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The Role of Cytochromes and Blue Copper Proteins in the Oxidation of Methanol and Methylamine in Organism 4025, an Obligate Methylotroph

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Organism 4025 contained only *b*-type and *c*-type cytochromes. In the absence of any *a*-type cytochrome oxidase it was concluded that respiration in these bacteria is catalysed entirely by way of the *o*-type cytochrome oxidase. After growth on methanol, the soluble protein contained cytochromes c_H and c_L, and a single blue copper protein of unknown function, 'azurin'. After growth on methylamine the soluble proteins were strikingly different. The total concentration of soluble cytochrome *c* was about 70% lower; cytochrome c_L was about 65%, and cytochrome c_H about 16% of the concentrations present after growth on methanol. Although some 'azurin' was still present, a second copper protein, amicyanin (94% of the total), was induced during growth on methylamine. The concentration of this protein was considerably higher than that of blue copper proteins measured in other bacteria and 35 times higher than the concentration in the facultative methylotroph *Pseudomonas* AM1 during growth on methylamine. It is the very high concentration of amicyanin that gave suspensions of methylamine-grown organism 4025 their blue-green colour. All of the soluble cytochrome *c*, and all of the methanol dehydrogenase, methylamine dehydrogenase and blue copper proteins were located in the periplasmic fraction. All these results are consistent with the conclusion that the main electron acceptor for methylamine dehydrogenase in organism 4025 is amicyanin.

INTRODUCTION

Organism 4025 is a non-pigmented, Gram-negative, obligate methylotroph unable to grow on methane but able to grow on methanol or methylamine (Johanides *et al.*, 1979; Vrdoljak & Froud, 1982). Although similar in many respects to other obligate methylotrophs (Anthony, 1982), this organism is unusual in its response to copper in the growth medium, maximum growth only occurring at high copper concentrations (Vrdoljak *et al.*, 1978). During preliminary investigations of growth of organism 4025 on methylamine it was observed that suspensions of this organism became blue-green in colour when aerated. This was subsequently shown to be due to the presence of soluble blue copper proteins which have been purified and characterized and shown to be amicyanin and 'azurin' (Lawton & Anthony, 1985). The amicyanin, but not the 'azurin', was shown to be an electron acceptor from methylamine dehydrogenase, as found previously in the pink facultative methylotroph *Pseudomonas* AM1, and in the obligate methylotroph *Methylomonas* J (Tobari & Harada, 1981; Tobari, 1984). Surprisingly, *Methylophilus methylotrophus*, an obligate methylotroph similar in most respects to *Methylomonas* J and organism 4025, contained only a small amount of blue copper protein similar to azurin during growth on methanol (Beardmore-Gray & Anthony, 1984), and no blue copper protein at all during growth on trimethylamine, when methylamine dehydrogenase activity is necessary (Burton *et al.*, 1983). Although this result suggests that blue copper proteins are not essential as electron acceptors for methylamine dehydrogenase this conclusion is difficult to evaluate in most methylotrophs because of the difficulty of measuring low concentrations of blue

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

copper proteins. In the present work, organism 4025 has been further investigated with respect to the role of cytochromes and blue copper proteins in methanol and methylamine oxidation. It proved to be an especially suitable organism for this purpose because the large amounts of blue copper proteins facilitated their measurements in crude extracts and in periplasmic and membrane fractions.

METHODS

Growth of bacteria and preparation of soluble extracts. Maintenance of cultures and growth of organism 4025, harvesting and sonication of bacteria, and preparation of soluble fractions were all done as described previously (Lawton & Anthony, 1985). Bacteria were always harvested at the end of the exponential phase. Membrane fractions were prepared as described by Froud & Anthony (1984*b*).

SDS-PAGE. This was done by the method of Laemmli & Favre (1973) as described previously (Lawton & Anthony, 1985).

Measurement of protein, cytochromes and blue copper proteins. These methods were all described by Lawton & Anthony (1985). The concentrations of *o*-type cytochrome oxidase were calculated from reduced + CO *minus* reduced difference spectra obtained after reacting membranes with CO for 2 min. The molar absorption coefficient used was 170 mM^{-1} cm^{-1} (Daniel, 1970; Wood, 1984). In order to express the concentrations of cytochromes and blue copper proteins in terms of bacterial dry weight (as in Table 1), suspensions of bacteria of known dry weight were broken by sonication, cell debris was removed by centrifugation at low speed (6000 ***g*** for 10 min), and the supernatant liquid was separated into membrane and soluble fractions as previously described (Lawton & Anthony, 1985; Froud & Anthony, 1984*a*). The absence of detectable cytochromes in the bacterial debris indicated that more than 90% of the bacteria were broken by sonication.

Measurement of enzymes. Methylamine dehydrogenase was measured as described by Lawton & Anthony (1985). Methanol dehydrogenase was assayed spectrophotometrically by using phenazine ethosulphate and 2,6-dichlorophenolindophenol essentially as described by Anthony (1971). The reaction mixture contained, in a 1 ml cuvette (10 mm light path): 50 mM-Tris/HCl, pH 9·0; 2·5 mM-methanol; 0·55 mM-phenazine ethosulphate and 22 μM-indophenol. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were measured by the methods of Lohr & Walker (1971) and King (1974) respectively.

The oxidation of ascorbate/TMPD was assayed polarographically by using a Clark-type O_2 electrode (Rank Bros., Bottsiham, Cambs., UK) in a 2 ml reaction volume. The reaction mixture contained ascorbate (2 mM) and TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) (0·2 mM) in 25 mM-MOPS buffer pH 7·0.

Preparation of spheroplasts and periplasmic fractions. A method based on that described by Jones *et al.* (1982) was used. Organism 4025 was grown on methylamine in 1 litre batch cultures, harvested, washed and resuspended in 20 mM-Tris/HCl buffer (pH 7·5), containing 750 mM-mannitol, to a final density of 5 mg dry weight ml^{-1}. Lysozyme (0·1 ml of 10 mg ml^{-1}) was added to 8·5 ml of the bacterial suspension and the mixture was gently agitated at 30 °C for 12 min. Volumes (0·1 ml) of 100 mM-EDTA were then added at 1 min intervals and the mixture was incubated for a further 5 min before adding 0·5 ml $MgCl_2$ (1·0 M) and 0·1 ml RNAase/DNAase mixture (10 mg ml^{-1}). After 2 min the mixture was rapidly cooled on ice and centrifuged at 15000 ***g*** for 15 min to yield a clear orange-pink supernatant layer (9·6 ml) (the periplasmic fraction) and a pellet of spheroplasts. These were suspended in either 8·5 ml of 20 mM-Tris/HCl buffer (pH 7·5) containing 750 mM-mannitol or in 8·5 ml of buffer without mannitol. In the presence of mannitol the spheroplasts were stable for several hours whereas rapid lysis occurred in its absence. The lysed spheroplasts were centrifuged at 150000 ***g*** for 60 min to produce a straw-coloured cytoplasmic fraction and a red-brown membrane fraction, which was resuspended in 20 mM-Tris/HCl buffer (pH 7·5).

RESULTS AND DISCUSSION

The membrane-bound respiratory proteins of methylamine-grown organism 4025

Reduced-minus-oxidized difference spectra of membranes of organism 4025 grown on methylamine were almost identical to those measured with membranes of methanol-grown bacteria in the present work and with those described by Vrdoljak & Froud (1982) and Vrdoljak *et al.* (1984). The peaks at 520 nm and 550 nm demonstrated the presence of cytochrome *c* and the shoulders at 522 nm and 558 nm indicated the presence of *b*-type cytochromes. There was no significant absorption at wavelengths greater than 580 nm, indicating that *a*-type cytochromes and blue copper proteins were absent. These results suggested that the oxidase in organism 4025 is an *o*-type oxidase. This conclusion was supported by the demonstration that 70% of the *b*-type cytochrome reacted rapidly with CO (80% of the total reaction occurring within 15 s); the trough

Table 1. *Effect of carbon growth substrate on the electron transport components of organism 4025*

Bacteria were grown on methanol or methylamine, membrane and soluble fractions prepared, and cytochromes and blue copper proteins determined as described in Methods. In order to determine the individual cytochromes *c* and blue copper proteins it was necessary to determine the proportions of each. This was done for the cytochromes by passage through DEAE-cellulose. The proportions of amicyanin and 'azurin' were assumed to be the same as those observed after preliminary purification of these proteins as described by Lawton & Anthony (1985). The results shown are from single batches of bacteria; very similar results were found for all other batches of bacteria tested (three to five batches).

Respiratory protein	Concentration of respiratory protein [pmol (mg dry wt bacteria)$^{-1}$]	
	In methylamine-grown bacteria	In methanol-grown bacteria
Membrane-bound cytochrome *c*	435	525
Membrane-bound cytochrome *b* (total)	443	559
Cytochrome *b* reacting with CO ('*o*-type oxidase')	261	331
Cytochrome *b* not reacting with CO	182	228
Soluble cytochrome c_H	53	340
Soluble cytochrome c_L	104	160
Amicyanin	2678	0
'Azurin'	179	418

in the reduced + CO *minus* reduced difference spectrum was at 430 nm as expected for *o*-type oxidases (Poole, 1983; Wood, 1984). After more prolonged exposure to CO (10 min), spectral changes indicated the presence of some cytochrome *c* able to react with CO, as previously demonstrated by Vrdoljak & Froud (1982) in methanol-grown organism 4025. The results in Table 1 show that the concentrations of membrane-bound cytochromes were not markedly affected by the substrate used for growth of the bacteria. They also show that organism 4025 contained concentrations of cytochromes similar to other methylotrophs growing in methanol (Anthony, 1975, 1982; Widdowson & Anthony, 1975; Keevil & Anthony, 1979; Cross & Anthony, 1980; Froud & Anthony, 1984*b*; Tobari, 1984; Vrdoljak *et al.*, 1984). The concentrations of cytochrome *b* and the *o*-type oxidase were similar during growth of organism 4025 on methanol and methylamine.

The soluble cytochromes c of organism 4025 grown on methanol and on methylamine

Although the concentrations of membrane-bound cytochromes were not markedly affected by the growth substrate (above), the concentrations of all soluble redox proteins were affected (Table 1, Fig. 1). The total concentration of soluble cytochromes *c* (absorption maximum 550 nm) in methylamine-grown bacteria was only about 30% of that produced during growth on methanol. More remarkable, however, was the change in proportion of the two types of soluble cytochrome *c* that occurred when the growth substrate was changed from methanol to methylamine. As shown previously (Vrdoljak & Froud, 1982; Lawton & Anthony, 1985), organism 4025 contains cytochrome c_H, having a high isoelectric point (pI 9·4), and cytochrome c_L, having a low isoelectric point (pI 3·8). As shown in Table 1, in methylamine-grown bacteria the concentration of cytochrome c_L was 65% of that in methanol-grown bacteria but cytochrome c_H was decreased to about 16%. Studies of a related obligate methylotroph (*Methylophilus methylotrophus*) have led to the proposal that cytochrome c_L is the electron acceptor from methanol dehydrogenase and that cytochrome c_H functions as the electron donor to the *o*-type cytochrome oxidase (cytochrome *co*) (Beardmore-Gray *et al.*, 1983; Froud & Anthony, 1984*a*; Anthony, 1985). The concentration of cytochrome c_H measured in organism 4025 grown on methanol was about 80% of that measured in *M. methylotrophus* grown on this substrate (Froud & Anthony, 1984*b*), but during growth on methylamine the concentration of cytochrome c_H decreased to about 12%. These results are consistent with the conclusion that, during methanol oxidation, cytochrome c_H may play the same role in both methylotrophs, mediating between the

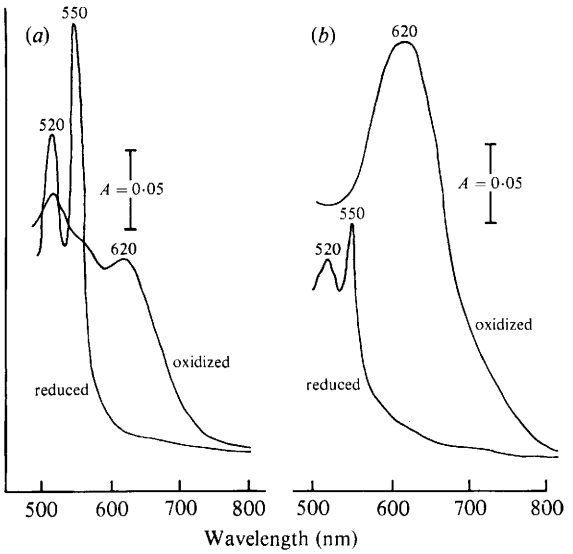

Fig. 1. Absorption spectra of crude soluble extracts of organism 4025. Extracts, prepared as described in Methods, were reduced with sodium dithionite or oxidized with potassium ferricyanide. Spectra (10 mm light path) were recorded at 20 °C. (*a*) Extract of methanol-grown bacteria (18 mg protein ml^{-1}); (*b*) extract of methylamine-grown bacteria (16·6 mg protein ml^{-1}).

cytochrome c_L and the *o*-type oxidase. The relatively low concentration of cytochrome c_H measured during growth of organism 4025 on methylamine suggests, however, that this cytochrome may play a less significant role during methylamine oxidation.

The blue copper proteins of organism 4025 grown on methanol and on methylamine

The appearance of methylamine-grown bacteria was strikingly different from that of methanol-grown bacteria. After growth on methanol, aerobic suspensions were pale pink, whereas the methylamine-grown bacteria were blue-green in colour. This difference was due to differences in the soluble components of the bacteria, as shown in Table 1 and Fig. 1. The peaks at 620 nm in both spectra were due to oxidized forms of blue copper proteins. During growth on methanol only one blue copper protein was produced. This was called 'azurin' by analogy with a blue copper protein found during growth of the facultative methylotroph *Pseudomonas* AM1 on methanol (Tobari, 1984). Its concentration was similar to that of azurins in other bacteria (for references, see below). The azurin in other bacteria functions in the reduction of nitrite during the anaerobic utilization of nitrate as a terminal electron acceptor instead of oxygen. Neither *Pseudomonas* AM1 nor organism 4025 is able to grow anaerobically with nitrate and so the function of their 'azurin' is unknown. Although it may eventually be found to play a role in methanol oxidation, it has been shown previously to have no effect on the methanol:cytochrome *c* oxidoreductase activity of methanol dehydrogenase of *M. methylotrophus* (Beardmore-Gray & Anthony, 1984).

The most marked effect of growth substrate on organism 4025 was the production of very high concentrations of amicyanin during growth on methylamine. It was 17 times the concentration of soluble cytochrome *c* and 8 times the total concentration of all the other soluble redox proteins (Table 1). This observation is clearly consistent with the conclusion that amicyanin is the electron acceptor for methylamine dehydrogenase in methylotrophs (Tobari & Harada, 1981; Tobari, 1984; Lawton & Anthony, 1985). It also explains the pronounced blue colour of whole cells of organisms 4025. What is not so obvious is why this organism produces so much amicyanin. The concentration of amicyanin in methylamine-grown organism 4025 was about 35 times that produced by methylamine-grown *Pseudomonas* AM1, the facultative methylotroph in which amicyanin and its role in methylamine oxidation was first described (Tobari & Harada, 1981). The concentration of amicyanin in organism 4025 was 10–50 times the concentration of

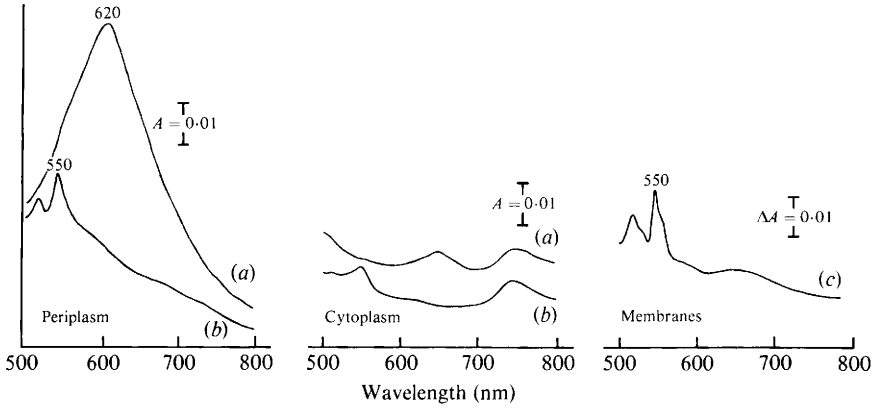

Fig. 2. Absorption spectra of membrane, cytoplasmic and periplasmic fractions of organism 4025 grown on methylamine. The fractions were prepared as described in Methods. Spectra (10 mm light path) of the periplasmic fraction (1·5 mg protein ml^{-1}) and of the cytoplasmic fraction (4·3 mg protein ml^{-1}) were recorded with water in the reference cuvette. The spectrum of the membrane fraction (5·2 mg protein ml^{-1}) is a reduced-minus-oxidized difference spectrum. (*a*) Spectrum of material oxidized with potassium ferricyanide; (*b*) spectrum of material reduced with sodium dithionite; (*c*) dithionite-reduced *minus* ferricyanide-oxidized difference spectrum.

blue copper proteins found in other bacteria (Sutherland & Wilkinson, 1963, Parr *et al.*, 1976; Cox & Boxer, 1978; Tobari, 1984). The only example that we have found of similarly high concentrations of a blue copper protein is that of rusticyanin, the periplasmic protein probably involved in the aerobic oxidation of ferrous to ferric ion in *Thiobacillus ferrooxidans* (Cox & Boxer, 1978; Ingledew, 1982).

When the amount of copper added to the growth medium was decreased from 1·0 to 0·1 mg ml^{-1} there was no change in the concentrations of soluble cytochromes *c*, but the concentrations of amicyanin (methylamine-grown bacteria) and azurin (methylamine-grown and methanol-grown bacteria) decreased by about 35%.

Location of electron transport components in methylamine-grown organism 4025

In order to determine the location of methylamine dehydrogenase, cytochromes *c* and blue copper proteins in organism 4025, methylamine-grown bacteria were fractionated by preparation of spheroplasts and periplasm, followed by controlled lysis of the spheroplasts (see Methods). SDS-PAGE demonstrated that the periplasmic fraction contained relatively few proteins. Of the six clearly distinguishable protein bands on gels, five corresponded in molecular weight to the reference proteins used: methanol dehydrogenase, methylamine dehydrogenase, cytochrome c_H, cytochrome c_L and amicyanin. However, because of the similarity in molecular weights of these cytochromes and blue copper proteins an unequivocal identification of these proteins by SDS-PAGE was not possible.

Spectra of the fractions showed that the blue copper proteins were located exclusively in the periplasmic fraction together with most of the soluble cytochrome *c* (Fig. 2, Table 2). Enzyme assays showed that methanol dehydrogenase and methylamine dehydrogenase were almost exclusively periplasmic and that glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were, as expected, cytoplasmic. Spectra of the membrane fraction showed that 40% of the cytochrome *c* of this organism was membrane-bound as found in other methylotrophs (Fig. 2). The oxidation of ascorbate/TMPD, assumed to be due to the *o*-type oxidase (see Froud & Anthony, 1984*a*), was catalysed exclusively by the membrane fraction.

The demonstration that soluble cytochromes *c* and the dehydrogenases for methanol and methylamine are located exclusively in the periplasmic fraction is consistent with previous observations in other methylotrophs (Alefounder & Ferguson, 1981; Jones *et al.*, 1982; Burton *et al.*, 1983; Kasprzak & Steenkamp, 1983; Carver & Jones, 1984). Clearly for amicyanin to be the

Table 2. *Location of respiratory chain proteins and marker enzymes in methylamine-grown organism 4025*

Periplasmic, cytoplasmic and membrane fractions were prepared, and enzymes and respiratory proteins determined as described in Methods. In these bacteria the soluble cytochrome *c* (periplasmic + cytoplasmic) was 34% cytochrome c_H and 66% cytochrome c_L; the blue copper protein was 94% amicyanin and 6% 'azurin'. The results shown are from a single batch of bacteria; almost identical results were obtained in a duplicate experiment.

	Distribution (% of total)		
Redox protein	Periplasm	Membranes	Cytoplasm
Glucose-6-phosphate dehydrogenase	0	3	97
6-Phosphogluconate dehydrogenase	0	4	96
Methanol dehydrogenase	92	2	6
Methylamine dehydrogenase	87	2	11
Ascorbate/TMPD oxidase ('cytochrome *o*')	0	100	0
Cytochrome *b*	0	100	0
Cytochrome *c*	47	40	13
Blue copper proteins	97	0	3

electron acceptor from methylamine dehydrogenase these proteins would have to be located in the same soluble compartment of the bacteria, and the results in Table 2 demonstrate that this is indeed the case.

It thus appears that the system for oxidation of methylamine in organism 4025 is similar to that for oxidation of methanol in O_2-limited *M. methylotrophus* in depending exclusively on soluble periplasmic proteins coupled to an *o*-type terminal oxidase. Whether or not any electron transport protein other than the blue copper proteins is required for this process in organism 4025, and whether or not the system in *Pseudomonas* AM1, which contains only an *a*-type oxidase, will prove to be exactly similar, remains to be seen. Of particular interest remains the nature of the system for methylamine oxidation in *M. methylotrophus*, which appears to have insignificant concentrations of amicyanin during growth on trimethylamine.

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