Journal of General Microbiology (1985), **131**, 155–163. *Printed in Great Britain*

The Effect of Copper Ions on Membrane Content and Methane Monooxygenase Activity in Methanol-grown Cells of *Methylococcus capsulatus* (Bath)

By STEPHEN D. PRIOR AND HOWARD DALTON*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

(*Received 21 August 1984*)

Methylococcus capsulatus (Bath) was grown in continuous culture with methanol (1·0%, v/v) as sole carbon and energy source. Cells grown on methanol exhibited differences in methane monooxygenase (MMO) activity which were dependent on the concentration of copper sulphate present in the growth medium; an increase in the concentration of copper in the growth medium enhanced both *in vivo* and *in vitro* MMO activity. The MMO activity in methanol-grown *Methylococcus capsulatus* (Bath) was always associated with the particulate fraction of cell-free extracts; at no time was soluble MMO activity detected. *In vitro* MMO activity was also stimulated by the addition of copper compounds to the assay system and the stimulation was shown to be pH-dependent. The concentration of copper sulphate in the growth medium also determined the intracytoplasmic membrane content of the cells, as judged by electron microscopy of thin-sections, which could be correlated with particulate MMO activity, although it is not possible at this time to say whether the increase in MMO activity seen is due to the increased membrane content or due to the copper ions *per se*.

INTRODUCTION

Methylococcus capsulatus (Bath) is an obligate methylotroph which can utilize either methane or methanol as its sole source of carbon and energy. Growth on methane involves oxidation of the substrate to methanol, a reaction catalysed by the enzyme methane monooxygenase (MMO). A series of oxidation reactions then convert the methanol to carbon dioxide via formaldehyde and formate. A proportion of the formaldehyde produced is assimilated into cellular biomass via the ribulose monophosphate pathway, the remainder being oxidized to provide energy for its assimilation.

Methanol can act as sole carbon and energy source for some methanotrophs but growth can be difficult to achieve since methanol is toxic even at concentrations as low as 0·01% (v/v) (Leadbetter & Foster, 1958; Stocks & McCleskey, 1964; Whittenbury *et al.*, 1970). It is possible to obtain growth on methanol either by preadaptation of the organism, which involves a gradual increase in the amount of methanol fed to the culture (Hou *et al.*, 1979*a*), or by growing the organism in chemostat culture under methanol limitation (Linton & Vokes, 1978).

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* CRL-Ml, and *Methylobacterium organophilum* CRL-26 on methanol caused the loss of MMO activity and they concluded that MMO was induced by methane (Hou *et al.*, 1979*b*). Other reports of growth of methanotrophs on methanol (Linton & Vokes, 1978; Hyder *et al.*, 1979; Best & Higgins, 1981) have shown that MMO activity was retained when cells were grown for periods of up to 9 months on methanol.

Abbreviations: MMO, methane monooxygenase; PAGGE, polyacrylamide gradient gel electrophoresis.

In a recent report, Best & Higgins (1981) showed that *Methylosinus trichosporium* OB3b could be maintained on methanol and that cells harvested from steady-state methanol-limited chemostat culture possessed MMO in the soluble fraction of cell-free extracts.

We have recently demonstrated that the intracellular location of the MMO from methane-grown *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b is determined by the copper : biomass ratio (Stanley *et al.*, 1983). When this value is low the soluble form of the enzyme predominates whereas at a high copper : biomass ratio the particulate form of the enzyme is preferentially present. Copper has also been shown to have an effect on the rate of oxidation of methane, the extent of intracytoplasmic membranes and the rate of growth in batch culture of *Methanomonas margaritae* (Takeda *et al.*, 1976; Ohtomo *et al.*, 1977; Takeda & Tanaka, 1980).

The purpose of the present investigation is to determine to what extent MMO is synthesized in methanol-grown *Methylococcus capsulatus* (Bath), how the concentration of copper in the growth medium affects the activity and intracellular location of the MMO, and to examine the effect of copper on the intracytoplasmic membrane content of the cells.

METHODS

Growth of Methylococcus capsulatus (Bath) and preparation of cell free extracts. Methylococcus capsulatus (Bath) was grown at 45 °C in a 2-l fermenter (L. H. Engineering, Stoke Poges, UK) on nitrate mineral salts medium (Whittenbury *et al.*, 1970). Cultures were initially grown on methane/air (1 : 4, v/v) as sole carbon and energy source and were adapted to growth on methanol by addition of 0·5% methanol to the medium and reducing the methane flow rate to zero over a period of 4 d. Subsequently the methanol concentration in the medium was raised to 1·0% (v/v).

Copper sulphate ($CuSO_4 . 5H_2O$) was added to the medium to give a final concentration of 0, 0·2, 0·6, 1·0, 1·2 and 2·0 mg l^{-1}. At each of the copper concentrations tested the culture was allowed to reach steady-state conditions before cells were removed for experimental procedures. The copper concentration was then increased in the medium and a new steady-state attained before the next samples were removed.

For whole-cell studies, cells were harvested by centrifugation (10000 *g* for 10 min) at 4 °C, washed twice in ice-cold 20 mM-sodium/potassium phosphate buffer pH 7·0 and resuspended to an OD_{540} of 20 (approximately 4 mg dry wt ml^{-1}).

Cell-free extracts for the soluble and particulate forms of the enzyme were prepared by slightly different methods. Cells were harvested by centrifugation at 10000 *g* for 10 min at 4 °C, washed in cold 20 mM-Tris/HCl 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 component C of the enzyme (Colby & 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 10000 *g* for 15 min. The cell-free extract was then separated into soluble and particulate fractions by centrifugation at 38000 *g* for 30 min.

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

MMO assay. MMO activity in cell-free 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 mg ml^{-1}. Propylene (2·5 ml) was added through a Suba-Seal and the flask equilibrated at 45 °C for 30 s prior to addition of NADH (5 mM). The particulate enzyme was assayed similarly but the buffer pH was 7·5 (the particulate enzyme has maximal activity at pH 7·5). The activity of the particulate MMO was also determined in the presence of copper compounds at pH 7·0 and 7·5.

Protein concentration was determined using the commercially available BioRad Protein Assay Concentrate.

Whole cell respiration studies. A Clark-type oxygen electrode (Rank Bros, Bottisham, Cambridge, UK) was used, with a reaction vessel of 3 ml capacity. All assays were carried out at 45 °C and contained 20 mM-sodium/potassium phosphate buffer pH 7·0 and sufficient whole cell suspension to give a final OD_{540} of 2·0 (0·5 mg dry wt cells ml^{-1}). 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).

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 carried out according to the method of Reynolds (1963) and the samples examined in a Joel 2 electron microscope.

SDS-polyacrylamide gradient gel electrophoresis (*SDS–PAGGE*). This was done using a linear gradient vertical slab gel and discontinuous buffering system (Laemmli, 1970). Polypeptide bands were visualized by staining with Coomassie Blue R250 (BDH).

Chemicals. Methane, ethylene and propylene were obtained from British Oxygen Co. Ltd, London, UK. Acetylene was obtained from Matheson Gas Products, Croydon, UK. Materials for gel electrophoresis were obtained from BDH. Electron microscopy materials were supplied by the following: uranyl acetate and osmium tetroxide, Polaron Equipment Ltd., Watford, Herts, UK, and Araldite epoxy resin by Emscope, Ashford, Kent, UK. All other chemicals were supplied by Fisons Scientific Apparatus.

RESULTS

Effect of addition of copper on methanol-grown Methylococcus capsulatus (*Bath*)

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 $CuSO_4.5H_2O$ l^{-1}).

When *Methylococcus capsulatus* (Bath) was grown on methane as the sole carbon and energy source an increase in the copper sulphate concentration in the medium led to an increase in cell density of approximately 25–30% as evidenced by increased OD_{540} and dry wt of cells (mg l^{-1}). 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 methanol-grown cells has also been observed in batch cultures of the obligate methylotroph *Methyanomonas margaritae* (Takeda & Tanaka, 1980).

Addition of copper to methanol-grown cells does, however, affect the activity of the MMO located in the particulate fraction of cell-free extracts. As the copper concentration in the growth medium was raised there was an increase in MMO activity as measured by the propylene epoxidation assay (Table 1). This activity was totally inhibited by addition of 0·1 mM-potassium cyanide to the assay showing that the enzyme activity was due to the particulate MMO rather than the soluble MMO since potassium cyanide is a potent inhibitor of the particulate but not the soluble enzyme (Stanley *et al.*, 1983). The particulate MMO was shown to exhibit maximal activity at pH 7·5 in the absence of copper in contrast to the soluble MMO isolated from cells grown on methane which as maximum activity at a pH value of 7·0. The addition of copper salts to the assay system at pH 7·0 enhanced propylene epoxidation activity as shown in Table 1 and below. This activity enhancement ranged from 60 to 80% above the activity measured without added copper and at very low concentrations of copper in the growth medium (less than 0·2 mg l^{-1}) it was only possible to detect *in vitro* particulate MMO activity by adding copper to

Table 1. *Effect of $CuSO_4$ on MMO activity in cell-free extracts of Methylococcus capsulatus* (*Bath*) *grown on methanol*

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

$CuSO_4$ in growth medium (mg l^{-1})	Particulate MMO specific activity [nmol propylene oxide formed min^{-1} (mg protein)$^{-1}$]	
	Normal assay (pH 7·5)	$CuSO_4$ in assay (pH 7·0, 0·4 mM-Cu^{2+})
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

Table 2. *Effect of adding $CuSO_4$ to the growth medium on whole cell oxidation rates of Methylococcus capsulatus grown on methanol*

Values given are means of two experiments. Included is the spread of results from the mean.

Substrate (concn, mM)	Oxidation rates [nmol O_2 consumed min^{-1} (mg protein)$^{-1}$]	
	No Cu^{2+} in growth medium	Cu^{2+} (1·2 mg l^{-1}) in growth medium
Methane (0·1)	61 ± 5	480 ± 22
Methanol (1·0)	130 ± 4	312 ± 10
Ethanol (1·0)	100 ± 10	158 ± 16
Formaldehyde (1·0)	94 ± 4	117 ± 4
Formate (1·0)	86 ± 7	94 ± 10
Ethylene (1·2)	0	12 ± 2
Propylene (1·5)	0	6 ± 3
Acetylene (1·2)	0	0

the assay system. There was no enhancement of activity when copper was added to the assay system at pH 7·5. Addition of the same concentration of copper sulphate (0·4 mM) to an active soluble cell-free extract of *Methylococcus capsulatus* grown on methane inhibited the epoxidation activity by 90% (Dalton *et al.*, 1984).

Addition of copper to the growth medium also led to a change in the oxidation rates of whole cells (Table 2). With no copper in the growth medium the rate of oxygen consumed when methane was added as a substrate was only 15% of the rate of oxygen consumption when 1·2 mg l^{-1} copper was present in the growth medium. The lowest concentration of copper in the medium was nominally zero (i.e. no copper was added), however, it is possible that traces of copper may have been present although all the chemicals used were of analytical grade.

Although alkenes are substrates for the MMO both *in vivo* and *in vitro* when suitable electron donors are supplied, neither ethylene nor propylene were oxidized here to any great extent by whole cells because the products of oxidation of these compounds cannot be further metabolized by the cell and so cannot supply the reducing equivalents necessary for continued MMO activity (Stirling & Dalton, 1979).

Assays were also performed with copper nitrate and copper chloride replacing copper sulphate. The results showed that copper chloride enhanced activity of the particulate MMO slightly more (12%) than copper sulphate and that copper nitrate was slightly less (26%) effective than the sulphate.

Although *Methylococcus capsulatus* showed whole cell MMO activity when grown on methanol with or without added copper, no MMO activity was detected in the soluble fraction of cell-free extracts. Purified components of the *Methylococcus capsulatus* soluble MMO (provided by Dr J. Lund of this department) were used to confirm that the soluble fraction of the cell-free extract did not lack activity due to the absence of a single component. Neither proteins A, B or C of the MMO complex nor any combination of these components reconstituted activity but addition of all three components to the soluble fraction gave activity and showed that there was no inhibitor of propylene oxidation present in the extract. It was also shown by Ouchterlony double-diffusion plates that the inactive soluble fraction exhibited no cross-reactivity to antibody raised to active component A of the soluble MMO (the antibody was provided by Dr M. P. Woodland of this department).

The change in the protein banding on SDS–PAGGE (Fig. 1) indicates that increasing the concentration of copper in the growth medium led to the synthesis of at least three major proteins which were either absent or present at very low levels in copper-deficient media. These proteins had molecular weights (46000; 35000; 25000) 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 (Stanley *et al.*, 1983).

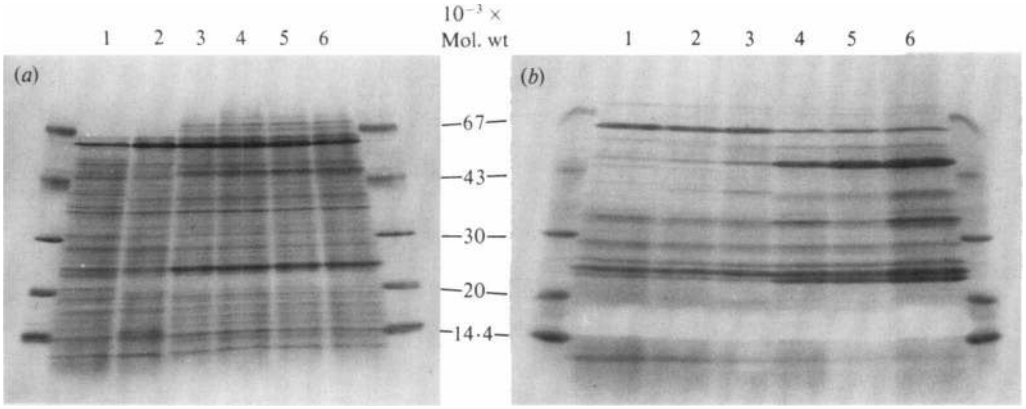

Fig. 1. SDS–PAGGE of cell free extracts from *Methylococcus capsulatus* (Bath) grown on methanol with different concentrations of copper sulphate in the growth medium. Soluble fractions (*a*) and particulate fractions (*b*) were prepared as indicated in Methods. Tracks 1–6, copper sulphate concentration (mg l^{-1}) in the growth medium as follows: 0, 0·2, 0·6, 1·0, 1·2, 2·0. Standards used were BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20000) and α-lactalbumin (14400). Proteins were stained using Coomassie Blue R250.

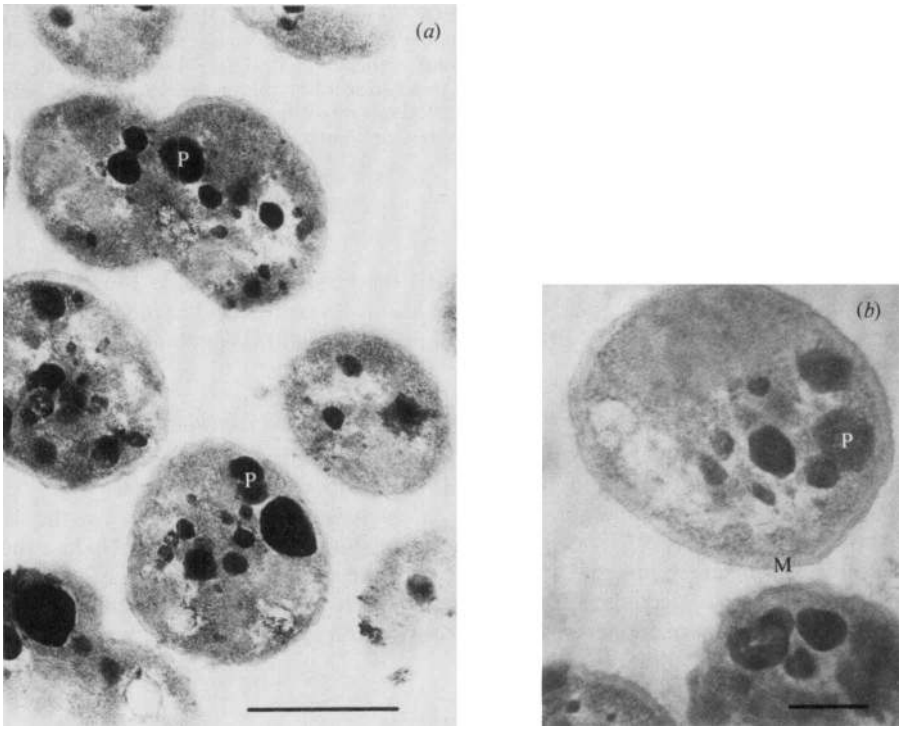

Fig. 2. Electron photomicrographs of thin sections of *Methylococcus capsulatus* (Bath) grown on methanol with no copper added to the growth medium. (*a*) Field view of cells displaying electron-dense particles (P) and lacking intracytoplasmic membrane arrays. (*b*) Thin-section of cells grown on methanol with no copper added to growth medium and showing the presence of electron-dense particles (P) and membranes occurring only at the cell periphery (M). Bar markers, 0·5 μm.

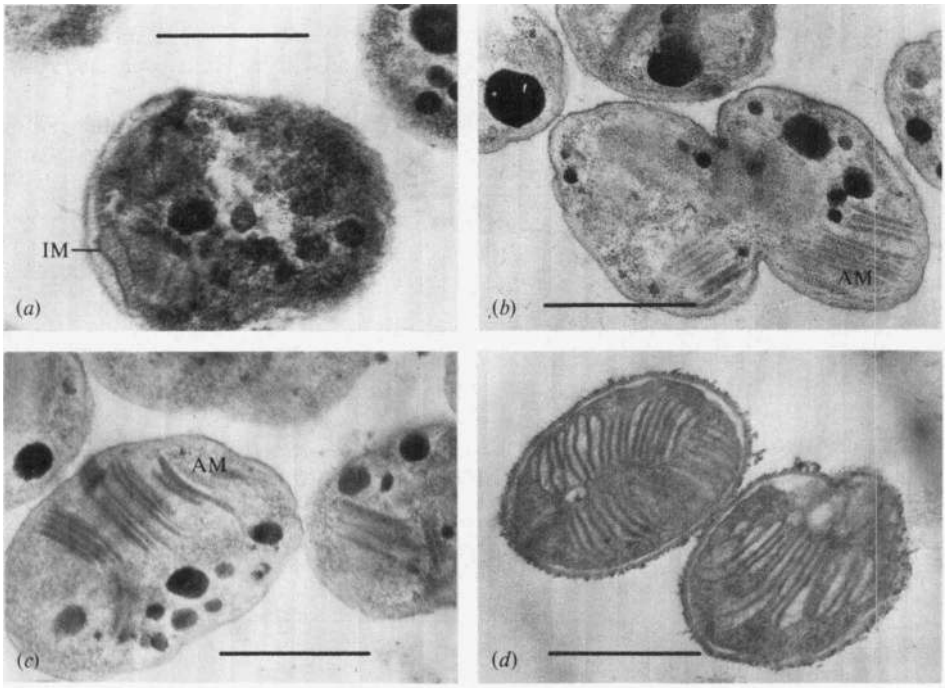

Fig. 3. Thin-section electron photomicrographs of *Methylococcus capsulatus* (Bath). (*a*) Cells grown on medium containing methanol and 0·2 mg copper sulphate l^{-1}. Intracytoplasmic membranes are visible (IM). (*b*, *c*) Cells grown on medium containing methanol and 0·6 mg copper sulphate l^{-1}. Intracytoplasmic membrane arrays (AM) are now clearly discernible within the cells. (*d*) Cells grown on medium containing 1·2 mg copper sulphate l^{-1} displaying large numbers of intracytoplasmic membranes and totally lacking the electron-dense particles found at lower concentrations of copper sulphate. Bar markers, 0·5 μm.

The protein banding on SDS gels also confirmed the absence of soluble MMO activity, as evidenced by the lack of protein bands which are known to correspond to the three subunits of protein A of the soluble MMO (Mol. wt 54000, 36000, 17000) (Woodland & Dalton, 1984).

Electron microscopy of methanol-grown Methylococcus capsulatus (Bath)

Thin-section electron microscopy of the cells grown at different concentrations of copper showed that at copper concentrations of 0·2 mg l^{-1} and above, the cells had very tightly packed arrays of intracytoplasmic membranes which increased in frequency as the copper concentration increased. At 2·0 mg copper l^{-1} in the medium the cells appeared to lose the tight packing and exhibit larger lumenal spaces between the membranes. With no copper in the medium the cells were of decreased size and totally lacked the membrane structures seen with the higher concentrations of copper; instead there appeared cyst-like organelles as shown in Figs 2 and 3.

DISCUSSION

Conflicting 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 (Leadbetter & Foster, 1958; Stocks & McCleskey, 1964; Whittenbury *et al.*, 1970). More recently methods have been published for the

adaptation of organisms to growth on methanol and several reports of methanol-grown methylotrophs are available (Linton & Vokes, 1978; Hou *et al.*, 1979*a*; Hyder *et al.*, 1979; Best & Higgins, 1981).

The results of our experiments prove that *Methylococcus capsulatus* is able to utilize methanol as sole source of carbon and energy and that such cells retain the ability to oxidize methane. In addition to oxidizing methane the cells can also oxidize a variety of other compounds. This agrees with the results of Linton & Vokes (1978) who maintained *Methylococcus* NCIB 11083 on methanol for 50 d without the cells losing methane-oxidizing activity. Similar results have been demonstrated with *Methanomonas margaritae* and *Meythylosinus trichosporium* OB3b (Takeda *et al.*, 1976; Best & Higgins, 1981).

Most of the work on the retention of methane-oxidizing activity when organisms are grown on methanol was done with whole cells. However, Best & Higgins (1981) have demonstrated *in vitro* MMO activity in the soluble fraction of cell-free extracts of *Methylosinus trichosporium* OB3b grown on methanol. In experiments reported here growth of *Methylococcus capsulatus* on methanol has demonstrated that MMO activity is due entirely to the particulate form of the enzyme, and that at no time during growth on methanol was the soluble form of the enzyme detected.

We have recently reported (Stanley *et al.*, 1983) that the intracellular location of the MMO in *Methylococcus capsulatus* and *Methylosinus trichosporium* OB3b grown on methane was dependent on the copper : biomass ratio. To determine whether the difference in localization of the MMO activity when cells are grown on methanol was also due to copper, the organism was grown at a variety of copper concentrations. The results show that particulate MMO was present in the cells at all concentrations of copper used in the experiment and that the activity of the enzyme increased with increasing copper concentrations in the growth medium.

The effect of copper on methylotrophs was first noted by Takeda *et al.* (1976), who demonstrated that when *Methanomonas margaritae* was grown on methane, growth was highly accelerated by the addition of copper ions; subsequently Ohtomo *et al.* (1977) and Takeda & Tanaka (1980) reported that in *Methanomonas margaritae* the presence of intracytoplasmic membranes was dependent on the addition of copper sulphate to the media and that in a copper-deficient media only vesicles at the cell periphery were present.

In the work presented here, the growth of *Methylococcus capsulatus* (Bath) on methanol, with increasing concentrations of copper sulphate, indicates that the MMO was always present in cells grown on methanol, but that at very low copper concentrations the activity is only detected by the addition of copper to the assay system. When copper was added to the growth medium the activity of the particulate MMO increased with increasing copper concentrations until at a concentration of 2·0 mg l^{-1} the copper began to inhibit the activity of the enzyme. This suggests that the particulate MMO in *Methylococcus capsulatus* (Bath) has an absolute copper requirement for activity. The question of whether the copper is directly involved with the enzyme or whether it is regulating the synthesis or activity of other proteins necessary for methane oxidation cannot at this stage be answered.

The addition of copper to cell-free extracts of *Methylococcus capsulatus* caused stimulation of the particulate MMO activity; this effect has also been observed in '*Methylosinus*' sp. CRL-15 (Patel *et al.*, 1979). The fact that this stimulation occurred immediately after addition of copper to the assay system suggests that the copper is acting as a cofactor or prosthetic group for a protein that is already present in the extract and is not required for *de novo* protein synthesis. The copper requirement of the cells for cell-free methane-oxidizing activity is also shown by the fact that cells grown on methanol in copper-deficient media only exhibit *in vitro* MMO activity when copper was added to the assay system. If copper plays a direct role in the oxidative activity of the particulate MMO then the stimulation of *in vitro* activity by copper at pH 7·0 and the lack of similar stimulation at pH 7·5 might be explained by a pH-dependent binding of copper to the enzyme. It has been shown that copper can bind to amino acid ligands and that this binding is pH dependent (Hallman *et al.*, 1971). It has been computed that at pH 7·4 98% of Cu^{II} was co-ordinated to cystine and histidine; in another experiment Perrin and Argawal (1973) reported that maximal binding of free Cu^{II} to pentaglycine occurred at pH 7·4. If, as in these examples,

copper exhibits maximal binding to the enzyme at pH 7·4 then the addition of extra copper to the assay system at this pH will not lead to any great increase in enzyme activity. At pH 7·0 a lower percentage of the available copper is bound to the enzyme causing a decrease in activity; raising the concentration of free copper by the addition of copper salts to the assay system can lead to an increase in the amount of copper binding to the enzyme and thus produce increased MMO activity. The isolation of a copper-requiring MMO from *Methylosinus trichosporium* OB3b was reported by Tonge *et al.* (1977) but a relationship between copper, MMO activity and localization of the enzyme was not shown at that time.

It is quite clear from the results presented in this work that the concentration of copper in the growth medium can exert a marked effect on the expression of whole cell MMO activity in methanol-grown cells. In this context therefore, it is relevant to note that Patel *et al.* (1978) and Hou *et al.* (1979*b*) did not observe any whole cell MMO activity in a variety of methanotrophs grown on methanol and concluded that the enzyme was induced by methane. Because the cells in their experiments were grown in batch culture containing only 5 μg $CuSO_4 . 5H_2O\ l^{-1}$ it is almost certain that such cells were copper-limited and therefore would not be able to express the particulate form of the enzyme which appears to have an absolute requirement for copper ions. When *Methylococcus capsulatus* and *Methylosinus trichosporium* OB3b are grown on methane and become copper-limited they express a soluble MMO (Stanley *et al.*, 1983). The results presented here and the lack of any activity in the experiments of Hou *et al.* (1979*b*) and Patel *et al.* (1978, 1979) suggest that most methanotrophs when grown on methanol under copper-limitation cannot express the soluble form of the MMO. This is in contrast to the results of Best & Higgins (1981) who reported that when *Methylosinus trichosporium* OB3b was grown on methanol, MMO activity was retained in the soluble fraction of cell-free extracts. This apparent anomaly might be explained by the fact that the cell-free extracts were prepared by different methods. Our experimental procedure involved the use of a French pressure cell to break the cells whereas Higgins' group used sonication. In a recent set of unpublished experiments we have shown that sonication of membrane fractions exhibiting particulate MMO activity can lead to release of activity into the soluble fraction. The possibility that the difference in localization may be due to the method of breakage is currently under investigation.

Thin-section electron microscopy of *Methylococcus capsulatus* (Bath) grown on methanol showed that in copper-deficient media the cells were slightly smaller than those grown in copper-containing media and that they totally lacked intracytoplasmic membranes. The cells appeared to have no internal membrane structures but had inclusions similar to the electron-translucent droplets described by Hyder *et al.* (1979) who grew *Methylococcus capsulatus* (Texas) on methanol. The lack of internal membranes when cells are grown on copper-deficient media but with methane rather than methanol as the carbon and energy source has also been observed with *Methanomonas margaritae* (Ohtomo *et al.*, 1977) and *Methylococcus capsulatus* (Bath) (S. D. Prior, unpublished observations).

The role of copper in the transition of cells from an intracellular structure lacking membrane arrays to one containing stacked arrays of intracytoplasmic membranes is not clear. The link between methane oxidation and intracytoplasmic membranes has been postulated (Hyder *et al.*, 1979; Takeda *et al.*, 1976; Takeda & Tanaka, 1980). The results presented here show that copper can exert an effect on both the levels of MMO and the presence or absence of intracytoplasmic membranes, but the question of whether the induction of enzyme leads to an increase in membrane content or vice versa, or even whether the two events are exclusive, cannot be answered.

We have shown here that the particulate MMO appears to be constitutively synthesized when cells are grown on methanol, a fact that is not too surprising when one considers that methanol is also a substrate of the MMO (Colby *et al.*, 1977) and that the amount and activity of the enzyme can be increased by the addition of copper to the medium of methanol-grown cells. It also shows that intracytoplasmic membranes increase in number in the presence of copper and may play an important role in the expression of MMO activity in *Methylococcus capsulatus* (Bath).

What is apparent from this study is that the conditions, and in particular the copper concentration, under which methylotrophs are grown can affect not only the levels of key enzymes in the cell but also the intracellular morphology.

This work was supported through a Science and Engineering Research Council Studentship to S.D.P.

REFERENCES

BEST, D. J. & HIGGINS, I. J. (1981). Methane-oxidising activity and membrane morphology in a methanol-grown obligate methanotroph, *Methylosinus trichosporium* OB3b. *Journal of General Microbiology* **125**, 73–84.

COLBY, J. & DALTON, H. (1978). Resolution of the methane monooxygenase of *Methylococcus capsulatus* (Bath) into three components. *Biochemical Journal* **171**, 461–458.

COLBY, J., STIRLING, D. I. & DALTON, H. (1977). The soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers and alicyclic aromatic and heterocyclic compounds. *Biochemical Journal* **165**, 395–402.

DALTON, H., PRIOR, S. D., LEAK, D. J. & STANLEY, S. H. (1984). Regulation and control of methane monooxygenase. In *Microbial Growth on C-1 Compounds, Proceedings of the 4th International Symposium*, pp. 75–82. Edited by R. L. Crawford & R. S. Hanson. Baltimore, USA: American Society for Microbiology.

HALLMAN P. S., PERRIN, D. D. & WATT, A. E. (1971). The computed distribution of copper (II) and zinc (II) ions among seventeen amino acids present in human blood plasma. *Biochemical Journal* **121**, 549–555.

HOU, C. T., LASKIN, A. I. & PATEL, R. (1979*a*). Growth and polysaccharide production by *Methylocystis parvus* OBBP on Methanol. *Applied and Environmental Microbiology* **37**, 800–804.

HOU, C. T., PATEL, R., LASKIN, A. I. & BARNABE, N. (1979*b*). Microbial oxidation of gaseous hydrocarbons; epoxidation of C_2-C_4 *n*-alkanes by methylotrophic bacteria. *Applied and Environmental Microbiology* **38**, 127–134.

HYDER, S. L., MEYERS, A. & CAYER, M. L. (1979). Membrane modulation in a methylotrophic bacterium *Methylococcus capsulatus* (Texas) as a function of growth substrate. *Tissue and Cell* **11**, 597–610.

KELLENBERGER, E., RYTER, A. & SECHAUD, J. (1958). Electron microscope study of DNA-containing plasma. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *Journal of Biophysical and Biochemical Cytology* **4**, 671–678.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–689.

LEADBETTER, E. R. & FOSTER, J. W. (1958). Studies on some methane-utilising bacteria. *Archive für Mikrobiologie* **30**, 91–118.

LINTON, J. D. & VOKES, J. (1978). Growth of the methane-utilising bacterium *Methylococcus* NCIB 11083 in mineral salts medium with methanol as sole source of carbon. *FEMS Microbiology Letters* **4**, 125–128.

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *Journal of Biophysical and Biochemical Cytology* **9**, 409.

OHTOMO, T., IIZUKA, H. & TAKEDA, T. (1977). Effect of copper sulphate on the morphologic transition of a strain of *Methanomonas margaritae*. *European Journal of Applied Microbiology* **4**, 267–272.

PATEL, R. N., HOU, C. T. & FELIX, A. (1978). Microbial oxidation of methane and methanol: isolation of methane-utilising bacteria and characterisation of a facultative methane-utilising isolate. *Journal of Bacteriology* **138**, 352–358.

PATEL, R. N. HOU, C. T., LASKIN, A. I., FELIX, A. & DERELANKO, P. (1979). Microbial oxidation of gaseous hydrocarbons. II. Hydroxylation of alkanes and epoxidation of alkenes by cell-free particulate fractions of methane-utilising bacteria. *Journal of Bacteriology* **139**, 675–679.

PERRIN, D. D. & ARGAWAL, R. P. (1973). Multimetal–multiligand equilibria: A model for biological systems. In *Metal Ions in Biological Systems*, vol. 2, pp. 176–180. Edited by H. Sigel. New York: Marcel-Dekker.

REYNOLD, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

STANLEY, S. H., PRIOR, S. D., LEAK, D. J. & DALTON, H. (1983). Copper stress underlies the fundamental change in intracellular location of methane monooxygenase in methane-oxidizing organisms: studies in batch and continuous cultures. *Biotechnology Letters* **5**, 487–492.

STIRLING, D. I. & DALTON, H. (1979). Properties of the methane monooxygenase from extracts of *Methylosinus trichosporium* OB3b and evidence for its similarity to the enzyme from *Methylococcus capsulatus* (Bath). *European Journal of Biochemistry* **96**, 205–212.

STOCKS, P. K. & MCCLESKEY, C. S. (1964). Morphology and physiology of *Methanomonas methanooxidans*. *Journal of Bacteriology* **88**, 1071–1077.

TAKEDA, K. & TANAKA, K. (1980). Ultrastructure of intracytoplasmic membranes of *Methanomonas margaritae* grown under different conditions. *Antonie van Leeuwenhoek* **46**, 15–25.

TAKEDA, K., TEZUKA, C., FUKUOKA, S. & TAKAHARA, Y. (1976). Role of copper ions in methane oxidation by *Methanomonas margaritae*. *Journal of Fermentation Technology* **54**, 557–562.

TONGE, G. M., HARRISON, D. E. F. & HIGGINS, I. J. (1977). Purification and properties of the methane monooxygenase enzyme system from *Methylosinus trichosporium* OB3b. *Biochemical Journal* **161**, 333–344.

WHITTENBURY, R., PHILLIPS, K. C. & WILKINSON, J. F. (1970). Enrichment, isolation and some properties of methane-utilising bacteria. *Journal of General Microbiology* **61**, 205–218.

WOODLAND, M. P. & DALTON, H. (1984). Purification and characterization of Component A of the methane monooxygenase from *Methylococcus capsulatus* (Bath). *Journal of Biological Chemistry* **259**, 53–59.