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An Improved Assay for Bacterial Methane Mono-oxygenase: Some Properties of the Enzyme from *Methylomonas methanica*

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Extracts of *Methylomonas methanica* catalyse the O_2- and NAD(P)H-dependent disappearance of bromomethane. The activity is unstable at 2°C but is stable at −70°C for several weeks. Bromomethane mono-oxygenase is particulate and is inhibited by metal-binding reagents, by compounds SKF 525A and Lilly 53325, by some metal ions and by acetylene. Evidence is presented that indicates that bromomethane mono-oxygenase is the enzyme responsible for methane oxidation *in vivo*.

Higgins & Quayle (1970) demonstrated that the initial oxidative attack on methane by washed suspensions of methylobacteria involves the incorporation of oxygen from molecular O_2. The subsequent observation of methane-stimulated NADH oxidation catalysed by extracts of *Methylomonas methanica* (Ferenci, 1974) or of *Methylococcus capsulatus* (Ribbons & Michalover, 1970; Ribbons, 1975) suggested that the enzyme responsible for this oxygenation is a mono-oxygenase. Nevertheless, a satisfactory routine assay for methane mono-oxygenase has not been published and previous workers have relied on indirect enzyme assays measuring methane-stimulated NADH disappearance spectrophotometrically or methane-stimulated O_2 disappearance polarographically (Ferenci, 1974; Ribbons & Michalover, 1970; Ribbons, 1975). The present paper describes a convenient and rapid assay for methane mono-oxygenase in which the disappearance of a soluble derivative of methane, bromomethane, is followed directly. Some properties of the enzyme from *Methylomonas methanica* have been investigated by using this assay.

Materials and methods

Compound SKF 525A (2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride) and compound Lilly 53325 [2,4-dichloro-(6-phenylphenoxy)ethylamine hydrobromide] were gifts from Smith, Kline and French, Welwyn Garden City, Herts., U.K., and Lilly Research, Windlesham, Surrey, U.K., respectively. 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. Hopkin and Williams, Chadwell Heath, Essex, U.K., supplied methanol (Ultrar). Analytical-grade metal salts (all chlorides except for $FeSO_4$ and $CdSO_4$), NH_4Cl, 8-quinolinol, NaCN, NaN_3 and bromomethane (liquid) were obtained from BDH Chemicals, Poole, Dorset, U.K. Other potential inhibitors and biochemicals were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Dr. T. Ferenci, Department of Microbiology, University of Sheffield, Sheffield, U.K., kindly provided a culture of *Methylomonas methanica*.

Organisms were grown at 30°C in shaken 2-litre conical flasks each containing 750ml of nitrate–mineral-salts medium (Whittenbury *et al.*, 1970) each connected to a football bladder containing about 1500ml of methane. Organisms were harvested after 24h incubation (when the E_{600} of the culture was 0.35–0.40), washed twice with ice-cold 25mM-sodium phosphate buffer, pH7.0, and resuspended in the same buffer containing 5mM-$MgCl_2$ at a concentration of about 200mg wet wt. of organisms/ml. Washed suspensions were broken by one passage through a French pressure cell at 103MPa (15000lb/in^2). The crude homogenate was centrifuged for 15min at 3000*g* and the supernatant (15–20mg of protein/ml) retained as the bacterial extract. Protein concentrations were measured with the Folin phenol reagent (Kennedy & Fewson, 1968) with crystalline bovine plasma albumin (fraction 5) as standard.

Bromomethane mono-oxygenase activity was determined as follows. Reaction mixtures contained in 2ml: 100μmol of sodium phosphate buffer, pH7.0; 10μmol of NADH; 2μmol of bromomethane; extract (0.75–1.50mg of protein). Reaction mixtures were contained in conical flasks (nominal volume 10ml) sealed with Suba-Seal stoppers and were incubated at 30°C in a reciprocating water bath at 90 oscillations/min. Initially, reaction mixtures contained all the components except extract. The flasks were shaken for 15min at 30°C and then a 0.2ml sample of the gas phase in each flask was injected into the gas chromatograph (Pye series 104, flame ionization detector) as a zero-time reading. Extract (50–100μl) was then injected into the flask through the stopper and further 0.2ml samples of the gas phase were injected into the gas chromatograph at timed intervals (usually 5, 10 and 15min). Controls

lacked extract or NADH or were incubated under an atmosphere of O_2-free N_2. Bromomethane was determined on a 2.1m column (internal diameter 4mm) of Porapak R (Waters Associates, Milford, Mass., U.S.A.) at 150°C with N_2 (30ml/min) as carrier gas. Peak heights were measured and compared with a standard curve obtained by taking 0.2ml samples of the gas phases in similar flasks maintained at 30°C containing 2ml of sodium phosphate buffer (50mM), pH7.0, and 0–2μmol of bromomethane. The rate of bromomethane disappearance in each experimental flask was calculated and the corresponding rate for the control flasks lacking extract substracted from it. One unit of enzyme is the quantity that catalyses the disappearance of 1μmol of bromomethane/min at 30°C under the conditions of the assay. Bromomethane oxidation by washed suspensions was measured as described above, but in the absence of NADH and with washed suspension replacing bacterial extract.

Substrate-stimulated NADH oxidation by methane, bromomethane or NH_4Cl was measured at 30°C in a Pye–Unicam SP.1800 double-beam spectrophotometer. Experimental and reference cuvettes contained in 1ml: 50μmol of sodium phosphate buffer, pH7.0; 0.15μmol of NADH; extract (0.3–0.75mg of protein). The extinction at 340nm was followed for 1–2min and then simultaneous additions of substrate solution and distilled water (equal volumes) were made to the experimental and reference cuvettes respectively. Additions were 0.2ml of methane-saturated water, 50μl of 0.1M-bromomethane or 50μl of 1M-NH_4Cl.

Results and discussion

Extracts of *Methylomonas methanica* catalysed the disappearance of bromomethane from reaction flasks only in the presence of O_2 and NADH (Fig. 1). The requirement for NADH excludes the possibility that the activity is due to the presence of unbroken bacteria in the extract. A linear relationship was observed between the rate of bromomethane disappearance and the amount of extract present (0.15–3.00mg of protein), suggesting that bromomethane mono-oxygenase comprises a single component or, as seems more likely, that the extract consists of a suspension of discrete multi-component enzyme complexes. The latter concept is supported by the observation that 76% of the activity in extracts was sedimented by centrifugation at 18000*g* for 30min. Specific bromomethane mono-oxygenase activities in extracts were 0.060–0.095 unit/mg of protein. Methane disappearance, although detectable, was very slow, presumably because of the poor solubility of this substrate.

Bromomethane mono-oxygenase activity was unstable at 2°C and 30–75% of activity was lost after 24h. The activity was completely stable to freezing, however, and extracts were stored as a routine at

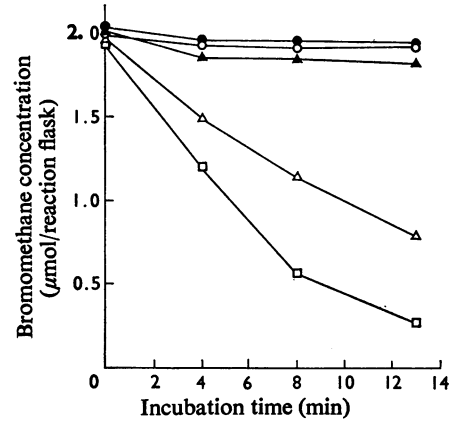

Fig. 1. *Oxygen-, extract- and NAD(P)H-dependent disappearance of bromomethane*

The disappearance of bromomethane was measured as described under 'Materials and methods'. □, Complete reaction mixture containing 100μl of extract (2mg of protein); ○, reaction mixture lacking extract; ▲, reaction mixture lacking NADH; ●, reaction mixture incubated under an atmosphere of O_2-free N_2; △, reaction mixture with NADPH (5mM) instead of NADH.

–70°C after rapid freezing in solid CO_2. Extracts stored in this manner retained full activity for several weeks.

The variation of enzyme activity with pH is shown in Fig. 2. Maximum activity was observed at pH6.9. Activity was independent of NADH concentration between 0.5 and 5.0mM, although higher concentrations were inhibitory. NADPH can replace NADH as electron donor and activity measured with NADPH (5mM) was about 60% of that measured with NADH (5mM) (Fig. 1). The apparent K_m for NADPH, determined from Lineweaver & Burk (1934) double-reciprocal plots, was 3.5mM. NADH could not be replaced by NAD^+ (5mM), $NADP^+$ (5mM), sodium ascorbate (5mM), GSH (5mM) or sodium dithionite (5mM).

Bromomethane mono-oxygenase activity was not stimulated by the addition to reaction mixtures of ATP (5mM), ADP (5mM), AMP (5mM), vitamin B_{12} (0.1–1.0mM), coenzyme B_{12} (0.1–1.0mM), tetrahydrofolic acid (0.1–1.0mM), sodium ascorbate (1mM), dithiothreitol (1mM) or GSH (1mM). Except for EDTA (19% inhibition at 5mM), all the metal-binding reagents tested were potent inhibitors, suggesting some metal ion involvement in bromomethane mono-oxygenase activity. Thus 8-quinolinol, neocuproine, sodium diethyldithiocarbamate, $\alpha\alpha$-dipyridyl and NaCN caused 59–81% inhibition at 0.01mM and thiourea, thioacetamide and NaN_3 caused 66–100% inhibition at 0.1mM. CO (15%, v/v, in air), hydroxylamine hydrochloride (1mM) and imidazole (1mM)

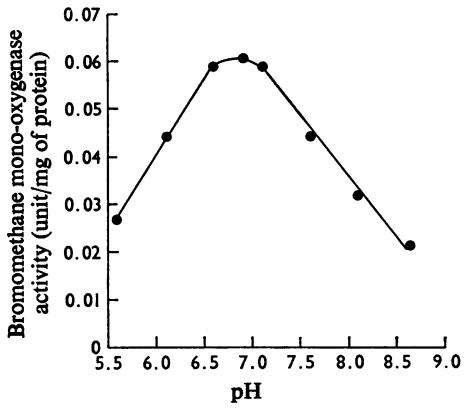

Fig. 2. *Effect of pH on bromomethane mono-oxygenase activity*

Enzyme activity was measured as described under 'Materials and methods' but with a range of 50mM-sodium phosphate buffers of different pH values. The pH of each reaction mixture was determined at the end of the experiment by using a glass electrode.

caused 68, 78 and 62% inhibition respectively. Compounds Lilly 53325 and SKF 525A, both of which inhibit some other mono-oxygenases (McMahon *et al.*, 1969; Hammond & Whyte, 1970; Hildebrandt, 1972), caused 75 and 25% inhibition respectively at 0.1mM. These results with inhibitors resemble those obtained by Hubley *et al.* (1975) with methane-oxidizing bacterial suspensions of *Methylosinus trichosporium* suggesting that the bromomethane mono-oxygenase activity measured *in vitro* is identical with the enzyme responsible for methane oxidation in whole bacteria.

The following compounds had no effect on enzyme activity: *N*-ethylmaleimide (5mM), sodium iodoacetate (5mM), 5,5'-dithiobis-(2-nitrobenzoic acid) (5mM), semicarbazide (1mM), isonicotinic acid hydrazide (1mM), isopropylisonicotinic acid hydrazide (1mM). The insensitivity of bromomethane mono-oxygenase to these thiol-binding reagents and carbonyl-binding reagents suggests that such groups are not involved in enzyme activity. A range of univalent, bivalent and tervalent metal ions was tested as inhibitors. Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} caused 24–90% inhibition at 1mM, whereas Hg^{2+} caused 62% inhibition at 0.2mM. K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and Co^{2+} had no effect on enzyme activity at 1–5mM concentration.

The effect of NH_4Cl on bromomethane mono-oxygenase activity was investigated in view of our observation of NH_4Cl-stimulated NADH oxidation catalysed by extracts of *Methylomonas methanica* (see below). Very high concentrations were required for inhibition (46% inhibition at 100mM), suggesting that the enzyme has a low affinity for this substrate. This is supported by the high concentration of NH_4Cl required to demonstrate NH_4Cl-stimulated NADH oxidation. Methanol (5mM), the supposed product of methane hydroxylation and an analogue of bromomethane, caused 76% inhibition.

Acetylene was a potent inhibitor of bromomethane mono-oxygenase activity, causing 100% inhibition at a concentration of 0.1% in air. The same concentration of acetylene also inhibits bromomethane oxidation by washed suspensions of *Methylomonas methanica* or *Methylomonas albus* and prevents the growth of *Methylomonas albus* in liquid culture on methane (20%, v/v, in air) but not on methanol (0.1%). Further, cultures of *Methylococcus capsulatus* grown on methane in the absence of fixed nitrogen reduce acetylene to ethylene in the presence of methanol, whereas the acetylene-reduction test is negative with methane as source of energy and reducing power (Whittenbury *et al.*, 1975). This is further proof that bromomethane mono-oxygenase is the enzyme responsible for the oxidation of methane *in vivo*.

Extracts of *Methylomonas methanica* catalysed bromomethane-dependent NADH oxidation measured as described under 'Materials and methods.' Specific activities agreed well with those determined from the rate of bromomethane disappearance in the bromomethane mono-oxygenase assay, indicting a stoicheiometric relationship between bromomethane and NADH disappearance, as would be expected of a mono-oxygenase-catalysed reaction. Extracts also catalysed methane- and NH_4Cl-stimulated NADH oxidation at rates 100 and 30% respectively of the rate with bromomethane as substrate. The following properties suggest that NH_4Cl-stimulated NADH oxidation is catalysed by bromomethane mono-oxygenase: (1) activity is proportional to extract concentration; (2) NADPH (0.15mM) does not replace NADH; (3) activity is totally inhibited by NaCN (15μM), $\alpha\alpha$-dipyridyl (40μM), compound Lilly 53325 (0.1mM), acetylene (1mM) or methanol (5mM) but is unaffected by isonicotinic acid hydrazide (1mM) or *N*-ethylmaleimide (5mM).

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Ferenci, T. (1974) *FEBS Lett.* **41**, 94–98
Hammond, R. K. & Whyte, D. C. (1970) *J. Bacteriol.* **103**, 607–612
Higgins, I. J. & Quayle, J. R. (1970) *Biochem. J.* **118**, 201–208
Hildebrandt, A. G. (1972) *Biochem. Soc. Symp.* **34**, 79–112

Hubley, J. H., Thomson, A. W. & Wilkinson, J. F. (1975) *Arch. Microbiol.* **102**, 199–202
Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
Kennedy, S. I. T. & Fewson, C. A. (1968) *Biochem. J.* **107**, 497–506
McMahon, R. E., Mills, J., Culp, H. W., Gibson, W. R., Miller, W. M. & Marshall, F. J. (1969) *J. Med. Chem.* **12**, 207–211
Ribbons, D. W. (1975) *J. Bacteriol.* **122**, 1351–1363
Ribbons, D. W. & Michalover, J. L. (1970) *FEBS Lett.* **11**, 41–44
Whittenbury, R., Phillips, K. C. & Wilkinson, J. F. (1970) *J. Gen. Microbiol.* **61**, 205–218
Whittenbury, R., Dalton, H., Eccleston, M. & Reed, H. L. (1975) in *Microbial Growth on C_1 Compounds: Proceedings of the International Symposium on Microbial Growth on C_1 Compounds* (Terium, G., ed.), pp. 1–11, Society of Fermentation Technology, Tokyo