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The 'methylamine oxidase' system of an obligate methylotroph

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The terminal respiratory oxidase was solubilized from membranes of organism 4025, an obligate methylotroph. The partially purified oxidase is probably a cytochrome *co*. It does not oxidize amicyanin, but it oxidizes 'azurin' and cytochromes c_H and c_L. By using a complete 'methylamine oxidase' system reconstituted from pure methylamine dehydrogenase, purified oxidase and soluble blue copper proteins and cytochromes, it was confirmed that amicyanin is essential for methylamine oxidation; it could not be replaced by 'azurin' or cytochrome c_H or c_L. It was shown that the usual mediator between amicyanin and the oxidase is cytochrome c_H, with 'azurin' able to replace it during growth at the high copper concentrations required for optimum growth of this unusual methylotroph.

INTRODUCTION

In methylotrophic bacteria the most widely distributed system for oxidation of methylamine during growth on this substrate as sole source of carbon and energy involves a 'methylamine oxidase' system consisting of a periplasmic quinoprotein methylamine dehydrogenase that is coupled by way of other periplasmic electron-transport proteins to a membrane-bound oxidase, which is either cytochrome aa_3 or an *o*-type oxidase called cytochrome *co* (Anthony, 1982, 1988). The primary electron acceptor for methylamine dehydrogenase in most bacteria is a specific inducible type I blue copper protein called amicyanin, first discovered by Tobari and his colleagues (Tobari & Harada, 1981; Tobari, 1984), and subsequently shown to have a periplasmic location and to accept electrons from methylamine dehydrogenase at a sufficiently high rate to account for the respiration rate *in vivo* (Lawton & Anthony, 1985*a*,*b*). The conclusion that amicyanin is the electron acceptor for methylamine dehydrogenase is supported by work with at least five very different methylotrophs, including the pink facultative methylotroph *Methylobacterium* and the obligate methylotroph *Methylomonas* J (Tobari & Harada, 1981; Tobari, 1984; Ambler & Tobari, 1985), the obligate methylotroph organism 4025 (Lawton & Anthony, 1985*a*,*b*) and the facultative autotrophs *Thiobacillus versutus* (van Houwelingen *et al.*, 1985) and *Paracoccus denitrificans* (Husain & Davidson, 1985, 1986; Husain *et al.*, 1986; Gray *et al.*, 1986; Lim *et al.*, 1986). Although amicyanin is the best (and usually only) electron acceptor for methylamine dehydrogenase it is not detectable in all methylotrophs containing methylamine dehydrogenase, the best example of this being *Methylophilus methylotrophus* during growth on trimethylamine (Burton *et al.*, 1983). In some bacteria methylamine dehydrogenase is able to react with the periplasmic cytochrome *c* (see Chandrasekar & Klapper, 1986; Fukumori & Yamanaka, 1987), but whether or not this has physiological significance is not yet certain.

Organism 4025 is an obligate methylotroph that requires copper for maximum growth and that contains very large amounts of two blue copper proteins during growth on methylamine; these are amicyanin and a second protein referred to as 'azurin'. It is similar to other methylotrophs in containing two periplasmic *c*-type cytochromes (Lawton & Anthony, 1985*a*,*b*); cytochrome c_H is a typical small basic cytochrome *c*, and cytochrome c_L is the specific electron acceptor for the quinoprotein methanol dehydrogenase (Anthony, 1986, 1988). It has previously been shown that all these periplasmic cytochromes and blue copper proteins from organism 4025 are able to interact with each other (Lawton & Anthony, 1985*b*). Preliminary observations indicate that the sole oxidase in organism 4025 is an *o*-type oxidase, possibly similar to the cytochrome *co* of *Methylophilus methylotrophus* (Froud & Anthony, 1984).

An investigation of the effect of copper on the growth of this organism on methanol and methylamine has shown that during O_2-limited continuous culture without added copper no amicyanin or 'azurin' could be detected, but that the cell density was only halved compared with that achieved in the presence of sufficient copper. A similar result was obtained when iron was omitted from a growth medium that contained sufficient copper for maximum growth on methylamine. In the presence of high concentrations of copper 'azurin' synthesis was induced so that its concentration was 3–5 times that of cytochrome c_H during growth on both methanol and methylamine. These results suggest that amicyanin might be replaced by cytochrome c_H in this organism in some growth conditions and that 'azurin' might sometimes replace cytochrome c_H as electron donor to the oxidase (Auton & Anthony, 1989). The present paper describes an investigation of these proposals in which the rates of interaction of purified cytochromes, blue copper proteins and oxidase were determined in reconstituted 'methylamine oxidase' systems.

METHODS

Growth of bacteria, preparation of extracts, and purification of methylamine dehydrogenase, soluble cytochromes, amicyanin and 'azurin'

Bacteria were grown in batch culture on methylamine exactly as described by Lawton & Anthony (1985*a*).

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After being harvested, they were resuspended in ice-cold 25 mM-Mops/NaOH buffer, pH 7.0, and were disrupted by sonication for 10 min in 1 min periods in a 100 W MSE Ultrasonic disintegrator. Whole cells and cell debris were removed by centrifugation at 6000 *g* for 10 min, and the supernatant fluid was centrifuged at 135000 *g* for 60 min at 0 °C. The supernatant was decanted and used for purification of soluble proteins as described by Lawton & Anthony (1985*a*).

Solubilization and purification of the oxidase, cytochrome *co*

The membrane fraction (1 g of protein) was homogenized in 25 mM-Mops/NaOH buffer, pH 7.0. Glycerol (20%, w/v) and the zwitterionic detergents CHAPS (20 mM) and Zwittergent (10 mM) were added (final concentrations in parentheses) to give a final protein concentration of 8 mg/ml. CHAPS is 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (from Sigma Chemical Co.) and Zwittergent is *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate (from Calbiochem). The suspension was sonicated for 10 s, then stirred from 30 min at 4 °C before centrifugation at 135000 *g* for 1 h. The resulting supernatant was filtered through a 0.22 μm-pore-size filter to remove membrane fragments, concentrated under N_2 on an Amicon XM30 membrane (30000-M_r cut-off) and subjected to anion-exchange chromatography on a Pharmacia Mono-Q f.p.l.c. column equilibrated in 25 mM-Mops/NaOH buffer, pH 7.0, containing 8 mM-CHAPS and 10% (v/v) glycerol. The column was washed with 350 mM-NaCl, and the oxidase was eluted with a gradient of 350–500 mM-NaCl. Active fractions were pooled, concentrated and further purified by gel filtration on a Pharmacia Superose-12 f.p.l.c. column equilibrated in the same buffer as used for ion-exchange chromatography. Active fractions were pooled and stored in liquid N_2. Spectra of the oxidase (0.15–0.2 mg of protein/ml) were recorded in 25 mM-Mops/NaOH buffer, pH 7.0, containing 10% (v/v) glycerol and 8 mM-CHAPS.

Assay of the oxidase

During purification the oxidase was assayed polarographically in a 2 ml reaction mixture with horse heart cytochrome *c* (100 μM) (Sigma type III) in the presence of ascorbate (2 mM) in 25 mM-Mops/NaOH buffer, pH 7.0, containing 6 mM-CHAPS and 1.6% (w/v) glycerol. For investigations of substrate specificity of the oxidase, it was measured spectrophotometrically in a 0.5 ml reaction mixture containing purified oxidase (37.5–150 ng of protein) and 25 mM-Mops/NaOH buffer, pH 7.0, containing 4 mM-CHAPS. Azurin oxidation was measured at 620 nm and cytochrome *c* oxidation was measured at 550 nm. The substrate proteins were first reduced with the minimum amount of dithionite and equilibrated into the assay buffer by passage through a Sephadex G-25 PD10 column.

Reconstitution of the 'methylamine oxidase' system

Reactions were measured in a 1 ml oxygen-electrode vessel at 30 °C in 25 mM-Mops/NaOH buffer, pH 7.0, containing 1.8 mM-CHAPS and 6% (w/v) glycerol. It was assumed that O_2 was fully reduced to water, consuming 4 electrons/molecule.

Assay of methylamine dehydrogenase, cytochromes, blue copper proteins and protein

The methods used were all those described by Lawton & Anthony (1985*a*), except for protein, which was determined by the bicinchoninic acid method as described by Smith *et al.* (1988).

SDS/polyacrylamide-gel electrophoresis

The methods were as described by Lawton & Anthony (1985*a*), M_r standards being components of the Sigma Dalton mark VII standard kit: lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde phosphate dehydrogenase, ovalbumin and bovine serum albumin. Haem proteins were stained as described by Thomas *et al.* (1976).

RESULTS AND DISCUSSION

Purification and characterization of the oxidase cytochrome *co*

Organism 4025 contains no *a*-type cytochrome, and it has been suggested that the sole oxidase in this organism is an *o*-type oxidase indicated by the presence of a CO-reactive cytochrome *b* in membranes of these bacteria (Vrdoljak & Froud, 1982; Lawton & Anthony, 1985*b*). In order to characterize a complete reconstituted 'methylamine oxidase' of organism 4025 it was first necessary to solubilize and purify the terminal oxidase from membranes of this organism.

Washed membranes were shown to oxidize horse heart cytochrome *c* at a high rate in a reaction non-competitively inhibited by azide (K_i 2–4 μM). A protein catalysing this activity was solubilized from these membranes by using a mixture of zwitterionic detergents (CHAPS plus Zwittergent) in glycerol as described in the Methods section. When Zwittergent was used separately, protein solubilization was successful but the oxidase was inactive; solubilization of active oxidase could be effected with CHAPS alone, but inclusion of Zwittergent increased both yield and specific activity by about 25%. By contrast with cytochrome *co* of *Methylophilus methylotrophus*, $MgCl_2$ did not aid solubilization and Triton X-100 could not be used because low concentrations led to very rapid loss of activity.

About 35% of the total oxidase was usually solubilized, and the sensitivity to azide of this solubilized oxidase was similar to that of whole membranes (K_i 2–4 μM). The oxidase was purified by anion-exchange chromatography on a Mono-Q column followed by gel permeation on a Superose-12 column with the Pharmacia f.p.l.c. system (see the Methods section for details). A 62-fold purification was achieved with a 10% yield. On SDS/PAGE the preparation contained two haem-staining peptides of M_r 28000 and 33000 plus four other peptides of higher M_r. Assuming that these were contaminants, the preparation was about 20% pure. An indication of the cytochrome content is given by the ratio of 1.7:1.0 measured for the absorbance at 280 nm compared with that of 410 nm in the spectrum of the untreated oxidase.

The spectrum of the oxidase was very similar in appearance to that of the pure cytochrome *co* of *Methylophilus methylotrophus*, having a peak at 550 nm and a shoulder at 560 nm, consistent with the conclusion that the oxidase contains cytochrome *b* and *c* components

Table 1. Oxidation of redox proteins by cytochrome *co*

Electron donor	Highest concn. tested (μM)	K_m value (μM)	$V_{max.}$ (μmol/min per mg of protein)
Amicyanin	24.4	–	0
'Azurin'	5.6	0.9	2.6
Cytochrome c_H	38.5	2.7	29.0
Cytochrome c_L	19.2	5.7	0.4

in equal proportions. The oxidase reacted rapidly with CO, the reaction being complete after bubbling CO through the preparation for 3 s. The (dithionite-reduced plus CO)-minus-(dithionite-reduced) difference spectrum had troughs at about 556 nm and 435 nm, indicating that the rapidly reacting species was the cytochrome *b* component. The cytochrome *c* component also reacted with CO, giving a trough at about 420 nm, but reaction was very slow, requiring 10 min rather than a few seconds.

A similar protein has been purified and characterized from another methylotroph. *Methylophilus methylotrophus*; it was shown to contain equal numbers of cytochrome *b* and cytochrome *c* subunits and was the first purified example of the type of oxidase now called cytochrome *co* (Froud & Anthony, 1984; Poole, 1988). It was able to oxidize cytochrome c_H at about 50 times the rate of cytochrome c_L. Although the oxidase from organism 4025 has been only partially purified, the evidence strongly suggests that it is another example of a cytochrome *co* and, although in the absence of a completely pure preparation the identification can be only tentative; for convenience it is referred to below as cytochrome *co*.

The oxidase was unable to oxidize amicyanin but was able to oxidize the other small respiratory proteins of organism 4025 (Table 1), the reactions following Michaelis–Menten kinetics. As found with the cytochrome *co* of *Methylophilus methylotrophus* (Froud & Anthony, 1984) and with cytochrome aa_3 of *Methylobacterium* AM1 (Fukumori *et al.*, 1985), the oxidase had a similar high affinity for both *c*-type cytochromes, but the rate was about 70 times higher for the typical small cytochrome c_H compared with the methanol dehydrogenase-specific cytochrome c_L. The highest affinity for any substrate tested was for 'azurin', although its $V_{max.}$ value was only about 10% of that with cytochrome c_H. The affinity of the purified oxidase for horse heart cytochrome *c* was almost identical with that measured for cytochrome c_H and was identical with that shown by membranes, before and after solubilization.

The partially purified cytochrome *co* of organism 4025 was non-competitively inhibited by NaN_3, the K_i value (2.6 μM) being similar to that measured with membranes and with the cytochrome *co* of *Methylophilus methylotrophus* (Froud & Anthony, 1984).

Reconstitution of 'methylamine oxidase'

The work with partially purified oxidase described above indicated that an electron-transfer mediator is required between the oxidase and amicyanin (during oxidation of methylamine) or cytochrome c_L (during oxidation of methanol). Both cytochrome c_H and 'azurin' are possible candidates for this role. In order to confirm

Table 2. Reconstitution of a 'methylamine oxidase' system

Reactions were in a 1 ml oxygen-electrode vessel containing 0.1 mM-methylamine, 0.8 μM-methylamine dehydrogenase and 8.2 nM-cytochrome *co*. In this system with 10.6 μM-amicyanin plus 12.5 μM-'azurin' and an equivalent amount of oxidase in non-solubilized membranes the rate measured was 115 mol of electrons/s per mol of cytochrome *co*. Reactions were started by addition of oxidase. The rate was doubled in all cases when the oxidase concentration was doubled.

Amicyanin	Cytochrome c_L	Azurin	Cytochrome c_H	Rate (mol of electrons/s per mol of cytochrome *co*)
–	–	5.6 μM	–	0
–	–	–	14.2 μM	0
–	62.7 μM	–	14.2 μM	0
13.8 μM	–	–	14.2 μM	32
13.8 μM	62.7 μM	–	14.2 μM	28
13.8 μM	–	1.3 μM	14.2 μM	40
13.8 μM	–	5.6 μM	–	115

that these proteins might be able to constitute a complete electron-transport chain from methylamine to O_2, such a 'methylamine oxidase' system was set up with the partially purified oxidase and pure electron-transfer proteins from organism 4025. The results, summarized in Table 2, confirmed that amicyanin could not be replaced by either cytochrome *c* or 'azurin' as electron acceptors for methylamine dehydrogenase, and that either cytochrome c_H or 'azurin' was essential for activity of the complete 'methylamine oxidase'. Of particular interest is that 'azurin' was able to replace cytochrome c_H completely and that a combination of this cytochrome plus 'azurin' gave a higher rate than the cytochrome alone. When partially purified oxidase was replaced by washed membranes containing an equivalent amount of oxidase (judged by the amount of CO-reactive cytochrome *b*) the rate of electron transport with 'azurin' replacing cytochrome *c* was exactly as measured with the partially purified oxidase.

Nature of the interaction between oxidase, 'azurin' and cytochrome c_H

Because 'azurin' and cytochrome c_H were both good donors to the oxidase, and good mediators in the complete system, their interactions with oxidase in the complete system was studied further. A partial 'methylamine oxidase' system was reconstituted with methylamine dehydrogenase, amicyanin and partially purified oxidase. The high amicyanin concentration (16 μM) gave the solution an intense blue colour that immediately disappeared on addition of methylamine. The complete 'methylamine oxidase' reaction was then initiated by addition of cytochrome c_H or 'azurin'. Amicyanin remained in its reduced form (colourless) during all reactions, indicating that the rate-limiting step being observed was the oxidation of cytochrome c_H or 'azurin'. The results given in Fig. 1 show that cytochrome c_H was oxidized in a single phase, the interaction being described by a single, high-affinity, binding site (K_m 36 nM; $V_{max.}$ 31 mol of electrons/s per mol of cytochrome *co*). At

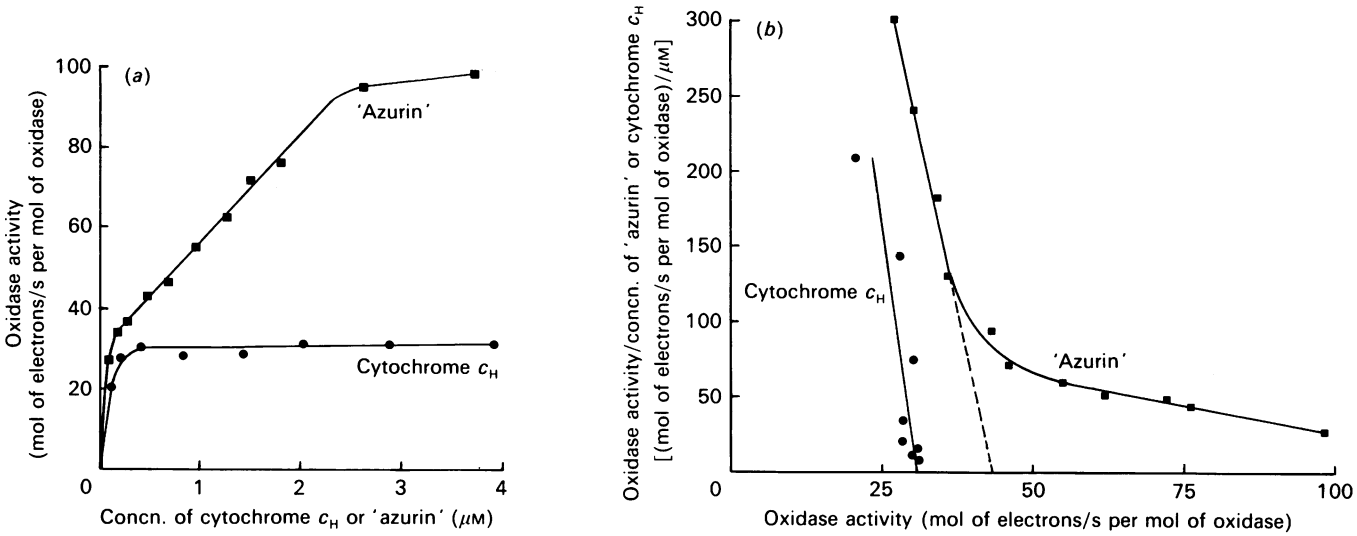

Fig. 1. Effect of 'azurin' or cytochrome *c* concentration on reconstituted 'methylamine oxidase' activity

All reactions were carried out in a 1 ml reaction volume in an oxygen-electrode vessel containing 25 mM-Mops/NaOH buffer, pH 7.0, with 1.8 mM-CHAPS and 6% (v/v) glycerol. The curves in (*b*) were drawn from the data presented in (*a*). Reaction mixtures contained methylamine (100 μM), methylamine dehydrogenase (0.8 μM), amicyanin (15.8 μM) and cytochrome *co* (8.2 nM). ●, Cytochrome *c*; ■, 'azurin'.

saturating concentrations of cytochrome c_H the rate was proportional to oxidase concentration. The K_m measured in this complete 'methylamine oxidase' system was considerably lower than that measured in the direct spectrophotometric assay (Table 1); this may have been due to competitive inhibition of the reaction by oxidized cytochrome in the direct assay, which would not have occurred in the complete system; such inhibition has been previously reported for oxidation of cytochrome *c*-551 and azurin by the oxidase of *Pseudomonas aeruginosa* (Barber *et al.*, 1976).

The results with 'azurin' were clearly different from those observed with cytochrome c_H, in that two distinct phases were seen (Fig. 1). The first phase reflects a high-affinity site (K_m 55 nM; $V_{max.}$ 43.5 mol of electrons/s per mol of cytochrome *co*); the second phase reflects a second, lower-affinity, site (K_m 800 nM; $V_{max.}$ 132 mol of electrons/s per mol of cytochrome *co*); this K_m value is identical with that measured in the direct spectrophotometric assay. Similar biphasic kinetics for oxidation of cytochrome *c* by bovine cytochrome *c* oxidase were first reported by Ferguson-Miller *et al.* (1976), the K_m values being 28 nM and 0.35–1 μM, which are very similar to those measured in the present work for oxidation of 'azurin' by cytochrome *co*.

It should be noted that, when measured with the complete 'methylamine oxidase' system, higher rates were obtained with 'azurin' than with cytochrome c_H, by contrast with rates measured in the direct (spectrophotometric) assay for the oxidation of electron donors by the partially purified oxidase, in which the rate of oxidation of cytochrome c_H was about 10 times that of 'azurin'.

Conclusions

The results of the work described in the present paper do not support the conclusion that amicyanin can be replaced by cytochrome *c* in organism 4025. The nature of the electron acceptor for methylamine dehydrogenase during growth in copper-deficient conditions when no amicyanin can be detected thus remains an open question.

The oxidase partially purified from organism 4025 contains *b*- and *c*-type cytochromes and is probably a cytochrome *co*, as previously described in *Methylophilus methylotrophus*. The maximum rate of electron transfer measured with this oxidase together with pure proteins in a 'methylamine oxidase' system was 115 mol of electrons/s per mol of cytochrome *co*, which is about 20% of that measured in whole bacteria. Clearly such calculations depend on many assumptions; but the fact that the values are the same order of magnitude confirms the physiological relevance of the reconstituted system.

The high rates of electron transfer in 'methylamine oxidase' systems containing 'azurin' strongly support the suggestion that 'azurin' might replace cytochrome c_H in those conditions when it is present at higher concentrations than cytochrome c_H. This arises when organism 4025 is grown in high copper concentrations (the optimum for methylamine being 3 mg of $CuSO_4,5H_2O$/l). In these conditions the concentration of 'azurin' is 5 times that of cytochrome c_H (Auton & Anthony, 1989), and so it is likely that the electron-transport chain for oxidation of methylamine does not involve soluble cytochromes but is dependent primarily on blue copper proteins. It is also probable that 'azurin' replaces cytochrome c_H during methanol oxidation when bacteria are grown at high copper concentrations. This may be relatively unimportant because the optimum concentration of copper for growth on methanol is lower than that required for growth on methylamine, and at the lower concentrations of copper similar amounts of cytochrome c_H and 'azurin' are synthesized. During conditions of optimum growth, therefore, the electron-transport chains for oxidation of methanol and methylamine in organism 4025 are likely to be as follows:

$CH_3OH \rightarrow$ methanol dehydrogenase $\rightarrow$ cytochrome $c_L \rightarrow$ cytochrome $c_H \rightarrow$ cytochrome $co \rightarrow O_2$

$CH_3NH_2 \rightarrow$ methylamine dehydrogenase $\rightarrow$ amicyanin $\rightarrow$ 'azurin' $\rightarrow$ cytochrome $co \rightarrow O_2$

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