Auracyanin A from the thermophilic green gliding photosynthetic bacterium *Chloroflexus aurantiacus* represents an unusual class of small blue copper proteins

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Abstract

The amino acid sequence of the small copper protein auracyanin A isolated from the thermophilic photosynthetic green bacterium *Chloroflexus aurantiacus* has been determined to be a polypeptide of 139 residues. His58, Cys123, His128, and Met132 are spaced in a way to be expected if they are the evolutionary conserved metal ligands as in the known small copper proteins plastocyanin and azurin. Secondary structure prediction also indicates that auracyanin has a general β -barrel structure similar to that of azurin from *Pseudomonas aeruginosa* and plastocyanin from poplar leaves. However, auracyanin appears to have sequence characteristics of both small copper protein sequence classes. The overall similarity with a consensus sequence of azurin is roughly the same as that with a consensus sequence of plastocyanin, namely 30.5%. We suggest that auracyanin A, together with the B forms, is the first example of a new class of small copper proteins that may be descendants of an ancestral sequence to both the azurin proteins occurring in prokaryotic nonphotosynthetic bacteria and the plastocyanin proteins occurring in both prokaryotic cyanobacteria and eukaryotic algae and plants. The N-terminal sequence region 1–18 of auracyanin is remarkably rich in glycine and hydroxy amino acids, and required mass spectrometric analysis to be determined. The nature of the blocking group X is not yet known, although its mass has been determined to be 220 Da. The auracyanins are the first small blue copper proteins found and studied in anoxygenic photosynthetic bacteria and are likely to mediate electron transfer between the cytochrome *bc*₁ complex and the photosynthetic reaction center.

Keywords: auracyanins; *Chloroflexus*; cupredoxin; evolution; primary structure

The primary structures of high potential iron–sulfur proteins (Thomson, 1985), ferredoxins (Bruschi & Guerlesquin, 1988), and especially small *c*-type cytochromes (Ambler, 1982, 1991) have been extensively studied in organisms belonging to the large family of photosynthetic bacteria. Small copper-containing proteins from the phototrophic families Chromatiaceae, Chlorobiaceae, and Rhodospirillaceae, however, have not yet been reported. The isolation and some spectroscopic characteristics of a first copper-containing peripheral membrane protein, originally called “auracyanin,” from the phototrophic green gliding bacterium *Chloroflexus aurantiacus*, were reported by Trost et al. (1988). The protein was isolated by initial salt treatment of isolated membranes. Upon a further step of purification by anion exchange chromatography, two other

peripheral membrane copper-containing proteins were isolated. They were called “auracyanin B1” (22 kDa) and “auracyanin B2” (18 kDa), relative to the first auracyanin isolated, which was given the name “auracyanin A” (McManus et al., 1992). The biochemical and physical properties for the B-forms are quite similar to each other, while auracyanin A is clearly distinct. In contrast to auracyanin A, the B-forms are nearly identical glycoproteins with B-2 having an N-terminal extension of eight residues (McManus et al., 1992). There is evidence from light-induced absorbance changes and ESR signals that auracyanin A is involved in the photosynthetic process of *C. aurantiacus* as an electron donor to the photoreaction center (McManus et al., 1992). The functional role for the two other forms is not known. Kinetic data indicate that all three proteins can donate electrons to a membrane-bound cytochrome *c*-554, the direct electron donor to the photosynthetic reaction center (McManus et al., 1992). Because *Chloroflexus aurantiacus* lacks soluble *c*-type cytochromes, it was therefore suggested that auracyanin A takes the function of soluble cyto-

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chrome c_2 found in purple photosynthetic bacteria (Trost et al., 1988; McManus et al., 1992). Blue copper proteins that transfer electrons from the cytochrome b_6f complex to the reaction center of photosystem I are the plastocyanins isolated from some cyanobacteria and eukaryotic green algae (Sandmann et al., 1983) and from most plants (Haehnel, 1986).

Whereas the sequences of the auracyanins B were reported earlier, first by direct protein sequence analysis (McManus et al., 1992) and later by gene sequence analysis (NCBI #U78046), the determination of the complete amino acid sequence of auracyanin A is reported here. We propose that the sequence is that of a blue copper protein type that is ancestral to both the known sequence classes of the azurins and the plastocyanins, and that the auracyanins are the first examples of a class of cupredoxins that is different from those proposed by Rydén (1984, 1988).

Results

The complete amino acid sequence of auracyanin A, shown in Figure 1, was obtained as follows.

N-terminal sequence analysis of the native protein resulted in PTH analyses with such low yields (only 1/100 of the amount supplied) that it was concluded that the protein was nearly fully blocked for Edman degradation. A first digest after carboxymethylation of the apoprotein was carried out using *Staphylococcus aureus* V8 Glu-C endoprotease. All the major peptides obtained from the reversed-phase high-performance liquid chromatography (RP-HPLC) separation (Fig. 2A) could be sequenced except fraction S3, suggesting that this fraction was the N-terminally blocked peptide of the protein. The broad peak 12 contained a major peptide of 33 residues covering the region Leu37–Glu69, of which

Fig. 1. Sequence map of auracyanin A from *C. aurantiacus*. The proposal is based on Edman degradation analyses of peptides obtained by proteolytic cleavages with Glu-C protease (S) and Lys-C protease (K), and by partial acid hydrolysis (AH) and cyanogen bromide cleavage (CN). The N-terminal sequence of the blocked peptides S3 and K3 was determined by fast atom bombardment mass spectrometric analysis indicated as (FAB-MS). X represents the unknown blocking group. Peptides S'3P and K'3P are peptides obtained after subdigesting S'3 and K'3 with a "Pro-C" endopeptidase. Peptide S'3X was obtained after nonspecific cleavage of the pyridylethylated apoprotein with Glu-C endoprotease.

Fig. 2. HPLC separation pattern of peptides obtained after cleavage of carboxymethylated apoprotein with Glu-C endoprotease (A) and pyridylethylated apoprotein with Lys-C endoprotease (B).

only the residue at position 60 could not be determined. Plasma desorption mass analysis (3,383.1 Da) indicated that peptide S11 was still four or five residues longer than could be determined by Edman degradation (2,959.5 Da).

The second digest, this time on the pyridylethylated apoprotein, with Lys-C endoprotease (Fig. 2B), established the sequence of auracyanin A from residue 27 to residue 135 by providing overlaps of at least two residues between the sequenced Glu-C peptides. The blocked N-terminal Lys-C peptide of auracyanin A was found in fraction K3. The mass data as well as some relevant results of amino acid analyses are given in the Miniprint Tables S.I-II (see Supplementary material in Electronic Appendix).

The C-terminal sequence of auracyanin A was established after cleavage of the carboxymethylated apoprotein with cyanogen bromide (Fig. S.IA) given the downstream position of the only cys-

teine residue in the protein. As expected, the two major fractions contained a large peptide (CN2) and a small peptide (CN3). The latter was found to be blocked. Another small peptide (CN1) apparently contained seven residues, of which the first three residues overlapped the last three sequenced residues of peptide S11. Analysis on a plasma desorption mass spectrometer indicated that CN3 (740.6 Da) had the same sequence as CN1 but with N-terminal pyroglutamic acid instead of glutamine. This conclusion was further supported by amino acid analysis of peptides CN1 and CN3 (Table S.II).

The sequence determination of the N-terminally blocked region of auracyanin A up to residue 26 proved to be extremely difficult. From amino acid analysis it was clear that the blocked peptides K3 and S3 differed by only four residues (1Ile, 1Ser, 1Gly, and 1Lys) and that they had no less than 5 serines and 11 glycines in common (Table S.II). In addition, the compositional analysis of S3 revealed the presence of one proline, one valine, two threonines, and one glutamic acid. The single glutamic acid in S3 was presumed to be the C-terminal residue of the peptide so that the existence of an N-terminal pyroglutamic acid residue could be excluded. An acetylated N-terminus was another possibility for the blocking group, which was tested by treating peptide S3 with methanolic HCl. Sequence analysis of the resulting peptide mixture S3M (Table S.III) resulted in information from the C-terminal region of the peptide although the yield from Edman cycle 4 onward dropped so dramatically that the precise sequence position in S3 could not be given at that moment. We were also unsuccessful in the digestion of auracyanin A with acylase I. It was then decided to submit peptides S3 and K3 to fast atom bombardment mass spectrometry. The spectrum, given in Figure 3, indicates that the molecular ion (MH^+) of S3 had a molecular mass of 1,941.6 Da. The corresponding protonated molecular mass calculated from the amino acid composition of peptide S3 is 1,721.7 Da (monoisotopic mass), which implies that the blocking group should be 219.9 Da. The first major peak in the fragmentation pattern of S3, aside from the one at 1,924.6 Da, which represents the loss of an OH-group from the parent ion, is located at 1,721.8 Da. This is exactly 219.8 Da less than the value for the parent ion. Therefore, the latter

Fig. 3. FAB-mass spectrum of peptide S3 recorded in the range m/z 1,350 to m/z 2,000. The differences between the main peaks correspond to the residue masses of the indicated amino acids.

value very likely represents the blocking group, temporarily called "X." In this case, the peak at the m/z value of 1,721.8 indicates the molecular ion without the blocking group. The other main peaks in the fragmentation pattern at m/z values of 1,664.5, 1,607.8, 1,551.3, and 1,494.2 represent fragment ions that each are shorter by a value of 57 Da, the residual mass of glycine. The difference of 87 Da between the peaks at 1,406.7 and 1,494.2, moreover, suggests that a serine residue follows the fourth glycine, so that the N-terminal sequence of peptide S3 could be proposed to be X-GGGGS- (Fig. 1). The same N-terminal sequence can also be deduced from the mass spectrum of peptide K3 (Fig. 4) although the fragmentation for the first five residues yielded relatively small peaks. The data are summarized in Table 1. The mass spectrum of K3, in addition, yielded sequence information beyond residue Ser5 with increasing size of the signals. From all these data, the N-terminal sequence could be interpreted as X-GGGSSGGSTGGSGS-.

We also attempted to further prove the sequence obtained from the mass spectrometric analysis by a chemical approach. After preparation of a new amount of peptide S3 from an *S. aureus* digestion of the carboxymethylated apo-auracyanin A (Fig. S.IB), we treated 24 nmol of the peptide comparable to S3, called S'3, with a proline specific endopeptidase. Sequence analysis of the resulting mixture S'3P indeed clearly indicated the Val19–Glu22 sequence, peptide S'3Pa (Table S.III), but also showed the presence of a peptide resulting from the cleavage of the Ser9–Thr10 bond, peptide S'3Pb (Table S.III). Due to the overlap of the residues 1, 3, 4, and 6 of S'3Pb with the sequence deduced from the

Table 1. Fast atom bombardment mass spectrometry fragmentation of blocked peptide K3 obtained after Lys-C endoproteinase digestion on pyridylethylated apoprotein^a

Fragment	Ion	Mass values	
		Theoretical	Experimental
G17–K26	y ₁₀	1,000.57	1,001.5
S16–K26	y ₁₁	1,087.60	1,088.2
G15–K26	y ₁₂	1,144.62	1,145.4
S14–K26	y ₁₃	1,231.65	1,232.5
G13–K26	y ₁₄	1,288.67	1,289.6
G12–K26	y ₁₅	1,345.70	1,346.6
G11–K26	y ₁₆	1,402.72	1,403.6
T10–K26	y ₁₇	1,503.77	1,504.7
S9–K26	y ₁₈	1,590.80	1,591.8
G8–K26	y ₁₉	1,647.82	1,648.8
G7–K26	y ₂₀	1,704.84	1,705.8
S6–K26	y ₂₁	1,791.87	1,792.8
S5–K26	y ₂₂	1,878.89	1,880.1
G4–K26	y ₂₃	1,935.92	1,937.0
G3–K26	y ₂₄	1,992.94	1,993.6
G2–K26	y ₂₅	2,049.97	2,051.0
G1–K26	y ₂₆	2,106.99	2,108.0

^aThe measured mass values correspond to the Y-series of fragments (Biemann, 1990), as shown in Figure 3.

Fig. 4. FAB-mass spectrum of peptide K3 in the range m/z 1,000 to m/z 2,350. Only the C-terminal fragments are indicated (Y_n and W_n). A number of X and Z ions are present as well, but are not indicated. The single letter codes for amino acids indicate the successive N-terminal neutral losses of the corresponding amino acid residues. The m/z values corresponding with Y-series are shown in Table 1.

mass spectrometric analysis of K3, the complete sequence of peptide S3 could be proposed. A further support for this proposal was found by sequence analysis of the peptide fraction that eluted in front of the blocked peptide S'3 obtained in the Glu-C digestion of the pyridylethylated apoprotein (Fig. S.IIC), labeled S'3X. This peptide apparently originated from an aspecific cleavage of the Ser6–Gly7 bond and yielded sequence information for 13 out of 16 Edman cycles (Tables S.III, S.IV). A similar enzymatic sub-digest of peptide K3 with the Pro-specific enzyme also resulted in cleavage of the Pro18–Val19 bond, by virtue of peptide K'3Pa from the digestion mixture K'3P (Table S.III). The Edman degradation, moreover, confirmed the sequence IleGlySerLys at the C-terminal side of the peptide in agreement with the difference in amino acid composition found between K3 and S3. Separation of the K'3 Pro-C cleavage peptide mixture by RP-HPLC (Fig. S.IID) yielded the peptide Val19–Lys26 in a pure form (K'3P6, Table S.III). Peptide K'3P7 could not be sequenced by Edman degradation and was very likely the uncleaved blocked N-terminal peptide of auracyanin A, according to the data from its amino acid composition analysis (Table S.IV).

After solving the problem of the N-terminal sequence, we confirmed several overlaps between K and S peptides from a final chemical cleavage of the protein by partial acid hydrolysis (Fig. 1), as well as some residues that had been identified only once in peptides from the other digestions. The last overlap to be proven between peptide K3 and K2 was obtained from fraction S'0 in the *S. aureus* digestion (Fig. S.IIB).

At this last stage of the work, the sequences of most of the peptides were confirmed by precise molecular mass determination using plasma desorption and/or electrospray ionization mass spectrometers. The experimental and calculated masses for the measured peptides are listed in Table S.I. We ultimately were also able to measure the exact mass of native auracyanin A using a laser desorption mass spectrometer (Fig. 5). The measured mass of 14,128 Da agrees very well with the calculated mass from the sequence without a copper atom (13,907.5 Da) supplemented with

the mass of the blocking group (220.5 Da). There is, therefore, no glycosylation present in the auracyanin A.

The nature and the chemical structure of the blocking group remain undetermined at present. We have not been successful in removing the group from the concerned peptides or from the protein by any of the enzymatic or chemical methods that have been mentioned above. With the knowledge of the N-terminal sequence of auracyanin, we may conclude, however, that a small fraction of the polypeptide chain was not blocked. The data from the sequence analysis of the native protein (Table S.V) can indeed be interpreted in relation to this sequence starting off with Gly1. Assuming an initial yield for the automated Edman degradation of 90%, we can calculate that about 1.5% of the native auracyanin A in our starting preparation was not blocked.

Discussion

Comparison of auracyanin A to other small blue copper proteins

The polypeptide chain of auracyanin A from *C. aurantiacus* contains in its C-terminal region three residues, at the positions 123(Cys), 128(His), and 132(Met), respectively, which are interspaced as in most of the small blue copper proteins where these residues serve as three of the four copper ligands. These features thus confirm the spectroscopic evidence (Trost et al., 1988; McManus et al., 1992) that auracyanin A belongs to this family of redox proteins. With its total length of 139 amino acids, auracyanin A is 10 residues longer than the largest azurin, and 37 residues longer than the largest plastocyanin known so far. These differences in length make it necessary to start from the known three-dimensional structures of small blue copper proteins if a proper alignment for auracyanin A has to be made, which takes mutual deletions and insertions into account. Figure 6 represents the structures from *P. aeruginosa* azurin (Norris et al., 1983), poplar plastocyanin (Guss & Freeman, 1983), and *Paracoccus denitrificans* amicyanin (Durley et al., 1993). The dominant secondary elements in these proteins are known to be eight β -strands that are structurally related. These strands, shown in different colors in Figure 6, are the basis of the alignment of the two consensus sequences given in Figure 7. The consensus sequence of the prokaryotic azurin is taken from the review article of Adman (1985), which, in the absence of others, considers only proteins from nonphotosynthetic bacteria. The consensus sequence of plastocyanin is taken from a paper that discusses the sequence of *Thiobacillus versutus* amicyanin in relation to the three-dimensional structure of poplar plastocyanin (Van Beeumen et al., 1991). The X-ray structure of amicyanin from *P. denitrificans* (Durley et al., 1993) has likewise been used in the alignment of auracyanin A. An important additional element in the discussion should be that a secondary structure prediction also suggests the presence of several β -strands in auracyanin A. As given in Figure 6, they do indeed occur in the prediction.

Taking all these elements together, we suggest the alignment of auracyanin A with the other copper proteins to be as given in Figure 7. It thereby becomes evident that His58 from auracyanin A is homologous to the fourth copper binding site that is located in β -strands D (His49 in the overall consensus numbering). Auracyanin as well as amicyanin has an N-terminal extension compared to plastocyanin and azurin, but the length of this extension is different for the two proteins (see below). If the extra N-terminal

Fig. 5. Laser desorption mass spectrum of native auracyanin A. The peak of 12,361 Da is from the internal standard (horse heart cytochrome *c*), which was used for the calibration of this spectrum. The numbers following the capital letter at the top of each peak represent the number of charges for that particular m/z peak.

Fig. 6. Simplified three-dimensional structures of amicyanin, plastocyanin, and azurin as deduced from the MacImdad software (Molecular Applications Group, Palo Alto, California). The evolutionary conserved β -structures are indicated by different colors: red, β A; yellow, β B; green, β C; blue, β D; orange, β E; pink, β F; light purple, β G; dark purple, β H. The three structures are drawn separately instead of superimposed for easy observation.

stretch is not considered, the identity of auracyanin A with azurin is 30.5%, and with plastocyanin it is 30.4%. If the conservative substitutions Phe/Tyr, Ile/Leu, Glu/Asp, and Ser/Thr are included, this amounts to 37.5 and 36.8%, respectively. Similarities

with individual azurins and plastocyanins do vary slightly ($\pm 5\%$), but do not change the general impression that auracyanin A is equally related to azurin and plastocyanin. Plastocyanin also occurs in the photosynthetic pathway of cyanobacteria, but none of

Fig. 7. Alignment of auracyanin A with plastocyanin and azurin consensus sequence, and with the sequence of amicyanin from *P. denitrificans*. Conserved consensus residues for each particular class of blue copper protein are indicated in red. Vertical lines between the sequences denote identical residues; vertical broken lines denote chemically related residues. The arrows indicate the four evolutionary invariant copper ligand residues in all small copper blue proteins and the presumed functionally identical residues in the auracyanin structure. Regions of β -strands are shown by underlining and are indicated by the letters β A, β B, β C, β D, β E, β F, β G, and β H under the alignment. The β -strands of plastocyanin are as discussed in Van Beeumen et al. (1991), and those of azurin are as given in Adman (1985). The β -strands and α -helices of auracyanin are those predicted by the Chou and Fasman method (1974). The sequences of auracyanin A and amicyanin both contain additional N-terminal sequences indicated by -. The numbering is made for the overall consensus sequence. Some peptide regions for which the exact comparative position is uncertain are indicated by brackets. The last four residues of the GVDASKIS sequence appear to form a small helix and are therefore tentatively aligned with a helix region (indicated as $\alpha 2$) in azurin.

these sequences has a higher similarity to auracyanin A than does plant plastocyanin (results not shown).

It is evident from the alignment that the number of residues between the potential β -strands D and E is comparable to that in azurin, but not to that in plastocyanin. This fact alone is sufficient to state that auracyanin A is not equally, but rather more related to azurin than to the photosynthetic protein plastocyanin.

Several other features of the sequence of auracyanin A, however, lead to the conclusion that the protein is also related to plastocyanin. First, auracyanin contains only a three-residues deletion between the presumed β -strands B and C, which is typical for the short connecting loop between these strands in plastocyanin. Second, there is a deletion of three residues in the connecting loop between strands C and D in plastocyanin vs. azurin, and a comparable deletion is also found at positions 44 and 45 in auracyanin (consensus numbering). Third, auracyanin A contains the sequence IRFKNN between positions 35 and 42, which is identical to that of the plastocyanin consensus sequence in five out of the six residues. Fourth, several of the fully conserved residues in plastocyanins are also found in auracyanin: Leu16, Phe18, Gly28, Gly112, Val134, and the Asn40–Asn41 sequence of which the homologues in plastocyanin constitute the last C-terminal part of β -strand C. Fifth, auracyanin, like plastocyanin, has no disulfide bridge in the N-terminal part of the protein, whereas azurin has such a bridge between positions 5 and 29 (consensus numbering). Sixth, an important feature of plastocyanin is the presence of a negative patch at position 58–60, which is completely absent in azurin and amicyanin (Colman et al., 1978; Guss & Freeman, 1983; Meyer et al., 1993). This negative patch is known to be a recognition or binding site between cytochrome *f* and plastocyanin (He et al., 1991; Modi et al., 1992a, 1992b). In auracyanin, the possible role of a similar patch may be fulfilled by the two glutamic acid residues located at positions 58 and 60, although we should be aware that the correspondence with the upper patch in plastocyanin may be coincidental. The auracyanin residues may, indeed, be on a different location on the surface of the protein from the upper negative patch of the plastocyanin. On the other hand, some conserved residues in plastocyanins that are not found in auracyanin are Leu9 (conservative substitution by Ile), Gly14 (absent), Pro20, Pro48, Phe53, Asp54, Pro62, Ser75, Gly 96, Gly125, Ala126 (absent), and Gly127.

The conclusion from the previous data that auracyanin has features of both plastocyanin and azurin also appears from a similarity count without considering the deletions and the insertions in region 55–85. There are 34 residues in common between auracyanin and plastocyanin vs. 29 residues between auracyanin and azurin. If substitutions of conservative nature are included (Fig. 7), these numbers amount to 41 and 36, respectively.

It has previously been demonstrated that amicyanin from the nonphotosynthetic bacteria *Thiobacillus versutus* (Van Beeumen et al., 1991), *Methylobacterium extorquens* AM1 (formerly called *Pseudomonas* AM1) (Ambler & Tobari, 1985) and *P. denitrificans* (Van Spanning et al., 1990) is more related to the photosynthetic plastocyanin than to the nonphotosynthetic azurin. Auracyanin has several residues in common with amicyanin that are not found in either plastocyanin or azurin, such as Glu22–Leu23, Gly28, Thr37, Ala43, Val 54, Pro92, Phe103, Ala111, Tyr116, and Val133. We do not think, however, that amicyanin is strongly related to auracyanin. Amicyanin has even fewer residues in the connecting region between the β -strands D and E by missing an identical or similar sequence to GVDASKIS of plastocyanin. More importantly, there

are only two residues between the copper ligands Cys118 and His123 as is the case in plastocyanin, whereas there are four residues in auracyanin and azurin.

The unusual N-terminal sequence of auracyanin A

Compared to plastocyanin and azurin, auracyanin and amicyanin both have an N-terminal extension, but of different length and of different nature. The composition of the 20 extra residues of amicyanin from *P. denitrificans* compared to the plastocyanin consensus sequence is not unusual, consisting of six alanines, two aspartic acids, two prolines, one lysine, one threonine, one isoleucine, two serines, two glutamic acids, one valine, one glycine, and one phenylalanine. In contrast, the 18 extra residues in auracyanin contain 11 glycine and 5 serine residues within the first 17 amino acids, with a series of 4 glycines as the starting sequence. Runs of consecutive glycine residues are known to occur in some eukaryotic proteins, for example, near the N-terminus of human calpain (Miyake et al., 1986) and in structural proteins of the plant cell wall where a seven Gly sequence is present (Condit & Meagher, 1986). Another case of a copper protein in which a glycine rich segment occurs is that of *Neurospora crassa* laccase, where the N-terminus contains a stretch of five consecutive glycine residues (Germann et al., 1988). A glycine rich N-terminal sequence is also found in the chlorosome CsmA polypeptide of *C. aurantiacus* but the number of residues is limited to three and starts only at position 3 (Wagner-Huber et al., 1988). We have no explanation for the unusual N-terminal sequence of auracyanin A other than to assume that it introduces a degree of flexibility such as might be required for a membrane-anchored protein interacting with several other membrane proteins in a large structural complex such as the photo-reaction center.

There is no information yet on the structure of the blocking group at Gly1 of auracyanin A. The mass data for the apoprotein and/or the N-terminal peptides indicate that the mass of the blocking group is 220 Da. Although we have no experimental evidence to make such an analogy, we would like to mention that 220 Da is exactly the value that corresponds to the structure acetyl-N-cysteine-S-glycerol, in which the sulfur atom is thioether linked to the glycerol, as in lipoproteins (Kawula et al., 1987). This structure, although esterified with fatty acids at the two remaining hydroxyl functions of the glycerol, also occurs at the N-terminus of the tetraheme reaction center cytochrome *c* from *Rhodospseudomonas viridis* (Weyer et al., 1987) and at the N-terminus of the azurin-like domain in the H8 protein of pathogenic *Neisseria*. We suggest that most of the auracyanin A is membrane bound, and that only the portion of the protein in which the fatty acids were cleaved was solubilized in our preparation.

Comparison to the auracyanins B

The two other small blue copper proteins from *C. aurantiacus*, the auracyanins B-1 and B-2 (McManus et al., 1992), have 36% overall sequence identity with auracyanin A, as can be calculated from the alignment given in Figure 8. Nearly all the residues that we discussed above in the comparison of auracyanin A with the three other copper proteins in Figure 7 also occur in the B-forms, so that the conclusions made are valid for the family of auracyanins as a whole. The two B-forms only differ by an N-terminal sequence stretch of eight residues, which suggests that they were released from the membrane by proteolysis. Both forms have another five

Fig. 8. Sequence alignment of the three different auracyanin forms. The data for the B-forms are the translated residues deduced from the gene sequence (NCBI #U78046), which corrects the data obtained on the protein themselves (McManus et al., 1992). Identical residues are boxed.

residues extension compared to the N-terminus of auracyanin A. The subsequent 19 amino acids of the auracyanins B are totally different from the unusual glycine-rich N-terminal sequence of auracyanin A. A two-residue and one-residue deletion in auracyanin A and a one-residue deletion in the auracyanins B are required to align the two forms of the protein. The one-residue deletion in auracyanin A results from the fact that there are only three residues between the His and the Met copper ligands (positions 123 and 128, consensus numbering, Fig. 7), whereas there are four in the auracyanins B. This is as also occurs in the plastocyanins whereas the prevalence of three residues between these ligands is typical for azurins. Likewise, as in azurins, there are four residues between the Cys and His copper ligands (positions 118 and 123) in both the auracyanins, whereas there are only two residues in the plastocyanins. On the basis of the two-residue insertion at the positions 63 and 64 (Fig. 8), the auracyanins B are unique in the family of the small blue copper proteins as a whole (Fig. 7). In the N-terminal regions 1–65 and 85–123, only 16% of the residues are identical, whereas the identity in the copper binding regions 66–84 and 124–155 is 58%. The high similarity in the copper-binding regions may explain why all three different forms can transfer electrons to the membrane-bound cytochrome *c*-554, a direct electron donor to the photosynthetic reaction center of *C. aurantiacus* (McManus et al., 1992). It should also be noted that auracyanin A, in contrast to the auracyanins B, is not glycosylated, despite the presence of the two possible glycosylation sites at positions 51 and 104 (Fig. 1).

Evolutionary position of auracyanin A

Auracyanin A studied here and the auracyanins B1 and B2 studied in the past (McManus et al., 1992) are the first examples of small blue copper proteins found in anoxygenic phototrophic bacteria, although the *Rhodobacter capsulatus* genome sequencing project

(<http://capsulapedia.uchicago.edu>) has recently identified a pseudo-azurin gene (RRCO2187). All the other cupredoxins were isolated from oxygenic cyanobacteria and plants (plastocyanins) and from nonphototrophic bacteria (azurins, pseudoazurins, and amicyanins). A phylogenetic tree built using the distance matrix method PROTDIST is given in Figure 9. The sequence alignments used to construct this tree (Fig. S.II) are based on the consensus sequence alignment given in Figure 7, which was based on the structural comparisons made in Figure 6. The tree is in general agreement with that obtained by Rydén and Hunt (1993), who suggested that an ancestor of azurin diverged to give rise to the amicyanin/plastocyanin/pseudoazurin branch, following the deletion of a large loop of residues. The phylogenetic tree presented in Figure 9 also suggests that an ancestor of azurin diverged to generate plastocyanin, amicyanin, and pseudoazurin. The clustering of azurins and plastocyanins is also in agreement with the structural similarity tree recently derived from a superposition of 23 cupredoxin domains (Murphy et al., 1997).

We can conclude from Figure 9 that the auracyanins clearly cluster together, and that they appear to be more related to the

Fig. 9. Phylogenetic tree from the distance matrix method PROTDIST. The number at the forks indicate the number of times the group consisting of the species that are to the right of that fork occurred among the trees, out of 100 trees.

plastocyanins than to the azurins. This conclusion is in agreement with biochemical data, which show auracyanins to have a role in photosynthesis similar to that of the plastocyanins. Auracyanins, indeed, have been suggested to be electron donors to the photo-reaction center of *Chloroflexus* (McManus et al., 1992), similar to plastocyanin, which is known to mediate electron transfer between P700 of photosystem PS1 and the cytochrome *b/f* complex. There is a fundamental difference, however, between the photosynthetic apparatus of plants and cyanobacteria, and that of *Chloroflexus*. Whereas the former use two different, but linked, photosystems (PSII and PSI), which according to a recent paper (Schubert et al., 1998) have a common evolutionary origin, *Chloroflexus* uses a single cyclic photosystem. On the basis of structural, kinetic, and reaction center sequence data, the cyclic system of *Chloroflexus* is closely related to the single photosystem of the purple bacteria (Blankenship, 1994), which is generally accepted, on the basis of its constituting cofactors (pheophytins and quinones) to resemble PSII of cyanobacteria and plants. The direct electron donor to the photosynthetic reaction center in most photosynthetic bacteria is a monoheme soluble cytochrome c_2 , which couples it to the *b/c*₁ complex. In some purple bacteria, the cytochrome c_2 delivers its electrons to an intervening reaction center bound tetraheme cytochrome *c*. In certain cases, a high potential iron-sulfur protein can also donate electrons to the reaction center bound cytochrome (Hochkoeppler et al., 1996). One of the unusual properties of *C. aurantiacus* is that although it does have a tetraheme cytochrome, it lacks a monoheme cytochrome *c*. Through its similarity with the plastocyanins, the presently determined sequence is consistent with the suggestion that auracyanin A fills the void in the probable electron transfer scheme of *Chloroflexus* due to absence of cytochrome c_2 (McManus et al., 1992).

The foregoing discussion, in our view, is not in contradiction with the fact that auracyanin A has a few structural features that clearly distinguishes it from plastocyanins, but which it rather has in common with the nonphotosynthetic azurins. It concerns the long stretch of residues that occurs as an alpha-helix between the beta-strands D and E in azurins (Fig. 6), and which are also predicted in auracyanin to be alpha-helical (Fig. 7). The phylogenetic tree shown in Figure 9 indicates that auracyanins may have branched very close to the base of the tree. In this evolutionary scenario, the peptide stretch between the beta-strands D and E in plastocyanins (and also in auracyanins) must have undergone a reduction in size, as well as a complete spatial reorientation (Fig. 6). Three-dimensional structural analysis will show whether the presumed helix in auracyanin A is oriented in the same way as in azurins.

It should also be noted that *Chloroflexus*, according to 16S rRNA analysis, is the earliest branching organism within the eubacteria that exhibits the photosynthetic phenotype (Woese, 1987). In having the characteristics of both the plastocyanins and azurins, the amino acid sequences of the auracyanins corroborate this finding; the fact that azurins have only been found in nonphotosynthetic proteobacteria is of no concern in this respect, because most of these bacteria are evolutionary related to the purple photosynthetic bacteria. The genetic resemblance of the reaction center genes of *Chloroflexus* to those of the purple bacteria has previously been explained by lateral gene transfer (Blankenship, 1992), although it has later been proposed that an evolutionary scheme for the pheophytin-quinone-type reaction center (PSII) would remove the need for invoking lateral gene transfer (Blankenship, 1994). The present analysis of the auracyanins does not add to the discussion on either one of the two possibilities, although it may be

reasoned that the detection of a blue copper protein in a purple bacterium favors the theory of gene transfer of the reaction center genes and of cotransfer of the copper protein gene. As mentioned above, the recent genome analysis of the purple nonsulfur bacterium *R. capsulatus* has shown that such a protein, a pseudoazurin (and thus not an azurin), may exist. In our phylogenetic tree (Fig. 9), pseudoazurins appear to be more related to plastocyanins than to the azurins. It would therefore represent yet another gene transfer event. Gene transfer must also have played a role in the evolutionary position of halocyanin, isolated from a supposed archaeobacterium. Halocyanin is located at the plastocyanin branch (Fig. 9), yet archaeobacteria, on the basis of 16S RNA, are said to be only distantly related to the eubacteria. It is evident, in our view, that a universal tree of life (Woese, 1987) based upon single genes cannot be representative of the whole genome when gene transfer is taken into account. The study of the evolution of organisms will require the careful analysis of complete genome sequences. The importance of gene transfer, especially in the early events of evolution when the "genetic temperatures" were very high, has recently also been stressed by Woese himself (1998).

Materials and methods

Derivatization of the apoprotein

Native protein was denatured with 6 M guanidium-HCl at pH 8.2 and treated with DTT to remove the copper. After removal of the metal, 86 nmol of apoprotein were carboxymethylated with iodoacetic acid following the method described by Crestfield et al. (1963). In a late stage of the work, 101 nmol of the apoprotein were derivatized with 4-vinylpyridine following the method of ABI User's Bulletin No.38 (1989) (Perkin-Elmer, Applied Biosystems Division, Foster City, California).

Proteolytic digests

Digestions of the apoprotein with *S. aureus* Glu-C endoproteinase (Miles, Slough, United Kingdom) were carried out three times on 20, 42, and 24 nmol of material using an enzyme/substrate ratio of 1/40 (mass/mass). Incubation times at 37 °C were, respectively, 16, 18, and 16.5 h, and the buffer used was 0.05 M NH₄Ac, pH 4.0. Digestions with Lys-C endoproteinase (Wako, Osaka, Japan) were also carried out three times on 36, 42, and 23 nmol of apoprotein, using an E/S ratio of 1/40 (mass/mass). The buffer used was 0.05 M or 0.1 M Tris-HCl, pH 9.0, and incubated at 37 °C for 4 h.

Subdigestion of the N-terminally blocked peptides S3 and K3 was carried out using a proline specific endopeptidase from *Flavobacterium meningosepticum* (Sekagaku Kogyo Co., Ltd., Tokyo, Japan) at an E/S ratio of 1/40 during 2 h at 30 °C in 0.1 M NH₄HCO₃, pH 7.0.

Chemical cleavage

About 12 nmol apoprotein with carboxymethylated cysteine underwent cyanogen bromide cleavage in 70% HCOOH at 30 °C for 24 h. The resulting reaction mixture was then diluted with 10 vol of Milli Q water and lyophilized. The lyophilisate was dissolved again with a small volume of water before it was separated by RP-HPLC.

Partial hydrolysis of about 15 nmol pyridylethylated apoprotein was carried out in 2% HCOOH at 110 °C for 2 h.

A subcleavage of 10 nmol peptide S3, brought onto a sequencer glass fiber filter of 1.2 cm diameter, was carried out using methanolic hydrochloride (sequencer reagent from Applied Biosystems, Foster City, California) during 16 h at 37 °C in liquid phase.

Peptide separations

Most of the separations of the proteolytic digests were carried out by RP-HPLC on a Vydac 2/4TP54 column of 4.6×250 mm (Vydac, Hesperia, California), and some separations were done on a PTC C-18 column of 2.1×220 mm. The solvents used to make the elution gradient were 0.1% trifluoroacetic acid in Milli Q water (solvent A) and 0.1% trifluoroacetic acid in 70% acetonitrile (solvent B). The course of the gradient is drawn on each of the figures. The chromatographic equipment consisted of a DuPont 870 three-piston pump controlled by a 8800 Gradient Controller, a Rheodyne 7120 injection valve with a 200 μ L sample loop, a DuPont UV spectrophotometer and a DuPont double pen recorder. The peptides obtained from the subdigest were likewise separated by RP-HPLC but now using a PTC C18 column of 2.1×220 mm. The chromatography was carried on a 130A Separation System (Perkin-Elmer, Applied Biosystems Division).

Sequence and amino acid analyses

Automated Edman degradation was carried out on a pulsed liquid sequenator with on-line analysis of the PTH-amino acids on a 120A Analyzer (Perkin-Elmer, Applied Biosystems Division). Reagents and solvents for sequence analyses were purchased from the sequenator manufacturer.

For amino acid analysis, we used the 420A Derivatizer with on-line analysis of the phenylthiocarbamyl derivatives at 254 nm on a 130A Separation System (Perkin-Elmer, Applied Biosystems Division). Samples were hydrolyzed for 24 h at 106 °C in the gas phase with 6 N HCl using 5×50 mm glass tubes placed in a hydrolysis vial (Millipore, Milford, Massachusetts).

Mass spectrometry analyses

Mass spectra were recorded on a JEOL JMS HX110 instrument equipped with the DA5000 data system. Fast Bombardment (FAB) was applied for desorption/ionization of the sample by use of the JEOL Xenon atom gun operated at 6 kV. The instrument was operated at 10-kV accelerating voltage. Glycerol was used as FAB-matrix. The starting m/z value of 1,000 was chosen to decrease the scan range and so to enhance the sensitivity.

Plasma desorption mass spectrometric analyses of some samples were performed on a Bio-Ion 20K instrument (BioIon, Uppsala, Sweden). Samples were applied on the in-house prepared nitrocellulose targets in 0.1% trifluoroacetic acid.

Native auracyanin was measured on a LASERMAT (Finnigan MAT, San Jose, California) at an accelerating voltage of 25 kV, using sinapinic acid as matrix.

Phylogenetic analysis

The comparison of auracyanin with the other blue copper proteins was done by aligning the sequences of some of the blue copper proteins using the program CLUSTALW and by the construction of phylogenetic trees. The sequences, their source, and their abbreviations used are as follows:

auracyanin A—*C. aurantiacus*—aurA cfx
 auracyanin B1—*C. aurantiacus*—aurB1 cfx
 azurin—*Neisseria gonorrhoea*—az nei.go
 azurin—*Pseudomonas fluorescens* (bio typeB)—az ps.flu
 azurin—*Pseudomonas denitrificans*—az ps.den
 azurin—*Pseudomonas aeruginosa*—az ps.aer
 azurin—*Bordetella bronchiseptica*—az bo.bor
 azurin—*Alcaligenes denitrificans*—az al.den
 azurin—*Alcaligenes faecalis*—az al.fae
 azurin—*Alcaligenes species*—az al.spe
 azurin—*Methylobacterium* j iso-2—az me.ji2
 plastocyanin—Spinach—pc spin
 plastocyanin—Potato—pc pota
 plastocyanin—Poplar (Lambdody)—pc pop
 plastocyanin—Green alga (*Enteromorpha prolifera*)—pc gre
 plastocyanin—*Chlorella fusca*—pc ch.fus
 plastocyanin—*Anabena variabilis*—pc an. var
 amicyanin—*P. denitrificans*—am pa.den
 amicyanin—*Thiobacillus versutus*—am th.ver
 amicyanin—*M. extorquens* AM1—am me. ex
 pseudoazurin—*M. extorquens* AM1—paz me.ex
 phytyocyanin—Stellacyanin—Lacquer tree—stc l.tre
 phytyocyanin—Cucumber basic protein—Cucumber—cbp cucu
 rusticyanin—*Thiobacillus ferrooxidans*—rsc th.fe
 halocyanin—*Natronobacterium pharaonis*—hc archi
 ascorbate oxidase—Zucchini squash—asc z.sq

The C-terminal domain of ascorbate oxidase, a distantly related blue copper protein, was included as an outgroup in the phylogenetic tree. The lengths of the sequences were adjusted by trimming so that no large N- or C-terminal overhangs existed. Phylogenetic analysis was carried out by the distance matrix method PROTDIST from the computer program PHYLIP 3.5c (Felsenstein, 1994). The bootstrap method was used to estimate the reliability of the results obtained in various phylogenetic analyses. The program SEQBOOT was used to generate multiple data sets by making random replacement of characters (amino acids), so that the resulting data set had the same size as the original. The phylogenetic trees were then analyzed by the program CONVERSE to calculate the percentage of replicates that gave a particular branch. An extra step was added for calculating the distance matrices from each of the replicate data sets. The bootstrapped data sets were run on the program NEIGHBOR and the output was used to run PROTDIST. Then the CONSENSE program was used to analyze the phylogenetic trees.

Supplementary material in Electronic Appendix

Supplementary material in electronic appendix Table S.I gives the mass spectrometric data for the peptides generated by several proteolytic cleavages of derivatized apoprotein. Table S.II reports the amino acid composition of five peptides crucial to the complete sequence determination. Table S.III gives quantitative data on the N-terminally blocked peptides K3 and S3 after they underwent chemical and/or enzymatic cleavages. Table S.IV summarizes the amino acid composition analysis data of blocked peptide K'3 after digestion with a Pro-specific proteinase. Table S.V gives the quantitative data for the Edman degradation of native auracyanin A. Figure S1 represents the HPLC separation of CNBr-peptides (A) and Glu-C peptides (B) from carboxymethylated apoprotein, of Glu-C peptides from pyridylethylated apoprotein (C), and of Pro-C

peptides from peptide K'3 (D). Figure S.II represents the alignment of auracyanins A and B-1 with several other blue copper proteins.

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