Biochem. J. (1977) **161**, 333–344
Printed in Great Britain

Purification and Properties of the Methane Mono-oxygenase Enzyme System from *Methylosinus trichosporium* OB3b

By GLYN M. TONGE,* DAVID E. F. HARRISON† and I. JOHN HIGGINS*
**Biological Laboratory, University of Kent, Canterbury CT2 7NJ, U.K.,*
and
†Woodstock Laboratory, Shell Research Limited, Sittingbourne ME9 8AG, U.K.

(*Received* 12 *July* 1976)

1. A three-component enzyme system that catalyses the oxidation of methane to methanol has been highly purified from *Methylosinus trichosporium*. 2. The components are (i) a soluble CO-binding cytochrome *c*, (ii) a copper-containing protein and (iii) a small protein; the mol.wts. are 13000, 47000 and 9400 respectively. The cytochrome component cannot be replaced by a similar cytochrome purified from *Pseudomonas extorquens* or by horse heart cytochrome *c*. 3. The stoicheiometry suggests a mono-oxygenase mechanism and the specific activity with methane as substrate is 6 μmol/min per mg of protein. 4. Other substrates rapidly oxidized are ethane, *n*-propane, *n*-butane and CO. Dimethyl ether is not a substrate. 5. The purified enzyme system utilizes ascorbate or, in the presence of partially purified *M. trichosporium* methanol dehydrogenase, methanol as electron donor but not NADH or NADPH. 6. Activity is highly sensitive to low concentrations of a variety of chelating agents, cyanide, 2-mercaptoethanol and dithiothreitol. 7. Activity is highly pH-dependent (optimum 6.9–7.0) and no component of the enzyme is stable to freezing. 8. The soluble CO-binding cytochrome *c* shows oxidase activity and the relationship between this and the oxygenase activity is discussed.

There have been several attempts in recent years to elucidate the mechanism of initial enzymic attack on methane by obligate methylotrophs. Initial indirect evidence for oxygenase involvement came from whole-organism ^{18}O-incorporation experiments (Leadbetter & Foster, 1959), and later more direct evidence came from the demonstration with ^{18}O that the oxygen in methanol derived from methane and excreted by two methylotrophs was derived exclusively from molecular oxygen (Higgins & Quayle, 1970). There followed reports of NADH- and oxygen-dependent methane oxidation by cell-free preparations from both *Methylococcus capsulatus* (Ribbons & Michalover, 1970; Ribbons & Higgins, 1971; Ribbons, 1975) and *Methylomonas methanica* (Ferenci, 1974; Ferenci *et al.*, 1975). Similar activity has been described for the substrate analogues of methane, CO (Ferenci, 1974; Ferenci *et al.*, 1975) and bromomethane (Colby *et al.*, 1975). The results of these studies are all consistent with an NADH-linked mono-oxygenase being responsible for methane oxidation. The purification of these enzyme systems has not proved possible owing to their apparent instability and particulate nature.

We have described the detection of a similar enzyme system in cell-free preparations of *Methylosinus trichosporium* that is both stable and can be readily solubilized from membrane preparations (Tonge *et al.*, 1975). Ascorbate and, in crude extracts, methanol can serve as alternative electron donors for the mono-oxygenase, indicating that the enzyme is not obligatorily NADH-linked. Evidence has been presented that the electron-donor component of the methane oxygenase system is a CO-binding soluble *c*-type cytochrome (Tonge *et al.*, 1974, 1975). This cytochrome, which is also a component of an electron-transport pathway, can be reduced by non-NADH-linked reactions involved in the further oxidation of methanol or ultimately by NADH derived from the oxidation of formate or endogenous storage compounds, via other electron-transport components (Higgins *et al.*, 1976*a*).

The present paper describes the purification of the components of the methane mono-oxygenase system and some of their properties.

Materials and Methods

Methods

Growth and maintenance of the bacterium. Methylosinus trichosporium OB3b, a gift from Professor R. Whittenbury (Department of Biological Sciences, University of Warwick), was maintained on mineral

salts/agar medium (Davey & Mitton, 1973) under a methane+air (50:50) atmosphere, subcultured every 3 weeks and stored at 4°C. It was grown on methane supplied as a methane+air mixture (50:50), at 30°C in a simple mineral-salts medium (Davey & Mitton, 1973) in O_2-limited continuous culture (dilution rate $0.06\,h^{-1}$). Organisms used for cytochrome purification were grown on the same medium, gassed with a methane+air mixture (50:50) at 30°C in a stainless-steel impeller-agitated Chemap fermenter (300 litres capacity; Chemap, Zurich, Switzerland) with pH control. Late-exponential-phase bacteria were harvested 48h after inoculation.

Preparation of cell-free extracts. Organisms were harvested by centrifugation (5000*g*, 30min, 4°C) and washed twice in sodium phosphate buffer (20mM, pH7.0) containing $MgCl_2$ (5mM). Organisms for preparation of membrane fractions were resuspended in the same buffer, and those for purification of the soluble *c*-type cytochrome were resuspended in imidazole/HCl buffer (50mM, pH7.0). Suspensions were cooled in ice and disrupted by ultrasonication (4×45s; MSE type 150W sonicator). Cell debris and unbroken organisms were removed by centrifugation (12000*g*, 10min, 4°C). The resulting extract was further centrifuged (150000*g*, 90min, 4°C), yielding particulate and supernatant fractions. The particulate material was resuspended in sodium phosphate buffer (20mM, pH7.0) containing $MgCl_2$ (5mM).

Assay of CO-binding cytochrome c. Cytochrome *c* concentration was calculated from dithionite-reduced minus ferricyanide-oxidized difference spectra by using a value of $17.3\times10^3\,cm^{-1}$ for the molar extinction coefficient ($A_{551-538}$) (Jones & Redfearn, 1967).

Assay of methane mono-oxygenase activity. Except when stated otherwise, methane mono-oxygenase was measured in cell-free preparations by determining methanol formation by g.l.c. in the presence of 150mM-sodium phosphate (to inhibit further oxidation of methanol) as described previously (Tonge *et al.*, 1975). Reaction mixtures contained methane (0.7μmol), oxygen (0.5μmol), ascorbate (4.5μmol), test protein (18–500μg) and sodium phosphate buffer, pH7.0 (450μmol) in a total volume of 3.0ml incubated at 30°C in a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). The formation of methanol was followed by rapid sampling and determining the methanol in these samples by g.l.c. in a glass (80–100 mesh) column (2.1m×4mm internal diam.) of Porapak Q in a Pye 104 gas chromatograph fitted with a flame ionization detector, at an N_2 flow rate of 40ml/min at 125°C. When assaying purified or partially purified samples free of methanol dehydrogenase activity, a lower phosphate concentration (20mM) was used. Reaction mixtures for investigating the properties of the purified enzyme contained protein 1 (12μg), protein 2 (3μg) and cytochrome c_{CO} (3μg).

Enzyme activity with higher alkane substrate analogues was measured by a similar procedure, by following, by g.l.c., the formation of the corresponding alcohols. Reaction mixtures contained ethane (0.35mM), propane (0.48mM) or butane (1.1mM). CO oxidation by purified methane mono-oxygenase was measured at 30°C by conventional respirometry in a Gilson respirometer (Gilson Medical Electronics, Middleton, WI, U.S.A.). Reaction mixtures contained, in a volume of 3ml, sodium phosphate buffer (36μmol), sodium L-ascorbate (15μmol) and enzyme protein (28μg), and flasks were gassed with a mixture of O_2+CO (50:50). CO_2 formation from CO was estimated by comparing gas uptakes in flasks with and without NaOH (0.2ml; 20%, w/v) in the centre well and by release of dissolved CO_2 after the completion of reactions by adding 0.2ml of 1M-H_2SO_4 from second side arms.

Measurement of gas concentrations and their uptake. The concentrations and utilization of methane and O_2 were determined by published methods (Tonge *et al.*, 1975).

Assay and purification of methanol dehydrogenase. Methanol dehydrogenase activity in cell-free extracts was determined by the procedure of Anthony & Zatman (1965) to assay the enzyme in *Pseudomonas* AM1, and it was partially purified by the procedure of Anthony & Zatman (1967).

Ultracentrifugation measurements. A Beckman model E analytical ultracentrifuge (Beckman–RIIC Ltd., Glenrothes, Fife, U.K.) was used to obtain sedimentation-velocity and molecular-weight data. The sedimentation coefficient (*s*) was determined by using a double-sector cell. Molecular weights were determined by using interference optics, by the meniscus-depletion method of Yphantis (1964). All measurements of photographic plates were made with a Projectorscope travelling microscope (Precision Grinding Ltd., Mitcham, Surrey, U.K.).

U.v.-visible spectrophotometry. Except for protein determinations, measurements were made in a Hitachi–Perkin–Elmer 356 dual-wavelength spectrophotometer (Perkin–Elmer Ltd., Beaconsfield, Bucks, U.K.).

E.p.r. (electron-paramagnetic-resonance) spectroscopy. Low-temperature spectra were recorded at 9GHz in a Varian E9 spectrometer (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.). For recording at 120K (liquid N_2), the microwave power was 50mW and the modulation was 10G, and these values were 100mW and 20G for work at 35K (liquid He). Samples were frozen initially by immersion in cold isopentane at 133K.

Determination of protein. Protein was measured by a modified biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard.

Determination of copper and iron. Extensively dialysed proteins were examined for metal content

by atomic absorption spectroscopy with a Pye–Unicam SP.90A instrument. Copper was also determined by the chemical method of Eden & Green (1940) as modified by Ventura & King (1951).

Polyacrylamide-gel electrophoresis. Purified proteins were examined by polyacrylamide-disc-gel electrophoresis (Shandon Scientific Co. Ltd., London N.W.10, U.K.) by the procedures of Gabriel (1971). Samples were run at both pH 8.3 (0.025 M-Tris/0.19 M-glycine buffer) and pH 4.5 (35 mM-β-alanine/13 mM-acetic acid buffer). Appropriate large-pore gels were prepared at pH 8.3 or 5.0 respectively and small-pore separating gels at pH 9.5 and 3.8 respectively. Electrophoresis was done at room temperature (22°C) with a constant current of 4 mA/tube until the marker (Bromophenol Blue or Methyl Green) was about 5 mm from the lower end of the gel. Proteins were stained by immersing the gels in 1% (w/v) Amido Black (George T. Gurr Ltd., London N.W.9, U.K.) in 7% (v/v) acetic acid for 1 h. Excess of dye was removed by immersion in 7% (v/v) acetic acid for 48 h. Relative band intensities were measured with a Joyce–Loebl u.v. scanner (Joyce–Loebl and Co., Gateshead, Co. Durham, U.K.).

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed by the method of Weber & Osborn (1969) at pH 7.0. Protein was stained with Coomassie Brilliant Blue [1.25 g in 454 ml of aq. 50% (v/v) methanol mixed with 46 ml of acetic acid], and gels were destained by incubating them for 24 h in a mixture of acetic acid (75 ml), methanol (50 ml) and water (875 ml). Standard marker proteins (mol.wt. in parentheses) used for calibration were bovine serum albumin (68000), carboxypeptidase (34600), horse heart cytochrome *c* (11700), insulin (5700), myoglobin (17200), ovalbumin (43000) and trypsin (23000).

Purification of the soluble CO-binding cytochrome c. The supernatant fraction of cell-free extracts after centrifuging at 150000*g* was treated with protamine sulphate (equal volume of 1%, w/v) and the resulting nucleoprotein precipitate removed by centrifugation. All subsequent procedures before adsorption chromatography were done in imidazole/HCl buffer (50 mM, pH 7.0) at 4°C. Pre-equilibrated DEAE-cellulose (Whatman DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.) was added to the supernatant (final concn. of ion-exchanger 15% w/v). The fraction was stirred for 10 min and then the DEAE-cellulose and bound protein were removed by vacuum-filtration before the remaining supernatant was concentrated by ultrafiltration under pressure, with an Amicon PM10 membrane (Amicon, High Wycombe, Bucks., U.K.), followed by further concentration with Aquacide II (Calbiochem Ltd., London W.1, U.K.). This concentrated material was then applied to the top of a column (52 cm × 2 cm) of Sephadex G-150 (Pharmacia Ltd., London W.5, U.K.) and eluted with buffer (Fig. 1). Fractions containing the cytochrome were pooled, concentrated with Aquacide II and further chromatographed on a column (42 cm × 2 cm) of Sephadex G-50 (Fig. 1). Cytochrome-containing fractions were again pooled and concentrated with Aquacide II and dialysed extensively against distilled water before being subjected to adsorption chromatography on a column (12 cm × 2.5 cm) of hydroxyapatite (Fig. 1). The cytochrome

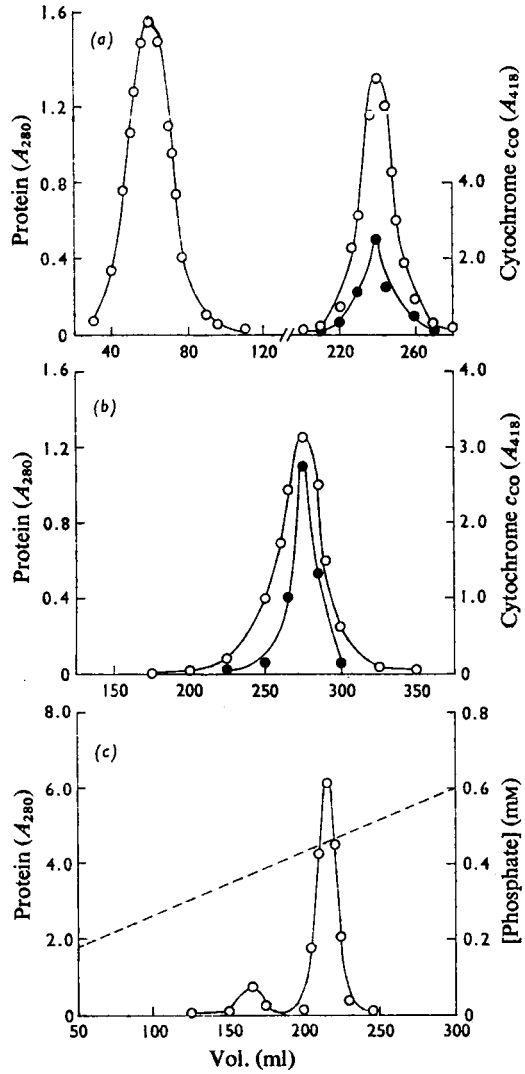


Fig. 1. *Purification of soluble CO-binding cytochrome c*
For details see the Materials and Methods section. Elution from Sephadex G-150 (*a*), Sephadex G-50 (*b*) and hydroxyapatite (*c*). ○, Protein; ●, cytochrome *c*; ----, sodium phosphate gradient.

was eluted with a linear gradient of sodium phosphate buffer, pH7.0 (0.1–0.5mM). Finally, cytochrome-containing fractions were pooled and dialysed against sodium phosphate buffer, pH7.0 (0.5mM).

Solubilization and separation of membrane-bound mono-oxygenase protein components. Cell-free particulate preparations (see above) were washed once with sodium phosphate buffer (20mM, pH7.0) containing $MgCl_2$ (5mM), and this buffer (buffer 1) was used throughout subsequent procedures. The resuspended particulate material was incubated (30°C, 1h) with 400μg of phospholipase D/ml (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.). The incubation mixture was cooled to 0°C, centrifuged (150000*g*, 90min) and the pellet discarded. The resulting supernatant fluid contained methane mono-oxygenase activity, which could only be measured after adding purified CO-binding cytochrome *c* (Tonge *et al.*, 1975). The supernatant was subjected to ultrafiltration under pressure through an Amicon PM10 membrane until filtration ceased (85% by volume of the solution had been extruded), yielding a high-molecular-weight residue (fraction 1 containing protein 1) and the filtrate containing low-molecular-weight material (fraction 2 containing protein 2). Recombination of these fractions plus addition of the cytochrome was necessary for methane mono-oxygenase activity.

Purification of protein 1. After ultrafiltration, protein 1 was dialysed against buffer 1 before being applied to a column (41cm×2cm) of Sephadex G-200 (Fig. 2). Active fractions were combined, concentrated with Aquacide II, dialysed against buffer 1 and further chromatographed on a column (45cm×2cm) of Sephadex G-75 (Fig. 2). Fractions were again combined, concentrated with Aquacide II and dialysed extensively against buffer 1.

Purification of protein 2. Protein 2 was concentrated by ultrafiltration through an Amicon UM2 membrane followed by Aquacide II treatment and then dialysed against buffer 1. This material was applied to the top of a column (39cm×2cm) of Sephadex G-25 and eluted in the void volume. After concentration with Aquacide II, the active material was then applied to a column (41cm×2cm) of Sephadex G-50, and active fractions (constituting the major peak in Fig. 2*c*) were combined and dialysed against buffer 1.

Specific activities during purification. The specific activities in Table 1(*b*) after the ultrafiltration step are based on the concentration of protein 1 only, since in order to ensure maximum specific activity excess of the other two protein components was used in assays. Absolute specific activity values are discussed below. Throughout the purification of proteins 1 and 2, the most purified *M. trichosporium* CO-binding cytochrome *c* (cytochrome c_{CO}) was included in assay mixtures (1.5μM). After separation of proteins 1 and 2 by ultrafiltration, assay mixtures used for following further purification of protein 1 contained, in addition to the cytochrome, partially purified protein 2 (ultrafiltration filtrate, 3μM). Sephadex G-75 eluate of protein (0.15μM) was then used in assay mixtures for following protein 2 purification.

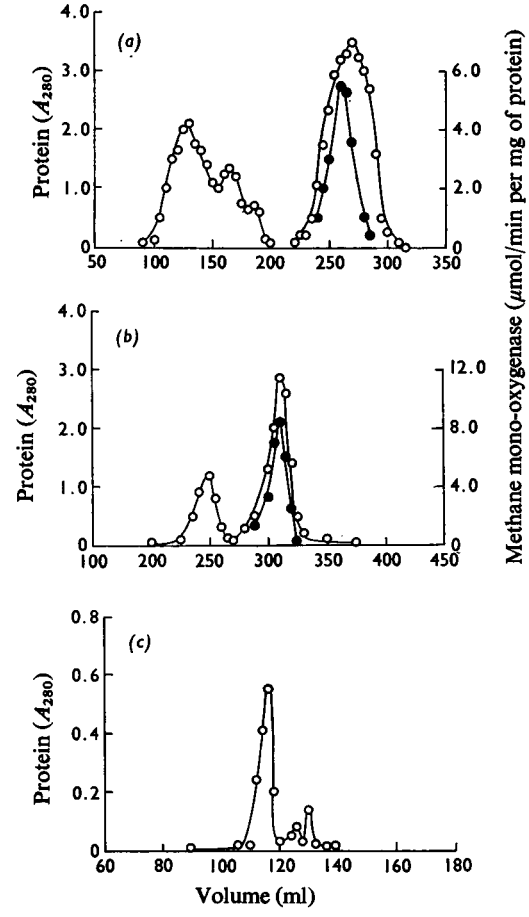


Fig. 2. *Purification of methane mono-oxygenase components (proteins* 1 *and* 2)
For details see the Materials and Methods section. Elution of protein 1 from Sephadex G-200 (*a*) and Sephadex G-75 (*b*). Elution of protein 2 from Sephadex G-50 (*c*). ○, Protein; ●, oxygenase activity.

Materials

Methane (technical grade) and CO (analytical grade) were obtained from British Oxygen Co. Ltd., London S.W.19, U.K., and ethane, *n*-propane, *n*-butane and dimethyl ether were purchased from BDH Ltd., Poole, Dorset, U.K. Methanol and reagents for polyacrylamide-gel electrophoresis were obtained from Fisons Ltd., Loughborough, Leics.,

Table 1. *Summary of the purification of methane mono-oxygenase components*
For details and discussion see the text.

(*a*) Purification of cytochrome c_{CO}

Procedure	Volume (ml)	Total protein (mg)	Cytochrome concn. (nmol/mg of protein)	Total cytochrome (mmol)	Recovery (%)	Purification (fold)
Crude extract	295	9400	0.97	9.1	100	0
150 000*g* soluble fraction	285	5700	1.6	9.1	99	1.6
Protamine sulphate supernatant	570	5120	1.7	8.8	99	1.7
DEAE-cellulose supernatant	238	667	11.6	7.7	85	11.6
Ultrafiltration residue	10	380	19.4	7.3	80	19.4
Sephadex G-150 eluate	40	84	80.5	6.9	77	80
Sephadex G-50 eluate	50	75	91.7	6.9	76	92
Hydroxyapatite eluate	15	66	104.3	6.9	76	104

(*b*) Purification of proteins 1 and 2

Procedure	Volume (ml)	Total protein (mg)	Specific activity (μmol/min per mg of protein 1 and/or 2)	Total activity (mmol/min)	Recovery (%)	Purification (fold)
Crude extract	218	10020	0.22	2.3	100	0
150000*g* particulate fraction	150	7660	0.28	2.2	99	1.3
Phospholipase D-solubilized fraction	132	370	5.3	2.0	89	23.8
Protein 1						
Ultrafiltration residue	10	290	6.8	2.0	87	30.9
Sephadex G-200 eluate (after concentration)	8	201	7.8	1.6	70	35.4
Sephadex G-75 eluate (after concentration)	11	165	9.0	1.5	66	40.5
Protein 2						
Ultrafiltration filtrate	81	69	9.0	1.5	66	40.5
Sephadex G-25 eluate (after concentration)	10	68	9.0	1.5	66	40.7
Sephadex G-50 eluate (after concentration)	10	64	9.1	1.5	66	41.3

U.K., and 3-amino-1,2,4-triazole, sodium L-ascorbate, dimercaptopropanol (British Anti-Lewisite), dithiothreitol, 2-mercaptoethanol, NADH, NADPH, *o*-phenanthroline, protamine sulphate, thioacetamide, thiosemicarbazide, thiourea, bovine serum albumin (fraction V), carboxypeptidase, horse heart cytochrome *c*, myoglobin, ovalbumin and trypsin were from Sigma. Allylthiourea was purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K., and insulin was from Boots Pure Drug Co. Ltd., Nottingham, U.K.

Compound SKF 525A (β-diethylaminoethyl 3,3'-diphenylvalerate) was a gift from Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K., and Lilly compound 18947 (2,4-dichloro-6-phenylphenoxyethyldiethylamine) and Lilly compound 53325 [2,4-dichloro-(6-phenylphenoxy)ethylamine hydrobromide] were gifts from Lilly Research Ltd., Windlesham, Surrey, U.K. All other chemicals were of the best commercial quality.

Results

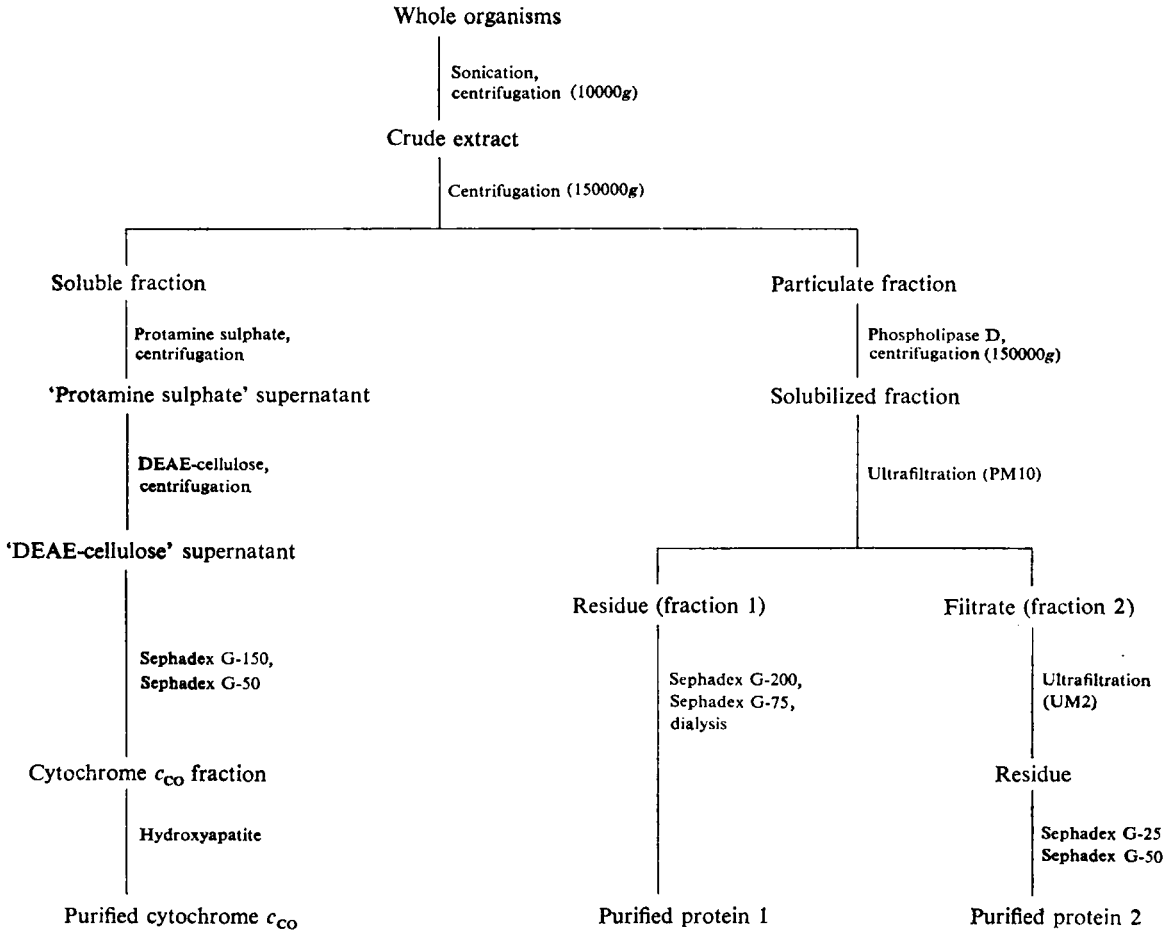
Purification of methane mono-oxygenase components

Three proteins have been purified from *M. trichosporium* cell-free extracts which, when combined in the presence of ascorbate and oxygen, catalyse the conversion of methane into methanol with high specific activity. The purification procedures are summarized in Scheme 1 and in Table 1.

After the ultrafiltration step (Table 1*b*), some (14% of the total) methane oxygenase activity was detectable in the ultrafiltration residue before addition of filtrate (protein 2) to the reaction mixture. This was presumed to be due to incomplete separation of proteins 1 and 2 at this stage.

Ultracentrifuge measurements and disc-gel electrophoresis

Cytochrome c_{CO} and protein 1 appeared homogeneous on ultracentrifugation, protein 1 giving a



Scheme 1. *Purification of methane mono-oxygenase components*

single symmetrical schlieren pattern (Fig. 3) with an $s_{obs.}$ value of 3.21×10^{-13}. There were linear relationships between log(fringe displacement) and the square of the distance from the axis of rotation for cytochrome c_{CO} (Fig. 4*a*) and protein 1 (Fig. 4*b*). These measurements gave mol.wt. values of 13500 and 48500 respectively. Sedimentation-velocity studies with cytochrome c_{CO} showed a single schlieren pattern, but the colour of the protein prevented satisfactory photographic reproduction. Ultracentrifuge determinations on protein 2 were not possible with available equipment because of its low molecular weight.

All three proteins gave single sharp bands after disc-gel electrophoresis at both pH8.3 in Tris/glycine buffer and pH4.5 in β-alanine/acetic acid buffer. Densitometer traces indicated that there may be small amounts (less than 3%) of contaminating proteins remaining.

Molecular-weight determination

The mol.wts. of cytochrome c_{CO}, protein 1 and protein 2 were estimated at 12500, 46000 and 9400 respectively both by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel-filtration chromatography. The first two values are in reasonable agreement with those obtained from sedimentation-equilibrium measurements. The mol.wt. of protein 1 was also estimated at 47000 from disc-gel electrophoresis of the native protein by using a Ferguson plot (Hedrick & Smith, 1968).

Spectral properties

The absolute visible-absorption spectrum of the

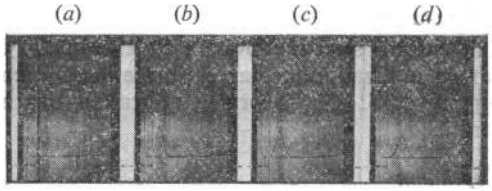


Fig. 3. *Ultracentrifuge pattern for protein 1 methane monooxygenase component*

The concentration was 13.0mg/ml in 20mM-sodium phosphate buffer (pH7.0) containing 5mM-$MgCl_2$. The photographs were taken at the following times (min) after the rotor had reached 52140rev./min: (*a*) 16; (*b*) 32; (*c*) 48; (*d*) 64. Temperature was 20°C. Sedimentation is from left to right.

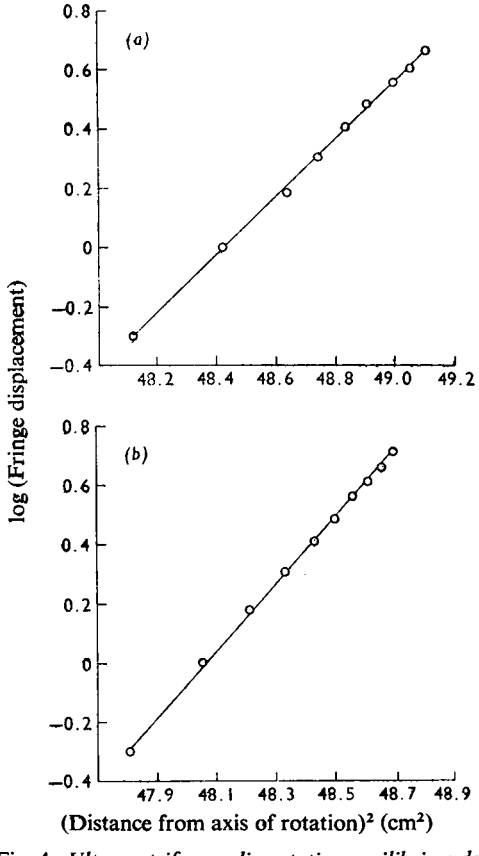


Fig. 4. *Ultracentrifuge sedimentation-equilibrium data*

Plot of log(fringe displacement) against the square of the distance from the axis of rotation for (*a*) CO-binding cytochrome *c* and (*b*) methane monooxygenase protein 1. Conditions were: (*a*) initial protein concentration, 1.0mg/ml in imidazole buffer (50mM, pH7.0, 52410rev./min, 16.25°C; (*b*) initial protein concentration 1.3mg/ml in sodium phosphate buffer (20mM, pH7.0) containing 5mM-$MgCl_2$, 29984rev./min, 17.55°C.

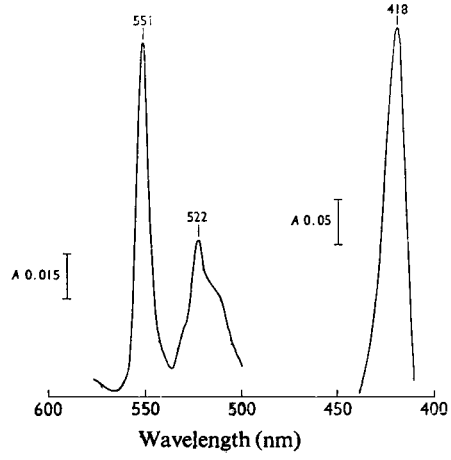


Fig. 5. *Visible-absorption spectrum of purified CO-binding cytochrome c*

The cytochrome (0.061mg of protein/ml) in sodium phosphate buffer (20mM, pH7.0) was fully reduced by adding a few crystals of sodium dithionite before recording the spectrum as described in the Materials and Methods section. The absorbance scale is indicated by vertical bars.

dithionite-reduced purified cytochrome is shown in Fig. 5. On the basis of a mol.wt. of 13000, the molar extinction coefficient ($A_{551-546}$) is $22.5\times10^3 cm^{-1}$. This value differs considerably from that calculated by Jones & Redfearn (1967) for *Azotobacter vinelandii* cytochromes c_4 and c_5 ($A_{551-538}$ of $17.3\times10^3 cm^{-1}$). In view of this new molar extinction coefficient the values for cytochrome concentration in Table 1 are overestimates by approx. 35%. The CO-binding spectrum of the purified cytochrome is shown in Fig. 6. Under the conditions described in the legend there was 66.5% binding, which is in close agreement with the value obtained in crude cell-free preparations (Tonge *et al.*, 1974). Proteins 1 and 2 did not show visible spectra.

Determination of iron and copper

The cytochrome c_{CO} contained 11.0μg of iron in 3.0mg of protein and a variable amount of copper (22.5μg in 16.5mg of protein to 7.8μg in 2.0mg of protein). This variation in copper content may reflect a loose association of the copper with the protein. The values suggest that the cytochrome contains one atom of iron per molecule and a variable amount of associated copper (0.3–0.8 atom per molecule). Protein 1 contained 8.9μg of copper in 7.5mg of protein, suggesting one copper atom per molecule.

Stability

All three components were totally inactivated by one cycle of freezing and thawing by a variety of

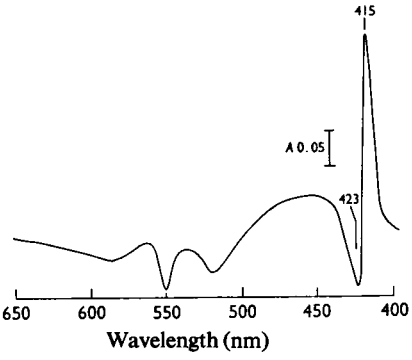


Fig. 6. *CO-binding difference spectrum of purified CO-binding cytochrome c*

The cytochrome (0.031 mg of protein/ml) in sodium phosphate buffer (20mM, pH 7.0) was fully reduced by adding a few crystals of sodium dithionite. CO was bubbled for 30s through one cuvette followed by incubation for 10min before the spectrum was recorded as described in the Materials and Methods section. The absorbance scale is indicated by the vertical bar.

freezing methods, including rapid freezing in liquid isopentane, liquid N_2 or liquid He or slow freezing to −22°C. The cytochrome c_{CO} was stable at 0–4°C and there was no deterioration in properties on storage under these conditions for 6 months. The remaining components of the oxygenase system were less stable, losing about 4 and 30% of activity after storage for 21 days at 0°C and 14 days at 4°C respectively.

Specific enzymic activity

In Table 1(*b*) specific oxygenase activities before ultrafiltration are expressed in terms of proteins 1 and 2 and after ultrafiltration in terms of protein 1 only. For consistency the specific activities during purification of protein 2 are expressed in terms of protein 1. The data have been presented in this way since the relative amounts of cytochrome c_{CO}, protein 1 and protein 2 required for maximum activity were not known during purification and large excesses of the cytochrome were used in all assays (see under 'Methods').

In order to establish a true specific-activity value it was necessary to determine the ratio of the individual components required for maximum specific activity. This was approx. 1:4:1 (cytochrome c_{CO}/protein 1/protein 2) on a weight basis, suggesting a molar ratio of 1:1:1 to be the optimum. Deviation from this ratio resulted in a decrease in specific activity. With the optimum ratio, the specific activity on the basis of protein 1 only is recorded in Table 1 as 9.1 μmol/min per mg of protein. However, since the other two components are essential, the specific activity on the basis of all three components is $9.1 \times \frac{2}{3}$ i.e. 6 μmol/min per mg of protein.

Stoicheiometry

The enzyme system showed a stoicheiometry for methane utilization/methanol formation/O_2 consumption of 5:4:7. Ascorbate utilization was not determined.

pH optimum

Enzyme activity was measured over a range (pH 6.4–7.6) of sodium phosphate buffers (20mM) containing $MgCl_2$ (5mM), but otherwise by using the standard methane oxygenase assay system (see under 'Methods'). The enzyme system was highly pH-dependent, the optimum being 6.9–7.0, and specific activities at pH 6.4 and 7.4 were 20 and 25% respectively of that at the optimum.

Substrate specificity

In addition to methane (specific activity 6 μmol/min per mg of protein), the enzyme system catalysed the oxygenation of the higher homologues, ethane, *n*-propane and *n*-butane, to the corresponding alcohols with specific activities of 5.5, 5.2 and 4.5 μmol/min per mg of protein respectively. CO was also a substrate, and in respirometric experiments (see under 'Methods') total gas uptake ($CO+O_2$) was always approximately twice the gas (CO_2) output, suggesting that the enzyme also functions as a mono-oxygenase when supplied with CO as substrate. The specific activity with this analogue was 1.7 μmol/min per mg of protein. There was no evidence that dimethyl ether could serve as a substrate.

Electron-donor specificity

In addition to ascorbate, NADH and NADPH were electron donors for partially purified enzyme. However, after subjecting protein 1 to gel filtration on Sephadex G-200, reduced nicotinamide nucleotides were no longer active. Presumably this was due to separation of protein 1 from solubilized intermediate electron carriers.

In view of the evidence that, in crude extracts, methanol could serve as a source of reducing power, via methanol dehydrogenase, for methane mono-oxygenase (Tonge *et al.*, 1975; Higgins *et al.*, 1976*a*) methanol was tested as electron donor for the purified oxygenase system. Ascorbate was omitted from standard assay mixtures, and methanol dehydrogenase (2.0mg), partially purified (15.6-fold) from *M. trichosporium*, was added together with methanol (5mM). Methane disappearance was followed by gas chromatography (see under 'Methods'), and a specific activity (based on methane mono-oxygenase protein only) of 0.8 μmol of methane utilized/min per mg of protein was recorded. The specific activity of the methanol dehydrogenase at the pH of the oxygenase assay (7.0) was 1.1 μmol/min per mg of protein, suggesting that the dehydrogenase was limiting the rate of the coupled reaction.

Table 2. *Effect of inhibitors on methane mono-oxygenase activity*

Oxygenase activity was measured by the standard procedure (see under 'Methods'), the reaction being initiated by adding methane-saturated buffer after preincubating the enzyme system with inhibitor for 3 min.

Inhibitor	Concentration (μM)	Inhibition (%)
KCN	1	65
2-Mercaptoethanol	100	85
Dithiothreitol	50	85
Allylthiourea	10	100
3-Amino-1,2,4-triazole	10	75
Thiourea	10	100
Thiosemicarbazide	1	85
Compound SKF 525A	100	82
o-Phenanthroline	100	58
Thioacetamide	10	92
Dimercaptopropanol (British Anti-Lewisite)	10	60
Lilly compound 18947	1000	100
Lilly compound 53325	1000	100

Cytochrome specificity

Since facultative methylotrophs when grown on C-1 compounds also contain large amounts of cytochrome c_{CO}, it was decided to determine whether a cytochrome c_{CO} purified from a facultative organism could replace *M. trichosporium* cytochrome c_{CO} in the mono-oxygenase system. The cytochrome was partially purified (20-fold) from methanol-grown *Pseudomonas extorquens* by a procedure identical with that used for the *M. trichosporium* cytochrome (Higgins *et al.*, 1976*b*). No methane mono-oxygenase activity was detectable when this cytochrome replaced that from *M. trichosporium* even though the purified cytochrome had retained its CO-binding properties and remained autoxidizable. Horse heart cytochrome *c* was similarly inactive.

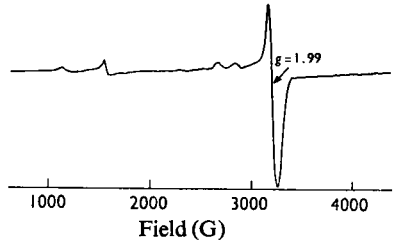


Fig. 7. *E.p.r. spectrum of protein* 1 *methane mono-oxygenase component*

The spectrum was measured on a sample containing 104mg of protein 1/ml at 35K as described under 'Methods'.

Effect of methane concentration

The apparent K_m for methane was 66μM and the apparent $V_{max.}$ was reached at a substrate concentration of approx. 340μM. The K_m for methane of a pseudomonad growing on methane was 26μM (Harrison, 1973).

Effect of anaerobiosis

No methanol was detectable when the standard assay for methane mono-oxygenase was done under anaerobic conditions.

Effect of inhibitors

Table 2 summarizes the effects of a variety of inhibitors on the mono-oxygenase system. The enzyme system is highly sensitive to cyanide, an effect distinct from inhibition by cyanide of cytochrome c_{CO} ascorbate oxidase activity, which is considerably less sensitive. A cyanide concentration of 500μM was required to inhibit the latter activity by 65%. A variety of chelating agents were also inhibitory, the most potent ones being efficient copper chelators (especially thiourea and to a lesser extent allylthiourea and thiosemicarbazide). Compound SKF 525A and Lilly compounds 18947 and 53325 have been shown to inhibit carotenoid hydroxylation in *Staphylococcus aureus* (Hammond & Whyte, 1970) and a cytochrome *P*-450 mono-oxygenase (Hildebrandt, 1972). These compounds, as well as thiosemicarbazide, also inhibit ammonia oxidation by *Nitrosomonas* (Hooper & Terry, 1973). Inhibitions by 2-mercaptoethanol and dithiothreitol are unusual effects and have also been observed for cell-free methane oxygenase activity in *Methylomonas methanica* (Ferenci *et al.*, 1975).

E.p.r. studies

The presence of copper ($g = 1.99$) in protein 1 was confirmed by e.p.r. spectroscopy (Fig. 7), although only a small proportion (approx. 10%) was detectable

by this method. A similar phenomenon occurs with tyrosinase (Vanneste & Zuberbühler, (1974). The cytochrome c_{CO} gave signals for both haem iron (a weak signal probably due to low-spin ferric iron) and copper, but protein 2 did not give an e.p.r. signal. On mixing the three protein components, there was an enhancement of the copper signal of about 30%, and on permitting the reaction to occur by supplying substrates there followed a decrease in this signal of about 50%. The significance of these changes remains uncertain in view of inactivation of the enzyme on freezing. Further studies by this technique await the development of a stabilization procedure.

Relationship between cytochrome c_{CO} ascorbate oxidase and methane mono-oxygenase activities

Previous studies showed that in cell-free extracts there was endogenous NADH oxidase activity. On addition of methane, this electron flow to oxygen apparently became redirected into a new oxygenase function, and there was indirect evidence that the cytochrome involved in both these functions was the cytochrome c_{CO} that has now been purified (Tonge *et al.*, 1975; Higgins *et al.*, 1976*a*).

The ascorbate oxidase activity of the purified cytochrome c_{CO} was measured polarographically by the standard methane mono-oxygenase assay system from which the other two protein components and methane had been omitted. In fresh preparations, the specific activity was about 50 μmol/min per mg of protein and the apparent K_m for oxygen about 250 μM. This is a rather high K_m for a physiological oxidase. Addition of the other two purified methane mono-oxygenase components did not affect this activity, but on the addition of methane to this complete oxygenase system most of the electron flow to oxygen became involved in the oxygenase reaction (on the basis of the amount of methanol formed). The rate of oxygen consumption measured for the cytochrome c_{CO} ascorbate oxidase activity was unchanged on adding the other oxygenase components plus methane. Therefore these additions cause a change in the reaction responsible for oxygen consumption without altering the overall rate of consumption. However, the stoicheiometry values (see above) suggest that this change in reaction mechanism is incomplete and that under standard methane mono-oxygenase assay conditions approx. 40% of electron flow is still via the oxidase function.

The cytochrome therefore can act as an oxidase at least *in vitro*, but is also a component of the oxygenase system. The ascorbate oxidase activity of the cytochrome was inhibited by cyanide (95% inhibition in the presence of 1 mM-KCN) and activity was totally lost after one cycle of freezing and thawing. There was no change in the visible-absorption spectrum as a result of adding methane to the pure cytochrome alone or in the presence of the other oxygenase components.

Discussion

The results show that a three-component enzyme system that catalyses the conversion of methane into methanol can be isolated and purified from *M. trichosporium*. Its properties suggest that it is a mono-oxygenase, and involvement of such a mechanism was suggested by various previous studies with cell-free extracts of several methane-utilizing bacteria (Ribbons & Michalover, 1970; Ribbons & Higgins, 1971; Ferenci, 1974; Tonge *et al.*, 1975; Colby *et al.*, 1975; Higgins *et al.*, 1976*a*). Although formally analogous to microbial enzyme systems involved in higher-alkane oxidation (Peterson *et al.*, 1967; Cardini & Jurtshuk, 1968) and benzene oxidation (Axcell & Geary, 1973, 1975), the properties of methane mono-oxygenase are quite distinct. The closest analogy appears to be with the corynebacterial *n*-alkane-oxidizing system (Cardini & Jurtshuk, 1968), which also involves a cytochrome (cytochrome *P*-450). However, in marked contrast with these other mono-oxygenases and in contrast with conclusions drawn from indirect studies with cell-free extracts of methylotrophs, the *M. trichosporium* methane mono-oxygenase is neither directly nor obligatorily NADH (NADPH)-linked. The immediate electron donor is the cytochrome c_{CO} component, which in the unsupplemented purified system can be reduced by ascorbate but not by NAD(P)H. In crude cell-free preparations and presumably *in vivo*, NADH can serve as electron donor via the electron-transport chain; electrons derived from non-NADH-linked reactions, especially the methanol and formaldehyde dehydrogenase steps, can also serve. This is demonstrated by the experiment in which methanol in the presence of partially purified methanol dehydrogenase could supply the reducing power for the purified enzyme system. This recycling of electrons was demonstrated earlier in crude cell-free preparations (Tonge *et al.*, 1975; Higgins *et al.*, 1976*a*). A conventional NADH-linked mono-oxygenase would not be consistent with observed cell-yield data (Harrison *et al.*, 1972; Van Dijken & Harder, 1975; Higgins *et al.*, 1976*a*; Barnes *et al.*, 1976; Hutchinson *et al.*, 1976), but involvement of electrons derived from the methanol and formaldehyde dehydrogenase reactions might well be consistent. In this respect there is also some evidence that ATP is generated during this electron recycling (Tonge *et al.*, 1976).

At this stage in the investigation there is little detailed information about the enzymic mechanism, but the cytochrome c_{CO} has been studied in greater detail than the other components of the system. Soluble

CO-binding *c*-type cytochromes have been found in a variety of micro-organisms (Weston & Knowles, 1973) and are found in especially high concentrations in methylotrophs (Tonge *et al.*, 1974; Anthony, 1975). There is strong evidence that the cytochrome c_{CO} is an electron-transport component essential for oxidation of methanol in *M. trichosporium* (Tonge *et al.*, 1975; Higgins *et al.*, 1976*a*) and in the facultative methylotrophs *Ps.* AMI (Anthony, 1975; Widdowson & Anthony, 1975) and *Ps. extorquens* (Higgins *et al.*, 1976*b*). In these facultative organisms the cytochrome c_{CO} is obligatory for methylotrophic growth (Anthony, 1975; Higgins *et al.*, 1976*b*), but not for non-methylotrophic growth (Anthony, 1975). It is noteworthy that the cytochrome from *Ps. extorquens* will not function in the methane mono-oxygenase system, although in other respects both cytochromes appear similar and to differ somewhat from that purified from *Ps.* AMI (Anthony, 1975). The significance (if any) of the copper found loosely associated with the cytochrome is uncertain, although cytochrome a_3 contains copper that is thought to be involved in the oxidase reaction (Beinert *et al.*, 1962; Van Gelder & Beinert, 1964).

The cytochrome c_{CO} clearly shows high oxidase activity *in vitro* in the absence of methane and the other oxygenase components. It has a redox potential in the normal range for physiological oxidases, but a rather high K_m value for oxygen. Whether the cytochrome has an oxidase function *in vivo* in addition to being a component of the methane oxygenase system remains unproven. However, there is indirect evidence that it is a physiological oxidase both in *M. trichosporium* and in other methylotrophs. In cell-free extracts of *M. trichosporium* methanol oxidase is always associated with the cytochrome c_{CO}, and indeed is found mainly in supernatant fractions in which the cytochrome c_{CO} is the only detectable cytochrome (Higgins *et al.*, 1976*a*). It seems likely that the cytochrome is reduced *in vivo* as a result of the further oxidation of methanol and that the cytochrome is then reoxidized in either an oxygenase or oxidase function, depending on growth conditions (Higgins *et al.*, 1976*a*).

Additional evidence for an oxidase function comes from cyanide inhibition patterns for ascorbate/*NNN'N'*-tetramethyl-*p*-phenylenediamine, NADH and methanol oxidase activities in extracts of *M. trichosporium* (G. M. Tonge & I. J. Higgins, unpublished work), which are closely similar to those reported for *Ps. extorquens* (Higgins *et al.*, 1976*a*), suggesting the presence of at least two terminal oxidases in these bacteria. The sensitivities of the methanol oxidase activity and the pure cytochrome c_{CO} ascorbate oxidase activity to cyanide are closely similar, whereas the methane mono-oxygenase activity is far more cyanide-sensitive. These data concur with inhibitor studies on whole organisms and cell-free extracts of *Methylomonas methanica* (Ferenci, 1976) in which methane oxidation was shown to be approximately 15 times more sensitive to cyanide than methanol oxidation.

Substrate specificity studies showed that the enzyme oxidizes other *n*-alkanes, which is consistent with results obtained with washed suspensions of obligate methylotrophs (Leadbetter & Foster, 1960) and cell-free extracts of *Methylococcus capsulatus* (Ribbons, 1975). The enzyme system also oxidizes CO at about one-third the rate of methane oxidation. Indirect evidence that methane mono-oxygenase can oxidize CO has been published (Ferenci, 1974; Ferenci *et al.*, 1975).

It is not possible to draw any definite conclusions about the detailed mechanism of oxygenation of methane from the data in the present paper. However, the cytochrome component binds both O_2 and CO, and the complete oxygenase system oxygenates both methane and CO. It is therefore possible that the carbon substrate is bound by the cytochrome, which would be analogous to part of the known mechanisms of several cytochrome *P*-450 mono-oxygenases (Orrenius & Ernster, 1974; Gunsalus *et al.*, 1974). It could be that binding of the carbon substrate is responsible for inhibiting the endogenous oxidase activity of the cytochrome. In cytochrome *P*-450 systems the cytochrome is responsible for O_2 binding, although the methane mono-oxygenase also contains a copper-containing component (protein 1) which may be the O_2-binding component. There is evidence for some other mono-oxygenases that copper proteins bind 'active' oxygen (Vanneste & Zuberbühler, 1974). Methane mono-oxygenase is extremely sensitive to a variety of chelating agents, especially those known to be effective copper chelators. This effect has also been demonstrated in whole organisms of *M. trichosporium* (Hubley *et al.*, 1975) and in *M. trichosporium* and *Methylococcus capsulatus* (Patel *et al.*, 1976). 2-Mercaptoethanol and dithiothreitol are also highly inhibitory, again possibly owing to interaction with copper. The inhibition of mammalian tyrosinase by dithiothreitol (Burnett, 1971) may also be due to interaction with enzyme-bound copper (Vanneste & Zuberbühler, 1974). Sensitivity of cell-free methane-oxidizing activity to thiol reagents has been reported previously for *Methylomonas methanica* (Ferenci *et al.*, 1975). Apparent changes in the magnitude of the e.p.r. signal during the reaction are consistent with copper involvement, but the finding must be interpreted with caution owing to the technical difficulties discussed in the Results section. The role of protein 2 is unclear, but it may be required to facilitate physical association between the cytochrome and protein 1.

It has been suggested that a mono-oxygenase may well not be involved in methane oxidation and that the mechanism may involve free radicals (Hutchinson

et al., 1976). The present paper presents strong evidence for mono-oxygenase involvement, although not excluding the possibility of the involvement of enzyme-bound free radicals in the reaction mechanism. However, there was no evidence for relevant free radicals from the e.p.r. experiments (though these could well have escaped detection under the conditions of the experiments), neither have we any evidence that dimethyl ether is a substrate for, or a product of, the reaction. Recently, soluble methane-oxidizing activity has been reported in crude cell-free preparations of *Methylococcus capsulatus*. This also appears to be a mono-oxygenase (Colby & Dalton, 1976).

G. M. T. thanks the Science Research Council for a CASE studentship. Part of this work was supported by grants to I. J. H. from the Science Research Council (GR/A/0214) and the Shell Grant Committee. We thank Dr. P. J. Geary for the ultracentrifuge studies, Dr. R. C. Bray for use of the e.p.r. spectrometer, Dr. D. J. Lowe and Mr. M. J. Barber for help with the e.p.r. experiments and Dr. J. W. Drozd for useful discussions.

References

Anthony, C. (1975) *Biochem. J.* **146**, 289–298
Anthony, C. & Zatman, L. J. (1965) *Biochem. J.* **96**, 808–812
Anthony, C. & Zatman, L. J. (1967) *Biochem. J.* **104**, 953–959
Axcell, B. C. & Geary, P. J. (1973) *Biochem. J.* **136**, 927–934
Axcell, B. C. & Geary, P. J. (1975) *Biochem. J.* **146**, 173–183
Barnes, L. J., Drozd, J. W., Harrison, D. E. F. & Hamer, G. (1976) in *Microbial Production and Utilisation of Gases* (H_2, CH_4, *CO*) (Schlegel, H. G., ed.), Goltze Druck, Gottingen, in the press
Beinert, H., Griffiths, D. E., Wharton, D. C. & Sanadi, R. W. (1962) *J. Biol. Chem.* **237**, 2337–2346
Burnett, J. B. (1971) *J. Biol. Chem.* **246**, 3079–3091
Cardini, G. & Jurtshuk, P. (1968) *J. Biol. Chem.* **243**, 6070–6072
Colby, J. & Dalton, H. (1976) *Biochem. J.* **157**, 495–497
Colby, J., Dalton, H. & Whittenbury, R. (1975) *Biochem. J.* **151**, 459–462
Davey, J. F. & Mitton, J. R. (1973) *FEBS Lett.* **37**, 335–337
Eden, A. & Green, H. H. (1940) *Biochem. J.* **34**, 1202–1208
Ferenci, T. (1974) *FEBS Lett.* **41**, 94–98
Ferenci, T. (1976) *Arch. Microbiol.* **108**, 217–219
Ferenci, T., Strøm, T. & Quayle, J. R. (1975) *J. Gen. Microbiol.* **91**, 79–91
Gabriel, O. (1971) *Methods Enzymol.* **22**, 565–578
Gornall, A. G., Bardawill, C. S. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–763
Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P. & Munck, E. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 559–613, Academic Press, New York
Hammond, R. K. & Whyte, D. C. (1970) *J. Bacteriol.* **103**, 607–612
Harrison, D. E. F. (1973) *J. Appl. Bacteriol.* **36**, 301–308
Harrison, D. E. F., Topiwala, H. H. & Hamer, G. (1972) in *Fermentation Technology Today, Proceedings No. IV of International Fermentation Symposium* (Terui, G., ed.), pp. 491–495, Society of Fermentation Technology, Osaka
Hedrick, J. L. & Smith, A. J. (1968) *Arch. Biochem. Biophys.* **126**, 155–164
Higgins, I. J. & Quayle, J. R. (1970) *Biochem. J.* **118**, 201–208
Higgins, I. J., Knowles, C. J. & Tonge, G. M. (1976*a*) in *Microbial Production and Utilisation of Gases* (H_2,CH_4, *CO*) (Schlegel, H. G., ed.), Goltze Druck, Gottingen, in the press
Higgins, I. J., Taylor, S. C. & Tonge, G. M. (1976*b*) *Proc. Soc. Gen. Microbiol.* **3**, 179
Hildebrandt, A. G. (1972) in *Biological Hydroxylation Mechanisms* (Boyd, G. S. & Smellie, R. M. S., eds.), pp. 79–102, Academic Press, New York
Hooper, A. B. & Terry, K. R. (1973) *J. Bacteriol.* **115**, 480–485
Hubley, J. H., Thomson, A. W. & Wilkinson, J. F. (1975) *Arch. Microbiol.* **102**, 199–202
Hutchinson, D. W., Whittenbury, R. & Dalton, H. (1976) *J. Theor. Biol.* **58**, 325–335
Jones, C. W. & Redfearn, E. R. (1967) *Biochim. Biophys. Acta* **143**, 340–353
Leadbetter, E. R. & Foster, J. W. (1959) *Nature (London)* **184**, 1428–1429
Leadbetter, E. R. & Foster, J. W. (1960) *Arch. Microbiol.* **35**, 92–104
Orrenius, S. & Ernster, L. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 215–244, Academic Press, New York
Patel, R., Hou, C. T. & Felix, A. (1976) *J. Bacteriol.* **126**, 1017–1019
Peterson, J. A., Kusunose, M., Kusunose, E. & Coon, M. J. (1967) *J. Biol. Chem.* **242**, 4334–4340
Ribbons, D. W. (1975) *J. Bacteriol.* **122**, 1351–1363
Ribbons, D. W. & Higgins, I. J. (1971) *Bacteriol. Proc.* 107
Ribbons, D. W. & Michalover, J. L. (1970) *FEBS Lett.* **11**, 41–44
Tonge, G. M., Knowles, C. J., Harrison, D. E. F. & Higgins, I. J. (1974) *FEBS Lett.* **44**, 106–110
Tonge, G. M., Harrison, D. E. F., Knowles, C. J. & Higgins, I. J. (1975) *FEBS Lett.* **58**, 293–299
Tonge, G. M., Drozd, J. W. & Higgins, I. J. (1976) *Proc. Soc. Gen. Microbiol.* **3**, 179
Van Dijken, J. P. & Harder, W. (1975) *Biotechnol. Bioeng.* **17**, 15–30
Van Gelder, B. F. & Beinert, H. (1964) *Biochim. Biophys. Acta* **189**, 1–24
Vanneste, W. H. & Zuberbühler, A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 371–404, Academic Press, New York
Ventura, S. & King, E. J. (1951) *Biochem. J.* **48**, lxi
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
Weston, J. A. & Knowles, C. J. (1973) *Biochim. Biophys. Acta* **305**, 11–18
Widdowson, D. & Anthony, C. (1975) *Biochem. J.* **152**, 349–356
Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317