Methylosinus trichosporium OB3b (Stanley et al., 1983; Burrows et al., 1984). If phosphate

buffers act as copper-binding agents then the amount of copper available for uptake by cells will be less than the level of copper added to the growth medium because of a 'sink' of copper ions which are bound to the buffer. This effect may be extremely important when cells are grown on very low levels of copper (i.e.  $<50 \mu\text{g l}^{-1}$ ). At this concentration the use of phosphate buffer might exert an indirect effect on the organism by causing a decrease in the availability of copper ions thus preventing expression of particulate MMO activity (see Chapter 4). It would be extremely difficult to determine how much copper was bound to phosphate buffer and how much was freely available for uptake by cultures of methylotrophs, and the possibility remains that the cells have copper uptake systems that are capable of overcoming the binding of copper to the buffer, but the influence of such systems should not be ignored.

#### 5.11 Does the Particulate MMO from *M. capsulatus* (Bath) contain copper?

As discussed in previous sections the suggestion that copper is involved in  $\text{CH}_4$  oxidation is not new (Harwood and Pirt, 1974; Hubley *et al.*, 1975; Tonge *et al.*, 1975, 1977), and results presented here have demonstrated that particulate MMO activity was inhibited by copper-chelating agents and, under certain conditions, was stimulated by the addition of copper ions, but this does not constitute sufficient evidence for the assertion that the enzyme is copper-containing. An enzyme can only be considered a "true metal enzyme" if the metal ion is

an essential participant in the catalytic mechanism (Malmstrom and Rosenberg, 1959). An unequivocal demonstration of such participation is usually extremely difficult to achieve and invariably requires purification of the enzyme. The characteristics of two groups of metal enzymes provide an operational distinction that (1) enzymes with strongly bound metals do not require the addition of metal ions to the assay medium and such additions have no effect on the activity (except for possible inhibition and non-specific activation), or (2) the activity of enzymes with dissociable metal ions is an explicit function of the concentration of free metal ions in solution (Malmström and Rosenberg, 1959). The term "non-specific" activation is used to cover all cases where addition of a substance leads to an increase in reaction rate without causing a change in mechanism; a simple example is provided by the influence of ionic strength on the rate. It would appear from this definition that the particulate MMO from M. capsulatus (Bath) falls into the latter class of metal enzymes (for evidence see Section 5.8). However, an enzyme cannot be classified as being metal ion-activated simply by showing that its activity is increased by the addition of metal ions to the assay system. This is a necessary, but far from sufficient criterion, since the metal ion can influence the reaction rate without playing an intrinsic role in the mechanism of the reaction. Such uncertainty is best resolved by purifying the enzyme and no claim of metal ion activation for an impure enzyme can be considered fully reliable. The situation in M. capsulatus must therefore remain unresolved until such time as purification of the enzyme is achieved (see Chapter 6), but preliminary results suggest that copper ions do play a role in determining the activity of the

particulate MMO, but the mechanism by which this is achieved is still not clear. It is interesting to note that copper has been identified as a metal ion frequently associated with oxidation reactions and that all of the known copper proteins, apart from the storage protein (thioneins), are involved, either directly or indirectly, in electron transfer to dioxygen. These proteins are listed in Table 5.9 and have recently been extensively reviewed by Malmstrom (1982) and Dreyer (1984).

The suggestion that the particulate MMO contains copper is not, therefore, an unreasonable prospect but it should be noted at this point that dioxygen activation can occur by combination of  $O_2$  with fully reduced flavin and also by binding  $O_2$  to reduced metal centres with metal ions other than copper (e.g.  $Fe^{2+}$  in cytochrome P-450 (Malmstrom, 1982)).

The role of copper in particulate MMO activity in M. capsulatus (Bath) is thus not clear at this time, but the two possibilities, (1) that copper is present in the active site of the enzyme and (2) that copper leads to expression of a novel electron transfer protein capable of providing reducing equivalents to the enzyme in the absence of NADH, remain primary goals for any research into methane oxidation by this organism.

Table 5.9      Functional Classes of Copper Proteins

<u>Classes of Functions</u>	<u>Cu Proteins</u>
I.    Electron transfer from Protein to $O_2$ without separation of charge (oxygen binding)	Hemocyanin
II.   Electron transfer from protein to $O_2$ with separation of charge (oxygen reduction)	
1. with liberation of all $O_2$ as:	
a) $H_2O_2$ ( $H_2O_2$ -forming oxidases)	Amine oxidase, Galactose oxidase
b) $H_2O$ ( $H_2O$ -forming oxidases)	Laccase, Coeruloplasmin, Ascorbate oxidase, Cytochrome oxidase
2. with insertion of at least 1 atom of $O_2$ into an organic molecule (oxygenases)	Tyrosinase, Dopamine hydroxylase
III.   Electron transfer between reduced $O_2$ species ( $O_2^-$ or $H_2O_2$ -dismutases, peroxidases)	Superoxide dismutase
IV.   Electron transfer between proteins (non-autoxidizable electron transferase, involved in electron transport chains with $O_2$ at one end)	Azurin, Amicyanin, Plastocyanin, Stellacyanin, Umecyanin, Kusticyanin

## 5.12 Substrate specificity of the Particulate MMO

### 5.12.1 Introduction

The ability of methane-utilizing bacteria to oxidize compounds other than methane has been recognized for many years. The first major study of the oxidation of substrates which could not support growth was performed by Leadbetter and Foster who in the period 1958-1960 observed that the organism Pseudomonas (now Methylobomonas) methanica when grown on methane was capable of oxidising ethane, propane or butane (Leadbetter and Foster, 1958, 1959, 1960). They called this phenomenon 'cooxidation', and they also noted that methane-grown suspensions of the organism did not produce significant amounts of carbon dioxide from these substrates indicating incomplete oxidation of these molecules, and alcohols, alkanolic acids and ketones were identified as products.

The period 1960 to 1976 yielded many reports of oxidation of simple substrates by a number of methanotrophic isolates, but the reports of stable in vitro MMO activity (Colby and Dalton, 1976; Tonge et al., 1977) not only increased our knowledge of the enzymology of methane oxidation but also led to a marked increase in the number and range of compounds which could be oxidized by these species (Colby et al., 1977; Stirling and Dalton, 1977). The oxidation of hydrocarbons by MMOs from a variety of microbes was comprehensively reviewed by Dalton (1980) who noted the oxidation of alkanes, alkenes, ethers, alicyclic, aromatic and heterocyclic hydrocarbons.

The soluble MMO from M. capsulatus (Bath) is the most extensively

characterized enzyme system amongst methane-oxidizing bacteria and has been shown to insert oxygen into a wide range of compounds (Dalton, 1980). The demonstration that this organism could express two forms of the MMO allowed for the determination of the substrate specificity of a membrane-bound MMO and also permitted a comparison to be made with the soluble enzyme from the same organism.

#### 5.12.2 Experimental

The experiments were performed as detailed in Materials and Methods. Control assays included the addition of potassium cyanide (1 mM), which acts as a potent inhibitor of the particulate MMO but does not inhibit soluble MMO activity, to eliminate the possibility of contamination with soluble enzyme, the use of boiled extract to ensure that the reaction was not simply a non-enzymic conversion, and determination of activity in the absence of reducing equivalents. The results are shown in Table 5.10 and it was immediately apparent from the results that the use of crude membrane fractions leads to difficulties due to the presence of enzymes in the extract which can utilize the products of MMO activity. The inability to detect significant levels of primary alcohols was presumably due to the presence of the non-specific methanol (primary alcohol) dehydrogenase in the assay system (Patel et al., 1972). The low values obtained for production of aldehydes may also be due to the influence of enzymic activity in the preparation, the enzyme in this case being aldehyde dehydrogenase (Patel et al., 1980). The enzymes are not present or are inactive in preparations of the soluble MMO from M. capsulatus due to the addition of potassium cyanide to the assay system. It is not, however, possible to use potassium cyanide in membrane

Table 5.10      Substrate specificity of the particulate MMO from  
M. capsulatus

<u>Substrate</u>	<u>Product</u>	<u>Rate of Product Formation</u>
		<u>[nmol min<sup>-1</sup> (mg protein<sup>-1</sup>)]</u>
Methane	Methanol	N.D.
Ethane	Ethanol	N.D.
Propane	Propan-1-ol	1.4
	Propan-2-ol	25.3
	Propanal	0.4
Butane	Butan-1-ol	2.3
	Butan-2-ol	14.3
	Butanal	0.0
Pentane	Pentan-1-ol	0.6
	Pentan-2-ol	9.4
	Pentan-3-ol	0.0
	Pentanal	0.2
Benzene	Phenol	0.0
Cyclohexane	Cyclohexanol	0.0
Propylene	Propylene Oxide	84.3

N.D. - Not Detected.

The experiments were performed as outlined in Materials and Methods.

fractions due to the extreme sensitivity of the particulate MMO to this compound (see Section 5.4).

It has been reported that in the presence of 200 mM phosphate buffer the oxidation of methane leads to the accumulation of methanol (Higgins and Quayle, 1970), and the assays for methane and ethane were therefore repeated at this higher concentration of buffer, but no new products were detected. This result confirms the observations of Colby *et al.* (1975) working on *Methylomonas methanica*; however in a recent publication Burrows *et al.* (1984) reaffirmed the accumulation of methanol at high phosphate concentrations suggesting that this effect may be limited to organisms like *Methylosinus trichosporium*. It is worth noting in this context that the mechanism by which methanol accumulates at high phosphate concentrations is not known and there is no evidence for direct inhibition of methanol dehydrogenase by high phosphate concentrations.

The particulate MMO from *M. capsulatus* (Bath) was shown in these experiments to catalyze both terminal and subterminal hydroxylation of propane, butane and pentane but was unable to catalyze the hydroxylation of alkanes of chain length greater than C5. The inability of cell extracts to oxidize benzene and cyclohexene confirms the results of Stirling *et al.* (1979) who examined a particulate fraction, which exhibited methane-oxidising activity, from the organism *Methylomonas methanica*. It would appear from these results that the rate of product formation from most of the 'interesting' substrates, such as methane, ethane, etc. requires purification of the enzyme to eliminate the

influence of the other enzymes present in the crude membrane fraction. It is, however, possible to compare the overall trend of substrate specificity in M. capsulatus (Bath) with published data from other methanotrophs.

#### 5.12.3 Comparison of substrate specificities of MMO's from a variety of methanotrophs

The data from experiments on methanotrophs that are capable of expressing particulate MMO activity demonstrates that the presence of this form of the enzyme limits the range of hydrocarbons which the enzyme can hydroxylate to a maximum chain length of C5 (pentane). The results also indicate that the rate of hydroxylation decreases as the chain length increases from C1-C5 (Hou et al., 1979b; Stirling et al., 1979; Pilyashenko et al., 1979). In addition, unlike soluble MMO, the particulate enzymes were unable to oxidize aromatic or alicyclic hydrocarbons. The pattern of a wide range of substrates being oxidized by the soluble MMO and a narrow range of substrates being oxidized by the particulate enzyme has now been demonstrated in Methylorubrum trichosporium OB3b (Hou et al., 1979b; Stirling and Dalton, 1979; Burrows et al., 1984), Methylobacterium organophilum CML-26 (Hou et al., 1979b; Patel et al., 1982) and Methylococcus capsulatus (Bath) (Stirling et al., 1979).

The reason why the particulate MMO's from these methylotrophs have a more limited substrate specificity than their soluble counterparts have not yet been established but there are several possibilities. The inability of the membrane-bound enzymes to oxidize compounds which have

a carbon chain length greater than C5 might be taken as evidence that the hydroxylase components of the two enzymes are different and that the conformation of the active site of the particulate enzyme will only permit the entry of carbon compounds with backbones of C5 or less. This might explain why alkanes of C6 and greater are not oxidized even in terminal-carbon positions. The other possible constraint on the size of molecules able to reach the active site of the enzyme might be the close association of the protein with the cell membrane. In this case, the active site of the particulate enzyme may be similar to the active site of the soluble MMO but the fact that the protein is embedded in the membrane only permits compounds of carbon chain length less than C5 to reach the active site of the enzyme.

The inability of the particulate MMO's to oxidize aromatic and alicyclic compounds might also be explained in terms of steric effects, as outlined above, but in addition a third factor should be considered, namely, the effect that these compounds have on cell membranes. The role of intracytoplasmic membranes in methane oxidation has not yet been resolved but it has been demonstrated that an increase in particulate MMO activity is accompanied by an increase in intracytoplasmic membrane content (Prior and Dalton, 1985). Aromatic and alicyclic compounds are noted for their ability to disrupt cell membranes, therefore, the addition of such compounds to preparations containing particulate MMO activity might lead to disruption of the cell membrane which may be essential for full enzymic activity. The reasons for the limited substrate specificity of the particulate MMO must therefore remain unresolved until this enzyme is purified and a direct comparison between

the two enzymes can be made.

One important point which emerges from the results described above is that the intracellular location of the MMO plays a crucial role in determining substrate specificity and the utmost care must be taken when examining previously reported data or when performing new experiments to ensure that the location of the MMO is known.

## CHAPTER 6

### Further Characteristics and Properties of the Methane Monooxygenase enzymes from *M. capsulatus* (Bath)

## 6.1 Partial Purification of the Particulate MMO from *M. capsulatus* (Bath)

The results of the experiments outlined in Chapter 5 on the particulate MMO from *M. capsulatus* have demonstrated some of the problems associated with working on a crude enzyme preparation. There is no doubt that in order to study the enzyme and to compare it with the soluble MMO, purification of the enzyme is a necessary step that must be achieved.

The first stage in the purification of a membrane-bound enzyme system usually involves solubilization of the enzyme to remove it from the membrane, this then allows the application of procedures such as column chromatography for routine purification. The major difficulty which occurs when enzymes are solubilized from the membrane is that removal of the enzyme results in loss of activity; despite the fact that solubilization may lead to inactivation an attempt at removing the enzyme from the membrane was made as outlined below.

### 6.1.1 Solubilization of the membrane-bound MMO

The only membrane-bound MMO which has been purified so far is the enzyme from *Methylosinus trichosporium* UB3b (Tonge *et al.*, 1975, 1977). The method used for isolation involved removal of the enzyme from the cell membrane by phospholipase treatment. Despite the fact that this procedure is reported to be no longer effective (Higgins *et al.*, 1981a) it was thought that this might be a good starting point for a study on *M. capsulatus*.

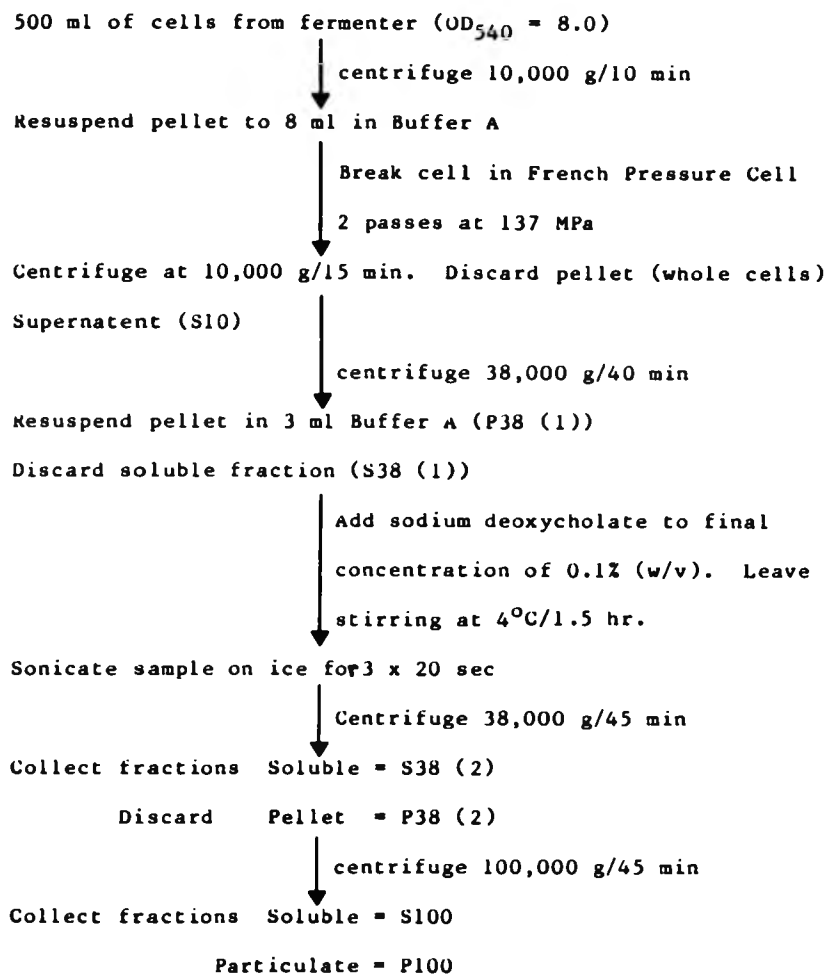
The procedure used was that of Tonge *et al.* (1977) but the resultant soluble and particulate fractions exhibited no MMO activity either singularly or in combination. The procedure was repeated on three occasions but at no time was any MMO activity detected. The same procedure was then applied to samples from *M. capsulatus* but with a variety of solubilizing agents (Triton X-100; Phospholipase C; lipase; octyl-  $\beta$ -D-glucoside and CHAPS [3-(3-cholamidopropyl dimethylammonio)-1-(2-hydroxy-1-propanesulphonate)]); but none of these effected solubilization of the activity from the membrane. At this point a new detergent was tried, this was a gift from the British Technology Group and was an N-D-glucose-N-methylalkanamide with the trivial name Mega-9. This detergent had been successfully used in solubilization of membrane-bound antibodies from plasma membranes (Hildreth, 1982).

Extracts were prepared from cells exhibiting particulate MMO activity and the membrane fractions incubated with Mega-9 (1% w/v) for 30 mins at 0°C before separating into soluble and particulate fractions by centrifugation at 38,000g for 45 min. The resultant fractions were assayed for MMO activity and the results showed that there was some activity (approximately 10% of the original MMO activity) in the soluble fraction, i.e. the detergent had caused partial solubilization of MMO activity from the membrane fraction. This procedure was repeated several times with the detergent concentration in the extract varying between 0.25-5% (w/v). The optimal range for the detergent appeared to be between 0.5-1% (w/v) but even under these conditions the yield of "solubilized" MMO activity was only 10% of the original activity in the membrane fraction. The "solubilized" enzyme activity was found to be

stimulated by the addition of copper ions to the assay system at pH 7.0, to have maximal activity in phosphate buffer at pH 7.5, and was totally inhibited by the addition of 0.1 mM potassium cyanide to the assay system. These are characteristics similar to the membrane-bound enzyme (see previous Chapters in this thesis) and suggested that the enzyme was not fundamentally changed by removal from the membrane. The major problems were the small amounts of material obtained during each procedure and the instability of the "solubilized" MMO activity. A large scale preparation which yielded 10 ml of "solubilized" enzyme was subjected to centrifugation at 100,000 g for 1 hr and the resultant fractions assayed for MMO activity. This showed that rather than remaining in the supernatant fraction, the "solubilized" enzyme activity was associated with the pellet fraction suggesting that the enzyme was not truly soluble but was associated with small membrane fragments that are only sedimented by high-speed centrifugation. The results with Mega-9 were thus misleading and rather than releasing the enzyme from the membrane the detergent was simply reducing the size of the membrane fragments to which the enzyme was bound, such that the routine centrifugation procedure used to separate soluble and particulate MMO activity (38,000 g for 45 min) was no longer effective.

The inability to solubilize the enzyme with Mega-9 prompted a search for other methods of preparation of membrane-bound enzymes. One promising method appeared to be that of May et al. (1984) who purified an epoxidase/hydroxylase enzyme from membrane fractions of Pseudomonas oleovorans. Their method was modified slightly and is outlined in Figure 6.1.

**Figure 6.1**      Procedure for Partial Purification of the Particulate  
MMO from *M. capsulatus* (Bath)



Buffer A

5 mM Pipes buffer, pH 7.0

The results obtained using this method are shown in Table 6.1, and demonstrate that this procedure was much more effective than any other method previously tried. The soluble fraction that was obtained after treatment of the membrane with the detergent exhibits MMO activity which was almost 50% of the activity of the starting material. The result of the high-speed centrifugation step indicates that, as was the case with Mega-9, the enzyme was not wholly solubilized but was found to be associated with small membrane fragments. The interesting point about this procedure was the increased activity and stability of the "solubilized" enzyme which occurs in the presence of added copper ions. In the absence of added copper ions the MMO activity, as evidenced by the epoxidation of propylene, was only maintained for approximately 8 mins (Figure 6.2), whereas in the presence of 0.04 mM  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  the activity of the enzyme was maintained for 40 mins and the yield of propylene oxide was increased almost ten-fold (Figure 6.2). The influence of copper ions on the "solubilized" enzyme was also evident in the P100 fraction although in this case the stabilization and increase in yield were less spectacular (Table 6.2).

The influence of copper ions on the samples indicates that these ions have an important role in particulate MMO activity confirming the findings of earlier experiments using crude cell extracts. The solubilization method outlined above appears to provide a useful starting point for attempts to purify the particulate MMO from M. capsulatus (Bath), but at this time the activity is still associated with membrane fragments and it remains a possibility that the enzyme will lose all activity when isolated from the membrane. The

Table 6.1      Particulate MMO activity in Partially Purified  
Fractions of M. capsulatus (Bath)

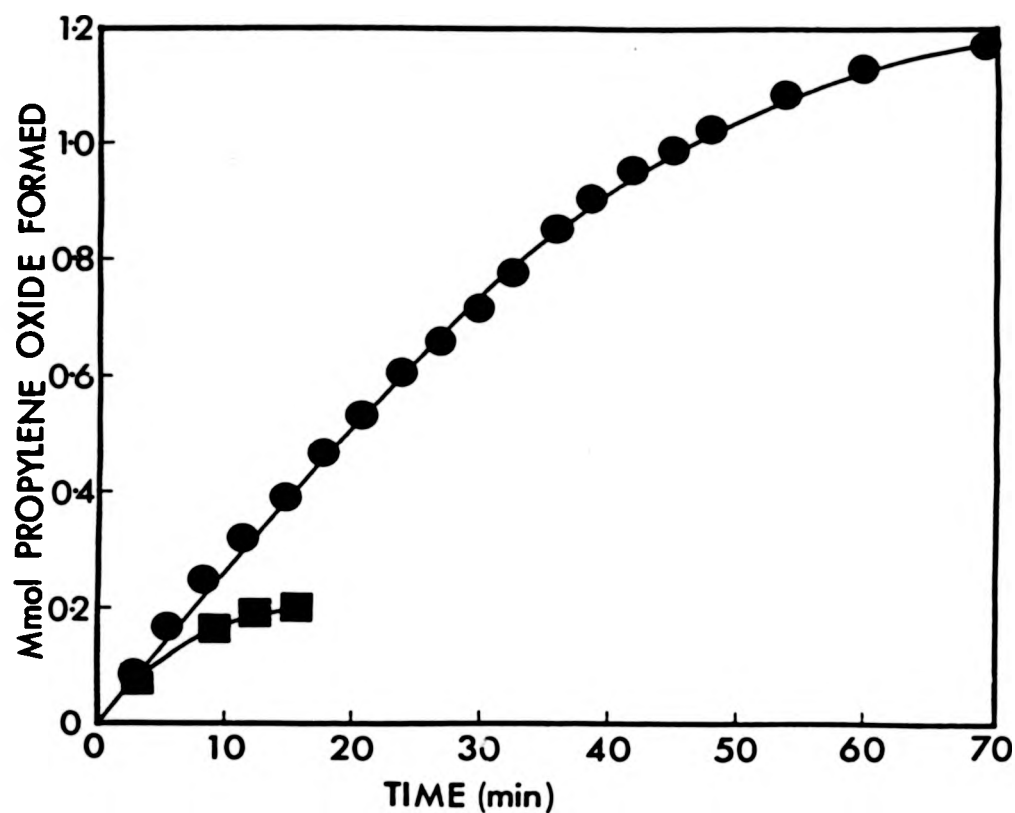
The fractions were obtained as outlined in Figure 6.1.

<u>Fraction</u>	<u>Specific Activity</u> (nmoles propylene oxide formed/ min./mg. Protein)
P38 (1)	114
S38 (1)	0
P38 (2)	16
S38 (2)	49
P100	21
S100	0

Table 6.2      The Effect of Copper ions on Particulate MMO activity  
in the P100 fraction of cell extracts

<u>Sample</u>	<u>Specific Activity</u> (nmoles propylene oxide formed/ min./mg. Protein)
P100    no addition	24
P100 + 0.2 $\mu\text{M}$ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	152
P100 + 0.4 $\mu\text{M}$ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	210

The P100 fraction was obtained as outlined in Figure 6.1.



**Figure 6.2**      The Effect of copper ions on partially purified particulate MMU activity

The rate of propylene epoxidation catalysed by partially purified particulate MMU was measured in the absence of copper ions (■) and in the presence of 0.04 mM copper sulphate (●) as indicated in the text.

purification of the enzyme is without doubt the most important short term goal of any study on the particulate enzyme and if purification can be effected, it should be possible to answer many of the questions raised by the work presented in this thesis; in particular it may determine whether the enzyme contains copper ions and how closely related this form of the enzyme is to the soluble MMO from the same organism.

#### 6.1.2 Further Purification of the Particulate MMO

Having achieved a partial purification of the enzyme from the membrane the problem of effecting complete solubilization needs to be considered. If we use the small membrane fragments (P100 fraction) as the starting point for further purification how might this be achieved? The one obvious strategy would be to persevere with the use of solubilizing agents and it is possible that one of the detergents already tested on the crude membrane fraction might be effective in solubilizing the enzyme from the much smaller membrane fragments. In view of the limited success so far achieved the most likely candidate for further testing is Mega-9; this detergent not only provided some solubilization of the enzyme from crude fractions but has already been shown to effect the release of antibody from membranes in an active form (Hildreth, 1982). The other possibility is to search for new detergents which have proven effective at solubilizing proteins from membranes; one such compound is lysolecithin. This detergent has been used to effect the release of an ATP-Pi exchangease in an active form from membrane fragments of the mitochondrial inner membrane (Komai *et al.*, 1973; Sadler *et al.*, 1974). If this proves ineffective there are any number of detergents available,

any of which may be effective at releasing the enzyme from the membrane. A second strategy might be the use of specific enzymes which might remove any membrane attached to the enzyme; such enzymes include phospholipases, lipases, etc. It remains a possibility that on removal from the membrane the enzyme might lose its functional activity. If this is the case it might be necessary to try and restore activity by using reconstituted membranes formed from commercially available compounds. This method has been applied with some success in the purification of enzymes associated with the mitochondrial membrane and may be the only way in which the particulate MMO activity can be studied.

## 6.2 The use of radiolabelled acetylene to identify the active site of the particulate and soluble MMO from *M. capsulatus* (Bath)

### 6.2.1 Introduction

Acetylene has been shown to act as a potent inhibitor of methane-oxidising activity in *Methylomonas methanica* (Colby *et al.*, 1975), *M. capsulatus* (Dalton and Whittenbury, 1976), and *Methylosinus trichosporium* (Stirling and Dalton, 1979). The first report of the inhibitory effect of acetylene on cell-free extracts of *M. capsulatus* demonstrated that 3% acetylene in the assay mixture was sufficient to totally inhibit MMO activity (Stirling and Dalton, 1977). In addition, it was reported that, of eight acetylenic compounds tested for inhibition of MMO activity, four were found to strongly inhibit methane oxidation (acetylene, propyne, but-1-yne and but-2-yne) but that

inhibition efficiency decreases not only with increasing chain length but also with shifting the acetylenic bond away from the terminal carbon to a subterminal position. Acetylene has been shown to inhibit cell-free methane oxidising activity in other methanotrophs including M. trichosporium OB3b (Scott et al., 1981a), M. capsulatus (Texas) (Stirling and Dalton, 1977) and both soluble and particulate forms of MMO in M. capsulatus (Bath) (Stanley et al., 1983).

The work of Stirling and Dalton (1977) showed that when acetylene inhibited the MMO activity in the soluble fraction of cell extracts of M. capsulatus (Bath) the concentration of the acetylene in the assay diminished. They further demonstrated that the apparent loss of acetylene was dependent on the concentration of the extract present, and on the presence of NADH and oxygen. Attempts to identify a product for the apparent acetylene oxidation were unsuccessful and it was suggested that the inhibitor may have bound to the enzyme complex. The demonstration that acetylene was a potent inhibitor of both particulate and soluble MMO (Stanley et al., 1983) suggested that it might be possible to use radiolabelled acetylene as a probe for the MMO enzyme.

#### 6.2.2 Inhibition of methane monooxygenase activity by acetylene

The effect of acetylene on MMO activity is shown in Table 6.3. Partial inhibition of soluble MMO activity occurred in the presence of acetylene alone or acetylene plus either oxygen or NADH. Total inhibition of MMO activity, however, required the presence of both NADH and oxygen. The results are also shown for MMO activity in the particulate fractions of cell extracts and are similar to the soluble system. At no time during

Table 6.3      Inhibition of Methane Monooxygenase activity  
by Acetylene

<u>Preincubation Conditions</u>	<u>Activity (%)</u>	
	<u>Soluble MMO</u>	<u>Particulate MMO</u>
Control (no preincubation)	100	100
+ O <sub>2</sub> (5 ml)	93.8	85.8
+ NADH (5 mM)	98.2	87.3
+ C <sub>2</sub> H <sub>2</sub> (30 µl)	44.6	32.1
+ C <sub>2</sub> H <sub>2</sub> + O <sub>2</sub> (30 µl/5 ml)	40.9	36.5
+ C <sub>2</sub> H <sub>2</sub> + NADH (30 µl/5 mM)	46.3	41.8
+ C <sub>2</sub> H <sub>2</sub> + NADH + O <sub>2</sub> (30 µl/5 mM/5 ml)	0	0

Samples were preincubated, under the conditions shown, for 7 min. at 45°C before removal of the gaseous phase by evacuation. Samples were then assayed for MMO activity using the routine procedure. Control samples had specific activity as follows:

Soluble MMO 38.3 nmol propylene oxide formed/min/mg.Protein  
Particulate MMO 58.1 nmol propylene oxide formed/min/mg.Protein

any of the assay procedures was a product of acetylene oxidation detected by gas chromatography suggesting that the oxidation of acetylene may lead to formation of an inhibitor/enzyme complex which has no further MMO activity. The possibility that acetylene or a product of acetylene oxidation causes inhibition by irreversible binding to the enzyme was investigated using  $^{14}\text{C}$ -acetylene.

#### 6.2.3 The binding of acetylene to methane monooxygenase

If acetylene was bound to the enzyme then by using radiolabelled acetylene it should be possible to visualize the protein:inhibitor complex by the use of polyacrylamide gel electrophoresis and fluorography. The experimental details are given in Materials and Methods.

The resultant polypeptide banding patterns on SDS-polyacrylamide gels showed that the binding of acetylene had no effect on the migration of proteins in the gel. The fluorographs, when developed, showed that the  $^{14}\text{C}$ -acetylene remained bound to the proteins, even after boiling in SDS, and confirms the theory that acetylene, or a product of acetylene oxidation, forms a strong bond with the enzyme proteins. The gel and resultant fluorographs are shown in Figure 6.3.

The soluble fraction of cell extracts of M. capsulatus (Bath) grown under conditions where soluble MMO activity was expressed (see Chapter 3), showed that the radiolabelled acetylene had bound to a single polypeptide of molecular weight 54,000. This polypeptide corresponded to the  $\alpha$ -subunit of Protein A of soluble methane monooxygenase (Woodland

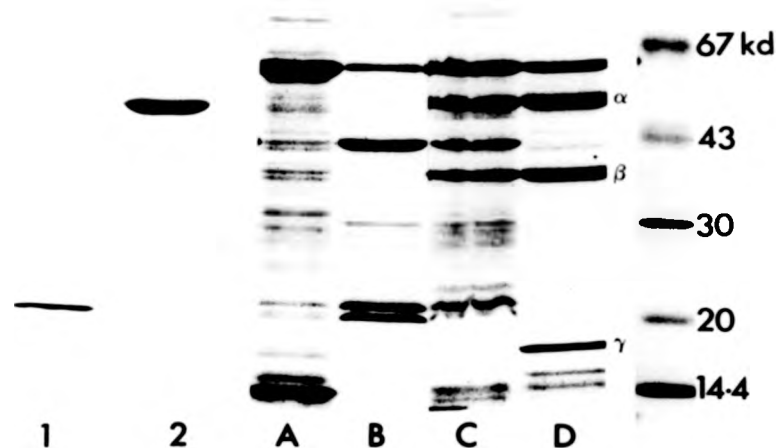


Figure 6.3      The binding of radiolabelled acetylene to proteins in  
cell extracts of *M. capsulatus* (Bath)

[ $^{14}$ C] acetylene binding to the active site of methane monooxygenase proteins. The figure shows the protein banding pattern on SDS-PAGE and the corresponding fluorographs which show that in each case acetylene is bound to a single protein of the MMO complex. Lane A, soluble fraction, high copper; lane B, membrane fraction, high copper. Lane C, membrane fraction, low copper; lane D, soluble fraction, low copper. Lane 1, fluorograph of proteins corresponding to lane B. Lane 2, fluorograph of proteins corresponding to lane D.

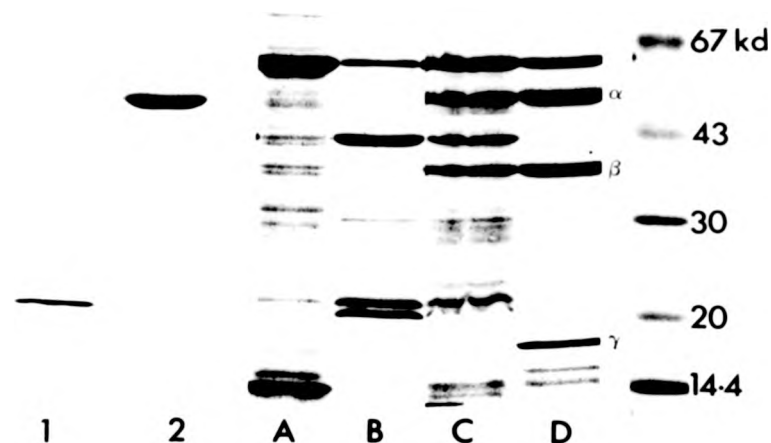


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and Dalton, 1984a). Experiments on the purified proteins of soluble MMO have shown that acetylene does not inhibit the NADH:acceptor reductase activity of Protein C of the enzyme, and does not therefore inhibit MMO activity by preventing the transfer of electrons from NADH to Protein A via Protein C (Prior and Green, unpublished observation).

The cells grown under conditions where they expressed particulate MMO activity (see Chapter 3) showed that radiolabelled acetylene again bound to a single polypeptide, but in this instance, the polypeptide was found in the particulate fraction of cell extracts and had a molecular weight of 26,000. This corresponded to one of three proteins which were induced at high copper:biomass ratios (Stanley *et al.*, 1983; Prior and Dalton, 1985; Chapter 3 this thesis) and which are thought to be associated with particulate MMO activity (Prior and Dalton, 1985).

The fact that acetylene binding occurs with specific proteins, and that these proteins are associated with methane monooxygenase activity suggests that the mode of inhibition of enzyme activity by acetylene must involve catalytic oxygenation of acetylene followed by binding to proteins, presumably at the active site of the enzyme.

#### 6.2.4 Is acetylene acting as a "suicide substrate" for the MMO?

Acetylene has been shown to act as a competitive inhibitor of whole cell methane oxidation by *Methylosinus* sp. Type 41 (DeBont and Mulder, 1976), and as a potent inhibitor of cell-free MMO activity (Stirling and Dalton, 1977; Scott *et al.*, 1981a; Stanley *et al.*, 1983). It has also been reported that acetylene inhibits the growth of the chemolithotroph

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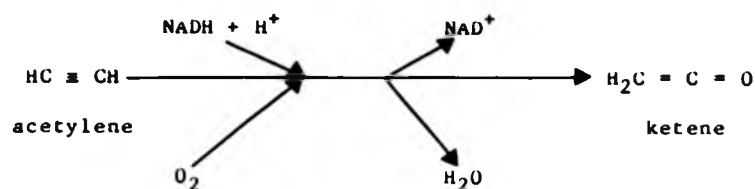
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Nitrosomonas europaea (Hynes and Knowles, 1978), possibly by inhibition of the enzyme ammonia monooxygenase (Hynes and Knowles, 1982). It has recently been demonstrated that acetylene acts as a suicide substrate for ammonia monooxygenase and that  $^{14}\text{C}$ -labelled acetylene can be used to label the protein concerned (Hyman and Wood, 1985); this experiment is comparable to the one outlined here. It is worth noting at this point that the substrate specificity and inhibitor sensitivity of ammonia monooxygenase is very similar to the particulate MMO from M. capsulatus (Bath) (Hyman and Wood, 1983) and in view of the fact that both enzymes show binding of acetylene to proteins of comparable molecular weights a comparison of the two enzyme systems may provide an interesting insight into the relationship between the methane- and ammonia-oxidising bacteria.

Suicide substrates are compounds that cause inactivation of enzymes due to the formation of transiently reactive intermediates of catalysis (Walsh, 1981, 1984). Often inhibition of enzymes by such compounds involves the irreversible binding of the intermediate to the active site of the enzyme. The acetylenic group is essentially inert and it is unlikely that it would act as a potent inhibitor of MMO activity in this form. It is however, possible that acetylene binds to the enzyme at the active site, and in the presence of NADH and oxygen, is oxygenated by the enzyme to produce a reactive intermediate that can interact with amino acids around the active site rendering the enzyme inactive. The hypothesis that it is a reactive intermediate formed from acetylene that causes inhibition of the enzyme explains why total inactivation requires NADH and oxygen, why acetylene disappears during the reaction and the

failure to detect a product of acetylene oxidation. The nature of the intermediate is not known but a possible candidate is thought to be ketene which is formed by hydroxylation of acetylene as shown below:



Ketene is a highly unsaturated and reactive compound which will react very readily with compounds containing hydroxyl and amino groups such as amino acids.

#### 6.2.5 Further experiments involving the use of $^{14}\text{C}$ -acetylene

The use of  $^{14}\text{C}$ -labelled acetylene as a probe for the active site of the MMO's from *M. capsulatus* may provide a method for identifying the amino acids associated with the active site of the proteins and may also provide a key to identifying the genes which code for the synthesis of these proteins.

The fact that the radiolabel is tightly bound to the proteins, even after fairly harsh treatment such as boiling in SDS, might allow for the identification of the amino acids around the active site of the enzyme. One method which might be employed in determining the composition of the amino acids in the active site is "peptide mapping". This method involves labelling the enzyme with  $^{14}\text{C}$ -acetylene, as outlined in Section 5.2 of this thesis, followed by limited proteolysis of the enzyme to

produce short polypeptide fragments. The fragment(s) which are near the active site can then be identified by detection of the  $^{14}\text{C}$ -radiolabel. Such detection would simply involve SDS-polyacrylamide gel electrophoresis followed by fluorography of the resulting gel bands to identify the radiolabelled polypeptide(s). The removal of polypeptides from gel slices is now a routine electrophoretic procedure and in this way the polypeptide fragment(s) which contain  $^{14}\text{C}$ -radiolabel can be readily obtained. Identification and the sequence of the amino acids in the polypeptides can then be determined by routine procedures. In this way the two enzymes from M. capsulatus can be compared with respect to the composition of their active sites. In addition, this technique may be applicable to MMU enzymes in other methanotrophs and might thus provide a method for identifying both the MMU enzymes and eventually the active site composition of many other methanotrophic bacteria.

Using the polypeptide sequence of the active site of the enzyme it should be possible to produce synthetic radiolabelled DNA probes that are specific for the amino acid sequence of the active site. Such probes can then be used to isolate the DNA from the organism and in this way the genes coding for expression of the enzyme may be identified. The use of  $^{14}\text{C}$ -acetylene to identify the active site of MMU enzymes might therefore prove one of the most valuable tools so far discovered for the determination of the biochemistry and genetics of methanotrophs.

### 6.3 Copper Uptake and Inhibition of Soluble MMU Activity by *M. capsulatus* (Bath)

#### 6.3.1 Introduction

The demonstration in this thesis that the addition of copper ions to a culture of *M. capsulatus* (Bath) grown under "copper-limited" conditions leads to the rapid inhibition of soluble MMU and induction of particulate MMU activity (Chapter 3.5), poses several questions about the role of copper in this organism. The most obvious questions concern the assimilation of copper into the cell and include, "how rapidly is copper assimilated by the cell?" and "what is the ultimate fate of the assimilated copper?" The former question can be answered by measuring the rate of copper uptake by cells as detailed in the following sections in this chapter, the latter question is much more difficult to answer as it requires detection of copper after assimilation and association with cellular components. The ideal method for following the fate of copper in the organism involves the use of a radioisotope of copper which can be readily detected either by scintillation counting or by fluorography. Unfortunately, the radioisotopes of copper are extremely short-lived and are very expensive to purchase. The isotope with the longest half-life is  $^{64}\text{Cu}$  which has a half-life of only 12.9 hrs. which makes experimentation very difficult and limits the number of experiments that can be performed with a given batch of isotope. In addition the cost of the isotope (£1000 for 25 mCi (minimum order), Amersham International plc, U.K.) places its use outside the scope of this project.

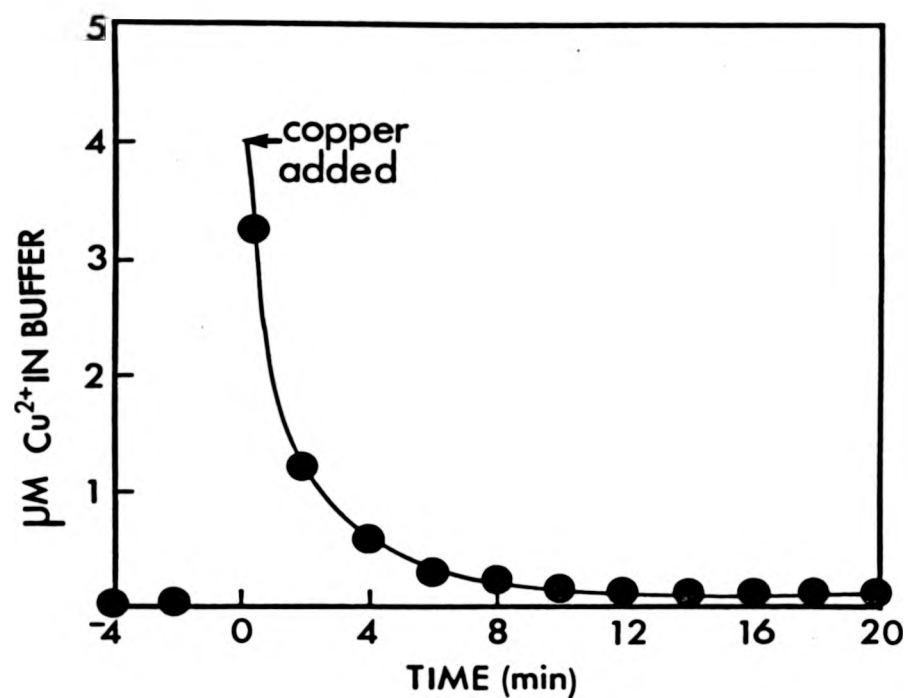
The other major area for research which could be performed without the

use of radiolabelled copper was the study of the inhibition of the soluble MMO that occurs when copper was added to a culture exhibiting soluble MMO activity. This is detailed in section 6.4.

#### 6.3.2 Copper uptake by *M. capsulatus* (Bath)

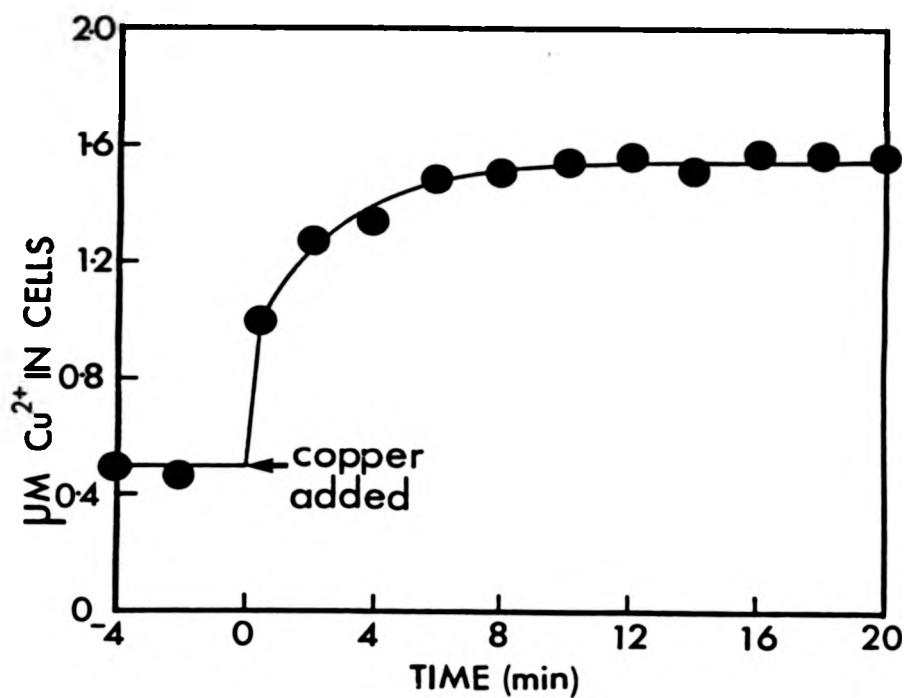
The organism was grown in chemostat culture on the low copper medium ( $0.2 \text{ mg l}^{-1}$ ) as described previously. All the experiments on copper uptake by the organism were performed in Pipes buffer (5 mM, pH 7.0) to minimise metal binding by the assay system (Norris and Kelly, 1977). Because of the problems associated with the use of radiolabelled copper isotopes measurements of the rate of copper uptake by the organism were made using atomic absorption spectrophotometry (Norris and Kelly, 1977). The experimental methods are detailed in Materials and Methods, and involved removal of cells from a copper-limited chemostat culture, resuspension of washed cells in Pipes buffer, addition of copper to the cell suspension, measurement of copper uptake by cells after separation of cells and buffer using a rapid filtration system. The results are shown in Figure 6.4a, b, c.

The results indicated that the loss of copper from the assay system (Figure 6.4a) occurs very rapidly suggesting that the cells are extremely efficient at assimilating copper from Pipes buffer. The accumulation of copper by *M. capsulatus* (Bath) was further investigated by examining the rate at which cells assimilated copper; the results are shown in Figures 6.4b and c. Metal accumulation by microorganisms generally comprises two phases: a rapid binding of cations to negatively-charged groups on the cell surface, and progressive, usually



**Figure 6.4a**      Copper uptake by cell suspensions of *M. capsulatus*  
(Bath) - Removal of copper ions from Pipes buffer

The concentration of copper in the buffer, after removal of the cells by rapid filtration, was determined by atomic absorption spectrophotometry. Copper sulphate (1 mg/l) was added to the cell suspension at the time indicated.



**Figure 6.4b** Copper uptake by cell suspensions of *M. capsulatus*  
(Bath) - Intracellular accumulation of copper ions in  
the presence of methane

The cells were harvested by rapid filtration, washed in 100 mM EDTA, and the intracellular concentration of copper ions determined by atomic absorption spectrophotometry. Copper sulphate (1 mg/l) was added to the cell suspension at the time indicated.

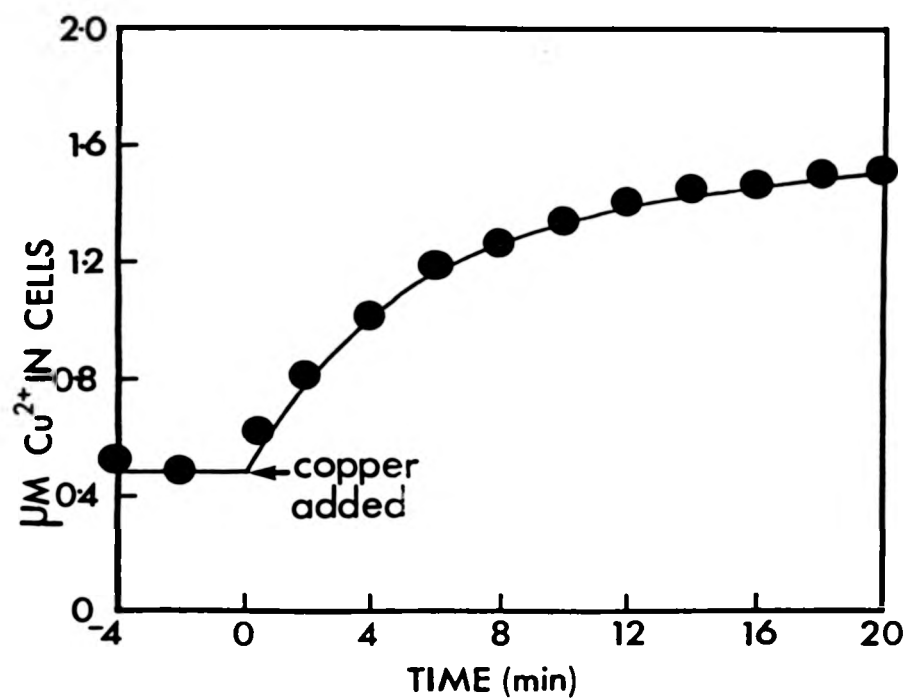


Figure 6.4c      Copper uptake by cell suspensions of *M. capsulatus*  
(Bath) - Intracellular accumulation of copper ions in  
the absence of methane

The experiment was identical to that described in Figure 6.4b except that methane was not present in the gaseous phase of the cell suspension.

metabolism-dependent, intracellular cation uptake (Norris and Kelly, 1977). The use of EDTA (100 mM) to wash the cells removes any copper which was loosely bound to the surface of the organism so that the copper levels measured in Figure 6.4 are indicative of copper which was intracellular and not bound to the surface of the organisms (Norris and Kelly, 1977).

When the uptake of copper ions was measured in the presence of methane at 30°C rather than the normal incubation temperature (45°C) the rate of assimilation was markedly decreased (Figure 6.5). The decreased copper uptake which occurs in the absence of methane (Figure 6.4c) and at a reduced temperature (Figure 6.5) suggests that the assimilation of copper by M. capsulatus (Bath) is energy-dependent, a fact that is also suggested by experiments in Section 6.4.2.

The rapid accumulation of copper within the cell in the presence of methane was equal to the time taken for the induction of particulate MMO activity observed when copper was added to chemostat cultures of M. capsulatus (Bath) which are expressing soluble MMO activity (Chapter 3 this thesis). Furthermore, the rapid assimilation of copper ions by the cells might also explain the rapid inhibition of soluble MMO activity that occurs when copper ions are added to cultures of M. capsulatus (Bath) expressing such activity (Dalton et al., 1984; Chapter 3, this thesis). The possible involvement of copper ions in the inhibition of soluble MMO activity was further examined and the results presented in the following section.

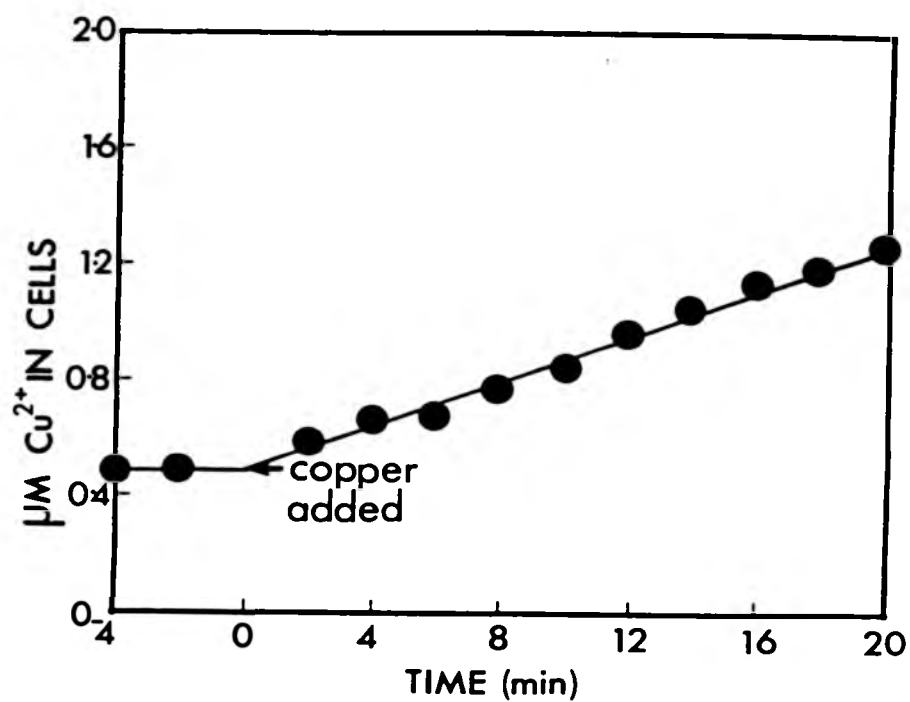


Figure 6.5      Copper uptake by cell suspensions of *M. capsulatus*  
(Bath) - The effect of temperature on the intracellular  
accumulation of copper ions

The experiment was identical to that described in Figure 6.4b except that incubation temperature was lowered from 45°C to 30°C.

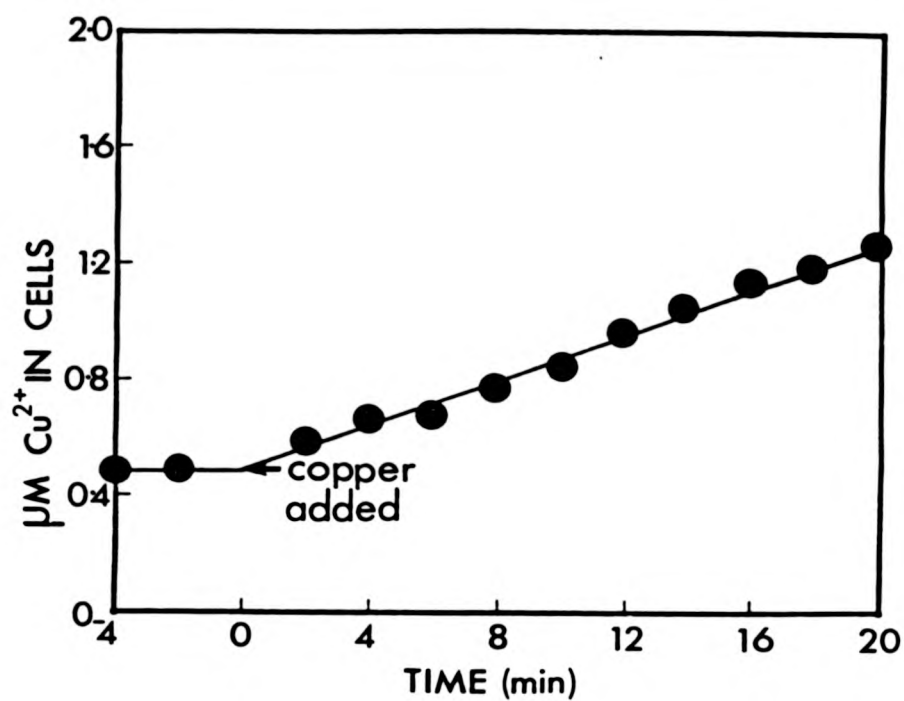


Figure 6.5

Copper uptake by cell suspensions of *M. capsulatus*  
(Bath) - The effect of temperature on the intracellular  
accumulation of copper ions

The experiment was identical to that described in Figure 6.4b except that incubation temperature was lowered from 45°C to 30°C.

#### 6.4 Inhibition of Soluble Activity by Copper Ions

The loss of soluble MMU activity upon addition of copper ions to cell suspensions of M. capsulatus (Bath) grown under copper-limited conditions was reported in Chapter 3 of this thesis and by Dalton et al. (1984). These workers also reported that copper ions were the only metal ions of eight that were tested, to show any inhibition of soluble MMU activity at metal ion concentrations up to 0.1 mM. The mechanism by which the soluble enzyme was inhibited was not reported, but experiments detailed in Chapter 3 showed that addition of copper ions to cells exhibiting soluble MMU activity did not lead to rapid loss of proteins associated with Protein A of the enzyme complex and suggested that the site of inhibition may be at the level of Proteins B and C. The effect of metal ions on soluble MMU activity was investigated to try and determine the specificity of the metal ions capable of inhibiting the enzyme and to investigate the site of inhibition of the soluble enzyme by copper ions.

##### 6.4.1 The effect of the addition of metal ions on in vivo and in vitro soluble MMU activity

The effect of various metal ions on soluble MMU activity are shown in Table 6.4. All the experiments were performed in Pipes buffer to prevent any metal binding by the assay system and to give the metal ions free access to the cells or the protein. Whole cell studies were performed with cell suspensions that were preincubated with sodium formate (5 mM) and the indicated metal ion for five min. prior to estimation of MMU activity to ensure that cells had sufficient time for

Table 6.4      Inhibition of soluble MMO by a variety of metal ions

The figures measure the percentage of specific activity relative to the rates measured with no metal ion addition (100%). Assays were performed as described in Materials and Methods. Soluble MMO assays contained 2 nmole protein A, 1.8 nmole protein B, 2 nmole of protein C and 5  $\mu$ mol NADH (100% = 88 mU/mg). NADH:acceptor reductase activity was measured with 0.1 nmole of protein C (100% = 30.5 U/mg) using the method of Colby and Dalton (1978). The metal ion concentration was held at 40 times the protein C concentration. For whole-cell studies metal ions were 10 mM except silver which was used at 1 mM. The sulphate salts of the metal ions were used except where indicated.

<u>Metal ion</u>	<u>Whole-cell</u>	<u>Soluble MMO</u>	<u>NADH:acceptor-reductase</u>
<u>added</u>	<u>suspension</u>	<u>% Rate</u>	<u>% Rate</u>
	<u>% Rate</u>		
No addition	100	100	100
Fe <sup>2+</sup>	67	108	83
Fe <sup>3+</sup>	63	88	98
Cu <sup>+</sup> (Cl <sup>-</sup> )	22	0	0
Cu <sup>2+</sup>	27	0	0
Cd <sup>2+</sup>	26	0	22
Ag <sup>+</sup> (NO <sub>3</sub> <sup>-</sup> )	20	0	2
Zn <sup>2+</sup>	64	48	80
Ni <sup>2+</sup>	36	116	91
Co <sup>2+</sup>	49	95	91

metal uptake. Cell-free studies were performed using purified components of the soluble MMO with NADH as the electron donor. The components were purified according to the methods of Woodland and Dalton (1984b) and Lund and Dalton (1985). The effect of metal ions on the independent dye reductase activity of Protein C of the soluble MMO complex was also investigated; these experiments were performed in conjunction with Dr. J. Green using the method of Colby and Dalton (1978).

Copper (I), copper (II), silver and cadmium ions were found to be good inhibitors of soluble MMO activity with both whole-cell suspensions and purified proteins; these metal ions were also effective at inhibiting NADH:acceptor reductase activity of Protein C. Nickel ions inhibited whole-cell MMO activity but did not affect the activity of the purified enzyme. This suggests that nickel may be preventing NADH production from formate in whole cells, and thus, indirectly causing a decrease in MMO activity. Zinc was found to be an inhibitor of purified MMO activity and also NADH:acceptor reductase activity, but was a poorer inhibitor of whole-cell activity. This may reflect difficulties in the uptake of this ion, or may be due to sequestration of the ion by the cell such that it is unable to act on MMO. These results should be contrasted to the effect of metal ions on particulate MMO activity as described in Chapter 5.

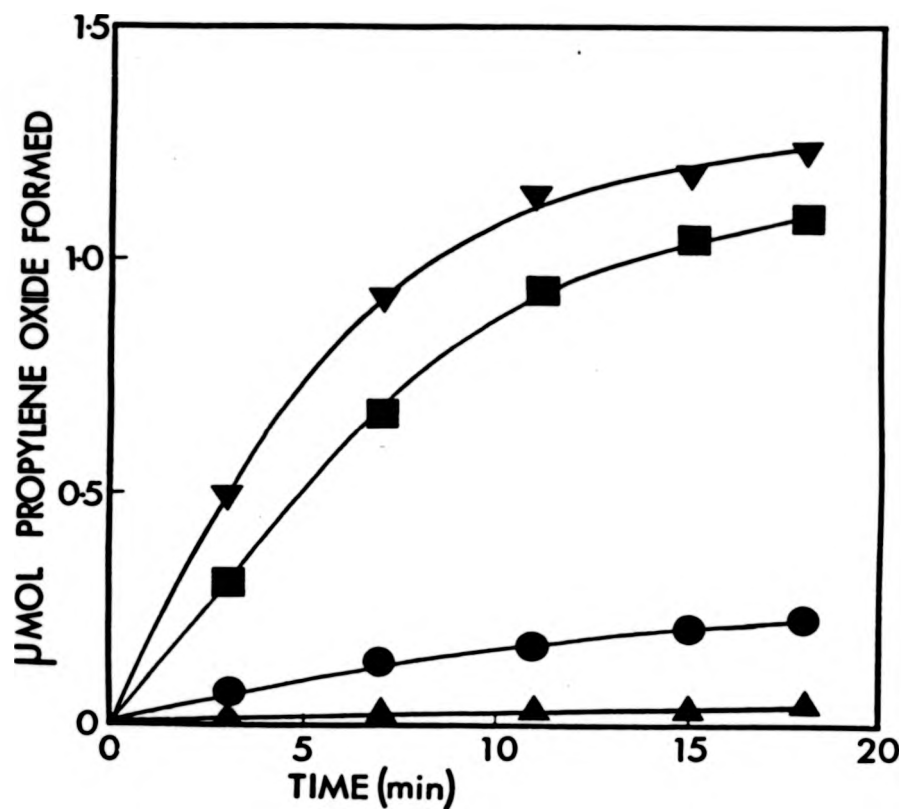
The inhibition patterns observed with purified soluble MMO and the NADH:acceptor reductase activity of Protein C were similar (Table 6.4) indicating that the site of metal ion inhibition was centred on Protein

C of the enzyme complex. It was also noted that cadmium and zinc appeared to be less effective inhibitors of NADH:acceptor reductase activity than of monooxygenase activity suggesting that these metal ions may act at more than one site on the enzyme.

#### 6.4.2 Inhibition of whole cell soluble MMO activity by copper

The demonstration that the addition of copper ions to cells exhibiting soluble MMO activity led to rapid inhibition of the soluble enzyme was discussed in Section 6.4. This rapid inhibition was investigated further in a series of experiments which were designed to determine whether the inhibition was energy dependent and also to determine whether the rate of inhibition was consistent with the results of studies on copper uptake by the organism which were discussed in Section 6.2.

Soluble MMO activity in M. capsulatus (Bath) requires NADH as the in vitro electron donor (Colby and Dalton, 1976); in whole cell suspensions NADH can be generated by the action of formate dehydrogenase on exogenous formate or formaldehyde dehydrogenase on formaldehyde (Colby and Dalton, 1976). Whole cell MMO assays were performed as described in Materials and Methods with the combinations of formate and copper sulphate indicated in the legend. The results are shown in Figure 6.6. The results indicate that the extent of inhibition of soluble MMO activity in whole cells by copper depends on the presence of formate and suggests that copper uptake by the cells requires energy. This confirms the findings outlined in Section 6.3 that the assimilation of copper into the cell is an energy-dependent reaction. The results shown in



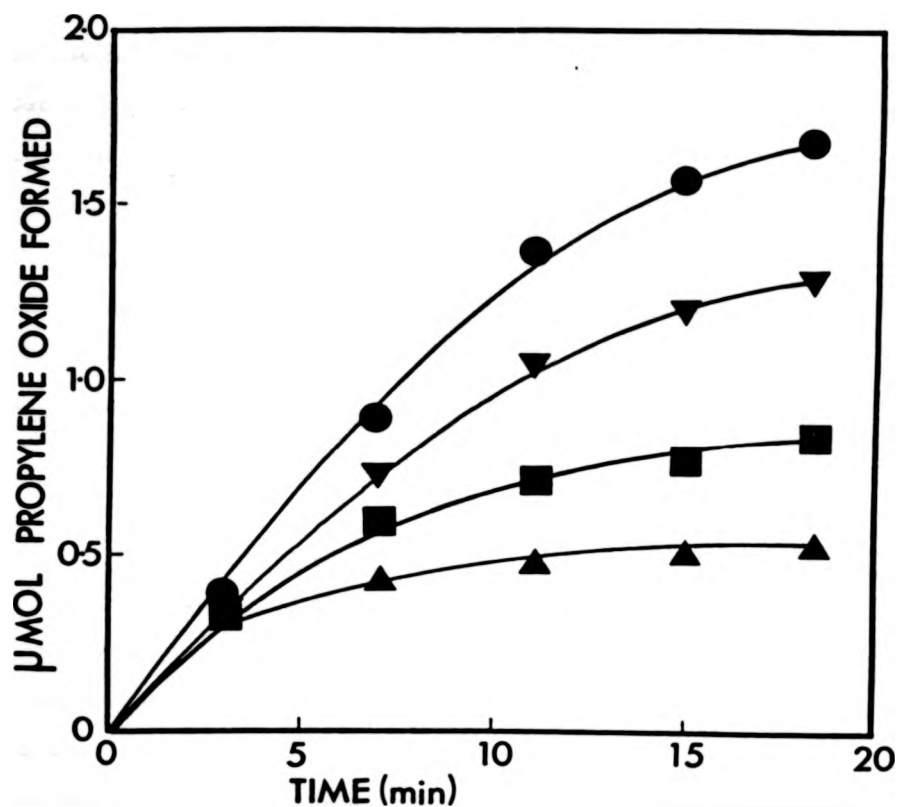
**Figure 6.6** Inhibition of whole-cell soluble MMO by copper in the presence and absence of formate

Whole-cell MMO assays were performed as described in Materials and Methods. The cells used in these assays were preincubated for 20 min. at 45°C with 5 mM formate (■), 1 mM copper sulphate (●), and 5 mM formate plus 1 mM copper sulphate (▲). A control experiment in which no addition was made to the preincubation mix is shown (▼).

Figure 6.6 show that copper uptake can occur in the absence of formate but that uptake, and hence inhibition, was greatly increased when an energy source was provided. The inhibition of the enzyme which occurs in the presence of formate without added copper was presumably either due to the instability of one or more of the soluble MMO components or to a loss of cell viability due to the toxic effect of formate on some other component of the cell.

In the absence of an exogenous energy supply (such as formate) the extent of inhibition of the soluble MMO by copper was found to be concentration dependent (Figure 6.7). The results showed that as the copper concentration in the assay system increased the extent of inhibition of the soluble MMO also increased. It is interesting to note that during the first 3 mins of the experiment copper in the assay system produced no significant difference in the rate of propylene oxide formation. This suggests that in the absence of an energy source the level of copper assimilation in this period of time is insufficient to inhibit the soluble enzyme. After 3 min the level of copper in the cell increased, due to diffusion of the metal ion into the cell, the soluble enzyme was inhibited and the rate of propylene oxide formation declined. The rate of diffusion is concentration dependent, hence the observation that the rate of inhibition increases as the concentration of the metal ion in the assay system increases.

These experiments complement the studies performed on copper uptake by M. capsulatus (Bath) (Chapter 6.3) and provide further evidence that the rapid inhibition of soluble MMO activity noted when copper ions were



**Figure 6.7**      Energy requirement for whole cell soluble MMO inhibition

Whole-cell MMO assays were performed as described in Materials and Methods. The assays were performed in the presence of copper sulphate at the levels indicated (●) 0 mM; (▼) 0.1 mM; (■) 1.0 mM; (▲) 10 mM. Propylene oxide formation was followed gas chromatographically at intervals over a 20 min period.

added to chemostat cultures of the organism (Dalton et al., 1984) was due to inhibition of the enzyme by copper.

The availability in our laboratory of the purified components of the soluble MMO from M. capsulatus (Bath) allowed for a detailed study of the mechanism by which copper inhibited this enzyme to be performed. The results outlined in Section 6.4.2 suggested that the site of inhibition of the enzyme might be Protein C which is known to have NADH:acceptor reductase activity (Colby and Dalton, 1979) hence a study was undertaken to determine the effect of copper ions on Protein C of the soluble MMO from M. capsulatus (Bath).

#### 6.4.3 The effect of copper ions on Protein C of the soluble MMO from M. capsulatus (Bath)

This study was performed in conjunction with Dr. J. Green of this laboratory and details can be obtained from Green, Prior and Dalton (1985) [Eur. J. Biochem. In Press].

The results demonstrated that Proteins A and B of the soluble MMO enzyme complex were unaffected by copper ions but the NADH:acceptor reductase activity of Protein C was irreversibly inhibited. The site of copper inhibition was shown to be primarily at the iron-sulphur centre of Protein C, with a secondary affect at the FAD centre when the copper:Protein C ratio is high. Copper appears to bring about the inhibition of soluble MMO by interacting with Protein C to disrupt the protein structure causing, firstly, the loss of the iron-sulphur centre, preventing the transfer of electrons from Protein C to Protein A (Lund

et al., 1985), and secondly, the loss of FAD preventing the protein from accepting electrons from NADH.

These results explain the rapid inhibition of soluble MMO activity which occurs on addition of copper ions to a culture of M. capsulatus (Bath) exhibiting soluble MMO activity. Furthermore, the demonstration that the mechanism of inactivation of soluble MMO is irreversible means that whether the particulate MMO is a copper-containing enzyme or not, (the evidence for this is discussed in Chapter 5), the cell has to synthesize an alternative MMO to the soluble enzyme in order to maintain growth at high copper levels.

## Chapter 7

### Coda

At the start of this project there was some controversy concerning the intracellular location of the enzyme methane monooxygenase (MMO) in the organisms Methylococcus capsulatus (Bath) and Methylosinus trichosporium OB3b. The results of a series of experiments which were outlined in this thesis demonstrated that, contrary to the information published thus far, the intracellular location of the enzyme was regulated by the copper availability in the growth medium of the organism concerned. The results showed that M. capsulatus (Bath) could express both a particulate and a soluble MMO enzyme and that the two enzymes were expressed differentially depending on the copper:biomass ratio in which the organism was grown. This copper-dependent regulation of the intracellular location of the MMO has since been confirmed in M. trichosporium OB3b. The mechanism by which the expression of the different proteins is regulated is not yet clear although evidence was provided which demonstrates that copper acts as an irreversible inhibitor of soluble MMO activity. The fact that copper inhibits the soluble MMO in this manner means that regardless of whether the two enzymes are different, (and the evidence presented in this thesis suggests they are) the organism must synthesize an alternative MMO when exposed to high levels of copper in order to continue growth.

The regulation of the two enzyme activities, and the fact that when grown on methanol M. capsulatus (Bath) expressed MMO activity which was exclusively associated with the membrane fraction of cell extracts, may be important in determining the organism and growth conditions used when a particular bioconversion is required. This is particularly relevant in view of the differences in substrate specificity exhibited by the two

enzymes and reported in this thesis.

Probably the most important short-term goal of any further study on methane oxidation by M. capsulatus is the purification of the particulate enzyme. To date the soluble MMO from this organism remains the most extensively characterized MMO from any methylotroph, and in order to compare the two enzymes the particulate enzyme must be removed from the membrane. The results presented here provide a starting point for solubilization of the enzyme and there is little doubt that until the enzyme is purified a direct comparison between the soluble and particulate enzyme from M. capsulatus cannot be achieved.

The use of radiolabelled acetylene to identify the active site of the soluble and particulate enzymes is potentially an extremely useful tool for the analysis of the MMO enzymes. The ability to bind a radiolabel to specific proteins means that using a variety of techniques, the proteins which constitute the active site of the enzyme can be identified and eventually sequenced. This technique when applied to M. capsulatus, will allow for a direct comparison to be made between the active sites of the soluble and particulate MMO enzymes. It may also prove useful in identifying and purifying the active site proteins from other methylotrophs and may even allow for a comparison to be made between the MMO from methylotrophic bacteria and the ammonia monooxygenase from Nitrosomonas sp. as these two enzymes are thought to be closely related. The identification of the active site proteins and their amino acid sequence might also allow for a genetic probe to be synthesized and in this way it may be possible to study the genetic

control of methane oxidation. Until now this has remained a problem because of the difficulties experienced in trying to produce mutants of methylotrophs that lack MMO activity.

In conclusion, the results presented here answer some of the questions about the intracellular location of the methane monooxygenase in M. capsultus (Bath), and in addition, provide several new areas for research in the field of methylotrophic bacteria.

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