Two distinct azurins function in the electron-transport chain of the obligate methylotroph *Methylomonas J*

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Methylomonas J is an obligate methylotroph although it is unable to grow on methane. Like *Pseudomonas* AM1, it produces two blue copper proteins when growing on methylamine, one of which is the recipient of electrons from the methylamine dehydrogenase. When grown on methanol, only the other blue copper protein is produced. We have determined the amino acid sequences of these blue copper proteins, and show that they are both true azurins. The sequences are clearly homologous to those of the proteins characterized from fluorescent pseudomonads and various species of *Alcaligenes*, and can be aligned with them and with each other without the need to postulate any internal insertions or deletions in the sequences. The iso-1 azurin, the one produced during both methanol and methylamine growth, shows 59–65 % identity with these other azurins, whereas the iso-2 protein shows only 47–53 % identity. The proteins show 52 % identity with each other. The two functionally equivalent blue copper proteins from *Pseudomonas* AM1 belong to two sequence classes that are quite distinct from the true azurins. Detailed evidence for the amino acid sequences of the proteins has been deposited as Supplementary Publication SUP 50151 (23 pages) at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1989) 257, 5.

INTRODUCTION

Methylomonas J (originally known as *Pseudomonas J*) was isolated from garden soil by selection on a medium containing methylamine as the sole carbon source (Matsumoto, 1978). It is an obligate methylotroph, and can grow on either methanol or methylamine, but cannot use methane. It was unfortunately not included in a recent taxonomic study of Gram-negative methylotrophic bacteria (Jenkins & Jones, 1987). The methylamine dehydrogenase has been purified and characterized (Matsumoto, 1978; Matsumoto *et al.*, 1978), and found to be very similar to the equivalent enzyme from the pink facultative methylotroph *Pseudomonas* AM1 (Shirai *et al.*, 1978). Both enzymes probably have covalently bound pyrroloquinoline quinone as prosthetic groups (Anthony, 1982). The electron acceptors of these methylamine dehydrogenases have been studied, and found to be blue copper proteins (Tobari & Harada, 1981; Tobari, 1984). The blue copper proteins from *Pseudomonas* AM1 have been sequenced (Ambler & Tobari, 1985). The protein that is the immediate electron acceptor from the dehydrogenase belongs to a new sequence class of blue copper proteins, the amicyanins, whereas the other one is a representative of another class of blue copper proteins, the pseudoazurins, now known to occur in other methylotrophic and denitrifying bacteria. X-ray-crystallographic studies (Petratos *et al.*, 1987) have shown that the three-dimensional structure of pseudoazurin is quite similar to that of the plastocyanins that function in oxygenic photosynthesis (Colman *et al.*, 1978).

We have now determined the amino acid sequences of the two blue copper proteins found in *Methylomonas J*

(Tobari, 1984), and have been surprised to find that they are both true azurins, a class of periplasmic electron-transport protein, representatives of which have been well characterized from fluorescent pseudomonads and from various *Alcaligenes* species (Horio, 1958; Sutherland & Wilkinson, 1963; Ambler & Brown, 1967; Ambler, 1971; Adman *et al.*, 1978; Norris *et al.*, 1983). Azurins have not heretofore been identified in methylotrophs, but sequence determination rather than amino acid analysis (Lawton & Anthony, 1985) is necessary before a blue copper protein can be safely assigned to one class or another.

EXPERIMENTAL

Preparation of blue copper proteins

Methylomonas was grown on a 0.5 % (w/v) methylamine hydrochloride medium containing 0.4 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /l, as described by Ambler & Tobari (1985), at 28 °C, for 2 days. The yield was 176 g of wet cells from 60 litres of culture. The azurins were isolated from sonically disrupted cells after $(\text{NH}_4)_2\text{SO}_4$ fractionation and chromatography on DEAE-cellulose, CM-cellulose and hydroxyapatite, gel filtration through Sephadex G-75 and finally isoelectric focusing. The preparation was performed in a cold-room at 0–5 °C. The buffers used were made by mixing 1 M- KH_2PO_4 and 1 M- K_2HPO_4 in the ratios 88:12 (v/v) for pH 6.0 and 39:61 (v/v) for pH 7.0 and then diluting to the required final concentration. The blue proteins were visible as coloured bands on columns from the CM-cellulose stage onwards, and if the colour faded through the reduction of the

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chromophore it was restored by the addition of the minimal amount of $0.1 \text{ M-K}_3\text{Fe(CN)}_6$.

Wet cells (176 g) that had been stored at -15°C were homogenized with 700 ml of 5 mM-phosphate buffer, pH 7.0, and the homogenate was then sonicated. The sonicated preparation was centrifuged at 14000 g for 40 min, and the precipitate was suspended in a further 700 ml of the same buffer and again sonicated and centrifuged. The two supernatants were combined, and fractionated with $(\text{NH}_4)_2\text{SO}_4$ at pH 7, and the 45–90 % saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction was dissolved in 15 ml of 5 mM-phosphate buffer, pH 7.0, and dialysed extensively against the same buffer, with small-pore-size dialysis tubing (Spectrapor; M_r cut-off 6000–8000). The solution was passed through a DEAE-cellulose (Whatman DE-32) column ($10 \text{ cm} \times 3 \text{ cm}$) equilibrated with 5 mM-phosphate buffer, pH 7.0, through which the blue proteins passed unretarded. They were concentrated by $(\text{NH}_4)_2\text{SO}_4$ fractionation (50–90 % saturation), dialysed against 5 mM-phosphate buffer, pH 6.0, and then adsorbed on a CM-cellulose (Whatman CM-32) column ($8 \text{ cm} \times 3 \text{ cm}$ diam.). Some cytochrome *c* was not adsorbed, but the two blue proteins were adsorbed, and one (iso-2 azurin) was eluted with 10 mM-phosphate buffer, pH 6.0, and the other (iso-1 azurin) with 25 mM-phosphate buffer, pH 6.0. The blue fractions were concentrated again by $(\text{NH}_4)_2\text{SO}_4$ fractionation followed by dialysis against 5 mM-phosphate buffer, pH 7.0, and then separately chromatographed on hydroxyapatite columns ($4 \text{ cm} \times 3 \text{ cm}$ diam.). The iso-1 azurin was eluted with a linear gradient of 5–125 mM-phosphate buffer, pH 7.0, and the iso-2 azurin was eluted with a linear gradient of 5–100 mM-phosphate buffer, pH 7.0. Each protein was then subjected to gel filtration through a Sephadex G-75 (superfine grade) column ($93 \text{ cm} \times 3 \text{ cm}$ diam.) equilibrated with 20 mM-phosphate buffer, pH 7.0, containing 0.1 M-NaCl . The main peak in each case was again concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis against water before final purification on a density-gradient isoelectric-focusing column (LKB 8100-1; vol. 110 ml). For the iso-1 azurin the main Ampholine was pH 8–11, and for the iso-2 azurin pH 6–8. The main peaks from each protein were dialysed against water, and checked for purity by u.v.-visible-absorption spectroscopy and by SDS/polyacrylamide-gel electrophoresis (Tobari, 1984). The proteins were mailed to Scotland freeze-dried at ambient temperature.

The yield from 100 g of wet cells was about $6 \mu\text{mol}$ of each of the azurins.

Amino acid sequence determination

The amino acid sequences were investigated by the methods that have been used to study the structures of bacterial cytochromes *c* (Ambler & Wynn, 1973; Ambler *et al.*, 1979, 1987) and blue copper proteins (Ambler & Tobari, 1985), and to similar standards. The proteins were digested with proteinases after denaturation by performic acid oxidation or reduction and *S*-aminoethylation, and the peptides produced were fractionated by gel filtration followed by high-voltage paper electrophoresis and chromatography. The peptides were analysed quantitatively for amino acid composition and purity. Amide groups were assigned from peptide electrophoretic mobilities and exopeptidase analysis.

The copper was not removed from the protein after

Table 1. Amino acid compositions of *Methylobomonas J azurins*

The hydrolysis values are derived from multiple analyses under different conditions. The sequence values are taken from the deduced sequences shown in Figs. 1 and 2.

	Amino acid composition (mol of residue/mol of protein)			
	Iso-1 azurin		Iso-2 azurin	
	Hydro- lysis	Sequence	Hydro- lysis	Sequence
Glycine	13.3	13	13.4	13
Alanine	14.0	14	12.1	12
Valine	16.6	17	10.0	10
Leucine	5.0	5	3.9	4
Isoleucine	1.9	2	3.8	4
Serine	6.1	6	11.9	12
Threonine	10.0	10	13.1	13
Aspartic acid	15.9	8	9.9	6
Asparagine		8		4
Glutamic acid	7.1	6	8.0	8
Glutamine		1		0
Phenylalanine	5.8	6	6.8	7
Tyrosine	2.0	2	4.0	4
Tryptophan	< 0.08	0	0.5	1
Cysteine	2.8	3	2.7	3
Methionine	4.8	5	4.8	5
Proline	1.9	2	4.6	5
Lysine	14.4	14	10.1	10
Histidine	4.1	4	4.9	5
Arginine	2.0	2	3.0	3
Total		128		129

denaturation, and its presence did not interfere with proteolytic digestion. The copper co-purified with small basic aromatic peptides such as T52a and H42a from iso-2 azurin (both Phe-Lys). The copper could be removed from these peptides by treatment with washed beads of Chelex-100 (Bio-Rad laboratories, Watford, Herts, U.K.). About 100 mg of resin was used for $0.2 \mu\text{mol}$ of peptide in 0.3 ml of 0.1 M-NH_3 , and the peptide was recovered without significant loss from the supernatant while the copper was bound to the resin.

RESULTS

The amino acid compositions of the azurin preparations are shown in Table 1. The evidence for the proposed amino acid sequences is summarized in Figs. 1 and 2. Details of the peptide purifications, analyses and sequence determination experiments are given in Supplementary Publication SUP 50151. The criteria for satisfactory results and the nature and format of the supplementary information are given in previous papers (Ambler & Wynn, 1973; Ambler *et al.*, 1979).

The amino acid sequence of the iso-1 azurin (Fig. 1) was deduced by characterizing the peptides from three digests: (a) $2.4 \mu\text{mol}$ of oxidized azurin digested with thermolysin; (b) $1.8 \mu\text{mol}$ of oxidized azurin digested with trypsin; (c) $1.6 \mu\text{mol}$ of oxidized protein digested with staphylococcal proteinase. An automatic sequencer degradation of the protein for the first 33 residues has been carried out by Dr. H. Matsubara (personal com-

Fig. 1. Amino acid sequence of *Methylomonas* J iso-1 azurin

Peptides derived by digestion with thermolysin (H) or staphylococcal proteinase (F) are shown above the sequence, and by digestion with trypsin (T) below the sequence. Peptides from sub-digests are labelled with a second letter indicating the secondary method of cleavage (C, chymotrypsin; D, pseudomonad proteinase). Continuous lines indicate quantitative amino acid analyses, substandard if marked *, and particularly bad if marked **. Dashed lines indicate peptides that were recognized as being present, but were not isolated in a pure state. The peptide lines are doubled where the sequence has been determined by the dansyl phenylisothiocyanate method, with the lower line broken at residues where the identification was substandard. C-Terminal residues identified as free amino acids after removal of the remainder by phenylisothiocyanate degradation are indicated by a vertical line joining the double lines at the end of the peptide. Peptides marked † were examined by carboxypeptidase A digestion, and those marked ‡ by aminopeptidase M digestion. A peptide that has been sequenced by using an automatic instrument is marked thus: □□□□□□.

Fig. 2. Amino acid sequence of *Methylomonas* J iso-2 azurin

The abbreviations and conventions used are explained in the caption to Fig. 1.

Fig. 3. Alignment of selected azurin sequences: (1) *Pseudomonas denitrificans* N.C.I.B. 9496 (Ambler, 1971); (2) *Methylobacterium J* iso-1; (3) *Methylobacterium J* iso-2; (4) *Neisseria gonorrhoeae* H.8 outer-membrane protein, azurin domain (Gotschlich & Seiff, 1987)

Residues identical in adjacent sequences are joined by a vertical bar. The residues whose side chains form the copper ligands are marked *. At positions where three out of the four sequences shown have the same residue, the odd residue is underlined. Percentage identities between the sequences are: (1)/(2), 65; (1)/(3), 52; (1)/(4), 52; (2)/(3), 52; (2)/(4), 56; (3)/(4), 40. The considerable divergence between sequences (3) and (4) should be noted.

munication); the results were in complete agreement with those of the present investigation (Fig. 1).

The amino acid sequence of the iso-2 azurin (Fig. 2) was also deduced by characterizing the peptides from three separate digests: (a) 2.0 μ mol of oxidized azurin digested with thermolysin; (b) 2.2 μ mol of oxidized protein digested with trypsin; (c) 1.6 μ mol of *S*-aminoethylated protein digested with staphylococcal proteinase.

The staphylococcal-proteinase digests of each protein were rather unsatisfactory, not all the expected peptides being isolated in adequately pure states. A contributory cause to these poor experimental results was that by mistake the proteins had first been 'digested' with staphylococcal penicillinase before being correctly digested with the proteinase.

DISCUSSION

Purity of proteins and accuracy of proposed sequences

The amino acid sequences of the protein preparations are in satisfactory agreement with the deduced sequences (Table 1). The protein preparations were homogeneous when examined by SDS/polyacrylamide-gel electrophoresis, with both azurins running in a position indicating a slightly higher M_r than horse heart cytochrome *c*. Each protein was also homogeneous on isoelectric focusing, with observed isoelectric points of 9.6 (iso-1) and 7.7 (iso-2) (Tobari, 1984). No peptides with properties that were incompatible with the proposed sequences were isolated from any of the digests. In the iso-1 azurin sequence we have no evidence for ascribing an amide group in residue 10 rather than residue 11, although mobility evidence indicates that one residue is aspartic acid and the other is asparagine. Dr. H. Matsubara (personal communication) has identified residue 10 as asparagine and residue 11 as aspartic acid, and these assignments are shown in Fig. 1.

Amicyanins: a functional or structural class of blue copper proteins?

Several methylotrophs, namely *Pseudomonas* AM1 (Tobari & Harada, 1981), *Methylobacterium J* (Tobari, 1984) and organism 4025 (Lawton & Anthony, 1985), have been shown to produce large quantities of two periplasmic blue copper proteins, and to use them in the oxidation of methylamine.

In *Pseudomonas* AM1, Tobari & Harada (1981)

showed that one of these proteins was the primary acceptor of electrons from methylamine dehydrogenase, and it passed these electrons on either to a soluble cytochrome *c* or to the other blue copper protein (Tobari, 1984). The primary acceptor protein, which was called 'amicyanin' after the organism in which it was discovered, is not produced if the cells are grown on methanol rather than methylamine. The amino acid sequences of the two blue copper proteins from *Pseudomonas* AM1 have been determined (Ambler & Tobari, 1985), and represent examples of two new sequence classes, distinct from plastocyanin and from the well-known azurins. *Pseudomonas* AM1 amicyanin is a 99-residue protein, with the copper ligands probably His-47, Cys-86, His-89 and Met-92. The second copper protein is of the sequence class for which the name 'pseudoazurin' has been proposed. Pseudoazurins are spectrally distinct from amicyanins and true azurins, having a subsidiary absorption maximum at 450 nm as well as the broad 600 nm band characteristic of the blue copper proteins. Pseudoazurins have been found in species of *Alcaligenes* and in other denitrifying and methylotrophic bacteria, and the three-dimensional structure of the protein from *Alcaligenes faecalis* strain S-6 has been determined (Petratos *et al.*, 1987).

In the present investigation, the amino acid sequences of the functionally equivalent proteins from *Methylobacterium J* have been determined, and both have been found to have sequences of the true azurin class (Fig. 3), which can be aligned with those from denitrifying pseudomonads and from *Alcaligenes* species without the need for any internal insertions or deletions. Azurins are proteins of about 130 residues (Ambler & Brown, 1967), with the copper ligands His-46, Cys-112, His-117 and Met-121 (Adman *et al.*, 1978; Norris *et al.*, 1983), and have a disulphide bridge between residues 3 and 26.

In *Methylobacterium J*, the iso-1 azurin was produced when the cells were grown on either methylamine or methanol, and it could not function as the primary acceptor to the methylamine dehydrogenase (Tobari, 1984). The iso-2 azurin was only produced when the cells were grown on methylamine, and was a good electron acceptor from the *Methylobacterium J* methylamine dehydrogenase, functioning in apparently the same way as amicyanin in *Pseudomonas* AM1.

We propose that the name 'amicyanin' be kept for the sequence class represented by the *Pseudomonas* AM1 methylamine dehydrogenase electron acceptor, and that proteins that are clearly of the same sequence class as *Pseudomonas aeruginosa* azurin (Ambler & Brown, 1967),

like the proteins involved in methylamine oxidation in *Methylomonas J*, are called 'azurins'. The structural elements in azurins do appear to have evolutionary stability, as illustrated in a recently discovered example where an azurin gene has been co-opted for a purpose other than producing a periplasmic electron-transport protein. An outer-membrane protein from *Neisseria gonorrhoeae* has been characterized as containing an azurin sequence (Fig. 3) preceded by a lipoprotein signal peptide and 39 residues that contain a common surface antigen epitope (Gotschlich & Seiff, 1987).

The *Methylomonas J* iso-1 azurin shows 59–65% sequence identity with the 12 azurins of known sequence from *Pseudomonas*, *Bordetella* and *Alcaligenes* species. The iso-2 protein has diverged more from the other azurins (47–52% identity) and is only 52% identical with the iso-1 protein. It is seemingly more divergent than the azurin segment of the *Neisseria* outer-membrane protein (49–57% identity with the other azurins), although all these proteins must fold to give very similar structures.

Protein sequences and methylotroph taxonomy

A taxonomic survey of the Gram-negative methylotrophs has been published recently (Jenkins & Jones, 1987), but not much progress has yet been made towards relating the various groups of methylotrophs to bacteria that cannot grow on C₁ compounds. Only a few methylotrophs have been examined by the method of 16 S rRNA oligonucleotide catalogues. The organisms that have been best studied metabolically, such as *Pseudomonas* AM1, *Methylophilus methylotrophus*, *Hyphomicrobium* and *Methylomonas J*, have not yet been assigned to subdivisions of the 'purple bacteria' by Woese or his associates. Azurins occur in the fluorescent pseudomonads (gamma subdivision; Woese *et al.*, 1985) and *Alcaligenes faecalis* N.C.I.B. 8156 (beta subdivision; Woese *et al.*, 1984b), whereas pseudoazurins are found in *A. faecalis* S-6 (Hormel *et al.*, 1986) and probably in *Paracoccus denitrificans* (Martinkus *et al.*, 1980), classed by Woese *et al.* (1984a) in the alpha subdivision. As well as the blue copper proteins, *Methylomonas J* produces at least two soluble cytochromes *c* (Ohta & Tobari, 1981), but as yet there is no evidence as to which sequence classes these cytochromes belong.

The sequence of the N-terminal 33 residues of the iso-1 azurin was determined in the laboratory of Dr. H. Matsubara (Osaka) before we started the present sequence investigation. His results are in complete agreement with our subsequently determined sequence, and were very helpful to us in planning our experiments. We thank Margaret Daniel for skilled and enthusiastic assistance. The work was supported by a grant from the Science Research Council to R. P. A. We thank the Wellcome Trust for providing the WELMET Edinburgh Protein Characterisation Facility, and Dr. L. A. Fothergill-

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