A Comparison of the Substrate and Electron-Donor Specificities of the Methane Mono-oxygenases from Three Strains of Methane-Oxidizing Bacteria

By DAVID I. STIRLING, JOHN COLBY and HOWARD DALTON
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

(Received 20 September 1978)

1. Methane mono-oxygenase from *Methylosinus trichosporium* has the same broad substrate specificity as the analogous enzyme from *Methylococcus capsulatus* (Bath); the enzyme from *Methylomonas methanica* is more specific. 2. Contrary to previous reports, NAD(P)H and not ascorbate is the required electron donor for the enzyme from *Methylosinus trichosporium*. 3. It is concluded that these three bacteria contain similar methane mono-oxygenases.

Methane mono-oxygenase from *Methylococcus capsulatus* (Bath) comprises three components (Colby & Dalton, 1978): (1) a large iron-containing protein (mol.wt. about 200000); (2) a small protein (mol.wt. about 20000); (3) a metalloflavoprotein (NADH-acceptor reductase) of mol.wt. 44600 containing FAD and a 2Fe–2S centre of the plant ferredoxin type (Colby & Dalton, 1979). NADH is the required electron donor (Colby & Dalton, 1976; Colby *et al.*, 1977). The analogous enzyme from *Methylosinus trichosporium* apparently differs markedly from this (Tonge *et al.*, 1977), although it also comprises three components. According to Tonge *et al.* (1977), sodium L-ascorbate is the required electron donor *in vitro* and NADH will not substitute for it. Less is known about the methane mono-oxygenase from *Methylomonas methanica* (Ferenci *et al.*, 1975; Colby *et al.*, 1975), but in crude extracts NAD(P)H is the only functional electron donor.

The substrate specificity of the methane mono-oxygenase from *Methylococcus capsulatus* (Bath) has been described in previous publications (Colby *et al.*, 1977; Dalton, 1977). It catalyses the hydroxylation of primary and secondary alkyl C–H bonds, the formation of epoxides from internal and terminal alkenes, the hydroxylation of aromatic compounds, the *N*-oxidation of pyridine, the oxidation of CO to CO₂ and the oxidation of ammonia to hydroxylamine. Comparatively little is known about the analogous enzymes from *Methylosinus trichosporium* and *Methylomonas methanica* in this respect.

The present investigations were undertaken to determine whether a common enzyme system is present in all methane-oxidizing bacteria. Our results, which in some cases conflict with previously published work, suggest that a similar enzyme occurs in the three bacteria we have studied so far.

Materials and Methods

Chemicals were obtained from the sources described by Colby *et al.* (1977). The cultures of *Methylomonas methanica* and *Methylosinus trichosporium* (OB3b) were supplied by Professor J. R. Quayle, University of Sheffield, Sheffield, U.K., and Dr. I. J. Higgins, University of Kent, Canterbury, U.K., respectively.

Growth of *Methylomonas methanica* and preparation of crude extract (12 mg of protein/ml) were done as described by Colby *et al.* (1975). *Methylosinus trichosporium* was grown at 30°C in continuous culture at a dilution rate of 0.05 h⁻¹ on ammonium mineral salts medium (Dalton, 1977) with methane (20%, v/v) in air as carbon source. The overflow from the culture vessel was collected aseptically and the bacteria were harvested by centrifugation at 4°C with a continuous-flow rotor. Bacteria were resuspended in 20 mM-sodium phosphate buffer, pH 7, containing 5 mM-MgCl₂ and deoxyribonuclease I (0.01 mg/ml) and broken by one passage through a French pressure cell at 137 MPa. Unbroken bacteria were removed by centrifugation at 4°C (20000g for 30 min) and the supernatant (crude extract, 37 mg of protein/ml) was immediately frozen by dropwise addition to liquid N₂ and stored at –70°C.

Enzyme assays were done as reported previously (Colby *et al.*, 1977) except that all incubations were done at 30°C. The following control assays were done: (1) containing boiled extract; (2) lacking NADH; (3) incubated anaerobically under argon; (4) containing 0.2 ml of ethyne, a specific inhibitor of bacterial methane mono-oxygenase (Colby *et al.*, 1975, 1977). The identification and estimation of oxidation products were done as described previously (Colby *et al.*, 1977). The hydration of epoxides with dilute HCl and the reaction of unsaturated com-

pounds with bromine were used as an aid to their identification (Colby *et al.*, 1977). The identification of some aldehyde products was confirmed by reducing them to the corresponding alcohol with yeast alcohol dehydrogenase (EC 1.1.1.1) and NADH. Carboxylic acids were estimated by gas chromatography of their methyl esters (Salanitro & Muirhead, 1975) after treatment with boron trifluoride/methanol.

Results

The substrate specificity of the methane mono-oxygenase present in crude extracts of *Methylosinus trichosporium* (Tables 1 and 2) was identical with that found previously with the isofunctional enzyme from *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977). The oxidation products observed were also the same except that further oxidation of alcoh-1-ol products to the corresponding aldehydes, and in some cases acids, occurred. The latter could not be prevented

by including 1 mM-KCN in the reaction mixtures as was done with extracts of *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977), because this compound is a potent inhibitor of cell-free methane mono-oxygenase activity in *Methylosinus trichosporium* (Tonge *et al.*, 1977). Similarly, high sodium phosphate buffer concentrations (up to 500 mM) in the reaction mixtures failed to prevent further oxidation of the alcoh-1-ols.

Fewer of the test compounds were oxidized by the methane mono-oxygenase in crude extracts of *Methylomonas methanica* (Tables 1 and 2). All the substituted methane derivatives except trichloromethane were oxidized, as were the n-alkenes and the ethers. Of the n-alkanes, only methane, ethane, propane, butane and pentane were oxidized and none of the cyclic compounds was a substrate. With those compounds that were substrates, the products of their oxidation were similar to those obtained with extracts of *Methylosinus trichosporium*. Again,

Table 1. *Compounds oxidized by crude extracts of both Methylosinus trichosporium and Methylomonas methanica*
Experimental details are given in the Materials and Methods section. Each compound was tested at various concentrations and the activities quoted were the highest observed. Specific activities are in $\mu\text{units/mg}$ of protein. Amounts of products are in μmol formed after 10 min incubation with 3.8 mg of extract protein. Products of methanol oxidation, i.e. formaldehyde, formate and CO_2 , were not determined so that specific activities for methane oxidation are minimum values. Abbreviation: n.d., not determined.

Substrate	Products	Crude extract of ... <i>Methylosinus trichosporium</i>		<i>Methylomonas methanica</i>	
		Amount	Specific activity	Amount	Specific activity
Methane	Methanol	1.88	50.8	2.79	73.3
Ethane	{ Ethanol	0.16	32.6	1.68	87.5
	{ Ethanal	0.25		1.65	
	{ Acetate	0.83		0	
Propane	{ Propan-1-ol	0.04	29.4	0	44.2
	{ Propan-2-ol	0.66		1.27	
	{ Propanal	0.42		0.41	
n-Butane	{ Butan-2-ol	0.60	33.5	0.54	17.5
	{ n-Butanal	0.42		0.13	
n-Pentane	{ Pentan-2-ol	1.12	34.7	0.38	17.5
	{ n-Pentanal	0.30		0.28	
Ethene	Epoxyethane	2.20	59.0	6.02	158.3
Propene	1,2-Epoxypropane	2.03	53.5	3.10	81.4
But-1-ene	1,2-Epoxybutane	1.69	44.4	3.83	100.4
<i>trans</i> -But-2-ene	{ <i>trans</i> -2,3-Epoxybutane	0.24	40.5	0.82	68.3
	{ <i>trans</i> -But-2-en-1-ol	0		0.38	
	{ <i>trans</i> -But-2-en-1-al	1.30		1.39	
<i>cis</i> -But-2-ene	{ <i>cis</i> -2,3-Epoxybutane	0.52	37.4	2.34	70.8
	{ <i>cis</i> -But-2-en-1-al	0.63		0.28	
	{ Butanone	0.27		0.06	
Dimethyl ether	n.d.	n.d.	25.8	n.d.	65.0
Diethyl ether	{ Ethanol	0.74	144.4	0	27.2
	{ Ethanal	4.60		1.04	
Chloromethane	n.d.	n.d.	29.0	n.d.	120.9
Dichloromethane	n.d.	n.d.	26.3	n.d.	43.2

Table 2. Compounds oxidized by crude extracts of *Methylosinus trichosporium*, but not by crude extracts of *Methylomonas methanica*

Details are given in the legend to Table 1.

Substrate	Products	Amount	Specific activity
n-Hexane	$\left\{ \begin{array}{l} \text{Hexan-1-ol} \\ \text{Hexan-2-ol} \\ \text{n-Hexanal} \end{array} \right\}$	$\left\{ \begin{array}{l} 0.17 \\ 0.28 \\ 0.48 \end{array} \right\}$	25.0
n-Heptane	$\left\{ \begin{array}{l} \text{Heptan-2-ol} \\ \text{n-Heptanal} \end{array} \right\}$	$\left\{ \begin{array}{l} 0.68 \\ 0.05 \end{array} \right\}$	19.2
Cyclohexane	Cyclohexanol	0.74	19.5
Benzene	Phenol	1.16	30.5
Styrene	Styrene epoxide	0.75	19.8
Toluene	$\left\{ \begin{array}{l} \text{Benzyl alcohol} \\ \text{Cresol} \end{array} \right\}$	$\left\{ \begin{array}{l} 0.39 \\ 0.12 \end{array} \right\}$	13.4
Pyridine	Pyridine N-oxide	n.d.	19.0
Trichloromethane	n.d.		27.0
2-Methylpropane	$\left\{ \begin{array}{l} \text{2-Methylpropan-1-ol} \\ \text{2-Methylpropan-2-ol} \end{array} \right\}$	$\left\{ \begin{array}{l} 0.31 \\ 0.33 \end{array} \right\}$	17.6

further oxidation of alcoh-1-ols occurred and could not be prevented with KCN, owing to inhibition of methane mono-oxygenase activity (Colby *et al.*, 1975), nor by high phosphate concentrations.

2-Methylpropane (isobutane) was tested with crude extracts of all three organisms. The results for *Methylosinus trichosporium* are shown in Table 2 and were similar to those observed in extracts of *Methylococcus capsulatus*. *Methylomonas methanica* extracts were inactive towards this substrate.

The ability of the following electron donors and hydroxylating agents to replace NADH as cofactor for the enzyme from *Methylosinus trichosporium* was tested: sodium L-ascorbate (1–50 mM), quinol (1–50 mM), sodium dithionite (1–50 mM), H₂O₂ (1–50 mM), sodium chlorite (1–50 mM), sodium periodate (1–50 mM) and NADPH (5 mM). A superoxide-generating system comprising milk xanthine oxidase (EC 1.2.3.2, 1 or 10 units), and xanthine (5 mM) was also tested. Only NADPH (65% of the rate with 5 mM-NADH) would substitute for NADH. Neither ox liver catalase (EC 1.11.1.6, 100 units) nor bovine superoxide dismutase (EC 1.15.1.1, 100 units) inhibited the methane mono-oxygenase activity with NADH as electron donor, confirming that neither H₂O₂ nor the superoxide anion is an intermediate in the reaction.

Discussion

The substrate specificity of the methane mono-oxygenase complex probably reflects the nature of the active site of the hydroxylase component. The identical range of substrates oxidized by the enzymes

from *Methylosinus trichosporium* and *Methylococcus capsulatus* (Bath) suggests that they have similar hydroxylases. Our results with crude extracts of *Methylosinus trichosporium* conflict with previously published results in several respects. According to Thomson (1974), whole organisms oxidize n-propane and n-butane to the corresponding alcoh-1-ol, aldehyde and acid. No accumulation of the alcoh-2-ols, as observed in the present study, was reported. Wilkinson (1975) reported that oxidation of diethyl ether by *Methylosinus trichosporium* occurs only at the terminal methyl group, yielding 2-ethoxyethanol, 2-ethoxyethanal and 2-ethoxyacetic acid. Our experiments with crude extracts reveal that oxidative attack occurs at the subterminal carbon atom, yielding ethanol and ethanal. Furthermore our observation that dimethyl ether was oxidized is in conflict with the results of Tonge *et al.* (1977).

The methane mono-oxygenase from *Methylomonas methanica* is more specific than the enzyme from the other two bacteria. However, the products formed from those substrates oxidized were the same, suggesting that the differences between the two types of enzyme are minor.

The electron-donor specificities of the methane mono-oxygenases provide some information on the accessory components of the enzyme complexes. The enzymes from *Methylococcus capsulatus* (Bath) and *Methylomonas methanica* have been shown to require NAD(P)H as donor (Colby *et al.*, 1975, 1977) and, in the present study, this has also proved to be the case with the methane mono-oxygenase from *Methylosinus trichosporium*. Previously Tonge *et al.* (1977) reported that ascorbate was the required electron donor for the latter enzyme, but those

authors have since been unable to repeat this observation (I. J. Higgins, personal communication). In our laboratory, repeated attempts with various ascorbate concentrations between 1 and 50 mM and with several different crude extracts failed to detect the ascorbate-mediated oxidation of methane, ethene or chloromethane.

Our investigations into the electron-donor and substrate specificities of the methane mono-oxygenases from three methane-oxidizing bacteria failed to reveal any major qualitative difference between them except for the more restricted substrate specificity of the enzyme from *Methylobacterium methanica*. One obvious difference remains, however, and this is the sensitivity of the enzymes from *Methylobacterium methanica* and *Methylobacterium trichosporium* to a wide range of chelating agents (Colby *et al.*, 1977; Tonge *et al.*, 1977), whereas the enzyme *Methylobacterium capsulatus* (Bath) is sensitive only to 8-hydroxyquinoline (Stirling & Dalton, 1977). As two of the three components of the enzyme from *Methylobacterium capsulatus* (Bath) have been shown to contain iron, this difference may only reflect variations in the accessibility of the metals that are probably catalytically important in all three enzymes.

This work was supported by a Science Research Council Grant to H. D.

References

- Colby, J. & Dalton, H. (1976) *Biochem. J.* **157**, 495–497
- Colby, J. & Dalton, H. (1978) *Biochem. J.* **171**, 461–468
- Colby, J. & Dalton, H. (1979) *Biochem. J.* in the press
- Colby, J., Dalton, H. & Whittenbury, R. (1975) *Biochem. J.* **151**, 459–462
- Colby, J., Stirling, D. I. & Dalton, H. (1977) *Biochem. J.* **165**, 395–402
- Dalton, H. (1977) *Arch. Microbiol.* **114**, 273–279
- Ferenci, T., Strom, T. & Quayle, J. R. (1975) *J. Gen. Microbiol.* **91**, 79–91
- Salanitro, J. P. & Muirhead, P. A. (1975) *Appl. Microbiol.* **29**, 374–381
- Stirling, D. I. & Dalton, H. (1977) *Arch. Microbiol.* **114**, 71–76
- Thomson, A. W. (1974) Ph.D. Thesis, University of Edinburgh
- Tonge, G. M., Harrison, D. E. F. & Higgins, I. J. (1977) *Biochem. J.* **161**, 333–344
- Wilkinson, J. F. (1975) in *Microbial Growth on C₁ Compounds: Proc. Int. Symp. Microbial Growth on C₁ Compounds* (Teriu, G., ed.), pp. 45–57, Society of Fermentation Technology, Tokyo