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The Purification and Characterization of the *o*-type Cytochrome Oxidase from *Methylophilus methylotrophus*, and its Reconstitution into a 'Methanol Oxidase' Electron Transport Chain

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The *o*-type cytochrome oxidase from methanol-grown *Methylophilus methylotrophus* has been solubilized and purified (15-fold) to homogeneity. The pure, active oxidase consisted of equal amounts of *b*-type and *c*-type cytochromes, corresponding to the two types of protein subunit seen on SDS-PAGE; these had molecular weights of 31 500 and 23 800, respectively. The active oxidase probably has two cytochrome *c* subunits and two cytochrome *b* subunits, both cytochrome types reacting with CO. The cytochrome *c* subunit did not correspond to either of the two soluble cytochromes *c* from *M. methylotrophus*. The pure oxidase complex oxidized TMPD very rapidly, having a V_{max} of 36 μmol O_2 min^{-1} (mg protein)$^{-1}$. It was inhibited non-competitively by azide (K_i, 1·13 μM) and KCN (K_i, 0·2 μM), the K_i values being similar to those measured during respiration by whole bacteria.

Of the two soluble cytochromes *c* from *M. methylotrophus*, cytochrome c_H was oxidized at 50 times the rate of cytochrome c_L, the turnover number with cytochrome c_H as substrate being 21 s^{-1}. The cytochrome *c* component of the oxidase was unable to act as electron acceptor from pure methanol dehydrogenase (soluble, or after solubilization from membranes). A complete 'methanol oxidase' electron transport chain was reconstituted from completely pure proteins: methanol dehydrogenase, cytochrome c_L and c_H and the cytochrome *c* oxidase complex. This 'methanol oxidase' showed the same sensitivity to inhibition as observed during methanol oxidation by whole bacteria.

INTRODUCTION

Methylophilus methylotrophus is the obligate methylotroph which, because it grows at high rates and yields, has been selected by ICI for production of single cell protein. It assimilates methanol by the ribulose monophosphate pathway and produces one molecule of reduced methanol dehydrogenase (MDH) plus two molecules of NADH during oxidation of methanol to CO_2. Because all the methanol utilized is metabolized by way of formaldehyde, more than 65% of electron transport to O_2 is likely to be by way of MDH, and less than 35% from NADH (Anthony, 1982). The MDH, a quinoprotein, interacts with the electron transport chain at the level of cytochrome *c*, thus bypassing the low redox potential part of the chain containing cytochrome *b*. As in other methylotrophs, the organism contains at least two completely distinct cytochromes *c*. These are designated cytochrome c_H, having a high isoelectric point and low molecular weight, and cytochrome c_L having a low isoelectric point and higher molecular weight (Cross & Anthony, 1980*a*, *b*; Beardmore-Gray *et al.*, 1982). It has been shown recently that cytochrome c_L is the physiological electron acceptor from MDH, cytochrome c_H being completely inactive in this respect (Beardmore-Gray *et al.*, 1983; Beardmore-Gray & Anthony,

Abbreviations: DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; MDH, methanol dehydrogenase; PES, phenazine ethosulphate; HQNO, hydroxyquinoline-*N*-oxide.

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1984). *Methylophilus methylotrophus* is able to synthesize two different oxidases, the relative amounts of each depending on the growth conditions. Cytochrome aa_3 is the predominant oxidase in bacteria grown in continuous culture under conditions of methanol-limitation; whereas in methanol-excess conditions (O_2- or NH_3-limitation) no cytochrome aa_3 is formed but an alternative *o*-type oxidase is induced 10-fold over the constitutive amounts always present. This was shown by the appearance of a new *b*-type cytochrome having a high midpoint redox potential ($E_{m,7}$ = 260 mV) and able to react with CO. The synthesis of this new potential oxidase occurred in parallel with a 10-fold increase in the rate of TMPD oxidation by membranes and with a large increase in sensitivity of respiration to azide (Cross & Anthony, 1980*b*). This oxidase was termed cytochrome *o* by analogy with other CO-binding, *b*-type cytochromes having an oxidase function in bacteria.

All the evidence from work with whole cells and membranes showed that the branch point to the two oxidases is at the level of cytochrome *c* and that both are able to function as the terminal oxidase for oxidation of both NADH and methanol.

The present work was initiated in order to answer the following questions: do both of the soluble cytochromes *c* react with oxidases; and are any redox components required for methanol oxidation other than MDH, soluble cytochrome(s) *c* and cytochrome oxidase? The oxidase chosen for study was the *o*-type cytochrome for two reasons: firstly, because the bacteria could be grown in conditions in which the cytochrome *o* was the sole oxidase; and secondly, because very few *o*-type oxidases have been purified in an active form suitable for characterization.

The present paper describes the purification and characterization of the *o*-type oxidase and its reconstitution into an active 'methanol oxidase' using pure MDH and soluble cytochromes *c* from *M. methylotrophus*. One of the most important conclusions from this work, that the oxidase contains both *b*-type and *c*-type cytochromes, has been reported recently (Carver & Jones, 1983).

METHODS

Chemicals. All chemicals were obtained from Sigma (including TMPD and PES) or BDH (including Triton X-100) except for the following: Breox B125, from Hythe Chemicals, Hythe, Southampton, UK; Methyl viologen, 3,3′,5,5′-tetramethylbenzidine, 2,3,5,6-tetramethyl-*p*-phenylenediamine and phenazine methosulphate, from Aldrich; Zwittergent SB 3-14, from Calbiochem; CM-cellulose and DEAE-cellulose, from Whatman; Sephadex chromatography media, from Pharmacia; Fractogel TSK HW 55 (S), from Merck; Bio-gel chromatography media from Bio-Rad; Coomassie brilliant blue R250 from Koch-Light.

Organism and growth conditions. Methylophilus methylotrophus (NCIB 10515) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, UK. The methods for growth, maintenance of stock cultures and monitoring of culture purity were as described previously (Cross & Anthony, 1980*a*, *b*), with the following modifications: stock cultures were maintained in 30% (v/v) glycerol/0·04% methanol at −15 °C. Bacteria were grown routinely on methanol (1%, v/v) in O_2-limited continuous culture at a dilution rate of 0·15 h^{-1} at 40 °C in a 3-l fermenter (L. H. Engineering Co, Stoke Poges, Bucks., UK) with a working volume of 2·2 l. The air flow rate was 1 l min^{-1} and the culture was stirred at 450 r.p.m. The concentration of O_2 in the culture was below the level of detection using a lead/silver O_2-electrode. KOH (1 M)/NaOH (1 M) was used for pH control and the antifoam agent was Breox B125. Bacteria were harvested in a Sharples Super Centrifuge (Sharples Centrifuges, Camberley, Surrey, UK) and washed at 4 °C in 25 mM-MOPS buffer (pH 7·0).

Preparation of soluble and membrane fractions. Bacteria (50–70 g wet wt) were homogenized in 100–200 ml 25 mM-MOPS buffer (pH 7·0) containing a few mg of deoxyribonuclease. They were disrupted by passing twice through a French pressure cell (Aminco, Silver Springs, Md., USA) at 100 MPa or by sonication (2 × 2 min). Whole bacteria and cell debris were removed by centrifugation at 6000 *g* for 10 min and the resulting crude extract centrifuged at 135000 *g* for 1 h to separate the membranes from the soluble fraction. The soluble fraction was stored at −18 °C and the membrane fraction was washed and resuspended in 25 mM-MOPS buffer (pH 7·0) and stored in liquid N_2.

Measurement of protein, cytochromes and MDH. Protein was determined by the method of Bradford (1976) using the reagent filtered as described by Spector (1978); crystalline bovine serum albumin (fraction V) was used as standard. Absorption spectra and cytochrome determinations were done as described previously (Cross & Anthony, 1980*b*). The assay system for MDH was based on that first described by Anthony & Zatman (1967), with PES as the primary electron acceptor and O_2 as the terminal electron acceptor at 30 °C in an O_2 electrode (Rank Bros, Bottisham, Cambs., UK). The reaction mixture contained (in a 2 ml volume): Tris/HCl, pH 9·0, 100 mM; NH_4Cl, 15 mM; PES, 0·5 mM; methanol, 7·5 mM; the reaction was started by addition of enzyme.

Purification of the soluble cytochromes c and MDH. These methods were exactly as described by Beardmore-Gray *et al.* (1983). The cytochrome c_L had a molecular weight of 17000 (see Cross & Anthony, 1980*a*).

Measurement of cytochrome oxidase activity. Oxidase activity was routinely assayed polarographically using ascorbate (2 mM) plus TMPD (0·2 mM) in 25 mM-MOPS at the pH optimum (7·0). The sensitivity of the oxidase to sodium azide was checked at the end of every experiment by addition of 4 μM-azide. Spectrophotometric assays of oxidase activity using reduced cytochrome *c* as substrate were done using a dual-wavelength spectrophotometer (Applied Photophysics, London, UK) by recording the decrease in absorbance at about 550 nm (using the exact absorption band for each cytochrome *c*), and a reference wavelength of 575 nm. Reduced cytochrome *c* was prepared immediately before use by reduction with sodium dithionite followed by passage down a Sephadex G-25 column. V_{max} and K_m values were obtained by linear regression analysis of the double reciprocal plots of rate against concentration of cytochrome *c*.

Solubilization of the oxidase. The method finally used is described below. The procedure used to assess the suitability of alternative solubilization procedures was as follows. Membranes were carefully dispersed into detergent (15 mg membrane protein ml^{-1}) and incubated at 0 °C for 30 min; insoluble membrane was separated by centrifugation of 135000 ***g*** for 30 min, resuspended in 25 mM-MOPS (pH 7·0) and assayed for activity using the ascorbate/TMPD system. The activity of the soluble fraction was assayed before and after overnight dialysis against 100 vols 25 mM-MOPS buffer (pH 7·0).

Purification of the cytochrome oxidase. All operations were done at 0–4 °C. The membrane fraction (1·2–2·4 g protein) was homogenized in 25 mM-MOPS buffer (pH 7·0). Triton X-100 and $MgCl_2$ were added sequentially to give final concentrations of protein, 15 mg ml^{-1}; MOPS buffer, 12 mM; Triton X-100, 2·25%, v/v; and $MgCl_2$, 100 mM. After 30 min gentle agitation this solution was centrifuged at 135000 ***g*** for 30 min. The red supernatant liquid, containing 70% of the oxidase activity, was dialysed in a beaker dialyser (Bio-Rad) for 1 h against 1 l 12 mM-MOPS buffer (pH 7·0) containing 0·5% Triton in order to remove the $MgCl_2$. The oxidase preparation was then clarified by centrifugation if necessary (135000 ***g***, 30 min) and passed down a column (5 × 5 cm) of DEAE-cellulose equilibrated with 12 mM-MOPS (pH 7·0) containing 0·5% Triton. The oxidase was not adsorbed and it was passed immediately down a column (11 × 3 cm) of CM-cellulose equilibrated with 12 mM-MOPS (pH 7·0) containing 0·1% Triton. The oxidase was not adsorbed, whereas MDH was tightly bound. The partially purified oxidase preparation was free from detectable quantities of contaminating cytochromes at this stage but a number of other proteins were observed on SDS-gel electrophoresis. (The oxidase appeared to be about 60% pure at this stage.) It was concentrated in a Chemlab concentration cell containing a Ulvac A50T membrane (50000 mol. wt cut-off; Chemlab Instruments, Hornchurch, Essex, UK) and applied in two portions to an upward flow column (80 × 2·4 cm) of Fractogel TSK HW 55 (S) in 12 mM-MOPS (pH 7·0) containing 0·2% Triton. The fractions with the highest specific activity were pooled and stored in liquid N_2.

The following procedures were shown to be ineffective for purification of the solubilized oxidase: acid treatment at pH 5·0; ammonium sulphate fractionation; ammonium acetate fractionation; fractionation with polyethylene glycol 6000; ion-exchange chromatography on QAE-Sephadex; preparative isoelectric focusing (pH 2–10, in 1% Triton, sample application at pH 7·0). The following procedures were shown to be ineffective for further purification of the oxidase after its partial purification on DEAE-cellulose: gel exclusion chromatography using Biogel A 0·5 or Biogel A50M; ion exchange chromatography on hydroxylapatite (Biogel HT) and affinity chromatography using immobilized cytochrome *c* (from *Saccharomyces cerevisiae*) by the method of Azzi *et al.* (1982).

Polyacrylamide gel electrophoresis. SDS-PAGE (12 or 15%) was performed as described by Laemmli & Favre (1973) at pH 8·3. Non-denaturing gel electrophoresis was done in the same buffer system using gels prepared with 4% monomer and with SDS replaced by Triton X-100 (0·5%) (Gennis *et al.*, 1982); or by using the buffer system (pH 7·22) described by Neville & Glossman (1974) with the SDS replaced with 0·5% Triton. When using this system the samples were run in both directions to ensure that all proteins would be separated and detected. Gels were stained for protein with Coomassie blue (Weber & Osborn, 1975) and for haem using 3,3′,5,5′-tetramethylbenzidine (Thomas *et al.*, 1976). Cytochromes were also visualized by UV transillumination (Katan, 1976). After electrophoresis in non-denaturing conditions the oxidase was also detected by its oxidation of TMPD in 25 mM-MOPS (pH 7·0) (de Vrij *et al.*, 1983). To determine the proportion of each protein in samples, peak integration was performed on gels stained with Coomassie blue using a Chromoscan 3 gel scanner (Joyce-Loebl, Gateshead, Tyne and Wear, UK). This method showed that the purified oxidase constituted more than 97% of the protein present, the baseline noise integrating to give 3% of the total integral. Molecular weights were determined by SDS-gel electrophoresis in the Laemmli system (above) using the following proteins as standards: insulin (5700), equine cytochrome *c* (11700), lysozyme (14000), myoglobin (17200), α-chymotrypsinogen (24500), ovalbumin (43000), pyruvate kinase (56000), methanol dehydrogenase (62000) and bovine serum albumin (67000).

Purification of membrane-bound MDH. The procedure was not optimized for a high yield of dehydrogenase; it was a by-product of the method used for purification of the oxidase. After application of the oxidase to the CM-cellulose column the dehydrogenase was tightly bound. It was eluted at 400 mM-NaCl during gradient elution using

0–600 mM-NaCl in 12 mM-MOPS buffer (pH 7·0) containing 0·5% Triton plus 1 mM-ascorbate. The dehydrogenase gave a single band on SDS-PAGE indicating that it was more than 90% pure. As found with the MDH from *Methylococcus capsulatus* (Wadzinski & Ribbons, 1975), the solubilized and purified enzyme was almost identical to the enzyme isolated from the soluble fraction (see Results).

Gel permeation chromatography. This was done using a TSK G3000 SW column (7·5 × 600 mm) obtained from LKB. The column was equilibrated with 12 mM-MOPS buffer (pH 7·0) containing 0·5% Triton, and the flow rate was 1 ml min^{-1}.

RESULTS

Solubilization of the oxidase

All the results below were obtained with the membrane fraction prepared from bacteria grown under conditions of O_2-limitation (methanol present in excess). As previously demonstrated (Cross & Anthony, 1980*b*) these bacteria contain only the *o*-type oxidase and no cytochrome aa_3. This was always confirmed in the present work for each batch of membranes prior to solubilization. The optimum conditions for solubilization involved the use of Triton X-100 as described below. Alternative methods involving treatment of the membrane fraction with sodium deoxycholate (0·016–1%, w/v) or with the zwitterionic detergent SB3-14 (0·01–2%, w/v) failed to solubilize active oxidase in yields greater than 3%. Treatments with Triton X-100 (0·16–6·8%, v/v) demonstrated that the optimum concentration for solubilization of active oxidase was 2·25% (v/v); pretreatment with a low concentration of Triton (less than 0·5%) prior to solubilization led to no increase in yield or specific activity. Lower yields of solubilized oxidase were obtained using Triton (2·25%, v/v) which contained KCl (0·1–1·0 M), or the potential stabilizing agents polyethylene glycol 600 (20%, v/v) or glycerol (10–20%, v/v).

The optimum solution for solubilization of active stable oxidase contained Triton X-100 (2·25%, v/v) and $MgCl_2$ (100 mM) in 12 mM-MOPS buffer (pH 7·0); the optimum final protein concentration was 15 mg ml^{-1}. Any significant change in solubilization method led to either poor solubilization or to unstable oxidase. The oxidase was very sensitive to alterations in pH, losing 70% of the original activity within 24 h at 4 °C, at pH 6·0 or pH 8·0; no loss of activity occurred over a 24 h period at 4 °C, pH 7·0.

Removal of the Triton after solubilization by treatment with Amberlite XAD-2 (Holloway, 1973) led to a complete loss of activity. Prior to purification it was necessary to remove the $MgCl_2$ by rapid dialysis; prolonged dialysis (overnight) led to considerable loss of activity. After removal of $MgCl_2$ the preparations were unstable until purified further on DEAE-cellulose.

Purification of the oxidase

Table 1 summarizes the purification of the oxidase; alternative, ineffective procedures are briefly described in Methods. After purification, the oxidase could be stored in liquid N_2, no inactivation occurring over a period of 2 months, and the enzyme was stable at 0–4 °C for at least 24 h. The oxidase, as prepared by the method outlined in Table 1, ran as a single narrow band during high-resolution gel permeation on a TSK G3000 SW column. It also ran as a single band during electrophoresis in non-dissociating conditions; only a single band could be detected by

Table 1. *The purification of cytochrome oxidase (co) from M. methylotrophus*

The procedure is fully described in Methods. Activities were measured with ascorbate/TMPD as substrate.

Purification step	Volume (ml)	Total activity (μmol O_2 min^{-1})	Specific activity (nmol O_2 s^{-1} mg^{-1})	Yield (%)	Purification (fold)
Membrane fraction	62	2108	14·5	100	1
Solubilized membrane	137	1532	21·5	73	1·5
DEAE-cellulose	194	1473	77·0	70	5·3
CM-cellulose	198	1456	119·0	69	8·3
Fractogel	44	635	211·0	30	14·7

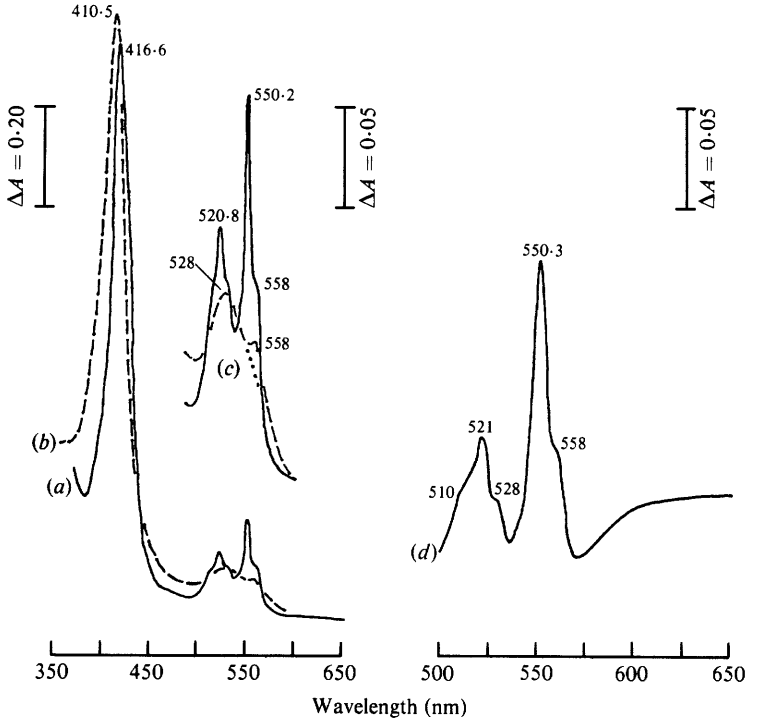


Fig. 1. Absorption spectra of the cytochrome oxidase. (*a*) Oxidase reduced with dithionite (——); (*b*) untreated oxidase (– – –); (*c*) oxidase after oxidation with ferricyanide (·····); (*d*) dithionite-reduced minus ferricyanide-oxidized difference spectrum of the oxidase. Spectra were measured at 20 °C in 12 mM-MOPS buffer (pH 7·0) (containing 0·2% Triton); the protein concentration was 0·6 mg ml^{-1} and the light path was 10 mm.

protein staining, by cytochrome fluorescence, by staining for haem with 3,3′,5,5′-tetramethylbenzidine (TMBZ) and by detecting TMPD-oxidizing activity. That the oxidase was more than 97% pure was shown by electrophoresis in dissociating gels containing SDS. Only two bands were observed and these corresponded to the two cytochrome components of the complete oxidase (see below). If it is assumed that no inactivation occurred during purification then the 14·7-fold purification indicates that the oxidase constitutes about 7% of the membrane protein.

Absorption spectra of the oxidase

The spectra in Fig. 1 show that the oxidase contains cytochrome *c* and cytochrome *b* components, absorbing at 550 nm and 558 nm, respectively. If the usual extinction coefficients for these cytochromes are assumed (see Methods) then these spectra show that the ratio of cytochrome *c* to cytochrome *b* in the complex is 1·2:1·0, suggesting that the pure oxidase complex contains equal amounts of *b*-type and *c*-type cytochromes.

On exposure of dithionite-reduced oxidase to air all of the cytochrome *c* became rapidly oxidized but only 50% of the cytochrome *b*. The significance of this observation is uncertain. Both cytochrome components reacted with CO although the cytochrome *b* component reacted more rapidly (Fig. 2). In this respect, the cytochrome oxidase complex was similar to other *o*-type cytochrome oxidases (see Discussion).

The cytochrome subunit composition of the oxidase

On SDS-PAGE (using 12% or 15% acrylamide) the oxidase dissociated into two components of molecular weights 23800 (±490, $n = 6$) and 31450 (±470, $n = 6$). As found by Carver & Jones (1983), both bands showed red fluorescence under UV-irradiation, and both stained with the TMBZ haem stain. Densitometer traces of gels after staining with Coomassie blue protein

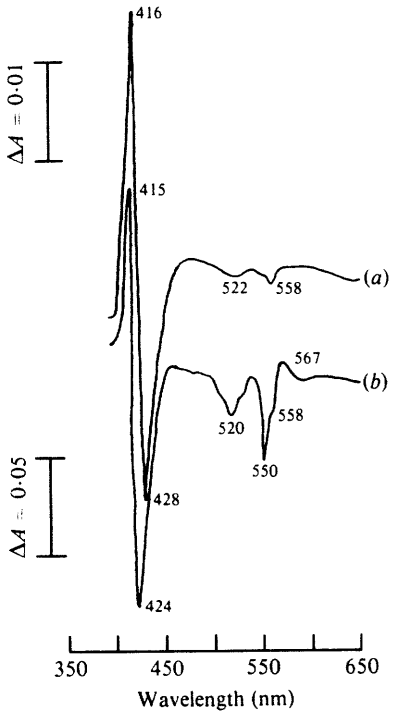


Fig. 2. Reaction of the oxidase with carbon monoxide. The (dithionite-reduced + CO) minus (dithionite-reduced) difference spectra were recorded at 20 °C after (*a*) 30 s and (*b*) 30 min exposure to CO. Spectra were measured at 20 °C in 12 mM-MOPS buffer (pH 7·0) (containing 0·2% Triton); the protein concentration was 0·2 mg ml^{-1} and the light path was 10 mm.

stain showed that the ratio of the larger component to the smaller component was about 1 :1·2. These results confirmed that the oxidase contained equal amounts of the two cytochrome components. They could be separated by very slow passage of the oxidase through a column of DEAE-cellulose equilibrated with 12 mM-MOPS (pH 7·0) containing a low concentration of Triton (0·1%). The smaller component was a *c*-type cytochrome and the larger component was a *b*-type cytochrome. Many preparations of the pure oxidase were made and in all cases the proportion of the *b*- and *c*-type cytochrome components was about 1 :1. Furthermore, whenever the two cytochrome components were separated all activity was lost.

On gel filtration of the oxidase through Fractogel HW 55(S), equilibrated with 12 mM-MOPS (pH 7·0) containing 0·2% Triton, the elution volume was identical to that of pure soluble MDH from *M. methylotrophus* (Ghosh & Quayle, 1981) suggesting that the native molecular weight of the oxidase is about 124000. If less than 10% of the estimated molecular weight is due to bound Triton then the oxidase probably exists as a tetramer containing two identical cytochrome *c* subunits plus two identical cytochrome *b* subunits.

The substrate specificity of the pure oxidase

The specific activity (based on the V_{max} value) of the pure oxidase measured with ascorbate/TMPD as substrate was 600 nmol O_2 consumed s^{-1} mg^{-1}; this corresponds to a turnover number of 262 nmol electrons nmol $oxidase^{-1}$ s^{-1}, assuming an oxidase molecular weight of 110500 (Table 2). This value compares with a value of 10–40 s^{-1} for the oxidase partially purified by Carver & Jones (1983).

Although TMPD is a convenient substrate for assaying the oxidase, the physiological substrate is likely to be one of the soluble cytochromes *c* shown to be present in *M. methylotrophus* (Cross & Anthony, 1980*a*, *b*). In order to investigate this possibility, the specificity of the

Table 2. *The oxidation of artificial electron donors and of various cytochromes c by the pure oxidase*

The oxidation of artificial electron donors was measured polarographically, and oxidation of cytochromes *c* was measured spectrophotometrically (see Methods for details of the assay systems). The rates are all expressed as nmol O_2 reduced s^{-1} (mg oxidase)$^{-1}$. It was assumed that four molecules of reduced cytochrome *c* are required to reduce one molecule of O_2. The concentrations of substrates used were as follows (the amount of oxidase is given in parentheses): 20–100 μM-horse heart cytochrome *c* (0·7 μg oxidase); 1·1–5·7 μM-cytochrome *c* from *S. cerevisiae* (0·7 μg oxidase); 1·2–4·8 μM-cytochrome c_H (0·23 μg oxidase); 1·2–7·3 μM-cytochrome c_L (3·5 μg oxidase). Hexaammineruthenium II/III is a contaminant of ruthenium red and is able to donate electrons directly to cytochrome aa_3 (Hochmann *et al.*, 1981); the concentration of ruthenium red used here was 35 μg ml^{-1}, with 143 μg of oxidase. Ascorbate (2 mM) was included in the reaction mixture during all investigations using TMPD, DMPD and ruthenium red.

Respiratory substrate	V_{max} value (nmol O_2 s^{-1} mg^{-1})	K_m value (μM)
TMPD	597	296
DMPD	833	4130
Hexaammineruthenium II/III	0	—
Cytochrome *c* (horse heart)	15·8	31·9
Cytochrome *c* (*S. cerevisiae*)	8·3	2·4
Cytochrome c_H (*M. methylotrophus*)	43·3	18·3
Cytochrome c_L (*M. methylotrophus*)	0·9	14·1

oxidase with respect to a range of electron donors was determined and the results summarized in Table 2. The most important conclusion to be drawn from these data is that, although affinity of the oxidase for both the soluble cytochromes *c* from *M. methylotrophus* was similar, the cytochrome c_H was oxidized 50 times faster than the cytochrome c_L, thus suggesting that the physiological electron donor to the oxidase is the smaller, basic cytochrome c_H. The turnover number for oxidation of this cytochrome by the pure oxidase is 21 nmol cytochrome *c* s^{-1} (nmol oxidase)$^{-1}$, assuming an oxidase molecular weight of 110500. Sparging the reaction mixture with 'white spot' N_2 (containing less than 5 p.p.m. O_2 – according to the manufacturer) led to a 50% decrease in activity of the oxidase, thus indicating that the K_m value for the pure oxidase must be very low (probably less than 1 μM). Complete removal of O_2 by passing the N_2 through the solution of Sweetser (1967) prior to sparging the reaction mixture completely prevented oxidation of cytochrome *c* by the added oxidase.

Inhibition of the oxidase

Previously it was shown that in *M. methylotrophus* grown under conditions of methanol-limitation the predominant oxidase is cytochrome aa_3; this oxidase is very sensitive to KCN (K_i 0·4–2·0 μM) but relatively less sensitive to azide (K_i about 50 μM). By contrast, cells grown under O_2-limitation contained only 'cytochrome *o*', which has a similar sensitivity to KCN as cytochrome aa_3 but which is much more sensitive to azide (K_i about 2 μM) (Cross & Anthony, 1980*b*). This was confirmed in the present work in which it was shown that during the oxidation of ascorbate TMPD by whole cells the K_i for azide was 1·1 μM.

The *o*-type oxidase retained its sensitivity to azide during purification, the K_i for azide inhibition during oxidation of TMPD being similar for whole cells (1·1 μM), membranes (3·5 μM), oxidase partially purified on DEAE-cellulose (1·25 μM), and completely pure oxidase (1·13 μM). The same was true for inhibition by KCN, the K_i values being, respectively, 0·3 μM, 0·17 μM, 0·16 μM and 0·2 μM. The similarity of these K_i values for whole cells and for oxidases at all stages of purification confirmed that the cytochrome oxidase purified in the present work was the *o*-type oxidase which operates during growth of *M. methylotrophus* under O_2-limitation. These results also suggest that the oxidase was not markedly altered during solubilization and purification.

Using membrane preparations, the inhibition by KCN was non-competitive with respect to substrate (TMPD, not shown), as was the inhibition of pure oxidase by both KCN and azide. A

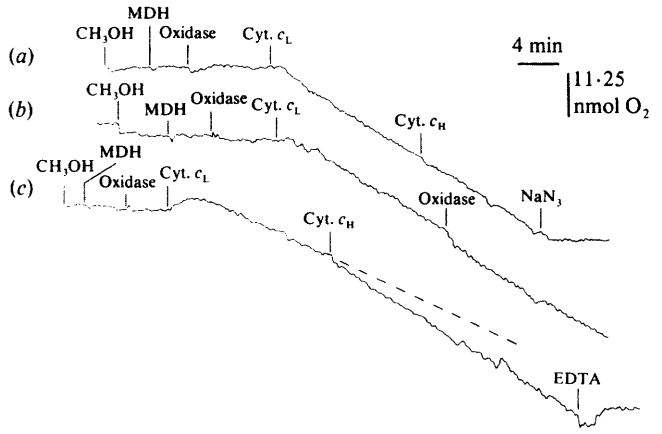


Fig. 3. The reconstitution of 'methanol oxidase' from pure MDH, pure *o*-type oxidase and soluble cytochromes *c* from *M. methylotrophus*. Oxygen consumption was measured polarographically. Proteins, substrates and inhibitors were added as indicated. The reaction volume was 2 ml and the following concentrations were used: MDH, 0·3 μM; cytochromes *c*, 0·32 μM; methanol, 7·4 mM; sodium azide, 200 μM; EDTA, 5 mM. The usual concentration of oxidase used was 0·3 μM; this assumes a molecular weight of 110500 (see text). In (*b*) addition of further oxidase led to a final concentration of 0·6 μM; and in (*c*) the oxidase concentration was 0·05 μM. The cytochrome c_L used in these experiments had a molecular weight of 17000.

similar conclusion with respect to KCN inhibition of the partially purified oxidase has been published by Carver & Jones (1983), although their preparation was less sensitive to KCN (K_i, 2 μM).

Although no thorough investigation of the inhibition by CO has been undertaken, it was shown that incubation of a reaction mixture with CO for 1 min was sufficient to inhibit the oxidase by 50% when using horse heart cytochrome *c* as substrate.

Although some inhibition was observed with high concentrations of EDTA (57% inhibition by 5 mM), the oxidase was shown to be insensitive to inhibition during oxidation of TMPD by 10 mM-salicylhydroxamic acid, 440 μM-hydroxyquinoline *N*-oxide (HQNO) and 900 μM-antimycin A. The insensitivity of the purified oxidase to HQNO, also observed by Carver & Jones (1983), suggests that HQNO inhibited TMPD oxidation by whole membranes (Cross & Anthony, 1980*b*) at a site not available on the Triton-solubilized oxidase complex.

Reconstitution of active 'methanol oxidase' from pure MDH, cytochromes c and cytochrome oxidase complex

It was shown previously that pure soluble MDH catalysed methanol-dependent reduction of cytochrome c_L but not of cytochrome c_H. This activity was assumed to be physiologically significant because optimum activity was measured at pH 7·0, it was independent of ammonia (the activator of dye-linked MDH) and it was inhibited by EDTA (K_i, 20 μM), a known inhibitor of methanol oxidation in whole bacteria (Beardmore-Gray *et al.*, 1982; Beardmore-Gray & Anthony, 1984). The rate of this *in vitro* reaction, however, was slow compared with that required to support the rate of respiration measured in whole bacteria. The observation that the active oxidase complex contains a cytochrome *c* component raised the possibility that this cytochrome *c* might be the true electron acceptor for MDH. The results in Fig. 3 show that this is not so; no O_2 consumption occurred when methanol was added to pure soluble MDH incubated with pure oxidase complex; furthermore, no activity was obtained when the soluble MDH was replaced by pure MDH originally solubilized from the membrane with Triton, then purified as described in Methods. This dehydrogenase was identical to the pure soluble enzyme with respect to sub-unit molecular weight (62000), binding to ion-exchange celluloses, turnover number and absorption spectrum. This 'membrane MDH' was active in catalysing methanol-dependent

reduction of pure cytochrome c_L but not cytochrome c_H – exactly as found for the soluble MDH (Beardmore-Gray *et al.*, 1983). The initial rate of reduction was eight times greater than with soluble MDH but accurate measurements were difficult because the rate rapidly diminished during the course of the reaction.

The demonstration that methanol oxidase activity could not be reconstituted from pure MDH plus the pure oxidase suggested that a separate cytochrome *c* must be essential for such reconstitution. This was confirmed by demonstrating that incubation of methanol, MDH and oxidase with pure cytochrome c_L led to 'methanol oxidase' activity (Fig. 3*a*). The cytochrome c_L could not be replaced by cytochrome c_H; this was as expected from the previous conclusion that MDH is specific for cytochrome c_L (Beardmore-Gray *et al.*, 1983). No 'methanol oxidase' activity could be measured in the absence of the cytochrome oxidase, methanol, MDH or cytochrome c_L; and all activity was abolished by addition of azide (200 μM) or EDTA (5 mM) (Fig. 3*a*, *c*) – as found in studies of methanol oxidation by whole bacteria.

Because cytochrome c_H was the preferred electron donor to the oxidase (see above), it was at first surprising that some reconstituted 'methanol oxidase' activity could be measured in the absence of cytochrome c_H (Fig. 3*b*). However, this occurred because cytochrome c_L was slowly oxidized by the oxidase and, in the conditions obtaining in Fig. 3*b*, the reduction of cytochrome c_L (not its oxidation) was the rate-limiting step. This was confirmed by showing that with less oxidase the oxidation of cytochrome *c* became rate-limiting and addition of the preferred electron donor, cytochrome c_H, increased the overall rate.

DISCUSSION

The present work has shown that the *o*-type oxidase from *M. methylotrophus* grown under conditions of O_2-limitation consists of two different cytochrome components; a cytochrome *c* and cytochrome *b* in equal proportions in a complex probably containing two subunits of each. The completely pure oxidase complex oxidizes TMPD at a higher rate than any *o*-type oxidase previously described, and it is very sensitive to azide, as are whole bacteria grown under conditions of O_2-limitation.

Cytochrome *o* was the name first given in 1959 by L. N. Castor and B. Chance to the 'CO-binding pigment' observed in *Staphylococcus aureus* (Chance 1953*a*, *b*; Castor & Chance, 1955; 1959). Because of their 'inability to classify the pigment in any of the typical cytochrome groups' they 'designated it cytochrome *o*' (Castor & Chance, 1959). Since then, many such cytochromes have been detected and designated cytochrome *o* and, indeed, this cytochrome appears to be the most widely distributed of bacterial cytochrome oxidases (Jurtshuk *et al.*, 1975; Poole, 1983). By analogy with other cytochromes, the name cytochrome *o* implies the involvement of a single haem type in oxidase function. Because this cytochrome has proved refractory to solubilization and purification, there has been no reconsideration of its nomenclature. However, the demonstration that both *b*- and *c*-type cytochromes are essential components of the completely pure oxidase from *M. methylotrophus* suggests that a more correct name for the oxidase described in the present work would be cytochrome *cb*; this would be directly analogous to cytochrome aa_3, cytochrome *cd* etc. However, for convenience of reference to the literature on 'cytochrome *o*' we propose the name cytochrome oxidase *co* for the oxidase described in the present work.

The only other *o*-type oxidases that have been purified in an active form, and characterized in any detail, are those from *Pseudomonas aeruginosa* (Yang, 1982; Matsushita *et al.*, 1982), *Rhodopseudomonas palustris* (King & Drews, 1976), *Azotobacter vinelandii* (Jurtshuk *et al.*, 1981; Yang *et al.*, 1979), *Rhodopseudomonas capsulata* (Hudig & Drews, 1982*a*, *b*) and *Escherichia coli* (Matsushita *et al.*, 1983; Kita *et al.*, 1984). The first three of these are similar to the oxidase described in the present work in a number of respects. Most importantly, they contain *b*-type and *c*-type cytochrome components and they have protein subunits of molecular weight 21 000–25 500 and 28 000–31 500; the cytochrome *b* component reacted with CO (as did the cytochrome *c* component), all oxidized high-potential substrates (TMPD or soluble cytochromes *c*); and all were inhibited by low concentrations of azide and cyanide. The purified cytochrome *o* from

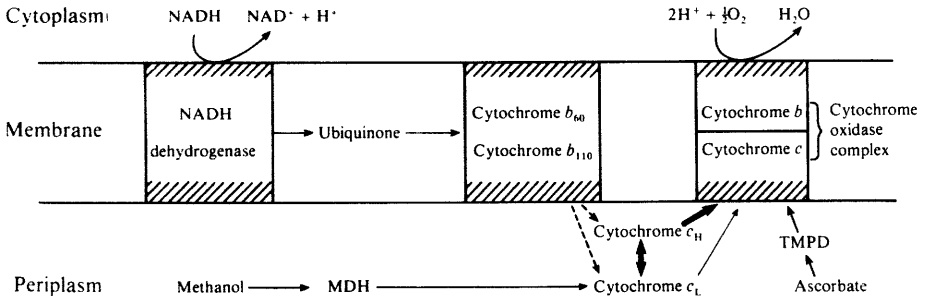


Fig. 4. Proposed pathway for electron transport in *M. methylotrophus* grown under conditions of O_2-limitation. Solid lines indicate reactions that have been demonstrated in these bacteria, the relative thickness indicating the probable relative importance of the routes for oxidation of the soluble cytochromes *c*. Broken lines are alternative routes for oxidation of cytochrome *b* which have not yet been investigated. It should be noted that the MDH, cytochrome c_H and cytochrome c_L are shown having a periplasmic location; this does not preclude the possibility that they are loosely bound to the periplasmic surface of the periplasmic membrane.

E. coli differed from these cytochrome oxidases in having two major components (M_r 34000; M_r 66000) both containing haem *b*, one of which (at least) being able to react with CO. This oxidase was also able to oxidize TMPD but the preferred substrate was the low potential substrate ubiquinol-1 (or reduced phenazine methosulphate). The oxidase from *R. capsulata* might be similar to the *E. coli* oxidase, being predominantly cytochrome *b* (only one component – M_r about 60000) but differing in not reacting with CO and in containing traces of cytochrome *c*.

It is probable that all active cytochrome oxidases contain at least two haem redox centres, and it may be that the 'cytochrome *o*' class will be found to encompass two different types of oxidase: the *M. methylotrophus* type, containing cytochrome *c* plus cytochrome *b* (cytochrome *co*); and the *E. coli* type, having only cytochrome *b* subunits (perhaps better called cytochrome *bo*).

The cytochrome *co* described in the present work is almost certainly the same as the oxidase partially purified from Triton-extracted membranes of methanol-limited *M. methylotrophus* by Carver & Jones (1983). Their most important conclusion was that the oxidase contains similar amounts of cytochrome *b* and cytochrome *c*. In this respect the two descriptions of the oxidase are similar, as are the conclusions with respect to molecular weights of the components and sensitivity to KCN (a non-competitive inhibitor). This demonstration that the same *o*-type oxidase is present in both O_2-limited and methanol-limited bacteria confirms our previous conclusion that, although the *o*-type oxidase is inducible to a higher level in carbon-excess conditions, it is always present to some extent even in carbon-limited conditions (Cross & Anthony, 1980*b*).

It should be noted that Carver & Jones (1983), from their work on the partially purified 'cytochrome *o*' of *M. methylotrophus*, 'identified' the *c*-type cytochrome in the complex as the soluble cytochrome c_L previously described (Cross & Anthony, 1980*a*; Beardmore-Gray *et al.*, 1982). This conclusion disagrees, however, with the evidence presented above; the cytochrome *c* component of the completely pure oxidase was not identical with the soluble cytochrome c_L during SDS-PAGE, and it was not reduced by MDH, whose natural electron acceptor is cytochrome c_L. Although these experiments strongly suggest that the cytochrome *c* in the oxidase complex is not cytochrome c_L, interpretation is difficult because of the presence of Triton, necessary in all work using the oxidase complex.

The work presented above, together with that described by Cross & Anthony (1980*b*), Jones *et al.* (1982) and Beardmore-Gray *et al.* (1983), suggests that electron transport in *M. methylotrophus*, when grown under conditions of O_2-limitation, is as described in Fig. 4. An important feature of this scheme is that MDH and cytochrome c_L together with the cytochrome *co* complex are sufficient for 'methanol oxidase' activity. Because cytochrome c_H is oxidized at 50 times the rate of cytochrome c_L, however, the electron transport chain between methanol and O_2 in whole organisms is likely to include both the soluble cytochromes *c*.

It will clearly be of interest to determine the roles of cytochrome c_H and cytochrome c_L as potential electron acceptors from cytochrome *b* during NADH oxidation (see Froud & Anthony, 1984); and as potential electron donors to the alternative oxidase, cytochrome aa_3, induced during growth in carbon-limited conditions.

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