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Some Properties of a Soluble Methane Mono-oxygenase from *Methylococcus capsulatus* Strain Bath

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

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Soluble extracts of *Methylococcus capsulatus* (Bath), obtained by centrifugation of crude extracts at 160000g for 1h, catalyse the NAD(P)H- and O_2-dependent disappearance of bromomethane, and also the formation of methanol from methane. Soluble methane mono-oxygenase is not inhibited by chelating agents or by most electron-transport inhibitors, and is a multicomponent enzyme.

Methane mono-oxygenase is apparently the enzyme responsible for the initial oxidative attack on methane in the three methane-oxidizing bacteria that have so far been studied, i.e. *Methylococcus capsulatus* (Texas) (Ribbons & Michalover, 1970; Ribbons, 1975), *Methylomonas methanica* (Ferenci *et al.*, 1975; Colby *et al.*, 1975) and *Methylosinus trichosporium* (Tonge *et al.*, 1975). In each case, methane mono-oxygenase activity is associated with the particulate membrane fraction of the bacterial extracts, although Tonge *et al.* (1975) were able to obtain active soluble preparations by treatment of their membranes with detergents, phospholipase D or ultrasonic radiation. In contrast, our particulate membrane preparations of *M. capsulatus* (Bath) consistently had no methane mono-oxygenase activity, as determined by following bromomethane disappearance (Colby *et al.*, 1975). The supernatant fractions obtained by centrifugation of the crude extracts at 160000g for 1h, however, catalysed bromomethane disappearance and, indeed, the accumulation of methanol from methane. This present paper describes some properties of the soluble methane mono-oxygenase from *M. capsulatus* strain Bath.

Materials and Methods

Sodium L-ascorbate, sodium formate, bovine plasma albumin (fraction V), DEAE-cellulose, NAD^+, NADH, NADPH and electron-transport inhibitors were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Acetylene and CO were supplied by Cambrian Chemicals, Croydon, Surrey, U.K., and methane (technical grade) by British Oxygen Co., London S.W.19, U.K. Methanol (Ultrar) was supplied by Hopkin and Williams, Chadwell Heath, Essex, U.K., and bromomethane by BDH Chemicals, Poole, Dorset, U.K. Other potential enzyme inhibitors were obtained from the sources described by Colby *et al.* (1975).

M. capsulatus (Bath) [described by Whittenburg *et al.* (1970)] was grown at 45°C in continuous culture at a dilution rate of 0.05h^{-1} on ammonium mineral salts medium (Whittenbury *et al.*, 1970), with methane (20%, v/v, in air) as a carbon source. Washed bacterial suspensions were prepared by centrifuging the culture (3mg dry wt./ml) or the overflow, washing the cell pellet once with 20mM-sodium phosphate buffer, pH 7.0, and resuspension in the same buffer containing 5mM-$MgCl_2$ (5ml of buffer per litre of original culture). Crude bacterial extracts were prepared by a single passage of the suspension through a French pressure cell at 137MPa (20000lb/in^2) followed by centrifugation at 5000g for 10min to remove unbroken bacteria. The crude extract was then centrifuged at 38000g for 1h, yielding particulate (P_{38}) and soluble (S_{38}) fractions. The S_{38} fraction was subsequently centrifuged at 160000g for 1h to give an intensely red particulate fraction (P_{160}) and a clear red supernatant (S_{160}). The protein concentration of the S_{160} fraction was about 50mg/ml as determined with the Folin–Ciocalteu reagent (Lowry *et al.*, 1951), with dried crystalline bovine plasma albumin as standard.

Methane mono-oxygenase activity was measured at 45°C either by following bromomethane disappearance essentially as described previously (Colby *et al.*, 1975) or by measuring methanol accumulation. In the latter case, the reaction mixtures contained, in 1ml: 50μmol of sodium phosphate buffer, pH 7.0; 5μmol of NADH; 0.5μmol of KCN (where present); 0–10mg of extract protein. Reaction mixtures were contained in conical flasks (volume 7ml), sealed with Suba-seal stoppers, in which 3ml of the gas phase was replaced by 3ml of methane. When very high enzyme activities were to be measured it was necessary to replace the air+methane mixture in the reaction flasks with O_2+methane (50:50). The flasks were incubated at 45°C on a shaking water bath at 90 oscillations/min. The reaction was started by adding the extract, and 5μl samples of the liquid phase were removed at timed intervals and injected into a gas

chromatograph (Pye series 104, flame ionization detector), equipped with a 2.1m column (internal diameter 4mm) of Porapak Q (Waters Associates, Milford, MA, U.S.A.), at 125°C, with N_2 (30ml/min) as carrier gas. The chromatograph was calibrated by using freshly prepared methanol solutions of known concentration and measuring peak heights.

Methanol oxidase activity was measured by following methanol disappearance aerobically in the absence of added electron acceptors. Reaction mixtures (1 ml) containing 50 μmol of sodium phosphate buffer, pH 7.0, and 0–10 mg of extract protein were incubated in conical flasks (7 ml) at 45°C on a shaking water bath. Reactions were started by adding the extract, and samples (5 μl) of reaction mixture were removed at timed intervals and their methanol contents determined by using a gas chromatograph as described above.

Results and Discussion

Crude extracts of *M. capsulatus* (Bath) catalysed bromomethane disappearance in the presence of NAD(P)H and O_2. Fractionation of the extract by centrifugation (see the Materials and Methods section) revealed that the bromomethane mono-oxygenase activity resided exclusively in the soluble S_{38} and S_{160} fractions; these soluble fractions also catalysed methanol accumulation from methane. Addition of the particulate P_{38} and P_{160} fractions back to the corresponding soluble fractions gave no enhancement of methane mono-oxygenase activity, whether measured by methanol accumulation or bromomethane disappearance. The soluble fractions retained full activity when stored at −70°C for several months and retained 75–100% of their activity at 0°C for 24h.

The S_{38} fraction and, to a lesser extent, the S_{160} fraction contained considerable methanol oxidase activity (typically 100 munits/mg of extract protein for the S_{38} fraction), which interfered with the measurement of methanol accumulation. This activity was not prevented by high phosphate concentrations (cf. Tonge *et al.*, 1975), but was totally inhibited by 0.5mM-KCN. Routine assays of methane mono-oxygenase activity by following methanol accumulation were therefore done with this inhibitor present.

Some properties of the soluble methane mono-oxygenase activity were investigated by using both methanol accumulation and bromomethane disappearance as the assay methods. Unless specified otherwise, the results quoted were obtained by using both assay methods. Activities measured with bromomethane as substrate were approximately half those obtained by measuring methanol accumulation from methane. Methane mono-oxygenase activity was highest at pH 6.5–7.0. NADH (5mM), NADPH (5mM) or an NADH-generating system consisting of NAD^+ (2mM), sodium formate (5mM) and endogenous formate dehydrogenase (EC 1.2.1.2; 280 munits/mg of extract protein) were necessary for activity. Highest activities were obtained with NADH or the NADH-generating system; NADPH was only 50% as effective as NADH at the concentrations used. Methanol (not tested by measuring methanol accumulation), ethanol and sodium ascorbate (each tested at 1, 5 and 25mM in the presence or absence of 0.5mM-KCN) all did not support methane mono-oxygenase activity. Crude extracts of *M. capsulatus* (Bath) and of *Methylomonas methanica* (Colby *et al.*, 1975) thus differ from those of *Methylosinus trichosporium* (Tonge *et al.*, 1975) in the range of electron donors that will support their methane mono-oxygenase activity. No methanol accumulation or bromomethane disappearance was observed when incubations were carried out anaerobically or in the presence of acetylene (0.1% in air), a potent inhibitor of methane oxidation but not methanol oxidation by whole cells.

The effect of various potential inhibitors on enzyme activity was tested. Whereas the active particulate preparations from *Methylomonas methanica* (Colby *et al.*, 1975) and from *M. capsulatus* (Texas) (Ribbons, 1975) were potently inhibited by chelating agents, as was methane oxidation by washed suspensions of

Table 1. *Effect of electron-transport inhibitors on methane mono-oxygenase activity*

Activities were measured as described in the Materials and Methods section. The results quoted were obtained by measuring methanol accumulation; very similar results were obtained when enzyme activities were determined by measuring bromomethane disappearance. Insoluble inhibitors were made up in reaction mixture at the final concentration and then subjected to ultrasonic radiation for 2–3 min to aid dispersion. For a description of the site of action of these inhibitors see Heinen (1972).

Inhibitor	Concentration (mM)	Inhibition (%)
NaN_3	1	0
KCN	1	0
CO	15% (v/v) in air	0
2,3-Dimercaptopropan-1-ol	1	58
	0.2	36
Ethyl carbamate (urethane)	1	35
	0.2	24
2-*N*-Heptyl-4-hydroxyquinoline *N*-oxide	1	5
Antimycin A	1	0
Rotenone	1	4
Amytal (5-ethyl-5-isopentylbarbituric acid)	4	0
Acriflavin	1	48
	0.2	32
Oligomycin	1	0

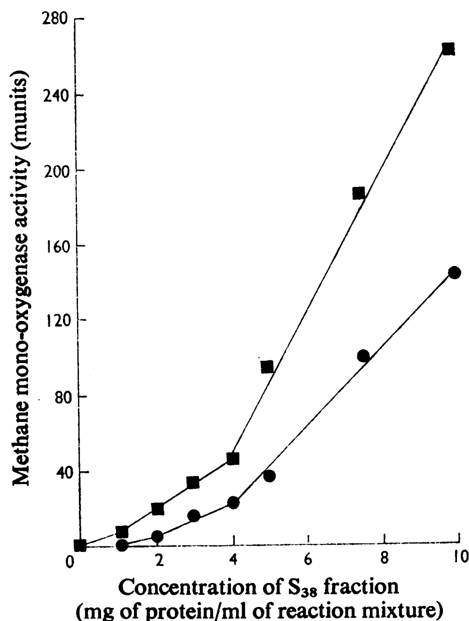

Fig. 1. *Non-linear relationship between methane mono-oxygenase activity and extract concentration*

Enzyme activities were determined as described in the Materials and Methods section. ■, Activity determined by measuring methanol accumulation; ●, activity determined by measuring bromomethane disappearance.

Methylosinus trichosporium (Hubley *et al.*, 1975), the soluble methane mono-oxygenase from *M. capsulatus* (Bath) was not significantly inhibited by $\alpha\alpha'$-bipyridyl, neocuproine, 8-hydroxyquinoline, thiourea, thioacetamide, KCN or NaN_3 (all at 1 mM). Neither was activity affected by compounds Lilly 53325 (1 mM), SKF 525A (1 mM) or CO (15%, v/v, in air), whereas each of these compounds inhibited the methane mono-oxygenase from *Methylomonas methanica* (Colby *et al.*, 1975). The effects of other potential enzyme inhibitors were similar to those observed previously with the latter enzyme.

A range of electron-transport inhibitors was tested in view of the observation by Tonge *et al.* (1975) that Amytal (4 mM) prevented NADH-mediated, but not ascorbate-mediated, methane oxidation by the particulate methane mono-oxygenase of *Methylosinus trichosporium*. This observation suggested to those authors that NADH is not the immediate electron donor, but that electrons are passed from NADH along the electron-transport chain to the physiological donor. Ribbons (1975) also observed inhibition of the particulate enzyme from *M. capsulatus* (Texas) by electron-transport inhibitors, suggesting that the same situation might exist in that organism. The results obtained with our soluble preparations are given in Table 1. Despite the rather weak inhibitions by dimercaptopropanol, acriflavin and urethane, the results suggest to us that the electron-transport chain is not involved in the passage of electrons from NADH to the soluble methane mono-oxygenase of *M. capsulatus* (Bath).

When methane mono-oxygenase activity was plotted as a function of the amount of soluble fraction present, a non-linear relationship was obtained irrespective of whether bromomethane disappearance or methanol accumulation was measured (Fig. 1). This relationship, which differs from the linear relationship observed with the particulate preparations from *Methylomonas methanica* (Colby *et al.*, 1975) and from *M. capsulatus* (Texas) (Ribbons, 1975), suggests that the enzyme is composed of more than one component. This was confirmed by the resolution of the S_{160} fraction into at least two components by passage through a column (8 cm × 4 cm) of DEAE-cellulose. Neither the material that was not retained on the column nor the material that was subsequently eluted from the column with 200 mM-NaCl was active when tested alone, but activity was restored by combining the two fractions.

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