The terminal respiratory chain of the methylotrophic bacterium *Methylophilus methylotrophus*

Mark A. Carver and Colin W. Jones*

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, England

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Cytochrome oxidase *o* has been isolated from the obligately aerobic, methylotrophic bacterium *Methylophilus methylotrophus* in the form of a cytochrome c_L–*o* complex. The latter is comprised of cytochrome c_L (M_r 21 000) and cytochrome *o* (M_r 29 000) in a 1–2:1 ratio, possibly in association with one or more minor polypeptides; the complex exhibits a high ascorbate–TMPD oxidase activity which is inhibited non-competitively by cyanide ($K_i \sim 2\ \mu M$). In contrast, the oxidation of methanol by whole cells is inhibited uncompetitively by cyanide ($K_i \sim 4\ \mu M$), thus indicating the involvement in methanol oxidation of cytochrome oxidase aa_3 rather than *o*.

Methylophilus methylotrophus *Cytochrome oxidase* o *and* aa_3 *Methanol* *Respiratory chain*

1. INTRODUCTION

The aerobic bacterium *Methylophilus methylotrophus* readily uses methanol as a source of carbon and energy, and is grown commercially by ICI as single cell protein. Whole cells oxidise methanol to formaldehyde via a respiratory chain methanol oxidase system comprised of methanol dehydrogenase, cytochromes c_H and/or c_L, and cytochrome oxidases aa_3 and/or *o* [1–4]; by analogy with *Methylomonas* J and *Pseudomonas* AM1, methanol oxidation may also involve a copper protein [5]. Methanol dehydrogenase and much of the *c*-type cytochrome are loosely attached to the periplasmic surface of the respiratory membrane, whereas the remainder of the *c*-type cytochrome and the cytochrome oxidases are firmly membrane-bound [6]. The methanol oxidase system catalyses proton ejection concomitant with electron transfer ($\rightarrow H^+/O$ = 2 g-ion H^+/g-atom O, $\rightarrow K^+/O = -2$ g-ion K^+/g-atom O) [3–7], and hence generates a protonmotive force which can be used to drive ATP synthesis [8].

As part of an investigation of electron transfer and energy transduction by the respiratory chain of *M. methylotrophus* we have isolated a cytochrome oxidase c_Lo complex, compared its sensitivity to cyanide with that of methanol oxidation in whole cells, and shown that the latter occurs predominantly via cytochrome oxidase aa_3 rather than *o*.

2. MATERIALS AND METHODS

2.1. *Whole cell experiments*

Methylophilus methylotrophus (NC1B 10515) was grown in methanol-limited continuous culture (D = 0.18 h^{-1}, 40°C) [7]. After harvesting, cells were washed once in 20 mM glycyl–glycine buffer (pH 7.0) + 140 mM KCl, then resuspended and assayed for respiratory activities in the same buffers at 40°C [7].

2.2. *Isolation of a cytochrome* c–o *complex*

A frozen paste of *M. methylotrophus* grown in

* To whom correspondence should be addressed

Abbreviations: HQNO, 2-*n*-heptyl-8-hydroxyquinoline-*N*-oxide; I_{50}, median inhibitory dose; SDS, sodium dodecylsulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

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methanol-limited continuous culture at 40°C was obtained from ICI Agricultural Division (Billingham). The paste was freeze–thawed twice and then stirred in the presence of bovine pancreatic deoxyribonuclease 1 (0.05–0.10 mg/ml; Sigma) until the viscosity was reduced to a minimum. The suspension was disrupted by two passages through a French pressure cell at 12000 lb/in.^{2}, then centrifuged at 2500 × *g* for 15 min to yield a dense supernatant which was subsequently centrifuged at 150000 × *g* for 1 h. This final supernatant was rich in cytochromes c_H and to a lesser extent c_L [1], whereas the pelleted membranes contained the remaining *c*-type cytochrome plus the *b*-type cytochromes (including cytochrome *o*) and cytochrome aa_3.

The pellet was resuspended in 10 mM Tris–HCl buffer (pH 7.5) + 0.5 M KCl (40 mg protein/ml) and recentrifuged to remove residual loosely-bound cytochrome *c*; the resultant membranes were resuspended in 10 mM Tris–HCl buffer (pH 7.5) + 1% (w/v) Triton X-100 (20 mg protein/ml), stirred for 2 h at 4°C and recentrifuged. The supernatant from this step contained cytochrome *c* and about 30% of the cytochrome *o* present in the original membranes (together with 0–15% of the cytochrome aa_3 depending on the exact degree of disruption and detergent extraction). Preparations with the lowest levels of cytochrome aa_3 were then fractionated with ammonium sulphate. The 25–35% fraction contained a translucent red-brown floating precipitate which was rich in cytochromes *c* and *o*, but also included a small amount of cytochrome aa_3 and a large amount of Triton. This floating precipitate was resuspended in a small volume of 10 mM Tris–HCl buffer (pH 7.5) + 0.2% (w/v) Triton X-100 to give an optically clear solution which was then dialysed overnight at 4°C against 50 vol. of the same buffer prior to loading on to a DEAE-cellulose (Whatman DE-52) column pre-equilibrated with this buffer. The cytochromes bound strongly to the column, but could be eluted with a linear gradient of 0–0.3 M KCl in 10 mM Tris–HCl buffer (pH 7.5) + 0.2% Triton X-100. Fractions rich in cytochromes *c* + *o* were eluted in 150 mM KCl. These were concentrated by ultrafiltration and then passed down a Sephadex G150 column pre-equilibrated with 10 mM Tris–HCl buffer (pH 7.5) + 0.2% Triton X-100. Effluents rich in cytochromes *c* + *o* were finally rechromatographed on DEAE-cellulose essentially as above, and fractions eluting at 150–200 mM KCl were collected and stored at −20°C.

2.3. *Other methods*

SDS–polyacrylamide gel electrophoresis was carried out at pH 8.0 using conventional procedures [9,10]. Gels were stained for protein with Coomassie blue or Kenacid blue (BDH), and for haem using 3,3′,5,5′-tetramethylbenzidine [11]; cytochromes *c* were identified from their red fluorescence under long-wavelength UV light [12]. Cytochrome spectra were recorded on a Pye-Unicam SP1800 split-beam spectrophotometer.

3. RESULTS AND DISCUSSION

A dithionite-reduced minus ferricyanide-oxidised difference spectrum of the cytochrome *c–o* complex isolated from *M. methylotrophus* (fig.1a) showed absorption maxima at 417–420, 552 and 558 nm indicative of *c*- and *b*-type cytochromes; positive identification of the latter as cytochrome *o* was obtained from a dithionite-reduced + CO minus dithionite-reduced difference spectrum (fig.1b) which showed absorption maxima at 417, 541 and 572 nm and minima at 430 and 558 nm.

SDS–polyacrylamide gel electrophoresis of the complex showed the presence of two major polypeptides (M_r 29000 and 21000) together with a variable population of minor polypeptides (M_r 74000, 60000, 50000, 40000 and 16500). The two major polypeptides both contained haem, as did the two smaller minor polypeptides when present. The 21000 M_r polypeptide was identified as cytochrome c_L [1] (the 16500 M_r minor polypeptide was probably a proteolysed form of cytochrome c_L); no evidence was obtained for the presence of cytochrome c_H in the complex. Repeated chromatography of the complex on DEAE-cellulose slowly removed cytochrome c_L, leaving the 29000 M_r polypeptide which was identified as cytochrome *o*.

The cytochrome c_L–*o* complex readily oxidised the artificial substrate ascorbate–TMPD, with a turnover rate of 10–40 s^{-1} (60–70 s^{-1} in whole cells). Removal of cytochrome c_L from the complex abolished ascorbate–TMPD oxidation, in-

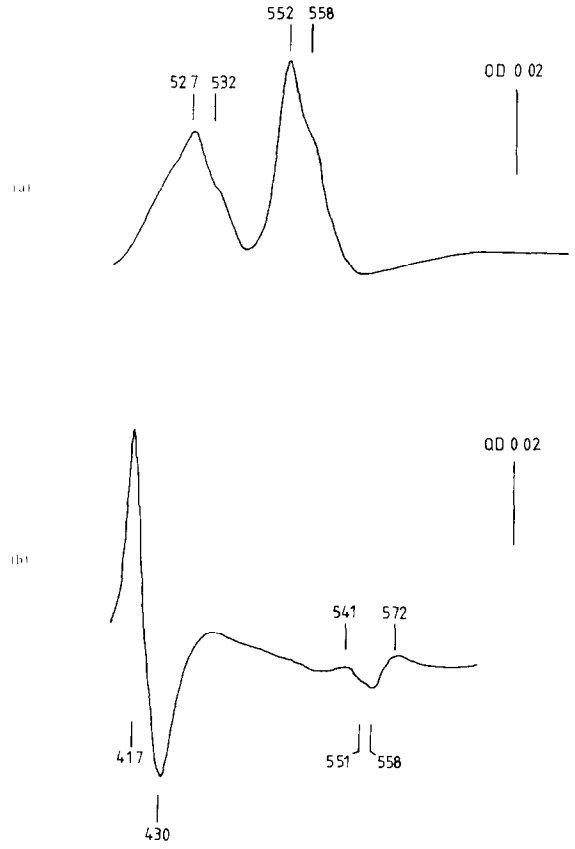

Fig.1. Absorption spectra of the cytochrome *c–o* complex from *M. methylotrophus:* (a) dithionite-reduced minus ferricyanide oxidised difference spectrum; (b) dithionite-reduced + CO minus dithionite-reduced difference spectrum (the sample was bubbled with carbon monoxide for 30 s).

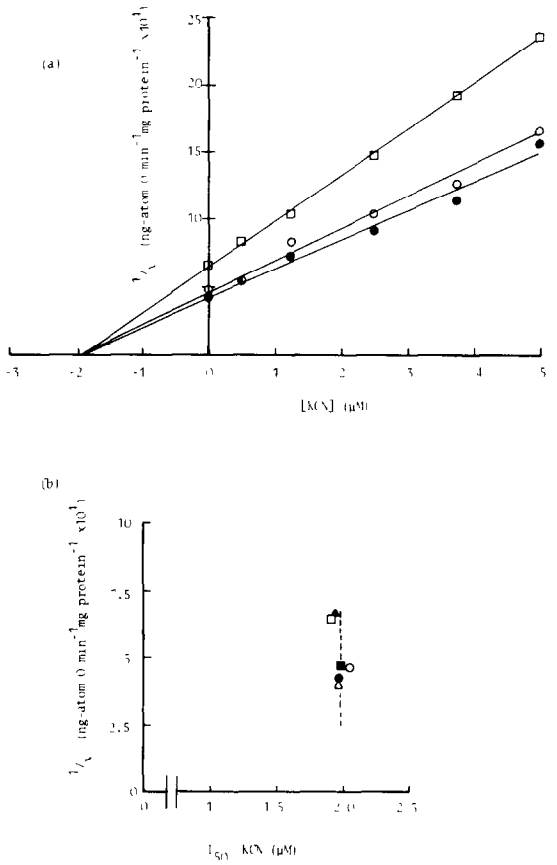

Fig.2. The effect of cyanide on the oxidation of ascorbate–TMPD by the cytochrome c_L–*o* complex from *M. methylotrophus*. The complex was assayed for ascorbate–TMPD oxidase activity in 10 mM Tris–HCl + 0.1% Triton X-100 buffer (pH 7.5), with ascorbate (2.5 mM) plus either 1000 μM (△), 500 μM (●), 180 μM (■), 150 μM (○), 62.5 μM (▲) or 50 μM (□) TMPD: (a) $1/v$ *vs.* [KCN] (Dixon plot); (b) $1/v$ *vs.* I_{50}. Rates of oxygen uptake were corrected for autoxidation. Lines were fitted by linear regression analysis.

dicating that cytochrome c_L is an essential component of the oxidase complex and that cytochrome *o* is unable to accept electrons directly from TMPD; in the absence of cytochrome *o*, cytochrome c_L showed no oxidase activity. Preparations with a cytochrome c_L:*o* ratio of 1–2:1 exhibited the highest activity, but further purification of the complex by chromatography on hydroxyapatite to remove the minor polypeptides severely diminished this activity. The identity and exact roles of the minor polypeptides are currently under investigation.

The ascorbate–TMPD oxidase activity of the cytochrome c_L–*o* complex was inhibited in a strict non-competitive manner by cyanide ($K_i \sim 2\ \mu$M; fig.2a,b). Activity was also inhibited by azide ($I_{50} \sim 10\ \mu$M), but largely unaffected by EDTA or HQNO (only 12% inhibition at 200 μM HQNO), a finding which does not support the suggestion [2] that HQNO inhibits cytochrome *o* in the respiratory chain of *M. methylotrophus*. Oxidase activity was also substantially inhibited by high concentrations of potassium chloride, potassium phosphate and ammonium sulphate (>50% inhibition by 20 mM ammonium sulphate), and care was therefore taken to remove contaminating salts at the end of the preparation procedure.

It is clear that cytochrome oxidase c_L–o has several features in common with other o-type bacterial cytochrome oxidases, notably those of *Azotobacter vinelandii* [13], *Pseudomonas aeruginosa* [14] and *Rhodopseudomonas palustris* [15]. All of these oxidases contain a CO-binding b-type cytochrome (cytochrome o, M_r 28000–30500) plus a c-type cytochrome, and are readily inhibited by low concentrations of cyanide and azide.

In order to investigate the physiological role of the cytochrome c_L–o complex in the respiratory chain of *M. methylotrophus*, the effect of cyanide on the oxidation of methanol by whole cells was examined in detail. Whole cells oxidise methanol with K_m 15–20 μM and at rates of up to about 300 and 1000 ng-atom $O.min^{-1}.mg$ dry wt^{-1} when prepared and assayed in glycyl–glycine buffer (pH 7.0) ± 140 mM KCl. It is therefore possible to examine cyanide inhibition of methanol oxidation over a wide range of electron transfer rates by varying the methanol concentration and the nature of the preparation/assay buffer. The results of such experiments showed unequivocably that methanol was inhibited in an uncompetitive manner by cyanide under all of the conditions tested (fig.3a). When the ordinate intercepts ($1/v$) from the Dixon plots were plotted against the abscissa intercepts ($K_i[1 + K_m/[S]]$; i.e., I_{50}) (fig.3b) a straight line relationship was obtained which passed through the origin (contrast fig.2b), thus confirming uncompetitive inhibition and indicating a K_i of about 4 μM. This result shows that the report of non-competitive inhibition [3] was in error.

In contrast, cyanide inhibition of ascorbate–TMPD oxidation by whole cells was uncompetitive only at extremely low electron flow rates (see also [3]); at higher rates inhibition was of either the non-competitive or mixed type (K_i 2–4 μM). Since uncompetitive and non-competitive inhibition of bacterial respiration by cyanide are characteristic of electron transfer via cytochrome oxidases aa_3 (or d) and o, respectively ([16,17]; unpublished), there is strong evidence that in whole cells of *M. methylotrophus* grown under methanol-limited conditions the oxidation of methanol occurs predominantly, perhaps exclusively, via cytochrome oxidase aa_3. In contrast, electrons from ascorbate-TMPD are clearly accessible to both cytochrome oxidase aa_3 and o with the preferred use of either oxidase probably depending on the

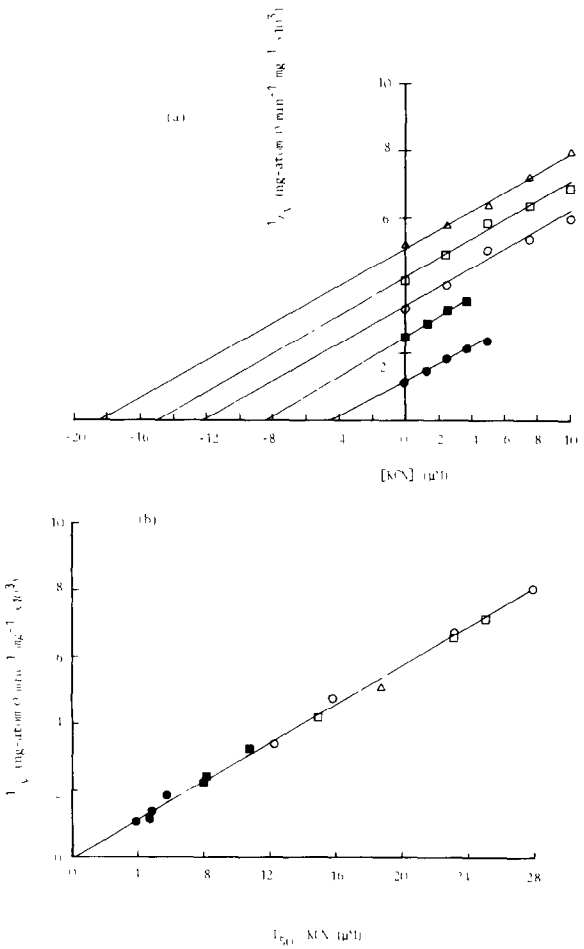

Fig.3. The effect of cyanide on the oxidation of methanol by whole cells of *M. methylotrophus*. Cells were prepared and assayed for methanol oxidase activity in either 20 mM glycyl–glycine buffer, pH 7.0 (closed symbols) or the same buffer supplemented with 140 mM KCl (open symbols). Methanol: 500 μM (●,○); 50 μM (■,□); 20 μM (▲). (a) $1/v$ *vs.* [KCN] (Dixon plot); (b) $1/v$ *vs.* I_{50}. The lines were fitted by linear regression analysis.

overall rate of respiration and on the exact ability of cytochromes c_L and c_H to interact with TMPD and each other; the mixed type of inhibition by cyanide which was observed on several occasions can easily be explained by kinetic models involving the partition of electron transfer between the two terminal oxidases (unpublished). Since cytochrome c_L is structurally and functionally associated with cytochrome oxidase o, it is tempting to speculate that cytochrome c_H may be specifically involved in

electron transfer from methanol dehydrogenase to cytochrome oxidase aa_3 (see also [2,4]). However, the exact roles of cytochromes c_H and c_L in the oxidation of physiological substrates remain to be determined.

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