The o-type oxidase of the acidophilic methylotroph Acetobacter methanolicus

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Membranes of the acidophilic methylotroph Acetobacter methanolicus contained only b- and c-type cytochrome and a CO-binding b-type cytochrome. An azide-sensitive oxidase that oxidizes cytochrome c and ascorbate/ TMPD was solubilized from the membrane with a mixture of CHAPS and Zwittergent₃₋₁₂ (1·7 fold increase in specific activity with 32% yield). The solubilized oxidase is unusually stable with respect to high ionic strength (200 mM-NaCl) and stable between pH 4·0 and 6·8. Of the two soluble c-type cytochromes from A. methanolicus only the typical class I cytochrome (cytochrome c_H) was a good substrate, as was equine cytochrome c_T . The oxidase was partially purified by anion-exchange chromatography but further purification proved impossible. The yield with respect to equine cytochrome c oxidation was 18%, with a 22-fold purification, but during purification most of the activity with respect to cytochrome c_H and TMPD was lost. Neutral phospholipids had little effect on activity of the oxidase but the charged phospholipids phosphatidylglycerol and phosphatidylserine stimulated activity up to about fourfold. It proved impossible to incorporate the oxidase into active lipoprotein vesicles. During the purification process the pH optimum for oxidation of cytochrome c_H was unchanged (pH 5·6) whereas that for oxidation of equine cytochrome changed from pH 9.5 to 7.5 and the sensitivity of the oxidase to azide changed from non-competitive to competitive during the purification process. The partially-purified oxidase contained only btype cytochrome, some of which was CO-reactive. It is proposed that the oxidase is a cytochrome co type of oxidase that loses its cytochrome c component during the purification process and is only able to oxidize c-type cytochromes if these can be formed into a 'reconstituted' cytochrome co with the partially-purified oxidase.

Introduction

The present paper describes work on an unusual o-type oxidase from the acidophilic methylotroph Acetobacter methanolicus, which grows at pH 4 on methanol as its sole carbon and energy source (Steudel & Babel, 1982; Steudel et al., 1980). As in other methylotrophic bacteria, methanol is oxidized by way of the quinoprotein methanol dehydrogenase, its specific electron acceptor cytochrome c_L , and a typical class I c-type cytochrome which is oxidized by a membrane oxidase (Anthony, 1982, 1986; Elliott & Anthony, 1988). Except for the oxidase all these proteins are periplasmic and so operate at pH 4, the pH of the growth medium. Preliminary observations suggested that this oxidase may be more stable to extremes of pH and salt concentration compared with cytochrome oxidases in other bacteria.

The bacterial o-type oxidases are defined loosely as oxidases having a CO-binding cytochrome b, which for

Abbreviations: CHAPS, 3-(3-cholamidopropyl)dimethylammoniol-propanesulphonate; TMPD, N, N, N', N'-tetramethyl-p-phenylenediamine.

convenience is usually referred to as cytochrome o and which is assumed to be the oxygen-reactive site. Cytochrome o was first described in Staphylococcus aureus (Chance, 1953; Castor & Chance, 1959) and the otype oxidases have since been shown to be the most widely distributed of the bacterial oxidases (Poole, 1983, 1988). They fall into two classes which differ fundamentally with respect to their structure and function (Poole, 1988). These are the cytochrome bo and cytochrome co classes, which contain haem b and haem c, respectively, as their second prosthetic group.

The substrate for cytochrome bo is ubiquinol, and its function is usually relatively azide-insensitive, the K_i for azide being 2–15 mM (Kita et al., 1984; Matsushita et al., 1987; Sone et al., 1990). By contrast, the substrate for cytochrome co is a class I periplasmic cytochrome c, whose oxidation is usually particularly sensitive to azide, the K_i for azide being less than 5 μ M (Froud & Anthony, 1984; Auton & Anthony, 1989). Cytochrome co may also be assayed by taking advantage of its ability to oxidize TMPD (usually together with substrate amounts of ascorbate).

The cytochrome bo of Escherichia coli is the only example of a functional ubiquinol oxidase of this type that has been characterized extensively (Kita et al., 1984; Saraste et al., 1988). E. coli is a facultative anaerobe which, in aerobic conditions, produces neither c-type nor a-type cytochromes, and it might be reasonable to assume that cytochrome bo would be found only in similar bacteria. However, that bacteria able to synthesize c-type cytochromes may also contain cytochrome bo has recently been demonstrated in Gluconobacter suboxydans, from which a ubiquinol-oxidizing cytochrome bo has recently been purified (Matsushita et al., 1987).

Much less is known about the second class of o-type oxidase, cytochrome co, although it has been purified from a number of bacteria, including Methylophilus methylotrophus (Carver & Jones, 1983; Froud & Anthony, 1984), Azotobacter vinelandii (Yang & Jurtshuk, 1978; Jurtshuk et al., 1981; Hunter et al., 1989), Rhodopseudomonas palustris (King & Drews, 1976), and Pseudomonas aeruginosa (Matsushita et al., 1982). Cytochrome co is sensitive to low concentrations of azide and cyanide, and it oxidizes the high-potential substrates cytochrome c and ascorbate/TMPD, but not the low-potential substrate ubiquinol. Most reports have described the use of ascorbate/TMPD as substrate for the oxidase and have not investigated oxidation of its physiological substrate, cytochrome c.

The present paper characterizes the o-type cytochrome of A. methanolicus. This study was initiated to consider the following questions. Does the greater stability of the oxidase to extremes of pH and salt concentration permit reproducible purification of a stable oxidase that can be used for further work on this important oxidase? Is the otype oxidase of this acidophile similar in type to other methylotroph o-type cytochromes with respect to its substrate specificity? And is the o-type oxidase similar to that from another acetic acid bacterium, Gluconobacter suboxydans, in being a cytochrome bo operating in an organism which is able to synthesize c-type cytochromes?

Methods

Organism and growth conditions. Acetobacter methanolicus MB58 is the type strain of the species (no. IMET 10945) in the culture collection of the Institute of Microbiology and Experimental Therapy of the Academy of Science of the GDR. It was the kind gift of Professor W. Babel (Institut für Biotechnologie, Leipzig, Germany). Stock cultures were maintained on methanol-containing minimal agar slopes at 4° C or in 30% (v/v) glycerol at -20° C. The defined growth medium was that described by Uhlig et al. (1986), containing methanol (1%, v/v).

Batch cultures of bacteria were grown at pH 4-0 as 500 ml cultures in 2-litre flasks, at 30 °C, on an orbital shaker (LH Fermentation), for about 24–36 h. They were harvested by centrifugation at 6000 g for 30 min, washed and resuspended (1 g wet weight ml⁻¹) in 20 mm-Tris/HCl pH 8-0.

Disruption and fractionation of bacteria. Bacteria were disrupted in an MSE Soniprep 150 ultrasonic disintegrator for 15 cycles each of 30 s sonication followed by 30 s cooling. Whole cells and cell debris were removed by centrifugation at $40000\,g$ for 15 min and membranes prepared by centrifugation of the resulting cell free extract at $100000\,g$ for 3 h. The red/orange pellet was washed by suspending it in 25 mm-MOPS buffer (pH 6·8) containing 500 mm-NaCl, followed by centrifugation for 1 h at $100000\,g$. The pellet was washed again in 25 mm-MOPS buffer (pH 6·8) and was centrifuged at $100000\,g$ for another 1 h. The pellet containing membranes was then resuspended in a minimum volume of the same buffer and stored in liquid nitrogen.

Solubilization of oxidase. Solubilized oxidase was prepared by stirring membranes (about 8-10 mg membrane protein ml⁻¹, final concentration) with detergent in 25 mm-MOPS buffer (pH 6·8) containing 10% (v/v) glycerol on ice for 30 min. The mixture was then centrifuged at $100000 \, g$ for 1 h to yield a pellet and supernatant, containing solubilized oxidase. The pellet was resuspended in the same buffer. Both the solubilized oxidase and pellet resuspension were stored in liquid nitrogen or at $-80\,^{\circ}\mathrm{C}$.

Partial purification of the oxidase. This was a single-step partial purification procedure. Solubilized membrane protein (24 ml containing 35 mg protein) in 25 mm-MOPS buffer (pH 6·8) containing 10% glycerol, 25 mm-CHAPS [3-(3-cholamidopropyl)dimethylammonio-1-propanesulphonate] and 8 mm-Zwittergent₃₋₁₂ was applied to a DEAE-Sepharose column (1·6 × 8 cm) pre-equilibrated with 25 mm-MOPS buffer (pH 6·8) containing 10 mm-CHAPS and 10% glycerol. The majority of the cytochrome c eluted without binding and no active oxidase was found in this eluate. Cytochrome oxidase was eluted with a stepwise NaCl gradient. Active fractions (eluted with 0·4 m-NaCl) were pooled and concentrated with a Centricon-10 (Amicon) before storage at -80 °C or in liquid nitrogen.

Anion-exchange chromatography on Q-Sepharose gave only 16-fold purification, compared with 22-25-fold purification obtained with DEAE-Sepharose. During attempts to purify the oxidase from solubilized membranes by hydroxyapatite chromatography, the oxidase activity was found to 'spread out' among eluted fractions and no purification was obtained. During hydrophobic interaction chromatography on octyl-Sepharose in 1.7 M-(NH₄)₂SO₄, the oxidase precipitated. Lower salt concentrations caused the protein to pass through the column without binding. In an attempt to purify further the oxidase from the eluate from DEAE-Sepharose it was exchanged into 0.2 mmdodecyl maltoside/MOPS buffer containing 0.8 m-(NH₄)₂SO₄ and applied to an octyl-Sepharose column for hydrophobic interaction chromatography. An orange band was eluted after a major protein band when the column was washed with the above buffer. However, when this was attempted on a larger scale, the orange band formed a precipitate and could not be eluted.

No activity was recovered after gel filtration on Superose-12 (Pharmacia) in the presence of 25 mm-MOPS/10% glycerol/10 mm-CHAPS/100 mm-NaCl, even though at least six protein bands were resolved.

Gel filtration on Superose-12 (Pharmacia) in the presence of 25 mm-MOPS (pH $6\cdot8$)/10% glycerol/ $0\cdot2$ mm dodecyl maltoside/100 mm-NaCl resolved the partially-purified oxidase into three major fractions, only one of which had a small amount of oxidase, but its specific activity was lower than that of the starting material, and no activity was recovered when this procedure was repeated on a larger scale.

Spectrophotometric assay of oxidase activity. The cytochrome oxidase activity of membrane fractions, solubilized fractions or partially-purified oxidase was determined spectrophotometrically using a SP8-400 UV/VIS dual-beam spectrophotometer (Pye-Unicam) with cytochrome c by recording the decrease in absorbance at about 550 nm (using the exact absorption band for each cytochrome c), and a reference wavelength of 600 nm in 25 mm-MOPS buffer pH 6-8. Reduced cytochrome c was prepared immediately before use by reduction with ascorbate followed by passage down a pre-packed PD-10 column (Pharmacia).

Polarographic assay of oxidase activity. Assays were carried out at 30 °C in 25 mm-MOPS (pH 6·8). A Clark-type oxygen electrode (Rank Bros) was used with a reaction volume of 2 ml. The electrode was calibrated using 2 ml of air-saturated buffer containing 445 nmol oxygen. The oxidase was assayed using an artificial electron donor, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) in the presence of excess ascorbate at concentrations of 0·2 mM and 2 mM respectively. When ubiquinol-1 (100 μ M) (a gift from Hoffman La Roche) was used as electron donor, the 1 ml reaction mixture contained 50 mM-Tris/HCl (pH 7·0), bovine serum albumin (1 mg ml⁻¹) and 0·05% Tween 20. Dithiothreitol (8 mM) was used to maintain the quinol in the reduced state.

Measurement of absorption spectra. Absorption spectra were recorded using a Shimadzu UV3000 dual-wavelength/double beam spectrophotometer. Unless otherwise stated, the scan speed was 100 nm min⁻¹, the spectral bandwidth was 2 nm, and the light path was 10 mm. Reduced-minus-oxidized difference spectra were recorded using samples that had been reduced with a few grains of solid sodium dithionite and oxidized with a few grains of ammonium persulphate. For the measurement of (reduced+CO)-minus-reduced spectra the contents of both the sample and reference cuvettes were reduced with sodium dithionite, and then CO gas was bubbled through the sample for 30 s.

Sources of c-type cytochromes. Cytochromes $c_{\rm H}$ and $c_{\rm L}$, from Methylophilus methylotrophus, Methylobacterium extorquens AM1 and A. metholanicus were purified as previously described (Cross & Anthony, 1980; O'Keeffe & Anthony, 1980; Elliott & Anthony, 1988). Cytochrome c_{550} from P. denitrificans and cytochrome c_{551} from P. aeruginosa were kind gifts of Dr Tony Long (this department) and Professor Colin Greenwood (University of East Anglia, UK), respectively. Equine cytochrome c was obtained from Sigma.

Measurement of protein and haem. Proteins were assayed by the bicinchoninic acid protein assay (Smith et al., 1988) with bovine serum albumin standards in detergent buffers. Haem was determined as the pyridine haemochrome as described by Fuhrhop & Smith (1975).

SDS-PAGE of proteins. Gels containing 10-15% (w/v) acrylamide monomer, with a 3% stacking gel, were used in the discontinuous system of Laemmli (1970) for the estimation of molecular masses of proteins and the purity of protein preparations. Proteins were stained with Coomassie Brilliant Blue, as described by Weber & Osborn (1975). Gels were stained for haem proteins with 3,3',5,5'-tetramethylbenzi-

dine (TMBZ) by the method of Thomas et al. (1976). Molecular masses were determined with Sigma Dalton Mark VII standard proteins.

Effects of phopholipids on oxidase activity and reconstitution into phospholipid vesicles. Phospholipids were prepared by drying known concentrations of phospholipid stocks under N₂ gas and resuspending the residue into a known volume of 20 mm-phosphate buffer pH (7.5) containing 0.1% Triton X-100, followed by brief sonication in a bathtype sonicator as described by Wong & Jurtshuk (1984). The effect of phospholipids was then determined by adding oxidase into mixtures of phospholipid and equine cytochrome c (20 nmol) in the spectrophotometric assay. Delipidation of the oxidase was attempted by gel filtration (Superose 12), the oxidase being eluted from the column with 25 mm-MOPS buffer containing 0.3 mm-dodecyl maltoside and 10% glycerol. In attempts to reconstitute partially-purified oxidase into phospholipid vesicles, oxidase (250 µl containing 50 µg protein) was mixed with sonicated phospholipid (in 100 µl 25 mm-MOPS buffer, pH 6.8, containing CHAPS, n-dodecylmaltoside or cholate) so that the lipid:protein ratio was between 10:1 and 1000:1. After 15 s sonication, the mixture was incubated on ice for 1 h before being diluted. 'Reconstituted oxidase' was separated from solubilized proteins by either passage down Sephadex G-50 column (1 × 5 cm) or centrifugation at 100000 g for 1 h.

Results

Characterization of the membranes of A. methanolicus

The spectra in Figs 1 and 2 show that membranes of methanol-grown bacteria contained only *b*- and *c*-type cytochromes (absorbance at 558 nm and 554 nm) and a CO-binding *b*-type cytochrome (cytochrome *o*) (trough at 432 nm). There was no evidence for a CO-binding *a*-type or *d*-type cytochrome (no absorbance above 570 nm).

TMPD was oxidized rapidly with a low affinity whereas cytochrome c was oxidized less rapidly, but its affinity for the oxidase was much greater (Table 1). The oxidase responsible was inhibited non-competitively by low concentrations of azide with both substrates. The kinetic constants for this inhibition, and the relative rates of oxidation of TMPD and cytochrome, are similar to those previously published for the cytochrome co of M. methylotrophus (Froud & Anthony, 1984).

The spectral characteristics, oxidation of high-potential substrates and the high sensitivity to azide indicate that the oxidase responsible for cytochrome oxidation in the membranes of *A. methanolicus* is an *o*-type oxidase of the cytochrome *co* type.

Solubilization of the o-type oxidase from A. methanolicus

Use of 2.25% (v/v) Triton X-100 in the solubilization yielded only 17.4% oxidase recovery compared with 70% solubilization obtained by Froud & Anthony (1984) using *M. methylotrophus*. A similar recovery (about 20%) was

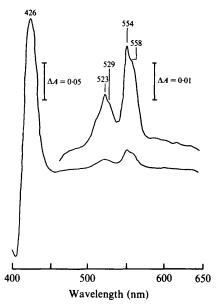


Fig. 1. Dithionite-reduced-minus-ammonium-persulphate-oxidized difference spectrum of the membranes of A. methanolicus. The difference spectrum of membranes (2.75 mg protein) suspended in 25 mm-MOPS buffer (pH 6.8) was recorded at room temperature by adding a few grains of sodium dithionite into the sample cuvette and ammonium persulphate into the reference cuvette.

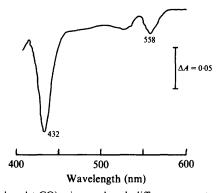


Fig. 2. (Reduced+CO)-minus-reduced difference spectrum of the membranes of A. methanolicus. Membrane suspension (as in Fig. 1 but with 3·25 mg protein) in both cuvettes was reduced with sodium dithionite and CO gas bubbled through the sample cuvette for 30 s at room temperature. The spectrum was recorded after a further 5 min.

observed when n-octylglucoside (2.5%), n-dodecyl maltoside (2%) or cholate (25 mM) was used to replace Triton X-100.

Deoxycholate (in 25 mm-MOPS buffer, pH 7·8), on the other hand, solubilized about 85% of the oxidase with a sevenfold increase in specific activity. This was partly due to the twofold stimulation of activity by the residual detergent in the assay system. However, the deoxycholate-solubilized oxidase was found to be difficult to manipulate. For example, when it was precipitated by 15% ammonium sulphate the precipitate could not be resolubilized and gel filtration (in 25 mm-MOPS/8 mm-CHAPS, pH 7·8) resulted in 70% loss of activity after one passage down a Sephadex G-25 column equilibrated in the above buffer.

Zwittergent₃₋₁₂ (30 mM), a zwitterionic detergent, which was used successfully in the purification of the o-and d-type oxidases of E. coli (Kita et al., 1984) and Klebsiella pneumoniae (Smith et al., 1990), solubilized almost all the cytochrome from the membranes, giving an orange-red soluble fraction and a creamy-white pellet. However, the soluble fraction showed very little activity and addition of 0.1% Tween 20 in the assay system did not result in higher activity.

CHAPS (30 mm), another zwitterionic detergent, solubilized 20% of the oxidase. Addition of 100 mm-MgCl₂ or KCl, and brief sonication (15 s) during solubilization, did not improve the yield, but addition of 8 mm-Zwittergent₃₋₁₂ increased the yield to 32%. No further improvement was obtained when a second round of solubilization of residual membrane was attempted using CHAPS (20 mm), cholate (0·2%) or Triton X-100 (0·25%). Changing the protein:detergent ratio with CHAPS/Zwittergent resulted in little change in the yield of solubilized oxidase, when using between 5 and 10 mg protein ml⁻¹.

The procedure eventually adopted involved solubilization in MOPS buffer (25 mm, pH 6·8) containing glycerol (10%), CHAPS (25 mm) and Zwittergent (8 mm) (see Methods for details). About 32% of active oxidase could

Table 1. Kinetic parameters for the oxidase at different stages during purification

Activities were determined as described in Methods at pH 6·8 in 25 mm-MOPS buffer. The types of inhibition by sodium azide are given in parentheses: C, competitive; NC, non-competitive.

| Substrate | Ascorbate/TMPD | | | Equine cytochrome c | | | |
|----------------------------|---|------------------------|--------------------|---|-------------------------------------|-------------------------------------|--|
| | V (μmol min ⁻¹ mg ⁻¹) | К _т (тм) | <i>К</i> ; (µм) | V (nmol min ⁻¹ mg ⁻¹) | <i>K</i> _m (μ M) | <i>K</i> _i (μ м) | |
| Membrane Solubilized | 5.6 | 0.63 | 35 (NC) | 31 | 13.0 | 8·8 (NC) | |
| oxidase Partially-purified | 3.7 | 2.20 | 100 (NC) | 53 | 8.3 | 24·0 (NC) | |
| oxidase | 12.8 | 12.50 | 104 (C) | 690 | 3.1 | 11·0 (C) | |

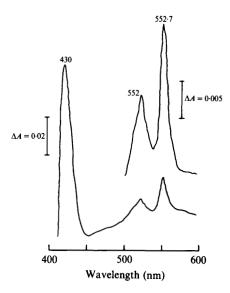


Fig. 3. Dithionite-reduced-minus-ammonium-persulphate-oxidized difference spectrum of the solubilized oxidase of *A. methanolicus*. The difference spectrum of solubilized oxidase (0.5 mg protein in 25 mm-MOPS buffer, pH 6.8, containing 25 mm-CHAPS and 8 mm-Zwittergent_{3.12}) was recorded at room temperature by adding a few grains of sodium dithionite into the sample cuvette and ammonium persulphate into the reference cuvette.

be routinely solubilized in this way, the oxidase having a 1.7-fold higher specific activity than that measured in the membrane preparation in the spectrophotometric assay. The solubilized oxidase could be stored indefinitely in liquid nitrogen.

Characterization of the solubilized oxidase

Identification of the cytochromes. Fig. 3 shows the presence of c-type cytochrome (α -peak at 552-7 nm). No 560 nm shoulder due to cytochrome b was detected, possibly due to large amounts of various membrane cytochromes c, but the CO-binding difference spectrum (Fig. 4) showed a 432 nm trough, similar to that seen in membrane preparations (Fig. 2), which is characteristic of a CO-reactive b-type cytochrome (cytochrome o).

Respiratory activity of the solubilized oxidase. The results in Tables 1 and 2 show that the solubilized oxidase had retained its activity with respect to oxidation of both TMPD and cytochrome c, and that inhibition by azide remained non-competitive in nature. The yield with respect to TMPD oxidation was only 13% compared with the 32% yield for cytochrome c oxidation.

Stability of the solubilized oxidase. In the absence of salt the oxidase was stable at 4 °C between pH 4 and 6.8, but 40% activity was lost over a 24 h period at pH 7.8. Over a period of 4 h at pH 6.8 (25 mm-MOPS buffer) in 200 mm-

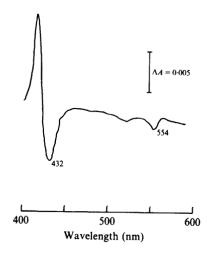


Fig. 4. (Reduced+CO)-minus-reduced difference spectrum of the solubilized oxidase of *A. methanolicus*. Solubilized oxidase (as in Fig. 3), in both cuvettes, was reduced with sodium dithionite and CO gas bubbled through the sample cuvette for 30 s at room temperature. The spectrum was recorded after a further 5 min.

NaCl there was a 50% increase in activity (measured with cytochrome c) which then remained constant for at least 3 d at 4 °C. This demonstrated that the oxidase from A. methanolicus is an exceptionally stable oxidase compared with the cytochromes co from other sources, which are very sensitive to changes of pH and salt concentration (e.g. see Carver & Jones, 1983; Froud & Anthony, 1984).

Single-step partial purification of the o-type oxidase

Table 2 summarizes the results of the partial purification of the oxidase by anion-exchange chromatography on DEAE-Sepharose. No further purification of the material eluting from DEAE-Sepharose was achieved by ion-exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography or gel filtration (see Methods).

Cytochrome composition of the partially-purified oxidase.

The dithionite-reduced-minus-ammonium-persulphate-oxidized difference spectrum of the partially-purified oxidase (Fig. 5) was both interesting and surprising as it demonstrated the presence of b-type cytochromes but no c-type cytochrome. This was confirmed by the low-temperature spectrum, which provided evidence for only one b-type cytochrome (Fig. 6). Some of the b-type cytochrome was CO-reactive, implying the presence of cytochrome o (Fig. 7).

Table 2. Purification of cytochrome oxidase from membranes of A. methanolicus

Solubilization and purification procedures, and activities, were determined as described in Methods. The volumes for each purification step were 6.5 ml, 13.9 ml and 7.7 ml; and total activities (nmol cytochrome oxidized min⁻¹) were 5507, 1752 and 690 for membranes, solubilized membranes and partially-purified oxidase respectively. A single purification step of anion-exchange chromatography on DEAE-Sepharose was used. This purification procedure was repeated many times, the results usually giving values within 10% of those presented below. Specific activities were measured in 25 mm-MOPS at pH 6.8 or in 50 mm-Tris/HCl (pH 7.0) for ubiquinol-1; they are expressed as nmol min⁻¹ (mg protein)⁻¹ for oxidation of equine cytochrome c and nmol O_2 consumed min⁻¹ (mg protein)⁻¹ for oxidation of ascorbate/TMPD or ubiquinol-1.

| Substrate | Cytochrome c | | | Ascorbate/TMPD | | | Ubiquinol-1 | | |
|----------------------------|-------------------|-----------|-------------------------|-------------------|--------------|-------------------------|-------------------|--------------|-------------------------|
| | Specific activity | Yield (%) | Purification (-fold) | Specific activity | Yield (%) | Purification (-fold) | Specific activity | Yield (%) | Purification (-fold) |
| Membrane Solubilized | 31 | 100 | 1.0 | 5 5 9 5 | 100 | 1.0 | 971 | 100 | 1.0 |
| oxidase Partially-purified | 53 | 32 | 1.7 | 3743 | 13 | 0.7 | 183 | 2.5 | 0.2 |
| oxidase | 690 | 18 | 22.3 | 12800 | 2 | 2.3 | 5 2 5 2 | 3.0 | 5.4 |

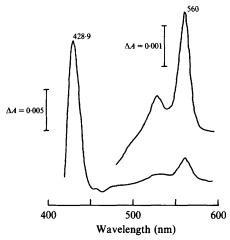


Fig. 5. Dithionite-reduced-minus-ammonium-persulphate-oxidized difference spectrum of the partially-purified oxidase of A. methanolicus. The spectrum of the oxidase (50 μ g protein in 25 mm-MOPS buffer, pH 6·8, containing 10% glycerol and 10 mm-CHAPS) was recorded at room temperature.

When a sample of the partially-purified oxidase was analysed by SDS-PAGE on a 15% acrylamide gel, no haem-staining band was present, confirming the absence of cytochrome c; protohaem IX dissociates from cytochrome b under denaturing conditions and does not show up in the haem staining whereas the covalently-bound haem in cytochromes c stains after gel electrophoresis (Goodhew et al., 1986). The absence of cytochrome c was further confirmed by preparing the pyridine haemochrome derivatives of the oxidase; the only haem present was haem b. These results suggest that the solubilized oxidase, if it is a cytochrome co, has lost the cytochrome c component during the partial purification.

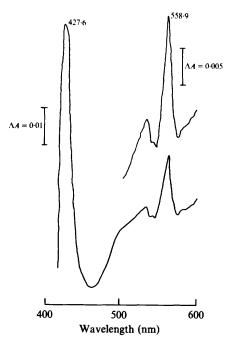


Fig. 6. Reduced-minus-oxidized difference spectrum at 77 K of the partially-purified oxidase from A. methanolicus. The spectrum of the oxidase (as in Fig. 5) was recorded at 77 K using 2 mm light path cuvettes, a spectral bandwidth of 0.5 nm and a scan speed of 50 nm min⁻¹.

Respiratory activities catalysed by the partially-purified oxidase

Oxidation of equine cytochrome c. In the spectrophotometric assay system at pH 6.8 (25 mM-MOPS buffer) the V value for cytochrome c oxidation was 690 nmol

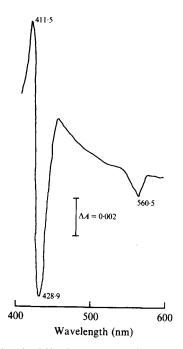


Fig. 7. (Reduced+CO)-minus-reduced difference spectrum of the partially-purified oxidase of A. methanolicus. Oxidase sample (as in Fig. 5) in both cuvettes was reduced with sodium dithionite and CO gas was bubbled through the sample cuvette for 30 s at room temperature. The spectrum was recorded after a further 5 min.

min⁻¹ mg⁻¹ and the $K_{\rm m}$ value was 3·1 μM (Table 1). The V value was about 20% of that measured with the cytochrome co of M. methylotrophus (Froud & Anthony, 1984). The K_i values for azide and cyanide were 11·0 μM and 4·8 μM respectively, inhibition being competitive with respect to cytochrome c, a result different from that obtained with the solubilized oxidase.

Oxidation of ascorbate/TMPD. The specific activity for TMPD oxidation at pH 6·8 (25 mm-MOPS buffer) was much higher than that for equine cytochrome c (Table 1), the V value being $12\cdot8 \,\mu\text{mol} \, O_2 \, \text{min}^{-1} \, \text{mg}^{-1}$, with a K_m value of $12\cdot5 \, \text{mm}$. The V value was about 30% of that measured with the pure cytochrome co from M. methylotrophus (Froud & Anthony, 1984). As found for cytochrome c oxidation, the inhibition by azide had changed during purification from being non-competitive to competitive in nature (K_i , $10 \, \text{mm}$) (Table 1). This differs from the cytochromes co of M. methylotrophus and organism 4025, where the K_i values were $1\cdot1 \,\mu\text{m}$ and $0\cdot2 \,\mu\text{m}$ respectively and the inhibition was non-competitive with respect to TMPD (Carver & Jones, 1983; Froud & Anthony, 1984; Auton & Anthony, 1989).

Although the specific activity for cytochrome c oxidation increased 13-fold during the single purification step, the specific activity with TMPD increased only 3-4-fold (Table 2).

Oxidation of ubiquinol. The oxidation of ubiquinol was measured polarographically in the presence of $100 \,\mu\text{M}$ -ubiquinone-1 plus 8 mm-dithiothreitol at pH 6·8 (25 mm-MOPS buffer). On solubilization, about 97% of the activity was lost, presumably due to lack of solubilized cytochrome bc_1 -complex; after partial purification, a 29-fold increase in specific activity was observed (Table 2).

Effect of phospholipids on activity of the partially-purified oxidase

As seen in Table 3, the neutral lipids phosphatidylethanolamine and phosphatidylcholine had very little effect, whereas phospholipids with or negatively-charged head groups (phosphatidylglycerol and phosphatidylserine) stimulated the oxidase activity up to fourfold at a lipid:protein ratio of 10:1.

Attempts to prepare active delipidated oxidase and to reconstitute the partially-purified oxidase into membrane vesicles using the gel filtration and dilution methods described by Gennis (1989) were unsuccessful. Although in some cases haemoprotein was incorporated into vesicles no oxidase activity was ever recovered.

Identification of a putative cytochrome c component of the oxidase

If the oxidase is a cytochrome co then its spectrum indicates that it must have lost the cytochrome c component during the ion-exchange chromatography. In an attempt to identify the 'lost' cytochrome c component, each eluted fraction from DEAE-Sepharose was added to the oxidase assay systems (with TMPD as substrate) to detect any stimulatory effect on oxidase activity. Only the cytochrome fraction which had not bound to DEAE-Sepharose was active, giving an eightfold increase in the ascorbate/TMPD assay and a twofold increase when cytochrome c oxidation was measured; it had no oxidase activity on its own. Boiling the stimulatory fraction for 10 min destroyed its activity, suggesting that the stimulation was not due to phospholipid.

SDS-PAGE of the stimulatory fraction revealed five haem protein bands (19, 21, 26, 48 and 72 kDa). The 21 kDa band was the only one that was absent from all those fractions that had failed to stimulate the activity of the partially-purified oxidase.

Ion-exchange chromatography with CM-cellulose or S-Sepharose at pH 5·5-6·5, or with DEAE-Sepharose at pH 8·0 in the presence or absence of detergent, failed to resolve the proteins in the stimulatory fraction. Gel filtration on a Superose 12 column in 20 mm-MOPS/100 mm-NaCl buffer (pH 6·8) containing 10 mm-

Table 3. Effects of phospholipids on the activity of the partially-purified oxidase

Activity was measured spectrophotometrically with equine cytochrome c (20 nmol) at pH 6·8 in 25 mm-MOPS buffer after pre-incubation of the oxidase with phospholipids for 15 min prior to assay. Duplicate experiments gave values within 10% of those given here, and when repeated with a different batch of partially-purified oxidase the values were within 20% of those given here, the trends and conclusions being identical. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylgycerol; PS, phosphatidylserine.

| Phospholipid (nmol) | Specific activity (nmol min ⁻¹ mg ⁻¹) | | | | | | |
|------------------------|--|-------------|-----------------------|-----|--|--|--|
| | Neutral ph | ospholipids | Charged phospholipids | | | | |
| | PE | PC | PG | PS | | | |
| None | 203 | 203 | 203 | 203 | | | |
| 20 | 196 | 234 | 804 | 443 | | | |
| 200 | 205 | 225 | 820 | 740 | | | |
| 2000 | 193 | 237 | 0 | 0 | | | |

CHAPS resolved three peaks absorbing at 405 nm. In order to determine which was the cytochrome c component of the oxidase, a large-scale preparation was required, but a clear separation of the cytochromes was not achieved when gel filtration was performed with a Sephadex G-75 column in 50 mm-MOPS/100 mm-NaCl (pH 7·0) containing 0·05% Triton X-100. However, only those fractions containing 19 and 21 kDa cytochromes showed a stimulating effect on the oxidase. Although these fractions also contained some larger haem proteins, the 21 kDa cytochrome was the only cytochrome that was absent from all fractions having no stimulatory effect. It was therefore concluded that the cytochrome c component of the oxidase might be this 21 kDa cytochrome.

The absorption spectrum of the fraction containing the 21 kDa cytochrome confirmed the presence of c-type cytochromes. The CO-binding difference spectrum, recorded after bubbling CO for 30 s, showed troughs at 551.4 nm and 427 nm, suggesting the presence of a CO-binding cytochrome c, similar to those present in the oxidase M. methylotrophus and organism 4025 (Froud & Anthony, 1984; Auton & Anthony, 1989).

Cytochrome specificity of the partially-purified oxidase

Because cytochrome $c_{\rm L}$ of A. methanolicus is rapidly auto-oxidized at about pH 7·0, the activity of the oxidase in the membrane was determined at pH 5·6; the relative rates of oxidation of cytochrome $c_{\rm H}$, equine cytochrome $c_{\rm and}$ cytochrome $c_{\rm L}$ (all at 10 μ M) were 45:23:1 respectively (Table 4). This confirms that cytochrome $c_{\rm H}$ is likely to be the physiological substrate for the oxidase of A.

methanolicus, as found with other methylotrophs, and the complete failure to oxidize cytochrome c_L after solubilization and purification confirms this. The oxidase also failed to oxidize the cytochromes c_L from Methylophilus methylotrophus and Methylobacterium extorquens AM1.

The specific activity of the oxidase with cytochrome $c_{\rm H}$ (10 µM) was 200 nmol min⁻¹ (mg membrane protein)⁻¹ and about 42% of this activity was obtained by solubilization. After purification on DEAE-Sepharose, the oxidase had lost most of its activity with cytochrome $c_{\rm H}$ but had retained 43% of its activity with equine cytochrome c (Table 4). The explanation for this is related to the dramatic change in pH optimum for equine cytochrome c after chromatography (see below). When measured at their pH optima, large losses of activity were observed for both c-type cytochromes, although more activity was lost with cytochrome $c_{\rm H}$ than with equine cytochrome c (Table 4).

The $K_{\rm m}$ values for equine cytochrome c and cytochrome $c_{\rm H}$ ($K_{\rm m}$ 2-2·5 μ M) were rather lower than those measured for the cytochrome co from Methylophilus methylotrophus (14-32 μ M). The V value at pH 5·6 (the pH optimum) was very low for cytochrome $c_{\rm H}$ (13 nmol min⁻¹ mg⁻¹), but very much higher for equine cytochrome c (700 nmol min⁻¹ mg⁻¹ at pH 7·5, its pH optimum). The relatively low value for cytochrome $c_{\rm H}$ is due to the remarkable loss of activity during ion-exchange chromatography. Addition of a small amount of reduced equine cytochrome c (0·4 μ M) to a reaction mixture containing 3·5 μ M-cytochrome $c_{\rm H}$ and 3·6 μ g oxidase increased the rate of oxidation of cytochrome $c_{\rm H}$ 5·4-fold. This suggests that equine cytochrome c acts as a replacement for the cytochrome c of the co complex,

Table 4. Oxidation of cytochrome c_H and equine cytochrome c

Activity was measured spectrophotometrically in 25 mm-buffer (MES at pH 5.6; MOPS at pH 6.8; CHES at pH 9.5). This purification procedure was repeated at least three times, the results usually giving values within 10% of those presented below. Assays contained 10 μ m-cytochrome and the amount of protein indicated. The values in parentheses are to give an indication of yield at each stage of the purification.

| Substrate pH | Total activity (μmol min ⁻¹) | | | | | | | |
|----------------------------|--|-------------|------------------|-------------|-------------|--|--|--|
| | Cytochrome c _H | | Cytochrome c_1 | | | | | |
| | 5.6 | 5.6 | 6.8 | 9.5 | 5.6 | | | |
| Membrane Solubilized | 7.0 (100%) | 3.46 (100%) | 6.4 (100%) | 32.0 (100%) | 0.15 (100%) | | | |
| oxidase | 2.93 (42%) | 0.80 (23%) | 1.34 (21%) | 6.7 (21%) | 0.014 (9%) | | | |
| Partially-purified oxidase | 0.014 (0.2%) | 0.35 (10%) | 0.60 (9%) | 0.11 (1.7%) | 0.00 (0%) | | | |

Table 5. Relationship between pH optima for various c-type cytochromes and their pI values, molecular masses and midpoint redox potentials

The 'pH optima' listed here refer to the pH tested between pH 5·2 and 9·5; the true optimum may be outside the range tested. The pI values, molecular masses and midpoint redox potentials are taken from the following sources: *Pseudomonas aeruginosa* from Horio *et al.* (1960); *Methylophilus methylotrophus* from Cross & Anthony (1980); *Methylobacterium extorquens* AM1 from O'Keeffe & Anthony (1980); equine cytochrome c from Pettigrew & Moore (1987).

| | 'pH | optimum' | | | |
|--------------------------|--|----------|-------------|-----------------------|---------------|
| Cytochrome | Solubilized Partially-purified oxidase oxidase | | pI value | Mol. mass (kDa) | E_{m7} (mV) |
| Cytochrome <i>c</i> -551 | | | | | |
| (P. aeruginosa) | 5.2 | >8.4 | 4.7 | 8-1 | 286 |
| Cytochrome c_{H} | | | | | |
| (A. methanolicus) | 5.6 | 5.6 | 5.8 | 9.0 | 224 |
| Cytochrome c_{H} | | | | | |
| (M. methylotrophus) | 5-2 | >8.4 | 8.9 | 8.5 | 373 |
| Cytochrome c_{H} | | | | | |
| (M. extorquens AM1) | 7.0 | 7.0 | 8.8 | 11.0 | 294 |
| Cytochrome $c_{\rm H}$ | | | | | |
| (Horse heart) | 9.5 | 7.5 | 10.5 | 12.5 | 250 |

enabling it to oxidize cytochrome $c_{\rm H}$ at a greater rate. When 0.5 mg of the 21 kDa cytochrome c fraction was used to replace the small amount of equine cytochrome c in the above 'reconstitution', the activity was increased about twofold, the lower increase compared with equine cytochrome c being due, presumably, to the small amount of the 21 kDa cytochrome c present in the fraction used.

pH optima for the oxidation of cytochromes c

The pH optimum for the physiological substrate (cytochrome c_H) was pH 5.6 and was unchanged during purification (Fig. 8a; Table 4). This exceptionally low pH optimum for an oxidase is probably related to the low pH optimum for growth.

The remarkable change in pH optimum from pH 9.5 to pH 7.5 for equine cytochrome c during purification of the oxidase (Fig. 8b) is consistent with the proposal that the nature of the oxidase markedly changed during purification on DEAE-Sepharose. If the cytochrome c component is lost then the pH optimum should tend to revert to the original if reconstituted with the oxidase cytochrome c. That this did occur was indicated by the observation that the pH optimum increased from pH 7.5 to 8.5 when a small amount (1 mg protein) of the fraction containing the 21 kDa cytochrome c, previously shown to stimulate oxidation of equine cytochrome c, was

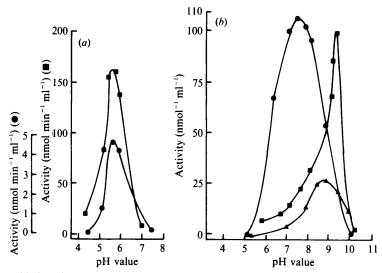


Fig. 8. pH optima for the oxidation of cytochromes c during oxidase purification. The pH optima were determined with (a) cytochrome c_H (10 μ M) and (b) equine cytochrome c (20 μ M) in 25 mM-buffer (formate, pH 4·0–4·2; acetate, pH 4·5–5·0; MES, pH 5·5–6·5; MOPS, pH 6·5–7·5; Tris, pH 7·0–9·0; CHES, pH 9·0–10·0). The assay system contained either solubilized oxidase (21 μ g protein) or partially-purified oxidase (7·4 μ g protein). \blacksquare , Solubilized oxidase; \blacksquare , partially-purified oxidase plus 21 kDa cytochrome c fraction.

mixed with oxidase (40 μ g) for 30 min prior to incubation in the oxidase assay (Fig. 8b).

The pI values of cytochrome $c_{\rm H}$ and equine cytochrome c are 5.8 and 10.5 and the pH optima were 5.6 and about 9.5 respectively. This suggested that the differences in the isoelectric points might reflect differences in the charged groups on the cytochromes c which might influence their binding or electron transfer reactions. It was thus of interest to investigate the extent of this correlation with other cytochromes c. The results (Table 5) show that there is no clear relationship between the pH optimum for each cytochrome and their pI values, molecular masses or midpoint redox potentials. They also show that there was no apparent correlation between pI value and the change observed in the pH optimum during purification.

Discussion

The work presented in this paper confirms that the oxidase of the acidophilic methylotroph A. methanolicus is an o-type oxidase, although further confirmation by the use of photochemical action spectroscopy remains desirable (Poole, 1988). The solubilized membranes contained no a- or d-type cytochromes and they oxidized the high-potential substrates cytochrome c and TMPD at high rates. Oxidation of these substrates was very sensitive to azide, the inhibition being of the noncompetitive type. The rates of oxidation of cytochrome c and TMPD by the partially-purified oxidase are similar

to those measured with the cytochromes co of Rhodopseu-domonas palustris (King & Drews, 1976), Pseudomonas aeruginosa (Matsushita et al., 1982) and Methylophilus methylotrophus (Carver & Jones, 1983; Froud & Anthony, 1984). All these results suggest that o-type oxidase solubilized from the membranes is of the cytochrome co type. A further similarity to the cytochrome co of M. methylotrophus is the observation that the preferred cytochrome substrate from A. methanolicus is the cytochrome c_H , activity with cytochrome c_L , the electron acceptor from methanol dehydrogenase, being negligible. This is consistent with conclusions drawn from work with other methylotrophs in which the oxidase is cytochrome co or cytochrome aa_3 (Froud & Anthony, 1984; Auton & Anthony, 1989; Fukumori et al., 1985).

After partial purification, the active preparation changed markedly. The only CO-binding cytochrome remained cytochrome o but there was no cytochrome c, and the only haem present was haem b. This suggests that the oxidase might be an atypical, azide-sensitive, cytochrome bo that is able to oxidize high-potential substrates. However, a more likely explanation is that it is a cytochrome co that has lost its cytochrome c, and in this respect it resembles some early descriptions of o-type oxidases which lost activity on purification, concomitant with loss of a cytochrome c component (e.g. Yang & Jurtshuk, 1978; Yang, 1985). The following dramatic changes in the properties of the oxidase after purification on DEAE-Sepharose argue strongly in support of this proposal. The most usual way of assaying oxidases of the cytochrome co type is to use the high-potential substrate

TMPD, which is assumed to pass electrons directly to the cytochrome c component of the oxidase. After purification of the solubilized oxidase, however, a high proportion of the TMPD oxidase activity was lost compared with activity with equine cytochrome c (Table 2). The K_m value for TMPD oxidation had increased sixfold and its sensitivity to azide had decreased; the K_i value had increased 100-fold and the mode of inhibition had changed from non-competitive to competitive (Table 1).

Another major change occurring during purification was the loss of almost all activity towards the physiological substrate, cytochrome $c_{\rm H}$, compared with equine cytochrome c (Table 4). Furthermore, although the pH optimum for cytochrome $c_{\rm H}$ oxidation remained unchanged, that for equine cytochrome decreased by 2 pH units, and its sensitivity to azide inhibition changed from non-competitive to competitive.

The most obvious explanation for these changes is that the partially-purified oxidase consisted only of the cytochrome o component and was only able to oxidize cytochrome c when this could become 'reconstituted' into an active cytochrome c. The equine cytochrome c was apparently more able to effect this reconstitution, which could presumably only occur at a lower pH than its usual pH optimum. This is further supported by the observation that a small amount of equine cytochrome c stimulated oxidation of the cytochrome c by the partially-purified oxidase. A fraction containing a 21 kDa cytochrome c, obtained from the solubilized oxidase during the purification step, was also able to stimulate the oxidase and to raise the pH optimum for oxidation of equine cytochrome c.

Although all the results of this work are consistent with the conclusion that the oxidase is a cytochrome co, it is worth noting that the distinctions between type of oxidase (cytochrome aa_3 , bo or co) and substrate specificity are not as well-defined as they once were; for example, there is considerable structural similarity between the cytochrome aa_3 from Paracoccus denitrificans, which oxidizes cytochrome c, and the cytochrome bo of E. coli, whose substrate is the low-potential substrate ubiquinol (Saraste et al., 1988; Raitio et al., 1990).

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