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The Soluble Methane Mono-oxygenase of *Methylococcus capsulatus* (Bath)

ITS ABILITY TO OXYGENATE *n*-ALKANES, *n*-ALKENES, ETHERS, AND ALICYCLIC, AROMATIC AND HETEROCYCLIC COMPOUNDS

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1. Methane mono-oxygenase of *Methylococcus capsulatus* (Bath) catalyses the oxidation of various substituted methane derivatives including methanol. 2. It is a very non-specific oxygenase and, in some of its catalytic properties, apparently resembles the analogous enzyme from *Methylomonas methanica* but differs from those found in *Methylosinus trichosporium* and *Methylomonas albus*. 3. CO is oxidized to CO_2. 4. C_1–C_8 *n*-alkanes are hydroxylated, yielding mixtures of the corresponding 1- and 2-alcohols; no 3- or 4-alcohols are formed. 5. Terminal alkenes yield the corresponding 1,2-epoxides. *cis*- or *trans*-but-2-ene are each oxidized to a mixture of 2,3-epoxybutane and but-2-en-1-ol with retention of the *cis* or *trans* configuration in both products; 2-butanone is also formed from *cis*-but-2-ene only. 6. Dimethyl ether is oxidized. Diethyl ether undergoes sub-terminal oxidation, yielding ethanol and ethanal in equimolar amounts. 7. Methane mono-oxygenase also hydroxylates cyclic alkanes and aromatic compounds. However, styrene yields only styrene epoxide and pyridine yields only pyridine *N*-oxide. 8. Of those compounds tested, only NADPH can replace NADH as electron donor.

The methane mono-oxygenase of *Methylococcus capsulatus* strain Bath is a multi-component enzyme that catalyses the NADH- and oxygen-dependent oxidation of methane to methanol. It is soluble in that enzyme activity is entirely associated with the supernatant fraction after centrifugation of bacterial extracts at 160000*g* for 1 h (Colby & Dalton, 1976). Similar activities have been demonstrated in extracts of three other methane-oxidizing bacteria. However, in its entirely soluble nature and in its resistance to inhibition by cyanide and most chelating agents, the enzyme from *M. capsulatus* strain Bath can be distinguished from those enzymes that have been reported in *M. capsulatus* strain Texas (Ribbons, 1975), *Methylomonas methanica* (Colby *et al.*, 1975; Ferenci *et al.*, 1975) and *Methylosinus trichosporium* (Tonge *et al.*, 1977). Moreover, the methane mono-oxygenases from *Methylomonas methanica* (Colby *et al.*, 1975) and from *M. capsulatus* strain Bath (Colby & Dalton, 1976) require NAD(P)H as electron donor and therefore differ from the NAD(P)H-independent three-component enzyme system purified from *Methylosinus trichosporium* by Tonge *et al.* (1977). The available evidence therefore suggests that the methane mono-oxygenases from different methane-oxidizing bacteria have somewhat different properties. The enzyme from *M. capsulatus* strain Bath is at present unique in being entirely associated with the soluble fraction of bacterial extracts, although this does not exclude the possibility that one or more components of the enzyme system are membrane-bound *in vivo*.

The methane mono-oxygenase from *M. capsulatus* strain Bath has been reported to oxidize bromomethane (Colby & Dalton, 1976), the enzyme from *Methylomonas methanica* has been reported to oxidize CO (Ferenci *et al.*, 1975), bromomethane and NH_4Cl (Colby *et al.*, 1975), whereas the purified methane mono-oxygenase system of *Methylosinus trichosporium* oxidizes CO, ethane, propane and butane (Tonge *et al.*, 1977). Nothing further is known about the substrate specificity of methane mono-oxygenases although a better understanding of the catalytic abilities of these enzymes would obviously be a valuable aid in the elucidation of their mechanism of action. The present paper gives the results of our investigations into the substrate specificity of the soluble methane mono-oxygenase of *M. capsulatus* strain Bath. The enzyme apparently possesses a very broad substrate specificity and catalyses a variety of different oxygen-incorporation reactions.

Materials and Methods

Materials

Hexan-2-ol, hexan-3-ol, heptan-1-ol, heptan-2-ol, heptan-3-ol, heptan-4-ol, octan-1-ol, octan-2-ol, octan-3-ol and octan-4-ol were gifts from British

Petroleum Ltd., Sunbury, Middx., U.K. Methane (technical grade) and CO (research grade) were obtained from British Oxygen Co., London S.W.19, U.K. Other potential enzyme substrates were the best grades available. Ethane, *n*-propane, *n*-butane, ethene (ethylene), ethyne (acetylene) and dimethyl ether were obtained from Cambrian Chemicals, Croydon, Surrey, U.K. Fisons Scientific Apparatus, Loughborough, Leics., U.K., supplied *n*-octane, benzene, phenol and diethyl ether; and ethanal (acetaldehyde) and butan-1-ol were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Pentan-3-ol, styrene, styrene epoxide, 1,2-epoxybutane and *trans*-2-buten-1-ol (crotyl alcohol) were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.; 2-ethoxyethanol was from May and Baker Ltd., Dagenham, Essex, U.K. Styrene was obtained free of stabilizer by vacuum distillation at about 40°C immediately before use. Toluene and 2-, 3- and 4-hydroxypyridines were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and 1,2-epoxypropane from Polaron Equipment, Watford, Herts., U.K. Bromine (AristaR), 3-chloroperbenzoic acid and all other potential enzyme substrates and products were obtained from BDH Chemicals, Poole, Dorset, U.K. Merck t.l.c. glass plates precoated with silica gel F-254 were supplied by Anderman and Co. Ltd., East Molesey, Surrey, U.K. Xanthine oxidase (grade 1, EC 1.2.3.2), catalase (ox liver, EC 1.11.1.6), superoxide dismutase (ox blood, EC 1.15.1.1) and NADH (grade 3) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Contaminating ethanol (10–20 mol/100 mol) was removed from 100 mM-NADH solutions in 20 mM-sodium phosphate buffer, pH 7, by washing with diethyl ether followed by evaporation under vacuum to remove the ether. Two or three washings were necessary to decrease the ethanol to negligible concentration (0.1–0.2 mol/100 mol).

Syntheses

trans-2,3-Epoxybutane and *cis*-2,3-epoxybutane were made by forming epoxides from the corresponding *cis*- or *trans*-but-2-ene with 3-chloroperbenzoic acid at room temperature (20°C). A conical flask (25 ml volume) sealed with a Suba-Seal stopper contained 3 ml of a 10% (w/v) solution of 3-chloroperbenzoic acid in dichloromethane. But-2-ene gas (*cis* or *trans*) was passed through the flask for a few minutes by means of syringe needles inserted through the stopper. A heavy precipitate was formed during the reaction and this was resuspended by the addition of a further 0.5 ml of dichloromethane when the reaction was completed. The suspension was centrifuged (2000 *g* for 10 min) to remove the precipitate and a 0.5 ml sample of the supernatant removed, washed twice with 0.5 ml of ice-cold satd. $NaHCO_3$ solution and then dried by adding a little anhydrous Na_2SO_4. The resulting dichloromethane solution of *cis*- or *trans*-2,3-epoxybutane was diluted with dichloromethane as necessary and then injected into the gas chromatograph for purposes of identification.

Growth of bacteria and preparation of soluble extracts

M. capsulatus strain Bath (Whittenbury *et al.*, 1970) was grown at 45°C in batch culture on ammonium/mineral-salts medium (Dalton & Whittenbury, 1976) in a 100-litre fermenter (L. H. Engineering Ltd., Stoke Poges, Bucks., U.K.). The fermenter was inoculated with 10 litres of a continuous culture, which had been grown as described previously (Colby & Dalton, 1976), and then harvested after 18 h (when the A_{540} of the culture was about 8) using a Westfalia continuous centrifuge (Westfalia Separator Ltd., Wolverton, Bucks., U.K.). The centrifuge was connected to the outflow of the fermenter via a stainless-steel cooling-coil immersed in ice. The organisms were washed once with ice-cold 20 mM-sodium phosphate buffer, pH 7, and then soluble extracts prepared as described previously (Colby & Dalton, 1976) except that the crude bacterial extract was centrifuged at 80000 *g* for 1 h instead of at 160000 *g* for 1 h. The soluble extract was immediately frozen in pellet form by dropwise addition to a Dewar flask containing liquid nitrogen, and the pellets were stored at −70°C.

Enzyme assays

Assays were done in conical flasks (7 ml internal volume) containing 1 ml of reaction mixtures and sealed with Suba-Seal stoppers. Liquid substrates were incorporated into the reaction mixtures, whereas gaseous substrates were added to the reaction flasks by replacing part of the gas phase with the same volume of substrate. Reaction mixtures (1 ml) contained: 50 μmol of sodium phosphate buffer, pH 7; 5 μmol of NADH; 0.5 μmol of KCN; soluble extract (2 mg of protein unless stated otherwise); test substrate (amount as indicated in the Tables). The flasks were incubated in a 45°C reciprocating water bath at 90 oscillations/min for 5 min and then the reaction was started by injecting freshly thawed soluble extract through the stopper.

The rate of oxidation of most test compounds was measured by following the appearance of products. Samples (5 μl) of reaction mixture (or, in the case of reaction flasks containing CO as substrate, 0.5 ml of gas phase) were injected into the gas chromatograph immediately after the addition of the soluble extract (zero time) and after 12 min of incubation (preliminary experiments showed that product formation from each test substrate was more or less linear with time over this incubation period). However, because the products of *n*-octane oxidation were insoluble, reaction mixtures incorporating this substrate were

first extracted with 1 ml of dichloromethane and then 5 μl of the dichloromethane layer was injected into the gas chromatograph. Specific activities were calculated from the total amount of products formed after 12 min of incubation (1 unit of activity is 1 μmol of product formed/min).

The oxidation of some other compounds was measured by following their disappearance from the gas phase or (with nitromethane, cyanomethane, methanol, trimethylamine and pyridine) from the liquid phase. Samples (either 5 μl of liquid or 50 μl of gas) were injected into the gas chromatograph at zero time, after 6 min of incubation and after 12 min of incubation. Specific activities were calculated from the amount of substrate removed after a 12 min incubation (1 unit of activity is 1 μmol of substrate used/min).

Each substrate was tested at various concentrations so as to determine the optimum conditions for its oxidation and the data in the Tables represent the highest activities observed for each. The values in the Tables were obtained by using the same soluble extract (protein concentration 40 mg/ml determined with the Folin–Ciocalteu reagent) for each test substrate, although the results were confirmed by using another extract prepared from a different batch of organisms.

Identification and estimation of oxidation products

Most products were identified and estimated by using a Pye series 104 flame-ionization gas chromatograph fitted with 2.1 m glass columns (internal diameter 4 mm) packed with Porapak Q (Waters Associates, Milford, MA, U.S.A.), with Chromosorb 102 (Johns–Manville, Denver, CO, U.S.A.) or with 5% (w/w) of Carbowax 20 M on Chromosorb W (60–80 mesh). Trimethylamine hydrochloride was estimated on a column of Porapak Q which incorporated a soda-lime pre-column housed in the injection heating block. Products were identified by comparing their retention times on each column with that of authentic standards and estimated by establishing a linear relationship between peak height (or in some cases peak area) and concentration for each compound. These three columns used in conjunction and operated isothermally at temperatures between 50° and 230°C with N_2 carrier-gas flow-rates of 15–60 ml/min achieved the separation and preliminary identification of all the volatile products observed except for CO_2 (see below).

The identification of some products was confirmed by treatment of reaction mixtures with 20 μl of HCl or with 5 μl of bromine for 5 min at 45°C followed by gas chromatography to determine whether the products still remained (cf. May & Abbott, 1973). Under these conditions bromine reacts with unsaturated compounds by addition whereas dilute HCl catalyses the hydrolysis of epoxides.

CO_2 formed from the oxidation of CO was estimated by using a Pye series 104 katharometer gas chromatograph fitted with a 2.1 m glass column (internal diameter 4 mm) packed with Porapak R (Waters Associates). The oven temperature was 50°C and the helium carrier-gas flow-rate was 30 ml/min. The katharometer was calibrated by using CO_2/air mixtures of known composition.

Tyrosine was estimated colorimetrically as described by Kaufman (1970). Pyridine *N*-oxide was identified by t.l.c. on silica gel as follows. After incubation, reaction mixtures containing 90 μmol of pyridine as test substrate were extracted with an equal volume of dichloromethane. The dichloromethane layer was taken off and evaporated to dryness. The residue was then redissolved in 0.1 ml of dichloromethane and spotted on to the chromatograms together with dichloromethane solutions of authentic pyridine *N*-oxide. The chromatograms were developed in either methanol or acetone and the R_F values for the reaction product in each solvent compared with those for authentic pyridine *N*-oxide.

Experimental and Results

Precautions taken to ensure the validity of the results

Crude soluble extract was used as the source of methane mono-oxygenase for the substrate-specificity and product-analysis studies because the instability of the enzyme system has so far precluded its purification. The further metabolism of methanol and of other primary alcohol products was prevented by using a soluble extract (methanol oxidase activity is particulate) and by inhibiting residual methanol oxidase activity with 0.5 mM-KCN (Colby & Dalton, 1976). In practice the particular soluble extract used in these studies had negligible methanol oxidase activity even in the absence of KCN. Primary alcohol dehydrogenase, which was present in the soluble extract in high specific activity when measured at pH 9 with added phenazine methosulphate and NH_4Cl (Anthony & Zatman, 1964), was not active under the conditions of the methane mono-oxygenase assays. The further metabolism of secondary alcohols and of other non-alcoholic products except formaldehyde was not observed; the latter is rapidly metabolized by crude soluble extracts even in the presence of KCN and in the absence of added cofactors other than NAD^+. In practice all the products observed were the result of a single oxidation step involving the incorporation of one oxygen atom into the substrate; they are therefore consistent with the operation of a mono-oxygenase.

Adequate controls were essential to ensure that the oxidations observed were catalysed by methane mono-oxygenase. The following controls were used: (i) reaction mixtures containing boiled extract to ensure that the oxidations were enzyme-catalysed;

(ii) reaction mixtures lacking oxygen or NADH to ensure that both these substrates were required i.e. to indicate that a mono-oxygenase was involved; (iii) reaction mixtures with and without KCN because many mono-oxygenases, unlike the methane mono-oxygenase of *M. capsulatus* (Bath), are inhibited by KCN; (iv) reaction mixtures containing ethyne (0.2ml) because the latter appears to be a specific inhibitor of methane mono-oxygenase in *M. capsulatus* strain Bath (Colby & Dalton, 1976; Dalton & Whittenbury, 1976). The results obtained using these controls are given in parentheses in the Tables and are in each case consistent with the oxidations being catalysed by methane mono-oxygenase.

Oxidation of substituted methane derivatives and of CO

The oxidation of the substituted methane derivatives was measured by following their disappearance from reaction flasks (Table 1) because no volatile products were detected by gas chromatography. This absence of detectable products can be accounted for by the instability of the corresponding 1-substituted methanol derivatives. Of the monohalogenated derivatives, chloromethane was oxidized as rapidly as methane (Table 2), bromomethane was oxidized less rapidly and iodomethane was not oxidized at all; i.e. there was a decline in the rate of oxidation with increasing size of the substituting halogen. Of the chlorinated derivatives, chloromethane and dichloromethane were oxidized as rapidly as methane itself, whereas trichloromethane was oxidized more slowly and tetrachloromethane was not oxidized. Thus up to two hydrogen atoms in methane can be replaced with chlorine atoms with no decrease in the observed rate of oxidation and complete substitution is required to prevent oxidation altogether.

Table 1. *Oxidation of substituted methane derivatives and of CO by soluble extracts of M. capsulatus (Bath)*

Specific activities with CO as substrate were calculated from the rate of CO_2 formation. Otherwise specific activities were calculated from the rate of disappearance of substrate and the products were not identified. Full experimental details are given in the Materials and Methods section. Specific activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, or with boiled extract. nd, Not done.

Substrate (μmol per reaction flask)	Specific activity (munits/mg of protein)
Chloromethane (1)	84 (0, 0, 80, 0, 0)
Bromomethane (1)	66 (0, 0, 66, 0, 0)
Iodomethane (1–3)	0
Dichloromethane (1)	82 (0, 0, 76, 0, 0)
Trichloromethane	35 (0, 0, 38, 0, 0)
Tetrachloromethane (1–3)	0
Cyanomethane (1)	33 (0, 0, 25, 0, 0)
Nitromethane (2)	45 (0, 0, 41, 0, 0)
Methanethiol (2)	64 (0, 0, 64, 0, 0)
Methanol (5)	246 (0, 20, nd, 16, 0)
Trimethylamine (2–4)	0
CO (134)	61 (12, 10, 56, 12, nd)

The cyano, nitro and thio derivatives of methane were oxidized more slowly than methane, whereas trimethylamine was not oxidized. Methanol was oxidized three times as rapidly as methane even though it is itself the product of methane oxidation. The apparent K_m for methanol was 0.95mM compared with 0.16mM for methane, indicating that methanol has a relatively poor affinity for the enzyme. Formaldehyde would be the expected product of methanol oxidation but its accumulation was not observed presumably because of its rapid metabolism by crude soluble extracts (see above).

Methane mono-oxygenase from *M. capsulatus* strain Bath also catalysed the oxidation of CO to CO_2 and this activity has also been observed with the enzymes from *Methylomonas methanica* (Ferenci *et al.*, 1975) and *Methylosinus trichosporium* (Tonge *et al.*, 1977). The small amounts of CO_2 formed in the control reaction flasks were also formed in controls lacking CO. This suggests that these low amounts of CO_2 were produced from endogenous substrates present in the extract.

Oxidation of C_1–C_8 n-alkanes to the corresponding primary and secondary alcohols

Methane mono-oxygenase catalysed the oxidation of *n*-alkanes of 1–8 carbon atoms (Table 2). C_1–C_5 alkanes were oxidized at comparable rates but there was a rapid decline in oxidation rate between pentane and octane. The enzyme is not a terminal hydroxylase: both 1-alcohols and 2-alcohols were produced from *n*-propane and *n*-butane indicating that both primary and secondary alkyl C–H bonds can be hydroxylated. The enzyme is apparently specific for the 1- and 2-alkyl carbon atoms, however, as there was negligible formation of 3- or 4-alcohols from pentane, hexane, heptane or octane.

Oxidation of internal and terminal n-alkenes

Methane mono-oxygenase catalysed the oxidation of the terminal alkenes ethene, propene and but-1-ene to the corresponding 1,2-epoxides (Table 3). Neither 2-propen-1-ol nor 3-buten-1-ol were formed from propene and but-1-ene respectively. The rate with ethene was almost twice that for methane oxidation but thereafter the rate of oxidation decreased with increasing carbon-chain length.

Internal alkenes were also oxidized. Thus *trans*-but-2-ene yielded a mixture of *trans*-2,3-epoxybutane and *trans*-2-buten-1-ol indicating that both the internal double bond and the terminal methyl group

Table 2. *Oxidation of C_1–C_8 n-alkanes by soluble extracts of M. capsulatus (Bath)*

Reaction mixtures contained 2mg of extract protein except for those with *n*-octane as substrate which contained 4mg of extract protein. Specific activities were calculated from the total amount of products formed after 12min incubation. The values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, or with boiled extract. Full details of the experimental methods are given in the Materials and Methods section.

Substrate (μmol per reaction flask)	Products (μmol formed in 12min)	Specific activity (munits/mg of protein)
Methane (134)	Methanol (2.02)	84 (0, 0, 85, 13, 0)
Ethane (134)	Ethanol (1.64)	68 (0, 0, 63, 13, 0)
Propane (134)	Propan-1-ol (0.65) Propan-2-ol (1.00)	69 (0, 0, 68, 0, 0)
Butane (134)	Butan-1-ol (1.10) Butan-2-ol (0.92)	77 (0, 5, 68, 0, 0)
Pentane (150)	Pentan-1-ol (0.49) Pentan-2-ol (1.26) Pentan-3-ol (<0.06)	73 (0, 0, 69, 0, 0)
Hexane (150)	Hexan-1-ol (0.60) Hexan-2-ol (0.36) Hexan-3-ol (<0.01)	40 (0, 0, 39, 0, 0)
Heptane (150)	Heptan-1-ol (0.14) Heptan-2-ol (0.51) Heptan-3-ol (<0.01) Heptan-4-ol (<0.01)	27 (0, 0, 27, 0, 0)
Octane (300)	Octan-1-ol (0.04) Octan-2-ol (0.39) Octan-3-ol (<0.01) Octan-4-ol (<0.01)	9 (0, 0, 9, 0, 0)

could be attacked. It is significant that the *trans* configuration was retained in both products precluding the formation of racemizable intermediates.

Three products were formed from *cis*-but-2-ene. Two were positively identified as *cis*-2,3-epoxybutane and 2-butanone but the third could not be conclusively identified as *cis*-but-2-en-1-ol because the authentic compound was not available as a reference marker. However, its retention times on gas-chromatographic analysis, which were similar to but not identical with those for *trans*-but-2-en-1-ol, and its reaction with bromine but not with dil. HCl (see Table 3), make its identification virtually certain. Again the *cis* configuration was retained in both the epoxide and the alken-1-ol. The explanation for the production of butanone only from the *cis*-isomer must await further study. It is unlikely to arise from contamination of the starting substrate with some other enzyme substrate because the amount of *cis*-but-2-ene present must be decreased to a value that affects the production of the other two products before the rate of butanone production is decreased. At present it is not possible to distinguish between its formation as a direct enzyme product and its subsequent formation by chemical or enzymic breakdown of one or the other products.

There was a marked difference between the rates of oxidation of the *cis*- and *trans*-but-2-enes: indeed *trans*-but-2-ene was oxidized more rapidly than any of the other alkenes tested except ethene. This suggests that the *trans* configuration is sterically favoured for oxidation by methane mono-oxygenase.

Oxidation of dimethyl ether and diethyl ether

Dimethyl ether oxidation was followed by measuring its disappearance from reaction flasks (Table 4)

Table 3. *Oxidation of C_2–C_4 n-alkenes by soluble extracts of M. capsulatus (Bath)*

Full experimental details are given in the Materials and Methods section. Specific activities were calculated from the total amount of products formed after 12min incubation. The values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, or with boiled extract. *, Product disappears from gas chromatograms after treating the reaction mixture with 20μl of HCl but remains after treatment with 5μl of bromine; †, product disappears after treating the reaction mixture with 5μl of bromine but remains after treatment with 20μl of HCl; ‡, product remains after treating the reaction mixture with either bromine or HCl.

Substrate (μmol per reaction flask)	Products (μmol formed after 12min)	Specific activity (munits/mg of protein)
Ethene (134)	Epoxyethane* (3.54)	148 (0, 13, 122, 12, 0)
Propene (134)	1,2-Epoxypropane* (2.10)	83 (0, 0, 83, 0, 0)
But-1-ene (134)	1,2-Epoxybutane* (1.19)	49 (0, 0, 49, 0, 0)
cis-But-2-ene (134)	*cis*-2,3-Epoxybutane* (0.61) *cis*-2-Buten-1-ol† (0.57) Butan-2-one‡ (0.20)	57 (0, 0, 51, 0, 0)
trans-But-2-ene (134)	*trans*-2,3-Epoxybutane* (0.77) *trans*-2-Buten-1-ol† (2.52)	141 (0, 0, 128, 0, 0)

and the products were not identified. It was oxidized three times as rapidly as methane at a rate similar to that for methanol oxidation. Diethyl ether was oxidized much more slowly, but in this case the products were identified as ethanol and ethanal which were formed in approximately equimolar amounts (Table 4). No 2-ethoxyethanol was formed, indicating that oxidation of diethyl ether occurs only at the carbon atom adjacent to the oxygen.

Oxidation of alicyclic, aromatic and heterocyclic compounds (Table 5)

Cyclohexane was oxidized to cyclohexanol with a specific activity somewhat higher than that for *n*-hexane. The ability of methane mono-oxygenase to hydroxylate such cyclic alkanes presumably reflects its ability to hydroxylate secondary alkyl C–H bonds. The aromatic ring of benzene was also hydroxylated, yielding phenol.

Toluene was tested as a potential enzyme substrate because it is the phenyl derivative of methane and the methyl derivative of benzene and therefore contains two different groups that could be hydroxylated. A mixture of benzyl alcohol and cresol was produced when the enzyme was incubated with toluene, indicating that both the aromatic ring and the methyl group were hydroxylated. The gas-chromatographic analysis did not distinguish between *o*-, *m*- and *p*-cresol. Styrene is analogous to toluene in being the phenyl derivative of ethane and also the vinyl derivative of benzene. Styrene was oxidized to styrene epoxide and, unlike toluene, yielded no ring-hydroxylated products.

Methane mono-oxygenase was tested for its ability to hydroxylate the aromatic ring of L-phenylalanine yielding L-tyrosine by assaying the product colorimetrically (Table 5). No tyrosine production was observed. This was not due to subsequent metabolism of the product because extracts did not catalyse the disappearance of tyrosine under the usual assay conditions.

Pyridine oxidation was detected and its rate estimated by observing the disappearance of pyridine from reaction flasks. However, no volatile products were detected by gas chromatography. Reaction mixtures were examined for non-volatile products by t.l.c. as described in the Materials and Methods section. A spot corresponding to pyridine *N*-oxide was found in complete reaction mixtures after 12min incubation. No 2-, 3- or 4-hydroxypyridine was found although these compounds could be readily

Table 4. *Oxidation of ethers by soluble extracts of M. capsulatus (Bath)*

Specific activities were calculated from the rate of disappearance of the substrate. However, the formation of ethanol and ethanal from diethyl ether was detected by analysing samples (5μl) of reaction mixtures before and after incubation. Full experimental details are given in the Materials and Methods section. Specific activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, and with boiled extract.

Substrate (μmol per reaction flask)	Products (μmol formed after 12min)	Specific activity (munits/mg of protein)
Dimethyl ether (4.5)	Not known	248 (0, 0, 227, 0, 0)
Diethyl ether (4.5)	Ethanol (0.51) Ethanal (0.57)	45 (0, 0, 45, 0, 0)

Table 5. *Oxidation of some alicyclic, aromatic and heterocyclic compounds by soluble extracts of M. capsulatus (Bath)*

Full details of the methods used are given in the Materials and Methods section. All reaction mixtures contained 4mg of extract protein. Except when pyridine was the substrate, specific activities were calculated from the total amount of products formed after 12min incubation. The rate of pyridine oxidation was determined by following pyridine disappearance from reaction flasks containing 3μmol of pyridine. Pyridine *N*-oxide was identified as the product of pyridine oxidation by t.l.c.; in this case reaction flasks contained 90μmol of pyridine as substrate. Specific-activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, or with boiled extract. nd, Not done; *, product disappears from gas chromatograms after treating the reaction mixture with 20μl of HCl or with 5μl of bromine; †, product disappears after treating the reaction mixture with 5μl of bromine but remains after treatment with 20μl of HCl; ‡, product remains after treating reaction mixtures with either 20μl of HCl or 5μl of bromine.

Substrate (μmol per reaction flask)	Products (μmol formed after 12min)	Specific activity (munits/mg of protein)
Cyclohexane (460)	Cyclohexanol‡ (3.0)	62 (0, 0, 62, 0, 0)
Benzene (450)	Phenol† (3.0)	62 (0, 0, 62, 0, 0)
Toluene (460)	Benzyl alcohol† (1.5) Cresol† (1.0)	53 (0, 0, 52, 0, 0)
Styrene (90)	Styrene epoxide* (2.3)	47 (0, 0, 37, 0, 0)
Pyridine (3, 90)	Pyridine *N*-oxide (nd)	29 (0, 4, 25, 0, 0)
L-Phenylalanine (2-16)	Tyrosine (0)	0

Table 6. *Effect of replacing NADH with other potential electron donors and hydroxylating agents*

Methanol formation from methane, and phenol formation from benzene, were measured as described in the Materials and Methods section except that cyanide was omitted from the reaction mixtures and NADH was replaced with the compounds indicated. The following compounds gave no activity when tested at 5 and 20mM in place of NADH with methane as substrate: sodium ascorbate, quinol, sodium dithionite, sodium borohydride, sodium chlorite, sodium periodate. n.d., Not done.

Test compound	Specific activity (munits/mg of protein)	
Substrate ...	Methane	Benzene
NADH (5mM)	85	62
NADPH (5mM)	49	nd
NADH (5mM)+superoxide dismutase (100 units)	87	62
Xanthine (5mM)+xanthine oxidase (0.1, 1 unit)	0	0
H_2O_2 (5, 20mM)	0	0
NADH (5mM)+catalase (100 units)	80	68

distinguished from pyridine *N*-oxide by t.l.c. No pyridine *N*-oxide was found in complete reaction mixtures at zero time, neither was it found after 12min incubation of the usual control reaction mixtures.

Electron donor specificity of methane mono-oxygenase

A variety of reducing agents was tested as electron donor but only NADPH could replace NADH (Table 6). The broad substrate specificity of methane mono-oxygenase suggests that the enzyme might act by generating a non-specific hydroxylating species such as H_2O_2 or superoxide anion. However, the failure of superoxide dismutase or catalase to prevent activity and the failure of H_2O_2 or superoxide (generated *in situ* from xanthine oxidase and xanthine) to support methane or benzene oxidation effectively excludes this possibility. Sodium chlorite and sodium periodate have been shown to support steroid hydroxylation by replacing NADPH in the cytochrome *P*-450 system of liver microsomal preparations (Hrycay *et al.*, 1975). Neither of these compounds supported methane oxidation by the methane mono-oxygenase of *M. capsulatus* (Bath).

Discussion

Methane mono-oxygenase of *M. capsulatus* (Bath) is not a terminal alkane hydroxylase as are, for example, the rubredoxin-containing alkane hydroxylase of *Pseudomonas oleovorans* (May & Abbott, 1973) and the cytochrome *P*-450 alkane hydroxylase of a diphtheroid bacterium (Cardini & Jurtshuk, 1970). Instead, methane mono-oxygenase oxidizes *n*-alkanes to mixtures of the corresponding 1- and 2-alcohols. Similarly the methane mono-oxygenase will form an epoxide from both terminal and internal alkenes, whereas the alkane hydroxylase from *Ps. oleovorans* will form epoxides from terminal alkenes only (May & Abbott, 1973). The enzyme systems from other methane-oxidizing bacteria have not been examined for these properties, but whole-cell studies suggest that the enzyme from *Methylomonas methanica* is also able to hydroxylate sub-terminal alkyl C–H bonds. Thus Leadbetter & Foster (1960) observed the formation of propan-1-ol, propionic acid and acetone when suspensions of *Methylamonas methanica* were incubated with *n*-propane; similarly butan-1-ol, butyric acid and 2-butanone were formed from *n*-butane. The mechanism for the production of the ketones is obscure, bearing in mind that the alcohol dehydrogenase present in this organism is specific for primary alcohols (Johnson & Quayle, 1964; Ferenci *et al.*, 1975). Nevertheless, the formation of the ketones does suggest that sub-terminal oxidation of the alkanes occurs. Different results were obtained by Thomson (1974) using two other methane-oxidizing bacteria *Methylomonas albus* and *Methylosinus trichosporium*. When incubated with *n*-propane or *n*-butane these organisms formed products characteristic of terminal oxidation only.

The sub-terminal oxidation of higher *n*-alkanes by micro-organisms is well-known (Markovetz, 1971). However, in most cases a random mixture of secondary alcohols and their oxidation products together with the primary alcohol are formed. For instance, *Ps. aeruginosa* transforms *n*-decane into a mixture of decan-1-ol and decan-2, 3-, 4- and 5-ols together with the corresponding ketones (Fredericks, 1967). Similar results were obtained by Klein *et al.* (1968) with an *Arthrobacter* sp. oxidizing *n*-hexadecane or *n*-pentadecane. Oxidation of long-chain fatty acids and *n*-alkanes specifically at the $\omega 1$ and $\omega 2$ carbon atoms has been observed, however, in the yeast *Torulopsis* sp. (Tulloch *et al.*, 1962; Heinz *et al.*, 1970). The hydroxylation of long-chain fatty acids at the $\omega 1$ and $\omega 2$ positions is also catalysed by the rat liver microsomal cytochrome *P*-450 system (Bjorkhem & Danielsson, 1970). However, in the latter case the different inhibitor sensitivities of the $\omega 1$ and $\omega 2$ hydroxylations (Ellin & Orrenius, 1975) and their different requirements for hydroxylating agents (Gustafsson & Bergen, 1976) suggest that the two types of hydroxylation are catalysed by different cytochrome *P*-450 species. There is no evidence at present to suggest that the 1- and 2-hydroxylations of C_5–C_8 *n*-alkanes observed in the present study are not due to the same enzyme system.

The mechanism for the oxidation of diethyl ether

by the methane mono-oxygenase of *M. capsulatus* (Bath) resembles that observed by Heydeman (1974) in bacteria isolated from diethyl ether enrichments. The sub-terminal carbon is hydroxylated yielding 1-ethoxyethanol as a hypothetical intermediate that dismutates to form ethanol and ethanal in equimolar amounts. This apparently differs from the mechanism of diethyl ether oxidation by two other methane-oxidizing bacteria *Methylomonas albus* and *Methylosinus trichosporium* (Wilkinson, 1975). Whole-cell studies indicated that these organisms oxidize diethyl ether solely at the terminal methyl group, yielding 2-ethoxyethanol, 2-ethoxyethanal and eventually 2-ethoxyacetate. This evidence, together with the observation that these two methane-oxidizing bacteria catalyse only terminal oxidation of *n*-propane and *n*-butane (Thomson, 1974), strongly suggests that they, unlike *M. capsulatus* (Bath) and probably *Methylomonas methanica*, contain an alkane hydroxylase that is specific for terminal methyl groups.

The methane mono-oxygenase of *M. capsulatus* (Bath) is a very non-specific enzyme system and many of its substrates show little or no structural resemblance to its substrate *in vivo*, methane. 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, and the oxidation of CO to CO_2. Moreover, some substrates can be attacked at more than one position e.g. *trans*- and *cis*-but-2-ene and toluene. Such behaviour resembles that of some cytochrome *P*-450-containing mono-oxygenases (Orrenius & Ernster, 1974), but the resistance of the methane mono-oxygenase to inhibition by CO (Colby & Dalton, 1976) suggests that it does not contain cytochrome *P*-450. The possibility that it contains a CO-binding cytochrome *c* of the type involved in the analogous methane mono-oxygenase system from *Methylosinus trichosporium* (Tonge *et al.*, 1977) cannot yet be excluded. However, these two enzyme systems do have very different electron-donor and inhibitor specificities (Colby & Dalton, 1976; Tonge *et al.*, 1977) and, as discussed above, also appear to have different substrate specificities. Investigations into the physicochemical properties of the enzyme system from *M. capsulatus* (Bath) will have to await the purification of its components.

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