In vivo Studies of Primary Alcohols, Aldehydes and Carboxylic Acids as Electron Donors for the Methane Mono-oxygenase in a Variety of Methanotrophs

By DAVID J. LEAK AND HOWARD DALTON*
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

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C₂ to C₄ primary alcohols and their corresponding aldehydes were oxidized by type I, type II and type X obligate methanotrophs. Reducing equivalents from each oxidation step could be utilized, in vivo, to stimulate methane mono-oxygenase activity. As neither oxidation step produces NADH directly, these observations are presented as evidence for reverse electron transport in methanotrophs. In type II methanotrophs, 5 mM-acetate, propionate and butyrate also stimulate methane mono-oxygenase activity apparently by inducing the breakdown of poly-β-hydroxybutyrate, subsequent metabolism of β-hydroxybutyrate giving rise to NADH.

INTRODUCTION

Whittenbury et al. (1970) recognized two classes of obligate methanotroph, types I and II, based on the structure and intracellular location of novel internal membranes. Subsequently this division has been substantiated by a number of biochemical characteristics, notably the different carbon assimilation pathways and the presence or absence of a complete TCA cycle (Colby et al., 1979; Higgins et al., 1981). However, extensive studies in this laboratory on the supposed type I organism Methylococcus capsulatus (Bath) have revealed the presence of some type II (Whittenbury et al., 1975) and some autotrophic (Taylor, 1977) characteristics, and accordingly this has been assigned to a third group, type X (Whittenbury & Dalton, 1981).

In obligate methanotrophs the primary step in methane metabolism is mediated by a mono-oxygenase (MMO), the resulting methanol being oxidized via formaldehyde and formate to carbon dioxide (Fig. 1) or assimilated at the level of formaldehyde into cell material (Colby et al., 1979; Higgins et al., 1981). NAD(P)H-dependent MMO activity has been demonstrated in cell extracts from each of the three types of methanotroph (Dalton, 1981), and the soluble MMO from Methylococcus capsulatus (Bath) purified and extensively characterized in this laboratory (Colby & Dalton, 1978; Woodland & Dalton, 1983). Although NAD⁺-linked formaldehyde and formate dehydrogenase activities have been demonstrated in methanotrophs (Patel & Hoare, 1971; Stirling & Dalton, 1978), theoretical studies suggest that organisms growing on methane would be limited by NADH supply unless methanol dehydrogenase could also supply reducing power to the MMO (Van Dijken & Harder, 1975; Anthony, 1978). However, no NAD⁺-linked methanol dehydrogenase has been found in methanotrophs. Tonge et al. (1977) originally reported that ascorbate or methanol could serve as electron donor for the MMO in extracts of 'Methylomonas trichosporium' OB3b, but these results have not been substantiated (Stirling & Dalton, 1979a; Scott et al., 1981).

In an attempt to discover the in vivo source of the electron donor for the MMO, Ferenci (1974) reported that carbon monoxide oxidation by whole cells of Methylomonas methanica was stimulated by ethanol but that there was no NAD⁺-linked alcohol dehydrogenase present in cell

Abbreviation: MMO, methane mono-oxygenase.

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extracts (Ferenci et al., 1975). It was suggested that NADH may be generated from ethanol oxidation by reversed electron flow or that an intermediate carrier between ethanol oxidation and MMO was present but lost in the preparation of cell extracts (Ferenci et al., 1975). Hazeu & de Bruyn (1980) also suggested that alcohol dehydrogenase could supply reductant for the oxygenase reaction in one type I and all type II methanotrophs and only very inefficiently in one type I and all type II organisms. The results were obtained using ethane as the oxygenase substrate which is known to be metabolized by methanotrophs to ethanol, acetaldehyde and sometimes acetate. Consequently one cannot readily differentiate between endogenously derived electron donors and those arising from the further metabolism of ethanol.

Following the observation that the MMO can oxidize a number of substrates other than methane, many of which cannot be further metabolized by these organisms (Stirling & Dalton, 1979b; Higgins et al., 1979), it has become possible to study MMO activity in whole cells without the inherent problems of further metabolism experienced with methane. Using such an assay, the oxidation of propylene to propylene oxide, we have investigated the ability of various compounds to act as electron donors for the MMO in a range of methanotrophs in order to further our understanding of electron transport to the MMO. Additionally the identification of electron donors other than C$_1$ compounds should be of use in any commercial process based on the co-oxidation properties of these organisms (Dalton & Stirling, 1982).

**METHODS**

*Growth of organisms and preparation of extracts.* Methylococcus capsulatus (Bath) was grown at 45 °C and all other organisms were grown at 30 °C as 500 ml batch cultures on nitrate mineral salts medium (Whittenbury et al., 1970) with methane in air (1:1) as carbon source. Cultures were grown for 3 d from a 0-2% (v/v) inoculum of a 3-d-old starter culture in the same medium. For whole cell studies bacteria were harvested by centrifugation (10000 g) 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 O.D$_{660}$ of 20 (approximately 5 mg dry wt ml$^{-1}$) unless otherwise stipulated in the text. For the preparation of extracts cells were harvested and washed as described, resuspended in 20 mm-Tris/Cl, 5 mm-MgCl$_2$, pH 7-0, and broken by two passages through a French Pressure Cell (Aminco, Md., U.S.A.) at 137 MPa. Whole cells were removed by centrifugation for 10 min at 6000 g and soluble and particulate fractions separated by centrifugation at 32000 g for 45 min.

*Whole cell assays.* Assays were performed in 7 ml conical flasks containing 1 ml cell suspension and sealed with rubber serum caps (Suba Seal, W. H. Freeman, Barnsley, U.K.). Potential electron donors were added after a 3 min incubation period, followed by 3 ml propylene, displacing an equal volume of air to give a final ratio of propylene/air of 1:1. All assays were performed in a reciprocating water bath at 90 oscillations min$^{-1}$ at 30 °C, except those with *Methylococcus capsulatus* (Bath) which were performed at 45 °C. The appearance of propylene oxide and disappearance of donors was followed by gas chromatography on 2:1 m × 4 mm i.d. Porapak Q (Waters Associates, Milford, Mass., U.S.A.) with flame ionization detection. Samples (5 μl) of the incubation mixture were analysed at regular intervals over a time course of 30-40 min. All analyses were conducted isothermally at temperatures within the range 150–190 °C and carrier gas (N$_2$) flow rates of 30–60 ml min$^{-1}$, the actual conditions depending on the donor in use. For the determination of acetate it was necessary to pretreat the column with a series of injections of 10 mm-acetate to reduce tailing and consequent variability. Acetone and propylene oxide were separated on 0.6 m × 2 mm i.d. Tenax GC (Enka N.V) at 120 °C. Quantification was by peak area integration using an HP 3380A integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

![Fig. 1. A generalized scheme for methane oxidation in methanotrophs. PQQ = pyrrolo quinoline quinone.](image-url)
**Enzyme assays.** Formaldehyde dehydrogenase activity was assayed spectrophotometrically essentially by the method of Johnson & Quayle (1964), following the reduction of NAD⁺ at 340 nm. Assays were conducted in 1-5 ml cuvettes at 30 °C, the reaction mixture (1-5 ml) consisting of 20 mM-sodium/potassium phosphate buffer pH 8.0, 2.5 mM-KCN, 0.67 mM-NAD⁺, 2 mM-reduced glutathione and various amounts of test protein. After 3 min incubation, the assay was initiated by the addition of 6.7 mM-formaldehyde. Non-specific aldehyde dehydrogenase was assayed spectrophotometrically by the method of Patel et al. (1980) following the reduction of 2,6-dichlorophenol-indophenol at 600 nm in the presence of phenazine methosulphate at pH 7.5, using 30 mM-propionaldehyde as substrate.

**Poly-β-hydroxybutyrate assay.** Poly-β-hydroxybutyrate was assayed by the method of Law & Slepecky (1961) on cell samples of 20–25 mg dry weight.

**Chemicals.** Methane (technical grade) and propylene were obtained from British Oxygen Co., London, U.K. All potential electron donors were the best grades available. C₁ to C₅ alcohols, aldehydes and acids (as sodium salts), valeraldehyde, hexaldehyde, benzaldehyde and propylene oxide were obtained from BDH. Heptaldehyde and octaldehyde were from Aldrich Chemical Co. Ltd, Gillingham, Dorset, U.K. Glyceraldehyde, glycolaldehyde and glyoxal (trimeric dihydrate) were from Sigma who also supplied the cofactors βNAD⁺ (grade III), reduced glutathione, 2,6-dichlorophenol-indophenol and phenazine methosulphate.

**RESULTS AND DISCUSSION**

**C₁ donors in Methylococcus capsulatus (Bath)**

Methanol, formaldehyde and formate all stimulated the oxidation of propylene by whole cell suspensions of *Methylococcus capsulatus* (Bath). Although the rate of propylene oxide formation was less with formaldehyde than with formate, the final yield was greater (Fig. 2) suggesting that both formaldehyde and formate dehydrogenases were supplying reducing equivalents to the MMO. This is consistent with *in vitro* observations that both dehydrogenases are NAD⁺-linked (Stirling & Dalton, 1978) and that NAD(P)H is the only effective electron donor for the MMO in this organism (Colby & Dalton, 1976). However, formaldehyde is also known to be a substrate for methanol dehydrogenase (Wadzinski & Ribbons, 1975a), an enzyme which, in all cases studied, is not NAD⁺-linked. Without being able to assess the contribution made by each enzyme to formaldehyde oxidation one cannot discount the possibility that methanol dehydrogenase could also be supplying electrons directly to the MMO. An additional complication arises when it is realized that methanol itself may be a substrate for the MMO (Colby et al., 1977) under these conditions. Therefore, the fact that 5 mM-methanol did not produce a greater yield of propylene oxide than did 5 mM-formaldehyde may simply reflect the lack of specificity in C₁ catabolic enzymes.

![Fig. 2. MMO activity of whole cells of *Methylococcus capsulatus* (Bath) measured by the epoxidation of propylene in the absence of added donor (○), or in the presence of 5 mM-methanol (●), 5 mM-formaldehyde (□), or 5 mM-formate (■). Endogenous MMO activity of the cells had been reduced by overnight incubation in nitrate mineral salts medium in the presence of propylene. Cells for assay were then washed and resuspended in 20 mM-phosphate buffer pH 7.0 to an OD₅₄₆ of 20.](image-url)
Fig. 3. (a) MMO activity of the whole cells of 'Methylomonas albus' BG8 (OD<sub>440</sub> = 20) measured by the epoxidation of propylene, in the absence of added donor (○) and in the presence of 5 mM-ethanol (●) or 5 mM-acetaldehyde (■). (b) The concentrations of ethanol (●) and acetaldehyde (■) during the MMO assay (a) stimulated with 5 mM-ethanol, and the concentration of acetaldehyde (■) during the MMO assay stimulated with 5 mM-acetaldehyde.

To resolve this problem the ability of methanol dehydrogenase to oxidize ethanol and higher primary alcohols was utilized, under the premise that the resulting aldehydes should not be substrates for either the NAD<sup>+</sup>-linked formaldehyde-specific dehydrogenase, or methanol dehydrogenase (Stirling & Dalton, 1978; Patel <i>et al.</i>, 1972; Patel & Felix, 1976).

**Ethanol and acetaldehyde as electron donors**

The oxidation of ethanol or acetaldehyde in the Type I organism 'Methylomonas albus' BG8 stimulated propylene oxide production in the MMO assay (Fig. 3). Qualitatively this finding was true for all organisms tested (Type I, Type I<sub>1</sub> and Type X), although quantitative differences in MMO rates and donor oxidation were evident. Ethanol disappeared rapidly in all cases, with a concomitant increase, followed by a slower decrease in acetaldehyde concentration. The rate of metabolism of acetaldehyde was identical whether generated from ethanol during the assay, or added as the donor (Fig. 3b).

The yield of propylene oxide with 5 mM-ethanol as donor was at least twofold higher than that with 5 mM-acetaldehyde in all cases, indicating that both ethanol oxidation and the further metabolism of acetaldehyde were providing reducing equivalents for the MMO. As there is no evidence for any primary alcohol dehydrogenase other than the non-specific methanol dehydrogenase in these organisms (see Higgins <i>et al.</i>, 1981), it must be concluded that this enzyme was channelling reducing equivalents from ethanol oxidation to the MMO. The observation that acetaldehyde metabolism could also stimulate propylene oxidation was surprising, especially in the case of Type I organisms, which do not have a functional TCA cycle (Davey <i>et al.</i>, 1972) capable of generating NADH from acetate. Although the NAD<sup>+</sup>-linked formaldehyde dehydrogenases previously characterized in methylotrophs cannot oxidize acetaldehyde (Stirling & Dalton, 1978) the evidence for acetaldehyde oxidation by methanol dehydrogenase is equivocal. Using purified preparations Wadzinski & Ribbons (1975<i>a</i>) found that acetaldehyde was oxidized, albeit slowly, by methanol dehydrogenase from <i>Methylococcus capsulatus</i> (Texas), whereas Patel <i>et al.</i> (1972) and Patel & Felix (1976) found no evidence for this with methanol dehydrogenase from the same organism or from 'Methylosinus sporium'. To resolve this discrepancy, the ability of higher aldehydes to drive the MMO has been examined.

**C<sub>3</sub> and C<sub>4</sub> alcohols and aldehydes as electron donors**

The ability of primary alcohols and aldehydes to stimulate propylene oxidation activity was extended by the observation that butan-1-ol and butyraldehyde were also effective electron donors in all methanotrophs. Propan-1-ol and propionaldehyde were also effective in those cases.
Table 1. Substrate specificities of formaldehyde dehydrogenase (NAD-GSH) and non-specific aldehyde dehydrogenase (PMS-DCPIP) from ‘Methylomonas albus’ BG8

Percentage activity based on 40 mM-formaldehyde (2.52 μmol min⁻¹ mg⁻¹) = 100%, and 40 mM-propionaldehyde (167 nmol min⁻¹ mg⁻¹) = 100%, respectively. Butyraldehyde and higher aldehydes were added as 1 or 2 μl samples without previous dilution.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NAD-GSH†</th>
<th>PMS-DCPIP‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde (0.67 mM, 4 mM, 40 mM)</td>
<td>100, 100, 100</td>
<td>ND, 2, 22</td>
</tr>
<tr>
<td>Acetaldehyde (0.67 mM, 4 mM, 40 mM)</td>
<td>0, 0, 0</td>
<td>1-4, 5, 70</td>
</tr>
<tr>
<td>Propionaldehyde (40 mM)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Butyraldehyde (1 μl per 1.5 ml)</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td>Valeraldehyde (1 μl per 1.5 ml)</td>
<td>ND</td>
<td>78</td>
</tr>
<tr>
<td>Hexaldehyde (1 μl per 1.5 ml)</td>
<td>ND</td>
<td>44</td>
</tr>
<tr>
<td>Heptaldehyde (2 μl per 1.5 ml)</td>
<td>ND</td>
<td>30</td>
</tr>
<tr>
<td>Octaldehyde (2 μl per 1.5 ml)</td>
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<td>10</td>
</tr>
<tr>
<td>Benzaldehyde (2 μl per 1.5 ml)</td>
<td>ND</td>
<td>2-5</td>
</tr>
<tr>
<td>Glyceraldehyde (20 mM)</td>
<td>0</td>
<td>106</td>
</tr>
<tr>
<td>Glycolaldehyde (40 mM)</td>
<td>0</td>
<td>112</td>
</tr>
<tr>
<td>Glyoxal* (40 mM)</td>
<td>0</td>
<td>146</td>
</tr>
</tbody>
</table>

† GSH, Reduced glutathione.
‡ PMS, Phenazine methosulphate; DCPIP, 2,6-dichlorophenol-indophenol.

ND, Not determined.

* Glyoxal = (CHO-CHO)₂H₂O, molarity based on monomer molecular weight.

Electron donors for methane mono-oxygenase

C₅–C₆ carboxylic acids as electron donors

Acetate (5 mM) stimulated propylene oxidizing activities in type II methanotrophs but not in type I or type X organisms. Although seemingly consistent with the presence of a functional TCA cycle capable of generating NADH in Type II organisms only (Davey et al., 1972), the amounts of propylene oxide produced in assays using high cell densities would have required stoichiometries of NADH production in excess of that provided by the TCA cycle alone (Fig. 4). This was especially true in assays with ‘Methylocystis parvus’ OB3b and ‘Methylosinus sporium’ OB3b. Two additional observations supported the suggestion that acetate-stimulated MMO activity may not be solely attributable to NADH from the TCA cycle. Firstly the TCA cycle examined. Butan-1-ol disappearance, butyraldehyde appearance and its subsequent disappearance were similar to that observed with ethanol and acetaldehyde (Fig. 3) except that butyraldehyde was oxidized more slowly in all cases.

Although Wadzinski & Ribbons (1975a) suggested that methanol dehydrogenase could oxidize acetaldehyde, it does not metabolize higher aldehydes (Patel et al., 1980) and cannot, therefore, be invoked to explain butyraldehyde oxidation. Furthermore, acetaldehyde and higher aldehydes are not substrates for any of the NAD(P)⁺-linked formaldehyde dehydrogenases previously characterized in methylotrophs (Stirling & Dalton, 1978). However, non-specific aldehyde dehydrogenases, capable of metabolizing these substrates, have recently been described in Hyphomicrobium spp. (Köhler & Schwartz, 1982) and in a Type II methanotroph (Patel et al., 1980). In both cases, assay of the enzyme required a dye-linked assay, the available evidence suggesting that, in vivo, it is linked to the respiratory chain via cytochrome c in a manner similar to that proposed for methanol dehydrogenase. Patel et al. (1980) have purified the non-specific aldehyde dehydrogenase from a type II methanotroph, and antibodies raised against it were shown to have some cross reaction with extracts from other methanotrophs. A similar enzyme has been partially purified in this laboratory from the type I methanotroph ‘Methylomonas albus’ BG8, separating it from an NAD⁺-linked formaldehyde-specific dehydrogenase requiring reduced glutathione as cofactor (D. J. Leak & H. Dalton, unpublished results). Substrate specificities are presented in Table 1.
Stimulation of MMO activity in 'Methylocystis parvus' (OD₅₄₀ = 90) by 5 mM-acetate. MMO activity was measured by the epoxidation of propylene in the presence (○) and absence (●) of 5 mM-acetate. The theoretical maximum rate of propylene oxide production (□) was determined from the rate of acetate disappearance and assumes the production of 3 mol propylene oxide per mol acetate oxidized via the TCA cycle.

Inhibitor fluorocitrate had no effect on acetate-stimulated propylene-oxidizing activity, and secondly both 5 mM-propionate and 5 mM-butyrate stimulated propylene-oxidizing activity although at a lower rate than acetate, with no significant reduction in carboxylic acid concentration.

An explanation for these observations came from the work of Thomson et al. (1976) who found that in Type II methanotrophs, acetate and higher carboxylate ions stimulated the breakdown of the storage material poly-β-hydroxybutyrate (PHB). The resulting β-hydroxybutyrate was subsequently dehydrogenated giving rise to acetoacetate and NADH, which presumably could be utilized by the MMO. A catalytic mechanism of this kind would be consistent with the observed dependence of propylene oxide yields on cell density. To confirm this the presence of PHB has been demonstrated in 'Methylocystis parvus' OBBP (0.8–0.9 µg mg⁻¹ dry wt) grown in 500 ml batch cultures. After 2 h incubation in the presence of 5 mM-acetate, with or without propylene, no PHB was detectable. In agreement with Thomson et al. (1976), no PHB was detected in the Type I methanotroph 'Methylomonas albus' BG8 grown under identical conditions.

In the studies of Thomson et al. (1976), acetoacetate derived from PHB was further decarboxylated giving rise to acetone which was detected by gas chromatography. Acetone from PHB breakdown has not been observed in these studies with cells from batch cultures, although it has been noted using cells of 'Methylocystis parvus' OBBP taken from continuous culture under oxygen limitation, conditions which would be expected to yield higher levels of cellular PHB (Scott et al., 1981). However, these differences may be a reflection of the difference between the pH value of the MMO assay (7.0) and the optimum for acetone production (5.0). The fate of acetate in these assays remains to be established. Thomson et al. (1976) did not observe acetate disappearance in their studies, while Wadzinski & Ribbons (1975b) observed incorporation of both acetate carbon atoms during growth of a Type II organism on methane supplemented with acetate. In the present study acetate disappearance was slow [≤ 7 nmol min⁻¹ (mg dry wt cells)⁻¹] but reproducible, and was observed in the presence and absence of propylene and also in the presence of propylene plus acetylene, a potent inhibitor of MMO activity. It is, therefore, unlikely that acetate metabolism is controlled by MMO activity per se. Determination of the ultimate fate of acetate under these conditions will require radiotracer studies.

Electron transport in methanotrophs

The ability of potential electron donors to drive propylene oxidation by the MMO in all of the organisms tested is summarized in Table 2. Results are presented as rates of MMO activity determined after 3 min with all donors at 5 mM. These are not maximum rates, especially those obtained with C₂ and higher aldehydes which only reach $V_{\text{max}}$ at 20–30 mM-donor, nor do they
Table 2. Effects of various C<sub>3</sub> compounds, C<sub>4</sub> compounds and formate on the rate of propylene oxide production (nmol min<sup>-1</sup>) from whole cells of methane oxidizing bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type</th>
<th>C,&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;OH</th>
<th>CH,&lt;sub&gt;3&lt;/sub&gt;CHO</th>
<th>CH,&lt;sub&gt;3&lt;/sub&gt;COO&lt;sup&gt;-&lt;/sup&gt;</th>
<th>C,&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt;OH</th>
<th>C,&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;7&lt;/sub&gt;CHO</th>
<th>C,&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;7&lt;/sub&gt;COO&lt;sup&gt;-&lt;/sup&gt;</th>
<th>HCOO&lt;sup&gt;-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methylococcus capsulatus</em> (Bath)</td>
<td>X</td>
<td>180</td>
<td>103</td>
<td>0</td>
<td>133</td>
<td>ND</td>
<td>ND</td>
<td>407</td>
</tr>
<tr>
<td>'Methyomonas albus' BG8</td>
<td>I</td>
<td>462</td>
<td>108</td>
<td>0</td>
<td>485</td>
<td>68</td>
<td>0</td>
<td>127</td>
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<td><em>Methylococcus methanica</em> A4</td>
<td>I</td>
<td>180</td>
<td>74</td>
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<td>108</td>
<td>ND</td>
<td>ND</td>
<td>380</td>
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<tr>
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<td>I</td>
<td>420</td>
<td>102</td>
<td>0</td>
<td>264</td>
<td>60</td>
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<td>453</td>
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<tr>
<td>'Methylobacter capsulata' Y</td>
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<td>220</td>
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<td>0</td>
<td>204</td>
<td>72</td>
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<tr>
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<td>II</td>
<td>96</td>
<td>76</td>
<td>61</td>
<td>100</td>
<td>0–10*</td>
<td>ND</td>
<td>660</td>
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<td>63</td>
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<td>25</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>413</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Stimulation often masked by high endogenous activity.

Electron donors for methane mono-oxygenase

serve as a guide to the stoichiometry of donor usage to propylene oxide production. However, they do serve as an approximate comparison of the effectiveness of different donors in different organisms. Some of these donors (e.g. formate) are capable of yielding NADH directly through specific dehydrogenases, while others (e.g. carboxylates in Type I<sub>1</sub> organisms) can yield NADH indirectly (Thomson et al., 1976). However, all the available evidence suggests that the oxidation of primary alcohols and C<sub>3</sub> and higher aldehydes is not directly coupled to the reduction of NAD<sup>+</sup>, but that their corresponding dehydrogenases pass electrons to the respiratory chain at the level of cytochrome c (O'Keefe & Anthony, 1980; Duine et al., 1979; Patel et al., 1980; Köhler & Schwartz, 1982; Beardmore-Gray et al., 1983). Confirmation that primary alcohols were being oxidized via methanol dehydrogenase was obtained from studies of KCN sensitivity in *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. In *Methylococcus capsulatus* (Bath) respiration driven by ethanol or methanol was inhibited by KCN with an \( I_{50} \) of 0.07 mM (approximately), while respiration driven by formaldehyde or formate was inhibited with an \( I_{50} \) of 0.6 mM and 0.54 mM, respectively. Results with *Methylosinus trichosporium* OB3b were similar. This suggested that the reoxidation of NADH produced by formate and formaldehyde dehydrogenases occurred through an oxidase which was relatively insensitive to KCN, whereas primary alcohols were oxidized by the cyanide-sensitive methanol oxidase.

Based on the assumption that NADH is the immediate electron donor for the MMO under these conditions, two possible explanations may be considered for the ability of cytochrome c-linked dehydrogenases to supply reducing equivalents to the MMO. (1) Either the primary alcohol dehydrogenase and non-specific aldehyde dehydrogenase are capable of producing NADH directly, \textit{in vivo}, or (2) they can yield NADH indirectly, by reversed electron transport. Alternatively, under the growth conditions utilized, NADH may not be the immediate electron donor \textit{in vivo}.

As previously discussed, there is no evidence from \textit{in vitro} experiments for the first of these possibilities. To be in contact with the cellular NAD/NADH pool this would presumably require the alcohol and aldehyde dehydrogenases to be cytoplasmic enzymes (unless an NADH shuttle system or pyridine nucleotide transport system was also postulated). Present evidence indicates that, in methanol-utilizers, methanol dehydrogenase is in fact periplasmically located (Alefounder & Ferguson, 1981; Jones et al., 1982).
If the oxidation of primary alcohols was producing NADH by reversed electron transport then, on the basis of existing evidence indicating that methanotrophs have classical aerobic respiratory chains (Davey & Mitton, 1973; Tonge et al., 1974) one would predict a maximum stoichiometry of 0.33 mol propylene oxide produced per mol alcohol/aldehyde utilized. The derivation of this figure assumes a respiratory chain translocating $6H^+\!/2e^-$ between NADH and cytochrome $c$ and a periplasmic methanol dehydrogenase capable of an effective net translocation of $2H^+\!/2e^-$ when linked to the respiratory chain (Netrusov & Anthony, 1979; Dawson & Jones, 1981) as depicted in Fig. 5. However, in this study stoichiometries of between 0.33 and 0.48 mol propylene oxide per mol ethanol utilized have been obtained with 'Methylocystis parus' OB BP (Type II), 'Methylomonas albus' BG8 (Type I), Methylomonas methanica PM (Type I) and 'Methylobacter capsulata' Y (Type I) indicating that, at least in these cases, the oxidation of 2 mol alcohol was sufficient to drive the epoxidation of 1 mol propylene by the MMO. It must, therefore, be concluded that if NADH was generated by reverse electron transport in these cases then either the stoichiometry of proton translocation is only $4H^+\!/2e^-$ for electron transport between NADH and cytochrome $c$, or the oxidation of primary alcohols translocates $4H^+\!/2e^-$ possibly through a proton pumping cytochrome $aa_3$.

The original claim that NADH was the only effective electron donor for the MMO (Colby & Dalton, 1976) was based on studies of the soluble MMO from Methylococcus capsulatus (Bath), and under these circumstances there is apparently no dispute that NADH is the immediate electron donor (see Higgins et al., 1981). However, where detectable, the MMO activity of all organisms studied here was either completely or mainly membrane bound when grown in 500 ml batch cultures. It is becoming apparent that the properties of membrane-bound and soluble enzymes from the same organism may differ considerably (Tonge et al., 1977; Stirling & Dalton 1979a, Scott et al., 1981) and we have recently demonstrated that in Methylococcus capsulatus (Bath) the coupling of primary alcohol oxidation to MMO activity is only seen in cells with membrane-bound MMO (Stanley et al., 1983). It is, therefore, conceivable that the immediate electron donor for the membrane-bound MMO may not be NADH (although NADH remains an effective donor under these circumstances in vitro), but some electron transfer protein which can accept electrons from methanol dehydrogenase and the non-specific aldehyde dehydrogenase without the involvement of NADH as an intermediate. Tonge et al. (1977) proposed that cytochrome $c_{oo}$ was the immediate electron donor for the membrane-bound MMO from 'Methylosinus trichosporium' OB3b, although this low molecular weight cytochrome has also been implicated in MMO substrate binding and oxidase activity (Higgins et al., 1981). These authors found that MMO activity driven by NADH was restored to washed, inactive membrane
preparations by adding back cytochrome $c_{550}$, suggesting that all reducing power for the MMO was being directed through this cytochrome. However, the stoichiometries observed in the present study suggest that this is unlikely. With formate as electron donor stoichiometries approaching 1 mol propylene oxide per mol donor utilized were observed with all organisms, whereas stoichiometries were consistently less than 0.5 with ethanol as a donor. The latter figure suggests that the coupling of ethanol oxidation to MMO activity might be less than 50% efficient (i.e. there may be alternative pathways of electron transport). However, as methanol dehydrogenase appears to be coupled directly to cytochrome $c_{550}$ (Duine et al., 1979, Beardmore-Gray et al., 1983) the same lack of efficiency should be observed with all electron donors if this cytochrome was the immediate electron donor for the MMO.

If some other electron transfer protein was the immediate electron donor, then these stoichiometries would be consistent with alternative pathways of electron transport from cytochrome $c_{550}$. However, on the basis of the aerobic respiratory chain already described these stoichiometries would be equally consistent with the immediate electron donor for the MMO being a component of the respiratory chain between NADH and cytochrome $c$ (e.g. an Fe/S protein), coupling of ethanol oxidation to MMO activity requiring reverse electron transport through two coupling sites. Such a configuration would be energetically more favourable than that of the soluble MMO and remains consistent with the evidence for the inhibition of NADH-driven membrane-bound MMO activity by the electron transport inhibitor, amytal (Tonge et al., 1975).

While the latter discussion has necessarily been generalized without reference to type of methanotroph or quantitative differences between organisms of the same type observed by previous authors (Hazeu & de Bruyn, 1980) we consider this a useful basis for further experimentation through which the nature of these differences may be resolved. Furthermore it becomes absolutely essential in these studies to clearly define the growth conditions used for such studies, since these can have profound effects on the overall electron transport system in operation (see Stanley et al., 1983).

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