The primary structures of *Pseudomonas* AM1 amicyanin and pseudoazurin

Two new sequence classes of blue copper proteins

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The amino acid sequences of two blue copper proteins from the pink facultative methylotroph *Pseudomonas* AM1 (N.C.I.B. 9133) were determined. They each consist of a single polypeptide chain and bind one copper atom. Amicyanin contains 99 and pseudoazurin 123 residues. Copper-binding sites, consisting of the side chains of two histidine, one cysteine and one methionine residues, can be recognized in each protein by analogy with azurin and plastocyanin, but the spacings of the ligand residues are different, and other sequence similarity is limited. Proteins that are in the pseudoazurin sequence class can be recognized in some strains of *Alcaligenes*, and probably also in *Paracoccus denitrificans*. Detailed evidence for the amino acid sequences of the proteins has been deposited as Supplementary Publication SUP 50130 (23 pp.) at the British Library (Lending Division), Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1985) 225, 5.

INTRODUCTION

Several small proteins are known that contain stoichiometric amounts of copper and are coloured blue. The colour is intense, and related to specific binding of one copper atom by each native polypeptide chain, and is lost if the protein is denatured. The protein-bound copper has a very characteristic e.p.r. spectrum. Such proteins are the bacterial azurins, plastocyanin from cyanobacteria and chloroplasts, and the non-chloroplast plant proteins stellacyanin and umeacyanin. Azurin and plastocyanin act, like cytochromes *c*, as soluble carriers in electron transport, and similar roles for the other small blue copper proteins are likely. The proteins have been intensively studied by spectroscopic methods, and have been the subject of structural investigation at the primary and tertiary levels. High-resolution X-ray crystallographic studies of plastocyanin and azurins have shown that in both classes of proteins the copper ligands are the side chains of two histidine, one cysteine and one methionine residues, arranged in the same order but with different spacing in the amino acid sequences. Both proteins have eight-strand β -barrel structures, although the lengths of corresponding strands differ between azurins and plastocyanins, and amino acid sequence similarity is limited. The structures, properties and relatedness of blue copper proteins have been recently reviewed (Lundeen, 1983; Rydén, 1984; Adman, 1985).

Amino acid sequence studies (Ambler, 1973, 1982) have shown that there are many structural classes of soluble cytochromes *c*, several of which have the same general 'cytochrome fold' (Dickerson, 1980), whereas others have quite different three-dimensional structures (Mathews, 1985). Examples of the same class may be found in quite different organisms, functioning in different types of electron-transport chains, and in addition cytochromes *c* of several different classes may

occur in the same organism. Tobari & Harada (1981) found that the occurrence of blue copper proteins in bacteria was similarly complex, and in the present paper we demonstrate the existence of two new classes of bacterial blue copper proteins, and discuss their distribution and structural relationship to azurin and plastocyanin.

Peel & Quayle (1961) isolated, as an airborne contaminant, a Gram-negative bacterium that could grow on C_1 compounds such as methanol, methylamine and formate as the sole carbon and energy source, and which they named *Pseudomonas* AM1. Taxonomic studies (Jenkins *et al.*, 1984) have shown that the organism is quite distinct both from the true pseudomonads and from obligate methylotrophs such as *Methylophilus methylotrophus* (the I.C.I. single-cell-protein organism), but that similar facultative methylotrophs that produce much pink carotenoid pigment are widespread (Anthony, 1982; Green & Bousfield, 1981).

Tobari & Harada (1981) studied electron transport in *Pseudomonas* AM1 when it was growing on methylamine, and showed that the electron acceptor for the methylamine dehydrogenase was a blue copper protein that they called amicyanin. This protein was not produced in significant amounts when the cells were grown on methanol, although a different blue copper protein was then synthesized. Further studies (Tobari, 1984) showed that this second protein, for which we propose the trivial name pseudoazurin, was synthesized along with amicyanin by *Pseudomonas* AM1 growing on methylamine, and the yield of pseudoazurin was enhanced if the growth medium contained additional copper.

In the present paper we report amino acid sequences for the amicyanin and pseudoazurin of *Pseudomonas* AM1, and show that they represent two new sequence classes of blue copper proteins, distinct from both azurin and plastocyanin.

EXPERIMENTAL

Preparation of blue copper proteins

Pseudomonas AM1 (= N.C.I.B. 9133) was grown on a methylamine medium, in 10-litre batches at 28 °C with 2.5 litres/min aeration, for 3 days, giving a yield of about 30 g wet wt. of cells. The medium, based on that of Jayasuriya (1955), contained (w/v): methylammonium chloride, 0.5%; Na₂HPO₄, 0.21%; KH₂PO₄, 0.14%; MgSO₄·7H₂O, 0.08%; CaCl₂·2H₂O, 0.001%; Fe₂SO₄·7H₂O, 0.0005%; MnSO₄·5H₂O, 0.00025%; Na₂MoO₄·2H₂O, 0.00025%; CuSO₄·5H₂O, 0.0001%; Silicone KM70 (Shin Etsu), 0.005% (v/v). Amicyanin and pseudoazurin were isolated from sonically disrupted cells by chromatography on DEAE-cellulose, hydroxyapatite and CM-cellulose, followed by gel filtration through Sephadex G-75, and finally by electrofocusing, as described by Tobari & Harada (1981) and Tobari (1984).

The preparation was carried out in a cold-room at 0–5 °C. The buffers used were made by mixing 1 M-KH₂PO₄ and 1 M-K₂HPO₄ 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 hydroxyapatite stage onwards, and if the colour faded through reduction of the chromophore it was restored by the addition of the minimal amount of 0.01 M-K₃Fe(CN)₆. Wet cells (100 g), which had been stored at –15 °C, were homogenized with 400 ml of 10 mM-phosphate buffer, pH 7.0, and the homogenate was then sonicated. The sonicated preparation was centrifuged at 14000 g for

40 min. The precipitate was homogenized in a further 400 ml of 10 mM-phosphate buffer, pH 7.0, sonicated and centrifuged, and the two supernatants were combined. This supernatant was fractionated with (NH₄)₂SO₄ at pH 7, and the 45–90% -saturated fraction was dissolved in 10 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 dialysed solution was clarified by centrifugation, and passed through a DEAE-cellulose (Whatman DE-32) column (10 cm × 3 cm diam.) equilibrated with 5 mM-phosphate buffer, pH 7.0. Cytochrome *c_L* (Tobari & Harada, 1981) was adsorbed, but the copper proteins passed straight through as a slightly turbid solution, which was subjected to (NH₄)₂SO₄ fractionation (50–90% saturation) and dialysis against 5 mM-phosphate buffer, pH 7.0. This solution was then applied to a hydroxyapatite column (6 cm × 3 cm diam.) equilibrated with 5 mM-phosphate buffer, pH 7.0. The blue proteins, cytochrome *c_H* (Tobari & Harada, 1981), methanol dehydrogenase and methylamine dehydrogenase (Anthony, 1982) were adsorbed at the top of the column. The column was eluted with a linear gradient of 5–100 mM-phosphate buffer, pH 7.0, the order of elution being amicyanin (about 20 mM), pseudoazurin (about 50 mM) and cytochrome *c_H* (about 80 mM). The dehydrogenases require a 10–500 mM gradient to elute them. The fractions containing the blue proteins were separately concentrated by (NH₄)₂SO₄ precipitation (50–90% saturation) and dialysed against 5 mM-phosphate buffer, pH 6.0, and then each was chromatographed on a CM-cellulose (Whatman CM-32) column (4 cm × 2 cm

Table 1. Amino acid compositions of *Pseudomonas* AM1 amicyanin and pseudoazurin

The hydrolysis values are derived from five analyses of different batches of protein hydrolysed under different conditions. Full details are given in Supplementary Publication SUP 50130. The sequence values are from the sequences of the proteins shown in Figs. 1 and 2.

Amino acid	Amino acid composition (residues/molecule)					
	Amicyanin			Pseudoazurin		
	Hydrolysis	Tobari (1984)	Sequence	Hydrolysis	Tobari (1984)	Sequence
Glycine	8.4	10	8	12.7	11	12
Alanine	10.1	11	10	10.3	10	10
Valine	11.0	13	11	14.0	14	15
Leucine	3.2	3	3	8.8	9	9
Isoleucine	3.0	3	3	3.0	3	3
Serine	5.1	6	5	2.8	3	3
Threonine	7.7	8	8	4.8	5	5
Aspartic acid	6.1	7	2	13.9	13	10
Asparagine			4			4
Glutamic acid	11.4	15	8	10.3	9	5
Glutamine			3			5
Phenylalanine	3.9	4	4	4.7	5	5
Tyrosine	2.9	2	3	2.9	3	3
Tryptophan	0.8	1	1	< 0.05	0	0
Cysteine	0.8	1	1	0.9	1	1
Methionine	2.9	2	3	6.8	5	7
Proline	7.3	8	8	6.6	6	7
Lysine	9.1	10	9	12.8	12	13
Histidine	3.1	3	3	3.2	3	3
Arginine	2.0	2	2	2.8	3	3
Total		(109)	99		(115)	123

Fig. 1. Amino acid sequence of *Pseudomonas* AM1 amicyanin

Peptides derived by digestion with thermolysin (H) are shown above the sequence, and by digestion with chymotrypsin (C) or trypsin (T) below the sequence. Peptides from subdigests are labelled with a second letter indicating the secondary method of cleavage (F, staphylococcal proteinase). Continuous lines indicate quantitative amino acid analyses, substandard if marked*, and particularly bad if marked**. Gaps are labelled where an identified peptide was lost before final characterization, or where no peptides were found. Under peptide lines, → indicates end groups and subsequent residues revealed by phenyl isothiocyanate degradation and identified by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) method, substandard if shown ->. C-Terminal residues of peptides isolated as free amino acids after removal of the remainder by phenyl isothiocyanate degradation are shown <->. Peptides marked ‡ were examined by digestion with aminopeptidase M.

Fig. 2. Amino acid sequence of *Pseudomonas* AM1 pseudoazurin

Peptides derived by cleavage of the protein with CNBr (X) are shown above the sequence. Otherwise the abbreviations and conventions used are explained in the caption to Fig. 1.

diam.) equilibrated with 5 mM-phosphate buffer, pH 6.0. Amicyanin was eluted with 20 mM-phosphate buffer, pH 6.0, and pseudoazurin with 12.5 mM-phosphate buffer, pH 6.0. Each protein was then subjected to gel filtration through a Sephadex G-75 (superfine grade) column (85 cm $\times$ 3 cm diam.) equilibrated with 20 mM-phosphate buffer, pH 7.0, containing 0.1 M-NaCl. The main peak in each case was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, dialysed against water, and applied in 5–15 ml to a density-gradient electrofocusing column (LKB 8100-1, 110 ml vol., with ampholytes: pH 9–11, 2.6 ml; pH 8–9.5, 0.24 ml; pH 6–8, 0.36 ml). Both amicyanin and pseudoazurin focused as two bands, the blue oxidized form and a colourless reduced form. The two forms were combined, converted completely into the oxidized state, dialysed against water, and checked for purity by u.v.-visible-absorption spectroscopy and by sodium dodecyl sulphate/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 1 μmol of amicyanin and 3 μmol of pseudoazurin. The yield of the latter protein was very low unless the medium contained at least 0.00001% added $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

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, 1984), and to similar standards. The proteins were denatured before digestion, either by performic acid oxidation or by reduction and *S*-aminoethylation. For the latter, the protein (2 μmol) was dissolved in 3 ml of 0.5 M-Tris/HCl buffer, pH 8.0, and 24 g of urea, 10 mg of EDTA and 25 mg of dithiothreitol were added. The mixture was kept at room temperature (20 $^\circ\text{C}$) for 16 h under N_2 , and then four 20 μl portions of ethyleneimine [Pierce (U.K.) Ltd., Cambridge, U.K.] were added at 10 min intervals. After the last addition, the mixture was incubated at 37 $^\circ\text{C}$ for 45 min, and the protein was then separated from the reactants by gel filtration through Sephadex G-25 [fine grade; column 25 cm $\times$ 1.5 cm diam., equilibrated with 5% (v/v) formic acid] and freeze-dried. After digestion, peptides were fractionated by gel filtration followed by high-voltage paper electrophoresis and chromatography, and then analysed quantitatively for amino acid composition and purity. Amide groups were assigned from peptide electrophoretic mobilities and exopeptidase analysis.

RESULTS

The amino acid compositions of the protein preparations are shown in Table 1. The evidence for the proposed amino acid sequences is summarized in Figs. 1 and 2. Details of the purification, analysis and sequence determination experiments are given in Supplementary Publication SUP 50130. 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 amicyanin (Fig. 1) was deduced by characterizing the peptides from three digests: (a) 3.0 μmol of oxidized protein digested with thermolysin; (b) 2.3 μmol of *S*-aminoethylated protein digested with trypsin; (c) 2.0 μmol of *S*-aminoethylated

Fig. 3. Sequence classes of blue copper proteins

The sequences are aligned on the cysteine residues known to be a copper ligand in plastocyanin and azurin, and presumed also to be so in amicyanin and pseudoazurin. These and the other three presumed ligands in each sequence are marked +. Also shown are the asparagine residues (N) that occur after the first ligand histidine in each sequence, and the proline residue (P) that is associated in sequence with the other three ligands. Aromatic residues that are largely or completely conserved in each sequence class are marked O, and the cysteine bridge in azurin is shown (C-C). The residues in azurin and plastocyanin are numbered according to the alignment given by Rydén (1984).

protein digested with chymotrypsin. An automatic Sequenator degradation of the protein for 29 residues has been carried out by Dr. H. Matsubara (personal communication); the results were in complete agreement with those of the present investigation (Fig. 1).

The amino acid sequence of the pseudoazurin (Fig. 2) was deduced by characterizing peptides from four digests: (a) 3.2 μ mol of oxidized protein digested with thermolysin; (b) 1.0 μ mol of native protein cloven with CNBr; (c) 1.8 μ mol of oxidized protein digested with trypsin; (d) 1.6 μ mol of oxidized protein digested with chymotrypsin. The chymotryptic digest was not very satisfactory, as there was incomplete cleavage at several sites, and poor characterization of the sequence from some parts of the sequence.

DISCUSSION

Purity of proteins and accuracy of proposed sequences

The amino acid compositions of the protein preparations are in satisfactory agreement with the deduced sequences (Table 1). The protein preparations were homogeneous when examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Tobari, 1984), and the values deduced for their M_r values before the sequences were known are in excellent agreement with those subsequently deduced from the sequences. These values were 10800 and 10710 for amicyanin and 13000 and 13380 for pseudoazurin; in each case the sequence-derived value for the apoprotein is given second. No peptides that were incompatible with the proposed sequences were isolated from any of the digests. The only unexpected proteolytic cleavage observed was partial hydrolysis of the Val-24-Arg-25 bond in the pseudoazurin (Fig. 2) in the unsatisfactory chymotryptic digest.

Sequence classes for blue copper proteins

The three-dimensional structures of azurin (Adman *et al.*, 1978; Norris *et al.*, 1983) and plastocyanin (Colman *et al.*, 1978) have been determined by X-ray crystallography, and this has enabled the residues that form the ligands to the copper atom to be recognized (Fig. 3). In azurin and plastocyanin, three of these residues, a cysteine, a histidine and a methionine, occur close together in the sequences. Similar clusters occur in both amicyanin and pseudoazurin, in each case containing the only cysteine residue in the polypeptide chain, and are likely to form the copper-binding sites. The fourth ligand is a histidine residue followed by asparagine in azurin and plastocyanin, and corresponding residues can be recognized in the amicyanin and pseudoazurin sequences (Fig. 3). The non-chloroplast plant blue copper proteins have some of these structural features (Rydén, 1984; Adman, 1985), but are not considered further in the present paper.

Both amicyanin and pseudoazurin seem to be more similar to plastocyanin in sequence than to azurin, and this similarity is most apparent in the *N*-terminal segments of the molecules, as far as the first copper-ligand histidine residue. In these regions the pattern of residues conserved in all or most plastocyanins can be seen in both amicyanin and pseudoazurin, and the sequences can be aligned with each other without the need to postulate many insertions or deletions.

The visible-absorption spectrum of *Pseudomonas* AM1

pseudoazurin has the broad maximum at about 600 nm common to all blue copper proteins, and also a subsidiary maximum at about 450 nm (Tobari, 1984). Iwasaki & Matsubara (1973) isolated an 'azurin' from *Achromobacter cycloclastes* I.A.M. 1013 that had this same type of absorption spectrum. Much of the amino acid sequence has been determined (Ambler, 1977), and it can be aligned with that of the *Pseudomonas* AM1 pseudoazurin without the need to postulate any internal insertions or deletions, and with about 50% identity between sequences. [We have been unable to grow strain I.A.M. 1013 since 1976 and it is for this reason that the sequence of its pseudoazurin remains incompletely determined. The genus *Achromobacter* is now considered to be part of *Alcaligenes* (Hendrie *et al.*, 1974).] Kakutani *et al.* (1981) isolated another protein of this spectral type from *Alcaligenes faecalis* strain S-6. The protein is an inactivating factor for a high- M_r copper-containing nitrite reductase. Dr. A. Tsugita (personal communication) has determined about two-thirds of the sequence of the molecule in one automatic degradation experiment. His results show that this protein is also a pseudoazurin. No internal insertions or deletions are needed to align the first 80 residues with the *Pseudomonas* AM1 protein, and there is more than 40% sequence identity. Martinkus *et al.* (1980) isolated an 'azurin' from *Paracoccus denitrificans* that has a pseudoazurin-type spectrum and only a single cysteine residue, and so is probably another representative of the class.

No other protein with a sequence similar to *Pseudomonas* AM1 amicyanin has yet been recognized. The blue protein from *Methylobacter* J (Tobari, 1984), which also functions as an electron acceptor for methylamine dehydrogenase, is now known to be a true azurin in sequence type (R. P. Ambler & J. Tobari, unpublished work). We propose that the name amicyanin is reserved for proteins of the sequence class shown in Fig. 3, and is not used to describe a specific catalytic activity.

Lawton & Anthony (1985) have recently reported the presence of two copper proteins in an obligate methylotroph, organism 4025. They report physical properties, and compositions for all amino acids except cyst(e)ine and tryptophan. It remains to be determined whether the proteins from this organism resemble those from *Pseudomonas* AM1 or *Methylobacter* J.

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