

## The role of blue copper proteins in the oxidation of methylamine by an obligate methylotroph

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Organism 4025, an obligate methylotroph, when grown on methylamine in the presence of a high concentration of copper, contained high concentrations of methylamine dehydrogenase and two blue copper proteins, amicyanin and an azurin-type protein; these were purified to homogeneity and characterized. The methylamine dehydrogenase is a basic protein (pI 8.8) and consists of light and heavy subunits ( $M_r$  14 100 and 43 000; total  $M_r$  112 000). This dehydrogenase differed slightly from other methylamine dehydrogenases in its absorption spectrum and in its lack of thermal stability. Amicyanin, the more abundant blue copper protein, had an  $M_r$  of 11 500, a midpoint redox potential of 294 mV at pH 7.0, and a much lower isoelectric point (pI 5.3) than other amicyanins. Its absorption maximum was 620 nm (7–24 nm higher than those of other amicyanins); its absorption coefficient (at 620 nm) was  $3.8 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ . The 'azurin' (6% of the blue copper protein) had an  $M_r$  of 12 500, a midpoint redox potential of 323 mV and a high isoelectric point (pI 9.4). Its absorption maximum was 620 nm, the absorption coefficient ( $16 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ ) at this wavelength being considerably greater than that of any blue copper protein described previously. The partially-purified soluble cytochromes  $c_H$  and  $c_L$  were similar to those of other methylotrophs. The interactions of the purified redox proteins were investigated in order to elucidate their role in methylamine oxidation. Methylamine dehydrogenase was able to donate electrons only to amicyanin, the rate of reaction being  $2.04 \text{ mmol/min per } \mu\text{mol}$  of methylamine dehydrogenase; this is sufficient to account for the rate of respiration in whole bacteria. The blue copper proteins were able to react rapidly with each other and with both the soluble cytochromes  $c$ .

Organism 4025 is a white Gram-negative obligate methylotroph, unable to grow on methane but able to grow rapidly on methanol or methylamine by way of the ribulose monophosphate pathway (Johanides *et al.*, 1979), and in these features it is similar to other obligate methylotrophs such as *Methylophilus methylotrophus* and *Methylomonas J* (Anthony, 1982, pp. 23–26). An unusual feature of organism 4025 is its response to copper in the growth medium, maximum growth being achieved only at higher copper concentrations (Vrdoljak *et al.*, 1978). During our preliminary investigations of this phenomenon, it was found that organisms grown on methylamine were blue-green in colour, indicating that they might contain very much higher concentrations of blue copper protein than any other methylotroph previously tested (S. A. Lawton & C. Anthony, unpublished work). It was

assumed that this blue copper protein either was similar in function to amicyanin, first described in the pink facultative methylotroph *Pseudomonas* AM1 (Tobari & Harada, 1981), or was similar to azurin, a protein usually associated with electron transport to nitrite, and also known to be present in some methylotrophs (Tobari, 1984). Amicyanin has been shown to be the primary electron acceptor for methylamine dehydrogenase in *Pseudomonas* AM1 and *Methylomonas J*, although it was said to be absent from trimethylamine-grown *Methylophilus methylotrophus* (Burton *et al.*, 1983).

The present paper describes an investigation of the blue copper proteins, present in high concentrations in organism 4025; these proteins were purified and characterized, and their interactions with each other and with cytochromes and methylamine dehydrogenase are described.

## Methods

### *Buffer solutions*

All buffer solutions used for preparation of bacterial extracts and for subsequent purification of blue copper proteins contained  $1.2 \mu\text{M}$ - $\text{CuSO}_4$ .

### *Growth of bacteria and preparation of soluble extracts*

Organism 4025 (ZMTF 4025), originally isolated on methanol by enrichment from soil (Vrdoljak *et al.*, 1977; Johanides *et al.*, 1979), was a gift from Dr. M. Vrdoljak (INA-Razvoj i Istrazivanje, Zagreb, Yugoslavia). Bacteria were grown on methylamine (0.5%, w/v) or methanol (1%, v/v) in batch culture on the medium of Amano *et al.* (1975) containing 5 ml of trace-element solution/l. This contained (g/l):  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2);  $\text{H}_3\text{BO}_3$  (0.003);  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  (0.02);  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.02);  $\text{Na}_2\text{MoO}_4$  (0.004);  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.53);  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.004).  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was added separately to give a final concentration in the growth medium of 1 mg/l. Growth was in stirred aerated 18-litre batch cultures in 20-litre glass vessels at  $30^\circ\text{C}$ . They were harvested by using a Sharples Super Centrifuge, washed twice at  $4^\circ\text{C}$  in 20 mM-Tris/HCl buffer, pH 8.0, and suspended in the same buffer. Bacteria (100 g wet wt.) suspended in 270 ml of buffer were disrupted by sonication (three 3 min periods), and membranes were removed by centrifugation at 380 000 g for 2 h.

### *Purification of methylamine dehydrogenase, amicyanin, 'azurin' and soluble cytochromes c from organism 4025 grown on methylamine*

The crude soluble extract was passed down a column (23 cm  $\times$  5 cm) of DEAE-cellulose equilibrated with 20 mM-Tris/HCl buffer, pH 8.0, containing  $1.2 \mu\text{M}$ - $\text{CuSO}_4$ . Methylamine dehydrogenase, amicyanin, 'azurin' and cytochrome  $c_{\text{H}}$  were not adsorbed, whereas cytochrome  $c_{\text{L}}$  was eluted by using a linear gradient of 20–300 mM-Tris/HCl buffer, pH 8.0. The failure of amicyanin to adsorb on the anion-exchange cellulose is noteworthy, because this protein was later shown to be acidic (pI 5.3). The fraction containing methylamine dehydrogenase, amicyanin, 'azurin' and cytochrome  $c_{\text{H}}$  was concentrated by ultrafiltration under  $\text{N}_2$  on an Amicon YM2 membrane (Amicon, High Wycombe, Bucks., U.K.) before it was subjected to gel filtration on a column (85 cm  $\times$  4 cm) of Sephadex G-150 equilibrated with 20 mM-Tris/HCl buffer, pH 8.0; this procedure separated the dehydrogenase from amicyanin, 'azurin' and cytochrome  $c_{\text{H}}$ . The fractions containing methylamine dehydrogenase were pooled and dialysed against 10 mM-potassium phosphate buffer, pH 7.0, and applied to a column (14 cm  $\times$  5 cm) of CM-cellulose equilibrated with the same buffer, and eluted (at 55–

65 mM-phosphate) by using a linear gradient of 10–100 mM-potassium phosphate buffer, pH 7.0. The pure dehydrogenase was dialysed against 10 mM-Mops/KOH buffer, pH 7.0, and stored at  $-17^\circ\text{C}$ .

The fractions containing amicyanin, 'azurin' and cytochrome  $c_{\text{H}}$  that had been separated from the dehydrogenase by gel filtration were pooled and dialysed against 10 mM-Mes/KOH buffer, pH 5.5, and concentrated by ultrafiltration under  $\text{N}_2$  on an Amicon YM2 membrane. This fraction was then subjected to cation-exchange chromatography on a Pharmacia FPLC Mono-S column equilibrated with 10 mM-Mes/KOH buffer, pH 5.5. 'Azurin' and cytochrome  $c_{\text{H}}$  were adsorbed, and were eluted together (at 310 mM-NaCl) by using a linear gradient of 0–1.0 M-NaCl in 10 mM-Mes/KOH buffer, pH 5.5. The 'azurin' produced by this method was about 98% pure, the single contaminating protein being cytochrome  $c_{\text{H}}$ .

Amicyanin, which was not adsorbed on the Mono-S column, was dialysed against 10 mM-Mops/KOH buffer, pH 7.0, and subjected to anion-exchange chromatography on a Pharmacia Mono-Q column equilibrated with the same buffer. Amicyanin was eluted (at 300 mM-NaCl) by using a linear gradient of 0–1.0 M-NaCl in 10 mM-Mops/KOH buffer, pH 7.0. The pure amicyanin was dialysed against the same Mops buffer and stored at  $-17^\circ\text{C}$ .

After the initial anion-exchange chromatography of the crude soluble extracts (above), the cytochrome  $c_{\text{L}}$  was concentrated by ultrafiltration under  $\text{N}_2$  as described above and dialysed against 20 mM-Mops/KOH buffer, pH 7.0, followed by gel filtration on a Sephadex G-50 column (76 cm  $\times$  2.5 cm) equilibrated with the same buffer. The pooled fractions containing cytochrome  $c$  were concentrated and subjected to further gel filtration on a Sephadex G-150 column (85 cm  $\times$  4 cm) equilibrated with 20 mM-Mops/KOH buffer, pH 7.0. The partially pure cytochrome  $c_{\text{L}}$  was stored at  $-17^\circ\text{C}$ .

### *Purification of 'azurin' from organism 4025 grown on methanol*

A crude soluble extract was prepared, as described above, from methanol-grown bacteria; this extract contained no amicyanin. It was then subjected to anion-exchange chromatography on DEAE-cellulose (15 cm  $\times$  6 cm) and gel filtration on Sephadex G-150 (85 cm  $\times$  4 cm) under conditions identical with those described for the purification of amicyanin. Because pure cytochrome  $c_{\text{H}}$  was difficult to purify, the fractions containing azurin and cytochrome  $c_{\text{H}}$ , obtained from Sephadex G-150 chromatography, were used for experiments with the methylamine dehydrogenase. The cytochrome  $c_{\text{H}}$  preparation contained

no dehydrogenases, amicyanin or cytochrome  $c_L$ ; experiments, with pure azurin, showed that methylamine dehydrogenase did not reduce azurin. The 'azurin' fraction from the gel-filtration column was then dialysed against 10 mM-potassium phosphate buffer, pH 6.0, before cation-exchange chromatography on a column (6 cm  $\times$  6 cm) of CM-cellulose equilibrated with the same buffer. 'Azurin' was eluted (at 40–50 mM) by using a linear gradient of 10–100 mM-potassium phosphate buffer, pH 6.0. The 'azurin' fraction was then dialysed against 10 mM-Mes/KOH buffer, pH 5.5, and applied to a Pharmacia FPLC cation-exchange column (Mono-S) equilibrated with the same buffer. 'Azurin' was eluted (at 750 mM-NaCl) by using a linear gradient of 0–1.0 M-NaCl in 10 mM-Mes/KOH buffer, pH 5.5. The pure protein was dialysed against 10 mM-Mops/KOH buffer, pH 7.0, and stored at  $-17^\circ\text{C}$ .

#### *Polyacrylamide-gel electrophoresis*

Sodium dodecyl sulphate/15% polyacrylamide-gel electrophoresis was performed as described by Laemmli & Favre (1973) at pH 8.3 and the gels were stained with Coomassie Brilliant Blue (R250) (Weber & Osborn, 1975). To determine the proportion of each protein in samples, peak integration was performed on gels stained with Coomassie Blue by using a Chromoscan 3 gel scanner (Joyce-Loebl, Gateshead, U.K.). This method showed that the purified methylamine dehydrogenase, amicyanin and 'azurin' constituted more than 96% of the protein present, no other peaks being visible, and the baseline integrating to 4% of the total integral.  $M_r$  values were determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the Laemmli system (above), with the following proteins as standards: insulin (5700), horse cytochrome  $c$  (11 700), lysozyme (14 000), myoglobin (17 200),  $\alpha$ -chymotrypsinogen (24 500), ovalbumin (43 000), pyruvate kinase (56 000), methanol dehydrogenase (62 000) and bovine serum albumin (67 000).

#### *Measurement of protein, methylamine dehydrogenase, amicyanin, 'azurin' and cytochromes $c$*

Protein was determined by the method of Bradford (1976), with the filtered reagent as described by Spector (1978); crystalline  $\gamma$ -globulin was used as standard. Methylamine dehydrogenase was assayed spectrophotometrically with phenazine methosulphate and 2,6-dichlorophenolindophenol as described by Eady & Large (1968), and polarographically with phenazine methosulphate, as the primary electron acceptor, and  $\text{O}_2$  at  $30^\circ\text{C}$  in an oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). The reaction contained (in a 2 ml volume) 25 mM-Mops/KOH buffer, pH 7.5,

0.5 mM-phenazine methosulphate and 10 mM-methylamine hydrochloride; the reaction was started by addition of enzyme or substrate.

Absorption spectra were recorded as described previously (O'Keeffe & Anthony, 1980), and the concentrations of blue copper proteins and cytochromes  $c$  were calculated from spectra by using the following wavelength pairs and millimolar absorption coefficients: azurin from *Pseudomonas aeruginosa* (625 nm–500 nm,  $3.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ ; Brill *et al.*, 1968); 'azurin' from organism 4025 (620 nm–500 nm,  $16 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ ); amicyanin from organism 4025 (620 nm–500 nm,  $3.8 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ ). Absorption coefficients for the cytochromes  $c$  of *Pseudomonas* AM1 and *Methylophilus methylotrophus* were taken from O'Keeffe & Anthony (1980) and Cross & Anthony (1980) respectively. The absorption coefficients for cytochromes  $c_H$  and  $c_L$  of organism 4025 were assumed to be the same as those for the *Pseudomonas* AM1 cytochromes  $c$ .

#### *Analytical isoelectric focusing*

This was performed with LKB 804-101 Ampholine polyacrylamide gels in the pH range 3.5–9.5 as described previously (O'Keeffe & Anthony, 1980).

#### *Amino acid analysis of the blue copper proteins*

This was done by Dr. M. Gore (University of Southampton) with a Rank-Hilger Chromaspec amino acid analyser after hydrolysis of the proteins (20 nmol) with 6 M-HCl at  $105^\circ\text{C}$  for 24 h in sealed evacuated ampoules. Norleucine was used as internal standard, and cytochrome  $c$  and bovine insulin were used to check recovery of amino acids.

#### *Measurement of midpoint redox potentials*

These were measured at pH 7.0 in 25 mM-Mops/KOH buffer at  $25^\circ\text{C}$  as described by Cross & Anthony (1980). Titration mixtures contained (in a total volume of 5.5 ml) 25 mM-Mops/KOH buffer, pH 7.0, 100 mM-KCl, 100  $\mu\text{M}$ -2,3,5,6-tetramethylphenylenediamine, 50  $\mu\text{M}$ -hydroquinone and 50  $\mu\text{M}$ - $\text{K}_3\text{Fe}(\text{CN})_6$ . Reductive titrations were performed with ascorbic acid, and oxidative titrations were performed with  $\text{K}_3\text{Fe}(\text{CN})_6$ .

#### *Assessment of redox proteins as electron acceptors for methylamine dehydrogenase*

Methylamine-dependent reduction by methylamine dehydrogenase of cytochromes and blue copper proteins was measured spectrophotometrically at  $22^\circ\text{C}$  at the wavelength maxima of the proteins as given above. The reaction mixtures contained (in 1 ml) 25 mM-Mops/KOH buffer, pH 7.0, pure methylamine dehydrogenase (15 nM), electron acceptor (concentration given in Table 3) and 10 mM-methylamine (added to start the reac-

tions). The specific activity of the dehydrogenase used in these experiments when measured with phenazine methosulphate as electron acceptor was 183  $\mu\text{mol}$  of dye reduced/min per  $\mu\text{mol}$  of dehydrogenase. Pure cytochromes  $c_H$  and  $c_L$  from *Methylophilus methylotrophus* were prepared as described by Cross & Anthony (1980) and were a gift from Dr. S. J. Froud (University of Southampton). Azurin from *Pseudomonas aeruginosa* was a gift from Dr. M. Gore (University of Southampton), and horse heart cytochrome  $c$  was obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.

## Results

### *Methylamine dehydrogenase of organism 4025*

The methylamine dehydrogenase was purified to homogeneity (Table 1), the specific activity of the pure protein being similar to that of other methylamine dehydrogenases tested (Eady & Large, 1968; Matsumoto, 1978; Shirai *et al.*, 1978). The results in Table 1 show that, as in other methylotrophs, the dehydrogenase constituted about 5% of the soluble protein of methylamine-grown bacteria. Its  $M_r$  value, as estimated by gel filtration, was about 112000, and it consisted of two types of subunit ( $M_r$  14000 and 43000), suggesting an  $\alpha_2\beta_2$  subunit complex similar to that of other methylamine dehydrogenases (Matsumoto *et al.*, 1978, 1980; Matsumoto & Tobar, 1978). The isoelectric point (pI 8.8) was similar to that of methylamine dehydrogenases from other obligate methylotrophs (Matsumoto, 1978). By contrast with the enzyme from *Pseudomonas* AM1, that from organism 4025 was not markedly heat-stable, 50% of activity being lost after 7 min at 80°C. As found for other methylamine dehydrogenases, its pH optimum was between pH 7.0 and 7.5.

Fig. 1 shows that the absorption spectrum of the pure dehydrogenase had an absorption maximum at 420 nm instead of the usual maximum at 430 nm; this presumably reflects a slightly unusual environment of the pyrrolo-quinoline quinone prosthetic group of this dehydrogenase (see de Beer *et al.*, 1980).

### *Soluble cytochromes c of organism 4025*

In order to investigate the potential activity of the soluble cytochromes  $c$  as electron acceptors from methylamine dehydrogenase, they were partially purified as described in the Methods section. The purified cytochrome  $c_L$  showed only one cytochrome band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and it contained no copper protein; the cytochrome  $c_H$ , although free of amicyanin and cytochrome  $c_L$ , was contaminated with 'azurin'. The cytochromes  $c_H$  and  $c_L$  from

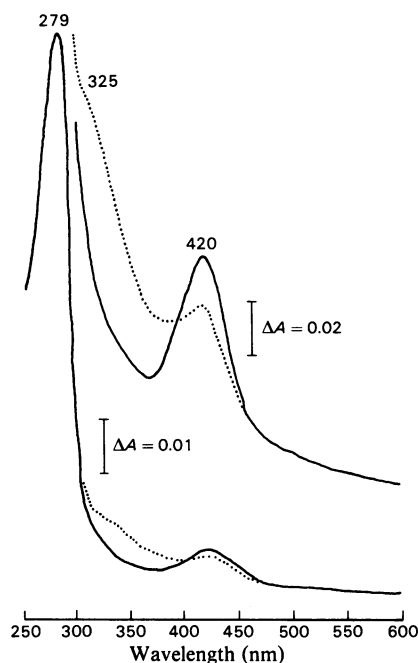


Fig. 1. Absorption spectra of pure methylamine dehydrogenase from organism 4025

The dehydrogenase was purified as described in the Methods section; after purification it was in the oxidized form. Spectra were recorded at 22°C in 25 mM-Mops buffer, pH 7.0, with the use of a 10 mm light-path; the concentration of dehydrogenase was 0.9 mg/ml. —, Spectrum of oxidized dehydrogenase; ····, spectrum of dehydrogenase reduced with 10 mM-methylamine.

Table 1. Purification of methylamine dehydrogenase from organism 4025

The dehydrogenase was assayed in an oxygen electrode with phenazine methosulphate as primary electron acceptor. Details of the assay system and of purification methods are in the Methods section.

Purification step	Volume (ml)	Total activity ( $\mu\text{mol}$ of $\text{O}_2$ /min)	Specific activity ( $\mu\text{mol}$ of $\text{O}_2$ /min per mg)	Yield (%)	Purification (fold)
Crude extract	270	410	0.034	100	1
DEAE-cellulose	400	313	0.106	76	3.1
Sephadex G-150	250	282	0.353	69	10.4
CM-cellulose	74	197	0.666	48	19.6

organism 4025 were similar in all respects examined to those from other methylotrophs (Anthony, 1982, pp 224–229). The cytochrome  $c_H$  had  $M_r$  12500 and pI9.4. The cytochrome  $c_L$  had  $M_r$  18000, pI3.8 and a midpoint redox potential of 284 mV.

#### Amicyanin of organism 4025

During growth on methylamine, organism 4025 contained two blue copper proteins, differing markedly in their isoelectric points. As shown below, methylamine dehydrogenase was specific for the acidic protein, which was therefore, by analogy with a similar protein from *Pseudomonas* AM1, called amicyanin (see Tobari & Harada, 1981). Synthesis of this protein was induced by growth on methylamine, being completely absent during growth on methanol (S. A. Lawton & C. Anthony, unpublished work).

The amicyanin from organism 4025 was purified to homogeneity as summarized in Table 2. Its  $M_r$  was shown by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to be 11500, and its isoelectric point (pI) was shown by isoelectric focusing to be 5.3, which is markedly lower than those of amicyanins from *Pseudomonas* AM1 (pI9.3) and *Methylomonas* J (pI7.7) (Tobari, 1984). Fig. 2 shows that the midpoint redox potential at pH7.0 was 294 mV. Fig. 3 shows the absorption spectrum of amicyanin, the copper-containing prosthetic group having an absorption maximum at 620 nm in the oxidized state, with an absorption coefficient of  $3.8 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ . The complex fine structure of the u.v.-absorption spectrum presumably reflects transitions of a limited number of tryptophan and tyrosine residues in individually

distinct environments within the protein, as has been shown for a number of other blue copper proteins (Iwasaki & Matsubara, 1973; Kakutani *et al.*, 1981).

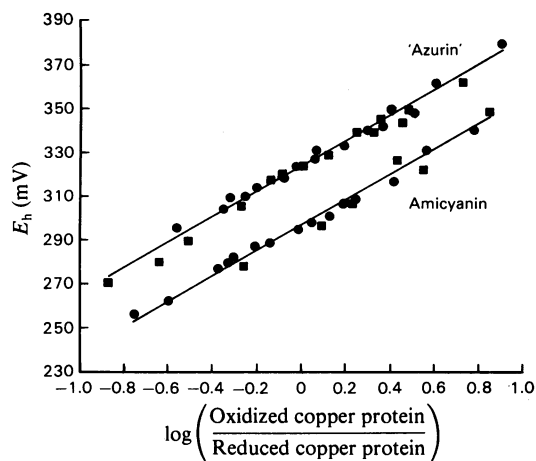


Fig. 2. Potentiometric titrations of amicyanin and 'azurin'. Amicyanin and 'azurin' were purified as described in the Methods section, and titrations were performed at 22°C in 25 mM-Mops buffer, pH7.0, as described in the Methods section. Oxidative titrations were performed with ferricyanide as oxidant (■); reductive titrations were performed with ascorbate as reductant (●). The lines on the Figure are theoretical curves for single-electron transfer reactions ( $n = 1$ ) described in the equation relating the measured redox potential ( $E_h$ ) to the standard midpoint potential ( $E_0$ ):

$$E_h = E_0 + 59 \cdot \log \left( \frac{\text{oxidized protein}}{\text{reduced protein}} \right)$$

Table 2 Purification of amicyanin and 'azurin' from organism 4025

Details of assay and purification methods are given in the Methods section. 'Azurin' was purified from methanol-grown bacteria, which contained no amicyanin. Amicyanin was purified from methylamine-grown bacteria, which also contained some 'azurin'. This was separated from amicyanin at the Mono-S stage, at which the ratio of amicyanin to 'azurin' was 15:1. This ratio was assumed to occur in crude extracts for the purposes of calculating yields and purification factors.

Purification step	Volume (ml)	Copper protein (nmol/mg of protein)	Total copper protein (nmol)	Yield (%)	Purification (fold)
<b>Amicyanin</b>					
Crude extract	80	1.53	5473	100	1
DEAE-cellulose	83	6.00	4441	69	3.9
Sephadex G-150	135	33.9	3065	56	22.2
Mono-S	77	76.5	2440	45	50.0
Mono-Q	83	96.8	2334	43	63.27
<b>'Azurin'</b>					
Crude extract	150	0.083	750	100	1
DEAE-cellulose	100	0.91	500	67	10.9
Sephadex G-150	75	2.37	398	53	28.5
CM-cellulose	84	17.26	311	41	208.0
Mono-S	92	81.3	292	32	978.6

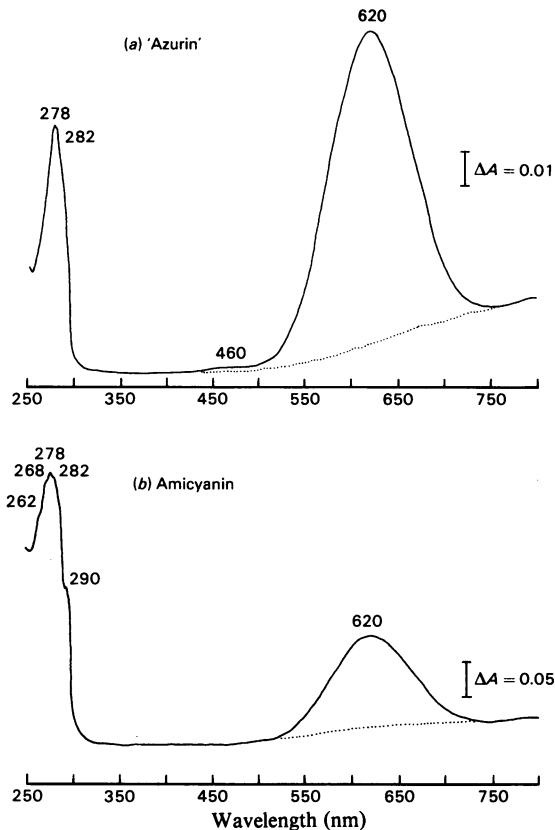


Fig. 3. Absorption spectra of blue copper proteins from organism 4025

Spectra were recorded at 22°C with the use of a 10 mm light-path. Although the blue copper proteins were in the reduced state in crude soluble fractions, after passage through DEAE-cellulose they were in the oxidized state. No oxidizing reaction was therefore necessary before the spectra of oxidized blue copper protein (—) were recorded. Reduced proteins were produced by treatment of blue copper proteins with dithionite (.....).

The amino acid composition of amicyanin was found to be as follows: Asp (18), Thr (6), Ser (7), Glu (5), Pro (3), Gly (7), Ala (8), Val (9), Met (7), Ile (2), Leu (4), Tyr (1), Phe (11), His (5), Lys (10) and Arg (3). The values in parentheses are the nearest integers. Tryptophan was not determined, but the single tyrosine residue observed in this amicyanin is consistent with the fine structure observed in the u.v.-absorption spectrum (see above). Although cysteine was not separately determined, it was observed as a peak during amino acid analysis; this corresponded to at least one cysteine residue per molecule of amicyanin. The amino acid composition of this amicyanin is clearly different from that of 'azurin' from organism 4025 (see below) and also different from that of

amicyanins and azurins from *Pseudomonas* AM1 and *Methylomonas* J (Tobari, 1984).

#### 'Azurin' of organism 4025

This basic blue copper protein was present during growth of organism 4025 on methylamine or methanol (S. A. Lawton & C. Anthony, unpublished work). It is similar in many respects to azurin from other bacteria, and so, for convenience, and to distinguish it from amicyanin, it is referred to as azurin-type or 'azurin'. It was purified to homogeneity as described in Table 2. Extracts of methanol-grown bacteria were used for its preparation in order to avoid complications due to the amicyanin always present in large amounts during growth on methylamine. Its isoelectric point was high (pI 9.4), as found for many other azurins (Sutherland & Wilkinson, 1963; Iwasaki & Matsubara, 1973; Kakutani *et al.*, 1981; Tobari, 1984), notable exceptions being the acidic azurins from *Pseudomonas aeruginosa* (Fee, 1975) and *Paracoccus denitrificans* (Martinkus *et al.*, 1980). The absorption maximum was 620 nm (Fig. 3) and the absorption coefficient at this wavelength was  $16 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ , a value that is considerably greater than those for other blue copper proteins (usually  $2\text{--}4 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ ). This result indicates that the environment, and perhaps the ligation, of the copper atom(s) in this azurin-type protein must be unusual and that it is worthy of further investigation in this respect. The  $M_r$  of 'azurin' from organism 4025 was 12500 and its midpoint redox potential at pH 7.0 was 323 mV (Fig. 2).

The amino acid composition was found to be as follows: Asp (15), Thr (8), Ser (5), Glu (8), Pro (1), Gly (6), Ala (9), Val (13), Met (6), Ile (4), Leu (5), Tyr (2), Phe (8), His (7), Lys (16) and Arg (3). Tryptophan was not determined. The values in parentheses are the nearest integers. Although cysteine was not separately determined, it was observed as a peak during amino acid analysis; this corresponded to at least one cysteine residue per molecule of 'azurin'. The amino acid composition of this 'azurin' is clearly different from that of amicyanin from organism 4025 (see above) and also different from the azurins and amicyanins from *Pseudomonas* AM1 and *Methylomonas* J (Tobari, 1984).

The 'azurin' described here (from methanol-grown bacteria) was identical with that partially purified from bacteria grown on methylamine with respect to  $M_r$ , pI and absorption spectrum.

#### Interaction of methylamine dehydrogenase and its potential electron acceptors

Methylamine dehydrogenase is a quinoprotein having as its prosthetic group pyrrolo-quinoline quinone (de Beer *et al.*, 1980; Anthony, 1982,

Table 3. Interactions of methylamine dehydrogenase from organism 4025 with electron-transfer proteins. The reduction of each potential electron acceptor was measured at 22°C in 25 mM-Mops buffer, pH 7.0, containing 15 nM-methylamine dehydrogenase, the reaction being started by addition of methylamine (20 mM). Details of the method and materials used are given in the Methods section.

Electron acceptor	Isoelectric point (pI)	Highest concentration tested ( $\mu\text{M}$ )	$K_m$ ( $\mu\text{M}$ )	$V_{\text{max}}$ ( $\mu\text{mol}/\text{min}$ per $\mu\text{mol}$ of enzyme)
Amicyanin (organism 4025)	5.3	—	20.0	2044
'Azurin' (organism 4025)	9.4	14.7	—	0
Cytochrome $c_H$ (organism 4025)	9.4	10.0	—	0
Cytochrome $c_L$ (organism 4025)	3.8	29.8	—	0
Azurin ( <i>Pseudomonas aeruginosa</i> )	4.9	—	32.3	545
Cytochrome $c_H$ ( <i>Pseudomonas</i> AM1)	8.8	27.9	—	0
Cytochrome $c_L$ ( <i>Pseudomonas</i> AM1)	4.2	8.9	—	0
Cytochrome $c_H$ ( <i>Methylophilus methylotrophus</i> )	8.9	10.2	—	0
Cytochrome $c_L$ ( <i>Methylophilus methylotrophus</i> )	4.3	9.9	—	0
Cytochrome $c$ (horse heart)	10.0	—	250	65

pp 200–206). Although it is usually assayed with an artificial dye (phenazine methosulphate) as electron acceptor, when first described by Eady & Large (1968) it was shown also to be able to react slowly with mammalian cytochrome  $c$ . Tobar & Harada (1981), using methylamine dehydrogenase from the pink facultative methylotroph *Pseudomonas* AM1, showed that a blue copper protein (named amicyanin), which was induced during growth on methylamine, was able to act as primary electron acceptor. This contrasts with methanol dehydrogenase, also having pyrrolo-quinoline quinone as its prosthetic group, which uses soluble cytochrome  $c_L$  as its electron acceptor. Table 3 summarizes the results of an investigation of the methylamine dehydrogenase of organism 4025 (an obligate methylotroph) with respect to these potential electron acceptors. It is clear that, although horse heart cytochrome  $c$  and azurin from *Pseudomonas aeruginosa* are able to react with methylamine dehydrogenase, the only physiological electron acceptor in this organism is the acidic blue copper protein, which we therefore refer to throughout this work as amicyanin. The  $K_m$  value for amicyanin was 20  $\mu\text{M}$ ; this is similar to the  $K_m$  value for amicyanin from *Pseudomonas* AM1.

When a low concentration of amicyanin (0.9  $\mu\text{M}$ ) was introduced into reaction mixtures containing methylamine dehydrogenase and high concentrations of those redox proteins not able to react directly with the dehydrogenase, these proteins became reduced at rates between 30% and 100% of the rate measured with amicyanin alone. This demonstrates that, in organism 4025, rapid electron transport can occur from amicyanin to the other blue copper protein ('azurin') and to the soluble cytochromes  $c$ . These cytochromes were also able to reduce rapidly the oxidized 'azurin'

from organism 4025, the rates of reaction being too great to measure in the conventional spectrophotometer.

## Discussion

The results described in the present paper show that, when grown on methylamine, the obligate methylotroph organism 4025 contains two high-potential blue copper proteins, amicyanin and an azurin-type protein. The amicyanin constituted 94% of the blue copper protein in these methylamine-grown bacteria, and was the only redox protein from organism 4025 able to act as electron acceptor from methylamine dehydrogenase. It differed from the amicyanin having the same function in the pink facultative methylotroph *Pseudomonas* AM1 in being an acidic protein, perhaps reflecting the importance of electrostatic interactions between the acid amicyanin and the basic dehydrogenase of organism 4025. In *Pseudomonas* AM1 an acid methylamine dehydrogenase (pI 5.2) reacts with a basic amicyanin having a pI of 9.3.

It has been shown (S. A. Lawton & C. Anthony, unpublished work) that the methylamine dehydrogenase, soluble cytochromes  $c$  and blue copper proteins are exclusively located in the periplasm of organism 4025. It can be calculated from Tables 1 and 2 that the dehydrogenase and amicyanin constitute 5% and 1.6% respectively of the soluble cell protein. If it is assumed that the periplasmic volume is 20% of the total bacterial volume, it can be shown that the concentrations of methylamine dehydrogenase and amicyanin in the periplasm are 180  $\mu\text{M}$  and 580  $\mu\text{M}$  respectively. The  $K_m$  of the dehydrogenase for amicyanin is 20  $\mu\text{M}$ , indicating that the enzyme is able to act at its maximum rate

(2.04 mmol of amicyanin reduced/min per  $\mu\text{mol}$  of dehydrogenase). Assuming that the soluble cell protein constitutes 25% of the dry weight of the bacteria, it can be calculated that the rate of respiration by way of methylamine dehydrogenase and amicyanin is 55.4 nmol of  $\text{O}_2$  consumed/min per mg dry wt. of bacteria. This is similar to the measured rate of respiration, by whole bacteria, with methylamine as substrate, which was usually between 100 and 150 nmol of  $\text{O}_2$  consumed/min per mg dry wt. Although such calculations can never be accurate, because they are based necessarily on many assumptions, this result does clearly demonstrate that the reaction between methylamine dehydrogenase and amicyanin is likely to be of physiological importance. This conclusion is supported by the observation that amicyanin is induced during growth on methylamine and that no other redox protein from organism 4025 was able to interact with methylamine dehydrogenase.

The results above demonstrated that amicyanin is able to donate electrons to the second blue copper protein, 'azurin', and to the soluble cytochromes  $c_H$  and  $c_L$ , and also that these proteins are able to react rapidly with each other. Thus, although amicyanin has been shown to be the unique electron acceptor from methylamine dehydrogenase, the route for electron transport between amicyanin and the cytochrome oxidase (cytochrome *o*) is not yet certain.

It should be noted that, although the present study clearly shows that the blue copper protein in such abundance in organism 4025 is mainly amicyanin, and that this is probably the only electron acceptor from methylamine dehydrogenase in this organism, no conclusion can be drawn with respect to the reason for the high concentrations of amicyanin produced in this organism compared with *Pseudomonas* AM1.

The demonstration that two obligate methylotrophs (organism 4025 and *Methylomonas* J) use amicyanin as electron acceptor from methylamine dehydrogenase suggests that the failure to observe blue copper proteins in the obligate methylotroph *Methylophilus methylotrophus* when grown on trimethylamine (Burton *et al.*, 1983) might be due to the difficulty of observing blue copper proteins in the presence of high concentrations of cytochrome *c*, and not due to an alternative system for electron transport from methylamine in that organism.

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