Inorganica Chimica Acta, 56 (1981) 149–156

Modeling Blue Copper Proteins: A Structure for Stellacyanin

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Received May 23, 1981

The loop of chelating residues places constraints on the metal ion environment in proteins. The high reduction potentials of the class of blue copper proteins that make possible their electron transfer function, as well as the variations in the reduction potentials of the different blue copper proteins, are related to this effect. A structure is proposed for stellacyanin on this basis with the aid of Chou-Fasman rules for secondary-structure assignment.

Introduction

The blue copper proteins from plant (plastocyanin) and bacterial (azurin) sources have intrigued inorganic chemists for some years [1]. Stellacyanin is a blue, single-copper protein that has similar physico-chemical properties to plastocyanin and azurin but which differs from them mainly in having a much lower electrode potential [1]. Of the electron transfer proteins stellacyanin has no methionine residue, which rules out this amino acid as an essential component of the copper active site. To date two other structures have been proposed for the copper core of stellacyanin, structures which are compared in the Discussion.

In this paper a different copper core and a folding scheme are proposed for the polypeptide chain of stellacyanin, based on the expectation that the tertiary structure of all of these proteins will be mostly conserved even if the ligands at the copper site are not. It is contended that the entatic state theory [2] of a tetrahedrally distorted copper site which does not change geometry during cycles of oxidation and reduction does not wholly explain electron transferability at the copper active site. In fact, the flattened tetrahedral geometry of the blue copper site has been found to be insufficient to explain the magnitudes of the reduction potentials of these proteins by Gray *et al.* [3].

Stellacyanin. The peptide backbone, cysteine-87 and histidine-92 as copper ligands, and the beta–carbon atoms of the residues in the segment bridged by the disulfide bond of cystine-87-93 which are cysteine-87, glycine, valine, proline, lysine, histidine and cysteine-93 are shown. The disulfide bond is in the lower center of the picture with cysteine-93 in the front and to the left. The six-residue chelate loop that connects the copper ion, cysteine-87 and histidine-92 forms the arch in the foreground. The copper ion is in the back of the disulfide bond and it is partially blocked by it in this view. Part of the imidazole ring of histidine-92 and its $-CH_2-$ group is seen through the arch. The chelate ring through the disulfide bond is in the lower front (copper, sulfur of cysteine-87, cysteine-93, histidine-92). A hydrogen atom of the imidazole ring of the second histidine ligand of copper (histidine-46) is visible in the back just under the sulfur atom of cysteine-87. The alpha-carbon and the backbone carbonyl oxygen of proline-90 are seen at the top of the arch in the forefront. A part of the proline ring is visible behind them above and below the arch. The $-CH_2-$group at the top left is the beta-carbon atom of a lysine side chain.

Procedure

With the crystallographic determination of the copper ion site in azurin [4] and plastocyanin [5], the characterization of copper environments in the blue copper proteins has gained a new dimension. It is now established, in agreement with McLendon and Martell's [6] earlier suggestion, that the CO_2-

TABLE I. Some chelate loops in a polypeptide segment that may exist in copper proteins are shown. It is assumed that one of the ligands is an imidazole ring nitrogen from a histidine side chain in all cases. A sulfur atom from cysteine or methionine, or an imidazole ring nitrogen from another histidine, was chosen as the second ligand. Non-blue copper proteins whose primary structures are known are included in the examples if there is information on the copper environment. The references are given in the parentheses.

Number of residues in chelate loop	Can it exist?	Does it exist?
2	no	no
3	yes	bovine superoxide dismutase(8); may exist in yeast superoxide dismutase (47).
4	yes	plastocyanin(5, 31, 46).
5	yes	azurin(4,9).
6	yes	plastocyanin(5, 31, 46); azurin(4, 9); may exist in stellacyanin (this work); may exist in tyrosinase (48).

TABLE II. Fragments of the primary structures of *Phaesolus vulgaris* (french bean) plastocyanin, *Pseudomonas aeruginosa* azurin and *Rhus vernificera* stellacyanin are shown [7, 10]. The known ligands of copper in azurin [4] and plastocyanin [5] and the suggested ligands in stellacyanin in this work and previously [10, 15, 16] are indicated by their numbers along the polypeptide chain. The conserved residues in various azurins and plastocyanins are underlined. The alignment that is shown brings together probable homologous residues (see also references 7, 10 and 16). For the one-letter notation, see the legend for Table III.

plastocyanin	*H-37 N* V *V F D* E . .
azurin	*H-46 N* W *V* L S T . .
stellacyanin	H-46 N V D K V T Q K N Y Q S C-59 . .
plastocyanin	*C-84* S – *P* – *H-87* E *G A G M-92* . .
azurin	*C-112* T *F P G H-117* S – A L *M-121* .
stellacyanin	C-87 G V P K H-92 C-93 D L G Q K V H-100 . .

terminal region of the polypeptide chain contributes three ligands to the copper site. These ligands are located several residues apart in strand 7 and at the turn of strand 8 [4, 5]. Remarkably, the copper environment shows resemblance to a copper chelate. Amino acid sequence data [7] and space-filling molecular models* (scaled to 1.25 cm per Angstrom and having standard bond lengths and angles) were used to construct the copper sites present in azurins and plastocyanins. Then, starting with no assumptions as to whether the segment is part of a helical, beta sheet, beta turn or random coil region, potential chelate loops that may form between a copper ion, a coordinated imidazole ring from a histidine residue and another imidazole nitrogen or a sulfur ligand from cysteine or methionine were checked. These loops are listed in Table I. These ligands were chosen because they have been shown to be present in azurin and plastocyanin by crystallography [4, 5], and also because they can all coordinate to copper(I) as well as copper(II) ions. According to Table I there is a limiting chelate loop size of three residues, with two intervening peptide links, between the coordinated atoms for a copper ion to be able to coordinate to the functional groups of the chosen amino acid side chains of the same polypeptide chain. This limiting loop size does not occur in the blue copper proteins, but does in bovine superoxide dismutase, whose crystal structure has been determined [8]. In this latter structure two histidine residues, separated by a valine, are both part of the coordination environment of its copper ion. A chelate loop of four residues is actually present in plastocyanin, a loop of five residues in azurin, and one of six residues in both azurin and plastocyanin (Table I).

The region of chelate loops involve in azurin (cysteine)$(x)_2$(proline)(x)(histidine)$(x)_3$(methionine), and in plastocyanin (cysteine)(x)(proline)(histidine)$(x)_4$-(methionine), where cysteine, histidine and methionine are three of the four copper ligands (see Table II). There appears to be no strain built into any of these loops (defined as distortion from planarity of trans peptide links in model-building), and interestingly the crystal structures of plastocyanin and azurin show rather strain-free, approximately tetrahedral copper(II) environments [9]. The homologous residues in the region of chelate loops at the CO_2-terminal end of the polypeptide chain and in the region of the histidine near the middle of the chain that acts as the fourth copper ligand are shown in

*CPK molecular models (The Ealing Corporation, South Natick, Massachusetts) are scaled to van der Waals sizes for approximately spherical atoms with standard bond lengths and bond angles for those atoms.

TABLE III. Chou-Fasman [11, 12] assignments of secondary structure for stellacyanin. The assigned structure for the segment is shown in the last column. The amino acid sequence data is from Bergman *et al.* [10]. The one-letter notation for amino acids that is employed was prepared by the IUPAC-IUB Commission on Biochemical Nomenclature and was published in *Pure Appl. Chem., 31,* 639 (1972).

Segment	Side chains	Chou-Fasman assignments for structural type		Final assignment for the segment
		helical	beta sheet	
1–6	TVYTVG	ihbihB	hHhhHi	beta strand
7–10	DSAG	iiHB	ibIi	beta turn
11–21	WKVPFFGDVDY	hIhBhhBihib	hbHbHHiiHih	beta strand
22–26	DWKWA	IhIhH	ihbhI	helical
27–30	SNKT	ibIi	bbbh	beta turn
31–40	FHIGDVLVFK	hhIBihHhhI	HbHiiHhHHb	beta strand
41–44	YDRR	biii	hiii	beta turn
45–51	FHNVDKV	hhbhiIh	HbbHibH	helical
52–55	TQKN	ihIb	hhbb	beta turn
56–59	YQSC	bhii	hhbh	beta strand
60–69	NDTTPIASYN	biiiBIHibb	bihhbHIbhb	random coil
70–73	TG(B)(B)	iB(i/b)(i/b)	hi(i/b)(i/b)	beta turn
74–80	RINLKTV	iIbHIih	iHbhbhH	beta strand
81–84	GQKY	BhIb	ihbh	beta turn
85–87	YIC	bIi	hHh	beta strand
88–91	GVPK	BhBI	iHbb	beta turn
92–106	HCDLGQKVHIN	hiiHBhIhhIb	bhihihbHbHb	beta strand
	VTVR	hihi	HhHi	
107	S	i	b	–

Table II. The primary structure of stellacyanin was recently determined [10]: bringing it into correspondence with the amino acid sequences of azurin and plastocyanin presents a problem, as the only cysteine residue in stellacyanin appears somewhere in the middle of the chain (Table II). Nor are there any residues close to cysteine-59 of stellacyanin that show homology to the cysteine-containing copper-binding site close to the CO_2-terminal end of the protein chain in azurin and plastocyanin [10]. As this portion of the chain contributes three ligands to the copper-binding site (strands 7 and 8 in azurin and plastocyanin [4, 5]), similar folding of the protein chain in stellacyanin would require that this end of the chain be also a copper-binding site. Here it is important to note that the constellation cysteine··· proline···histidine···methionine··· is conserved in all blue single-copper proteins whose primary structures have been determined [7], and is also conserved in stellacyanin which lacks methionine in the form (cysteine-87)$(x)_2$(proline-90)(x)(histidine-92)-(cysteine-93) [10]. Space-filling models indicate that the cysteine-87 end of the disulfide cystine-87-93 (but not cysteine-93) can form a strain-free bond to copper (as defined in this paper), assuming histidine-92 to be a ligand on the basis of homology (Table II). To check for the possibility that the histidine-46, cysteine-59, cysteine-87 ends of the disulfide and histidine-92 can actually coordinate to the same copper center in stellacyanin, a secondary structure was assigned to the polypeptide chain on the basis of Chou-Fasman rules [11, 12]. Using the assignments of Table III, the protein backbone was built with perfect tetrahedral (sp^3) and trigonal planar (sp^2) angles as an approximation to the suggested structure.

Results

The secondary structure assignments shown in Table III allow the protein chain to fold into a beta-barrel structure (Fig. 1) as in azurin and plastocyanin [4, 5]. This brings cysteine-59, histidine-46, histidine-92 and cystine-87-93 into a copper environment close to the top of the barrel. The proposed tertiary structure consists of seven beta strands, rather than eight, as was found to be present in azurin and in plastocyanin [4, 5]. In stellacyanin, in contrast to both azurin and plastocyanin, the histidine ligand that is not in the CO_2-terminal region appears to be part of a helical section, rather than a beta strand. The large chelate loop between histidine-46 and cysteine-59 is envisioned in Fig. 1 to be connected to the

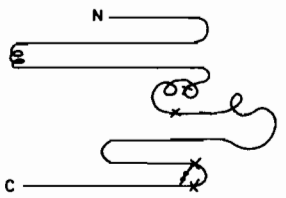

Fig. 1. The Folding Scheme for Stellacyanin. (X) indicates an expected copper ligand (histidine-46, cysteine-59, cysteine-87, histidine-92). The disulfide bond that is between cysteine-87 and cysteine-93 is indicated by (–•–•–). To check the scheme that is shown, the backbone of stellacyanin was built with plastic tubing cut to the approximate bond lengths [17] as indicated:

C_{alpha}–$C_{carbonyl}$: 1.53 Å (3.0 cm)
$C_{carbonyl}$–N_{amide}: 1.32 Å (2.6 cm)
N_{amide}–C_{alpha}: 1.47 Å (3.0 cm)

Exactly tetrahedral angles for virtual alpha-carbon atoms and exactly trigonal planar angles for virtual carbonyl–carbon and amide–nitrogen atoms of the peptide bond were used.

other two ligands of the CO_2-terminal region by an outside random coil region followed by beta strands. The assignment of cysteine-87 as the last residue of a short beta strand in Table III, with the intervening glycine, valine, proline and lysine residues forming a beta turn and histidine-92 as the first residue of the last beta strand, allows a chelate loop to form between cysteine-87, histidine-92 and the copper ion (see photograph). This folding scheme places the disulfide bond such that the copper ion may be exposed to solvent in that direction, which would explain the higher kinetic accessibility of stellacyanin [13]. The presence of the disulfide in the coordination environment of copper leads to the copper-cysteine-histidine loop, as discussed, and also forms an 11-atom chelate ring* between copper and the coordinated atoms (photograph). The effect of this is to orient the copper ion and histidine-92 with respect to the last two beta strands shown in Fig. 1. This observation is consistent with the rhombic EPR spectrum of stellacyanin [1] and is also supported by the ENDOR study of Hoffman *et al.* [14] who found that stellacyanin must have two asymmetrically arranged imidazole ligands. As the NMR study by Hill and Lee [15] indicates that there are only two histidine ligands in stellacyanin, a copper core of histidine-46, histidine-92, cysteine-59 and cysteine-87 end of cystine-87-93 is consistent with all experimentally determined facts about stellacyanin.

*In this paper a chelate loop is considered to have formed when donor atoms from two protein residues along a single polypeptide chain (usually separated by a few peptide bonds) ligate to a metal center. All other chelate rings that may form, such as by hydrogen-bonding are called chelate rings.

Discussion

Proposed Copper Cores for Stellacyanin

The reason why the reduction potential of stellacyanin is much lower than those of plastocyanin and azurin (+184 mV for *Rhus vernificera* stellacyanin as compared to +330 mV for *Pseudomonas aeruginosa* azurin and +347 mV for *Phaesolus vulgaris* (french bean) plastocyanin [3]) requires an explanation not provided by the entatic state theory of Vallee and Williams [2]. The ligand field stabilization energies of these proteins are very similar, calculated to be -6152 cm^{-1} for *Rhus vernificera* stellacyanin, -6113 cm^{-1} for *Phaesolus vulgaris* (french bean) plastocyanin, and -6885 cm^{-1} for *Pseudomonas aeruginosa* azurin for a tetrahedral copper site with a small tetragonal distortion in all three of these proteins [3]. Thus, the lower reduction potential of stellacyanin must arise from some fact other than the immediate geometry of the copper ion. The two previous models for the copper core of stellacyanin suggested that the substitution of another ligand for methionine in stellacyanin might account for its lower reduction potential [15, 16]. These models will now be described.

In the model of Ryden and Lundgreen [16], the single free cysteine (cysteine-59) and, on the basis of homology considerations, histidine-46 and histidine-92 were proposed to be copper ligands, as in this paper. Ryden and Lundgreen [16] suggest that, in the evolutionary development of this protein, the lack of a methionine residue in the CO_2-terminal copper-binding region may have been compensated by a third histidine ligand (histidine-100). This replacement of the (CuN_2S_2) core of azurins and plastocyanins by a (CuN_3S) core in stellacyanin was considered to lower its reduction potential. Model-building of this portion of the protein backbone indicates that a chelate loop can form about copper with histidine-92 and histidine-100 as ligands, if the disulfide does not coordinate. However, NMR evidence indicates that there are two histidine ligands in stellacyanin [15], and if histidine-46 is a ligand based on homology (Table II), then either histidine-92 or histidine-100 can not be a ligand. Again on the basis of homology (Table II), histidine-92 is likely to be the copper ligand.

In the model of Hill and Lee [15], who carried out the NMR investigation referred to, histidine-46, histidine-92, cysteine-87 and cysteine-93 (with no disulfide bond) are suggested to be the four ligands, giving a (CuN_2S_2) core with a charged cysteine residue replacing methionine, which is presumed to lower the reduction potential of stellacyanin. This

argument assumes that the disulfide bond forms in the apoprotein after the removal of copper. As disulfides in proteins are quantitated by taking the difference between the number of cysteine residues before and after breaking any disulfide bonds that may be present, the two possibilities can not be distinguished [17].

This model is unlikely to be correct for two major reasons. As indicated in Table I, an unstrained chelate loop can not form between copper and two protein residues (such as a histidine and a cysteine) that are located next to each other. Assuming such a bond to form, with cysteine-93 at a large distance from copper to relieve strain in the protein backbone, the chelate loops through each of these cysteines may account for the rhombicity of the EPR spectrum of stellacyanin, as suggested in [15]. However, the expansion in the radius of the copper ion after reduction would increase the strain in the structure rather than lower it. Also, this model is unable to explain why the single free cysteine of stellacyanin is not a ligand.

In the model proposed in the present paper, maximum similarity to the copper-binding regions of plastocyanins and azurins was assumed. Therefore, the region of an intact disulfide (cystine-87-93) which includes histidine-92 was expected to be a major copper-binding site. Assuming only one end of the disulfide to be a copper ligand on limiting loop size considerations (Table I), the disulfide was considered to replace the methionine ligand of azurin and plastocyanin. The (CuN_2S_2) core that may form in this way (with histidine-46, cysteine-59, histidine-92 and cysteine-87 as the ligands) is qualitatively different, due to the presence of the disulfide, to the methionine-containing (CuN_2S_2) cores of azurins and plastocyanins.

There is some support for a disulfide ligand in stellacyanin in the resonance Raman data of Ferris *et al.* [18]. Also, as was pointed out above, if the direction of access to copper is through the disulfide bond (see Fig. 1) rather than through the histidine ligand in the CO_2-terminal region [4, 5], this may make the copper ion of stellacyanin more accessible to reactants, as has been previously observed [13].

Copper-sulfur bond lengths in two model compounds and in the blue copper proteins

Two copper(II)-thioether complexes have been synthesized [19, 20] and characterized crystallographically in both +1 and +2 oxidation states of copper [21–23]. Both of these compounds have high positive reduction potentials not usually observed in copper(II) complexes. In square pyramidal (perchlorato)(1,8-bis(2-pyridyl)-3,6-dithiaoctane)copper(II) perchlorate [21], the copper(II)–sulfur bond lengths are 2.311 and 2.316 Å, and copper(II)–nitrogen bond lengths are normal (2.011 and 2.008 Å). As is often the case in square pyramidal copper(II) complexes [24], the copper(II) ion is displaced (by 0.25 Å) from the equatorial plane of sulfur and nitrogen donors towards the apical perchlorate ligand. The copper(II)–oxygen(perchlorate) bond length is 2.264 Å. In tetrahedral (1,8-bis(2-pyridyl)-3,6-dithiaoctane)copper(I) hexafluorophosphate [21], the copper–sulfur and copper–nitrogen bond lengths are changed to 2.345 Å for copper(I)–sulfur and 2.042 Å for copper(I)–nitrogen. The reduction potential of the copper(II) complex is +0.58 V.

In contrast to the open-ring dithioether complex just described, the closed-ring complex, (1,4,8,11-tetrathiacylotetradecane)copper(II) diperchlorate is perfectly square planar, with SCuS angles of 90° for all adjacent sulfur donors [22]. The copper(II)–sulfur bond length is 2.303 Å and is very similar to those in the open-ring dithioether copper(II) complex. When the closed-ring copper(II) complex, which has a reduction potential of 0.45 V [20], is reduced a polymeric tetrahedral structure results, in which three of the four sulfur atoms remain bonded to the same copper ion [23]. These SCuS bond angles are now shifted from 90° to 88.8 and 106.3° for adjacent sulfur atoms (The remaining SCuS angle was 180° in the copper(II) complex and is changed to 129.7° in the tetrahedral copper(I) complex). These copper(I)–sulfur bond lengths are 2.260, 2.338 and 2.327 Å. The fourth site in the irregular tetrahedron around copper is occupied by a sulfur atom from another ligand molecule at a slightly longer bond length of 2.342 Å. The average value of the SCuS angle this sulfur makes with the other three sulfur donors is 110°. Thus, the change in geometry at the copper ion from equatorial sulfur donors to approximately tetrahedral sulfur donors is accompanied by a 0.031 Å lengthening in the copper–sulfur bond length (and a similar change in the copper–nitrogen(pyridil) bond lengths) in the open-chain complex. In the closed-chain complex which can not accommodate a tetrahedral structure, there is a more drastic change in the copper ion environment which is not reflected in the copper-sulfur bond lengths (there is a lengthening of 0.014 Å in the average copper–sulfur bond length upon reduction). The magnitudes of these copper–sulfur bond lengths are similar to those observed in other copper(II) and copper(I) complexes with sulfur ligands which fall, usually, in the range 2.3–2.4 Å when coordinated equatorially in copper(II) compounds and tetrahedrally in copper(I) compounds. With charged thiolato ligands, a shorter copper–sulfur bond length is expected in the copper(II) complex with the same ligand. On the other hand, it has been suggested that in thioether complexes, the presence of empty pi antibonding orbitals on sulfur may compensate for the change in the radius of the

copper ion in going from +2 to +1 oxidation state, and a somewhat longer copper–sulfur bond length may actually be observed in the copper(II) complex [25]. A longer average copper–sulfur bond length is found for example in (perchlorato)(1,8-bis(2-pyridyl-3,6-dithiaoctane)copper(II) perchlorate as compared to that in its copper(I) complex as discussed above (0.031 Å longer), and also in bis(2,5-dithiahexane) complexes of copper(I), copper(II), and an intermediate complex [26] in which the copper–sulfur bond length is essentially unchanged in the intermediate complex and is lengthened by 0.057–0.091 Å in the copper(II) complex from 2.263 to an average bond length of 2.337 Å. However, the authors' suggestion [26] that this may reflect the result of the change in the coordination geometry of copper from tetrahedral to distorted octahedral is probably valid. In the more rigid closed-chain tetrathioether copper(I) complex, also discussed above, the copper–sulfur bond lengths are normal (any strain being absorbed into the chelate rings), and are shorter by 0.014 Å from the average copper-sulfur bond length in the reduced structure. This is almost certain to result from the exigencies of bonding of this closed-chain tetradentate ligand. Thus, despite the various arguments that may apply in specific cases, the steric limitations around copper remain the deciding factor. This point is likely to apply to copper cores in proteins as well.

The copper–ligand bond lengths in azurin have been studied by ESCA [27, 28]. A three-wave fit with two different copper–sulfur bond lengths did not affect the copper–nitrogen bond length (1.97 Å) but changed the single copper–sulfur bond length (reported to be 2.12 Å in the two-wave fit) to 2.10 and 2.24 Å [27]. Of these, 2.24 Å is a somewhat short bond length likely to be associated with the methionine ligand in azurin. The remarkably short 2.10 Å copper–sulfur(cysteine) bond is unusual (The parametrization used in the ESCA study on oxidized azurin gave the bond lengths, for potassium (hydrotris(3,5-dimethyl-1-pyrazolyl)-borate(*p*-nitrobenzenethiolato)cuprate(−1), with a tetrahedral (CuN_3S) core [29], of 2.05 Å (copper(I)–nitrogen(pyrazolyl)) and 2.19 Å (copper(I)–sulfur(thiolate))). In the reduced protein, a 0.1 Å lengthening of the copper–sulfur(cysteine) bond length was reported [28], bringing this bond length closer to the normal range.

The very short copper(II)–sulfur(cysteine) bond length was also found to be present in stellacyanin and plastocyanin [30]. This bond distance was corroborated by the crystal structure determination of plastocyanin at 1.6 Å resolution at 16% refinement [31]. It appears, therefore, that this unusually short cysteinyl sulfur to copper(II) bond is present in blue single-copper electron transfer proteins which are all characterized by very intense charge transfer bands in the visible region due to this cysteinate ligand [32].

The copper(II)–sulfur(methionine) bond length in plastocyanin differs from that in azurin significantly, and is about 2.9 Å long [31]. Thus, the bonding of methionine to copper in these two proteins is not equivalent despite the similarity of the copper environment both with respect to geometry and to ligands [3–5]. This, it is suggested, may arise from the differences in the chelate loops that form around copper (see Table I). The copper(I)–sulfur(methionine) bond may reasonably be expected to be shorter than 2.9 Å in reduced plastocyanin.

The differences in the coordination environment of stellacyanin, both with respect to the ligands and to chelate loops, make conclusions about this protein difficult to make from what is found to be the case for azurin or plastocyanin. It may be expected that cysteine-59 gives rise to the intense charge transfer bands in the 600-nm region and is bound to the copper(II) ion at a very short bond distance. If, as appears likely, this short bond length is maintained in azurin and in plastocyanin by the two chelate loops about copper, hydrogen-bonds in the very large loop between histidine-46 and cysteine-59 (some of which may connect to the CO_2-terminal region) may form chelate rings which may account for this very short copper(II)–sulfur(cysteine-59) bond length (note that in plastocyanin the side chain carbonyl of asparagine-38 forms a hydrogen-bond to the backbone amide of serine-85, thus forming a chelate ring [31] in addition to the two loops listed in Table I).

As for a disulfide ligand in stellacyanin, only two compounds of copper(II) that contain ligands with disulfide bonds have been characterizedy by crystallography. These are tetrasodium (glutathione disulfide)dicuprate(4–) hexahydrate [33] and diaquabis-(*D*-penicillamine disulfide)dicopper septahydrate [34]. In both compounds, which are octahedral, the disulfide bond bridges between axial sites of the two copper(II) ions at distances equal to or somewhat larger than the sum of the van der Waals radii of the sulfur atom and the copper(II) ion, so that the copper(II) ions may only be described as interacting weakly with the sulfur atoms [33, 34]. On the other hand, several copper(I)–disulfide complexes have been characterized by crystallography that actually show copper(I)–sulfur(disulfide) bonds at distances of 2.3–2.4 Å [35–38]. It appears likely, therefore, that a disulfide to copper(II) bond in stellacyanin may be long, and if disulfide is a ligand in stellacyanin (as is being proposed in this paper) this bond forms as a result of the tertiary structure of the protein (Table III and Fig. 1) which may be said to make it feasible for the copper(II) ion to coordinate to one end of the disulfide. The resulting chelate loop through the protein backbone

between cysteine-87 and histidine-92, and the chelate ring through cystine-87-93 and histidine-92 (photograph), very likely result together in the rhombic EPR spectrum that is associated with stellacyanin, contrasting with the axial EPR spectra of azurin and plastocyanin [1]. In reduced stellacyanin, a copper(I)–sulfur(disulfide) bond with a length close to the normal range may be expected. This would suggest that, as electron transfer occurs, the copper(II)(N_2S_2) core makes adjustments in bond lengths and angles to those in the copper(I)(N_2S_2) core. As this core is attached to a fairly rigid protein backbone that does not change its conformation substantially, the regions of attachment which are the chelate loop regions may be expected to show changes (in the conformation of side chains as well as small adjustments in dihedral angles of the protein backbone) in response to the change in the oxidation state of the copper ion. This makes the sizes of the chelate loops and the nature of the amino acid side chains in these loops (some of which act as copper ligands) important in trying to understand the copper core of the electron transfer proteins. There is in fact a substantial difference in the complexing of copper ions to actual protein chains as compared to small peptide complexes of copper(II). The crystal structures of complexes, such as the glycylglycinato complex of copper(II) [39], show a square planar or square pyramidal copper ion which is displaced from the least squares plane of the equatorial ligands and in which the equatorial ligands themselves are displaced from the least squares plane upwards and downwards [40]. Bonding through the amino nitrogen and carboxyl oxygen, as well as deprotonated peptide nitrogen, results in the displacements that were pointed out [40]. Complexes with such bonding have not been found in copper proteins even when the copper(II) environment is square planar or square pyramidal, as the crystal structure of bovine superoxide dismutase shows [8]. The mode of bonding in copper proteins, probably as a result of the constraints imposed by the protein structure, is through functional groups of various side chains that are in favorable locations for coordination to occur. This point becomes particularly evident in building the protein backbone.

Although superoxide dismutase is not a blue copper protein, a great deal is known about the copper ion environment in this protein through physicochemical and crystallographic characterization. As this protein undergoes oxidation–reduction in its functioning, the accompanying changes in the copper ion environment may indicate what may happen in the blue copper proteins. The copper core, which contains four imidazole(histidine) ligands two of which form a minimum size chelate loop as pointed out in Table I and one of which as imidazolate, bridges to the zinc ion of the bovine superoxide dismutase subunit [8], undergoes reduction by the breaking of the imidazolate bond to copper (which remains bound to zinc) [41]. This allows the approximately square planar copper(II) ion environment to become tetrahedral, with the chelate loop remaining intact in the reduced protein. The reduction potential of the square planar copper center in superoxide dismutase is as high as it is in the tetrahedral blue copper proteins (*ca.* +0.40 V [42]).

As the blue copper proteins function by outer-sphere electron transfer [43], the chelate loops around copper, as well as the immediate copper environment, may be expected to remain intact. The flexibility gained in superoxide dismutase by the breaking of one bond must be present already in the chelate loop environment of the copper ion, as no bonds are broken or made (except possibly some hydrogen-bonds in the region around copper, which will become known when the crystal structure of reduced plastocyanin, undertaken by Freeman and colleagues, is completed).

As shown in Tables I and II, the chelate loops that form about copper in azurin and plastocyanin differ both with respect to the sizes of the two loops and to the nature of the amino acid residues in the loop area, except for those that act as copper ligands and for a proline residue between cysteine and histidine. This proline facilitates bonding to copper by allowing the beta strand to make a turn. Although there have been attempts to determine the particular conformations of amino acid side chains in the vicinity of metal ions, which may allow complexation by studying metal ion interactions with amino acids or with small peptides (see, for example, reference 44), the actual conformations of the side chains in a protein are likely to be affected differently by the presence of a copper ion. A study of the relaxation of the 625-nm band in azurin has indicated a 1.6 ± 0.2 picosecond time constant for reverse charge transfer and a fast transient relaxation process which takes less than 0.5 picosecond [45]. The authors [45] suggest that rearrangement of ligands around copper when its oxidation state changes is facile (not rate-determining for electron transfer). One mechanism by which this may occur in azurin is by the apparent change in the structure of the protein backbone between histidine-117 and methionine-121. This portion of the protein chain was found to have a helical character [9]. Adman [9] suggested that different helical forms (an alpha-helix or a 3_{10}-helix) might be present in oxidized and reduced forms of azurin. This would undoubtedly affect the conformations of the side chains in this region. Some shift in the conformations of the residues in the first loop (between cysteine-112 and histidine-117) may also be expected. The presence of a proline residue adds flexibility to this loop in azurin and also in plastocyanin (cysteine-84 to histi-

dine-87). The second loop in oxidized plastocyanin (histidine-87 to methionine-92) contains a beta turn [46]. In this portion there are two invariant glycine residues (Table II) that may be expected to impart the required flexibility to this loop. As shifts in the backbone of the chelate loops are expected to be accompanied by changes in the conformations of the side chains in the loop area, it may be inferred that the sizes of the chelate loops formed, their number, and the nature of the amino acid residues in the loops may all play a role in determining the reduction potentials of the blue copper proteins. The differences in the various blue copper proteins with respect to these factors may then account for the variations observed in the reduction potentials. It is suggested that the lower reduction potential of stellacyanin for example is mainly caused by the differing chelate loop structure in this protein, and only to a lesser extent by the difference in the ligands to copper.

Acknowledgement

This work was carried out while the author is seeking political asylum in the Federal Republic of Germany.

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