Protein Science (1996), *5*:2175–2183. Cambridge University Press. Printed in the USA.
Copyright © 1996 The Protein Society

A missing link in cupredoxins: Crystal structure of cucumber stellacyanin at 1.6 Å resolution

P. JOHN HART,[1,2] ARAM M. NERSISSIAN,[2] REINHOLD G. HERRMANN,[3]
ROBERT M. NALBANDYAN,[4] JOAN SELVERSTONE VALENTINE,[2] AND DAVID EISENBERG[1,2]
[1]UCLA-DOE Laboratory of Structural Biology and Molecular Medicine, University of California, Los Angeles, California 90095
[2]Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095
[3]Botanisches Institut der LMU Munchen, Menzinger Str. 67, D-80638 Munchen, Germany
[4]Institute of Biochemistry, Academy of Sciences, Yerevan 375044, Armenia

(Received June 25, 1996; Accepted August 23, 1996)

Abstract

Stellacyanins are blue (type I) copper glycoproteins that differ from other members of the cupredoxin family in their spectroscopic and electron transfer properties. Until now, stellacyanins have eluded structure determination. Here we report the three-dimensional crystal structure of the 109 amino acid, non-glycosylated copper binding domain of recombinant cucumber stellacyanin refined to 1.6 Å resolution. The crystallographic R-value for all 18,488 reflections ($\sigma > 0$) between 50–1.6 Å is 0.195. The overall fold is organized in two β-sheets, both with four β-strands. Two α-helices are found in loop regions between β-strands. The β-sheets form a β-sandwich similar to those found in other cupredoxins, but some features differ from proteins such as plastocyanin and azurin in that the β-barrel is more flattened, there is an extra N-terminal α-helix, and the copper binding site is much more solvent accessible. The presence of a disulfide bond at the copper binding end of the protein confirms that cucumber stellacyanin has a phytocyanin-like fold. The ligands to copper are two histidines, one cysteine, and one glutamine, the latter replacing the methionine typically found in mononuclear blue copper proteins. The Cu-Gln bond is one of the shortest axial ligand bond distances observed to date in structurally characterized type I copper proteins. The characteristic spectroscopic properties and electron transfer reactivity of stellacyanin, which differ significantly from those of other well-characterized cupredoxins, can be explained by its more exposed copper site, its distinctive amino acid ligand composition, and its nearly tetrahedral ligand geometry. Surface features on the cucumber stellacyanin molecule that could be involved in interactions with putative redox partners are discussed.

Keywords: azurin; cucumber basic protein; cupredoxins; glutamine copper ligand; stellacyanin; phytocyanins; plastocyanin; X-ray crystallography

Stellacyanins, mononuclear copper-containing glycoproteins of unknown function, are members of the "blue" or "type I" copper protein family. Proteins in this family are characterized by intense electronic absorption bands in the visible spectrum ($\lambda_{max} \cong 600$ and 450 nm), which give them their characteristic blue to blue-green color (Adman, 1985; Lu et al., 1993). For those cases where function has been established, they act as freely diffusible electron transfer proteins (Adman, 1991; Canters & Van de Kamp, 1992; Solomon et al., 1992; Solomon & Lowery, 1993; Wuttke & Gray, 1993; Malmström, 1994; Guckert et al., 1995). These proteins have collectively been named cupredoxins by analogy with the iron-containing electron transfer proteins termed ferredoxins (Adman, 1985, 1991). Blue copper centers also occur in multicopper blue oxidases (ceruloplasmin, ascorbic acid oxidase, laccase) and in copper-containing nitrite reductases. In such multicopper proteins, the type I copper center typically accepts an electron from an external electron donor and subsequently shuttles it to another metal center within the same protein for use in catalysis.

The coordination environment of copper in stellacyanins differs dramatically from that of other cupredoxins. High-resolution X-ray crystallographic studies have revealed that most cupredoxins have a defining structural motif consisting of a nearly trigonal planar $Cu(His)_2(Cys)$ center with a fourth, more remote axial ligand provided by a thioether sulfur from a Met side chain (Baker, 1988; Petratos et al., 1988; Guss et al., 1992; Durley et al., 1993). [Azur-

Reprint requests to: David Eisenberg, Molecular Biology Institute, University of California at Los Angeles, Box 951570, Los Angeles, California 90095; e-mail: david@pauling.mbi.ucla.edu.

Abbreviations: AMC, Amicyanin; AZ, *Alcaligenes denitrificans* azurin; AZM121Q, *Alcaligenes denitrificans* azurin M121Q mutant; CBP, cucumber basic protein (plantacyanin); CST, *Cucumis sativus* stellacyanin; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; PAZ, Pseudoazurin (*Alcaligenes faecalis* S-6); PCY, Poplar plastocyanin; RMS, root mean square; STC, *Rhus vernicifera* stellacyanin.

in is found to have an additional, fifth axial ligand weakly bonded to copper, a carbonyl oxygen from a glycine residue (Baker, 1988).] These four liganding amino acid residues, two His, one Cys, and one Met, are conserved in all available sequences of blue copper proteins other than stellacyanins. By contrast, *Rhus vernicifera* (Japanese lacquer tree) stellacyanin (STC), the first-discovered and most intensively studied stellacyanin, contains no Met at all, and it has for some time been assumed that it uses another amino acid as an axial ligand (Peisach et al., 1967; Bergman et al., 1977) (see below).

STC additionally has been found to differ from other mononuclear blue copper proteins in that it undergoes redox reactions with small inorganic complexes or electrodes at unusually fast rates. These observations have led to the prediction that the STC copper center is considerably more solvent exposed than those of other cupredoxins (Wherland et al., 1975; Holwerda et al., 1976; Mauk et al., 1980; Tollin et al., 1986). In support of this conclusion, electron spin echo envelope modulation (ESEEM) studies have indicated a greater accessibility to solvent for STC relative to azurin (Mims et al., 1984). It was also suggested that the fast redox rates observed for STC might be attributed to an enhanced interaction with the glassy carbon electrode due to hydrophobic interactions (Ikeda & Sakurai, 1994).

Because X-ray crystallographic data for stellacyanins have been lacking, only conjectural models based on amino acid sequence analysis have been available. From such studies, it was suggested that a sulfur atom from a disulfide bridge in the vicinity of the copper binding site contributed to copper coordination (Wherland et al., 1988). It was subsequently suggested that the carbonyl oxygen or, at high pH, the amide nitrogen of a Gln residue functions as the fourth ligand to the copper atom, in place of the Met usually found in cupredoxins (Peisach et al., 1982; Fields et al., 1991; Thomann et al., 1991; Strange et al., 1995). Figure 1 shows a sequence comparison of cucumber stellacyanin (CST) copper binding domain with that of *Rhus vernicifera* stellacyanin (STC) and cucumber basic protein (CBP; also known as plantacyanin). The levels of sequence identity of copper binding domains of CST with STC and CBP are 34 and 33%, respectively (expressed as fractions of the shorter polypeptide). The two stellacyanins have Gln at the position corresponding to the axial copper ligand (amino acid 99 in cucumber stellacyanin), while CBP has Met. Further support of this Gln ligand hypothesis came from the finding that the replacement of the Met ligand by Gln in an engineered azurin alters some of the physical properties of the copper site making them more similar to those of stellacyanin (Romero et al., 1993).

Rhus vernicifera stellacyanin has long been an intriguing candidate for high resolution X-ray structural analysis due to its unusual sequence, spectroscopic, and electrochemical properties relative to other cupredoxins (Nersissian et al., 1996). Unfortunately, the gene for STC has not been cloned, and efforts to crystallize the protein in many laboratories have been thwarted, probably because of heterogeneity caused by glycosylation [approximately 40% of its molecular weight (Peisach et al., 1967)] and by polyploidy (Obokata et al., 1993) and other post-translational modification (Nersissian et al., 1996). To bypass these obstacles, we crystallized a recombinant stellacyanin from *Cucumis sativus* (cucumber) after removal of the non-copper binding domains and the glycosylation sites by site-directed mutagenesis (Nersissian et al., 1996). Here we report the refined 1.6 Å resolution X-ray crystal structure of the 109 amino acid, non-glycosylated copper binding domain of the cucumber stellacyanin protein.

Results

Quality of the final model

The refined CST model consists of 110 amino acid residues, 852 protein atoms, 109 water molecules, and a single copper ion. An N-terminal Met residue (Met^0) is incorporated as a translation initiator in the protein expression system used (Nersissian et al., 1996). All atoms are present in the final structure with the exception of the Met^0 and Asn^{109} side chains because their density was not observed. These two residues were thus modeled as glycine. Amino acid residues Ser^{65}–Glu^{70} have weak electron density due to high thermal parameters. Temperature factors average 25.44 and 27.82 $Å^2$ for backbone and all atoms, respectively. Notably high temperature factors for a protein that diffracts to 1.6 Å are found for the N-terminal two residues (values of roughly 40 $Å^2$), the disordered region of the variable strand (values between 80 and 90 $Å^2$), and the C-terminal two residues (values of roughly 70 $Å^2$).

RMS deviations from ideality for bond lengths and angles are 0.018 Å and 2.50°, respectively. Ninety-seven percent of the amino acid residues have ϕ and ψ angles that fall in the allowed regions of a Ramachandran plot (Ramachandran & Sasiskharam, 1968). Three residues, Asn^{67}, Asp^{68}, and Val^{69}, amino acids located in the poorly defined variable strand region described above, fall outside the allowed regions. The maximum overall RMS coordinate error is 0.17 Å, as estimated from a SIGMAA plot (Read, 1986) and 0.20 Å, as estimated from a Luzzati plot (Luzzati, 1952). Because all the measured data have been used without a σ cutoff, the actual

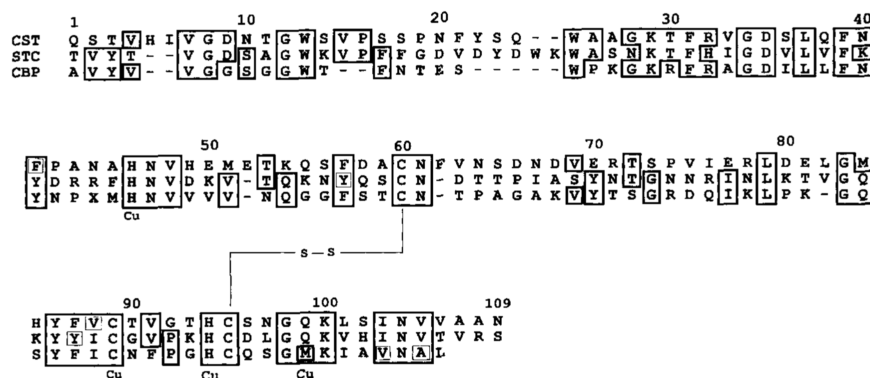

Fig. 1. Alignment of the amino acid sequences of the copper binding domains of cucumber stellacyanin (CST) (Nersissian et al., 1996), *Rhus vernicifera* stellacyanin (STC) (Bergman et al., 1977), and cucumber basic protein (CBP) (Murata et al., 1982). Residues that are identical in two or more of the sequences are boxed. Dashes indicate gaps introduced to allow optimal alignment. Symbols used to indicate side chain character are: (Cu) the known or postulated copper ligands; -S-S- disulfide bridge between the residues Cys^{60} and Cys^{95}. Numbering refers to the amino acid positions in cucumber stellacyanin.

average error must be lower than this, as the SIGMAA and Luzzati analyses assume that all the discrepancies between Fo and Fc result from errors in the model. We estimate that for β-strands, loops around copper site, all internal side chains, and other well-defined parts of the structure, the positional error is 0.1 Å or less. In support of this, copper–ligand bond distances varied less than 0.08 Å during the course of the refinement (no stereochemical restraints imposed).

Overall protein fold

As in other cupredoxins, *C. sativus* stellacyanin has an overall Greek key β-barrel topology. The refined structure is similar to the phytocyanin CBP (Guss et al., 1996), as expected from the use of molecular replacement. CST and CBP α-carbon atoms superimpose with an RMSD of 1.10 Å for 88 target pairs. CST has two α-helices and eight β-strands [as determined in SETOR (Evans, 1993)], with the strands organized into two β-sheets. Strand numbers 1, 2, 4, and 6 form the first β-sheet, and strand numbers 3, 5, 7, and 8 form the second. Strand #2 forms only one hydrogen bond to strand #4 in sheet #1. The second β-sheet is unusual in that it is highly distorted, exhibiting a severe degree of twist. The two sheets come together to form a flattened β-sandwich structure. Figure 2 illustrates the protein structure in ribbon format. The copper binding site is located at the "northern" or top end of the molecule [taking the description used for the related plastocyanin structure (Guss & Freeman, 1983)]. The CST ligand residues are an "upstream" histidine (His^{46}), which comes from the loop connecting β-strands #4 and #5, and a Cys-His-Gln triad (Cys^{89}, His^{94}, Gln^{99}), coming from a single loop positioned between β-strands #7 and #8. As in CBP (Guss et al., 1996), the disulfide bridge in CST between residues 60 and 95 does not play a role in copper coordination, but does anchor the C-terminus of a small helix (residues 53–59) and the so-called "variable" strand (residues 62–73) to the rest of the molecule.

Copper binding site

The CST copper coordination geometry can best be described as distorted tetrahedral, with Cu-His^{46}, Cu-Cys^{89}, and Cu-His^{94} ligand distances of 1.96, 2.18, and 2.04 Å, respectively. Andrew et al. (1994) have described the distance of the copper from the plane of the strong ligands as a feature allowing comparison of the copper binding sites in cupredoxins. In CST, the copper ion resides 0.33 Å out of the nitrogen-sulfur-nitrogen equatorial plane formed by the liganding atoms of these three residues in the direction of the axial ligand. The axial liganding atom, the ϵ-oxygen or ϵ-nitrogen of Gln^{99}, is at a distance of 2.21 Å from the copper ion, one of the shortest axial bond distances found to date in any naturally occurring cupredoxin. We modeled the ϵ-oxygen as the liganding atom, although this could not be distinguished from the ϵ-nitrogen by the electron density. However, the position of the aromatic ring of Trp^{13} relative to the non-liganding ϵ-atom of Gln^{99} provides an important clue as to its identity. The center of the plane formed by Trp^{13} CG, CD1, CD2, CE2, and NE1 atoms is 3.25 Å from the non-liganding ϵ-atom of Gln^{99}. If the NE2 atom of Gln^{99} is the non-liganding atom, its hydrogen atoms would point directly into the negatively charged "hole" formed by the π electrons of the atoms of the Trp^{13} ring. An ϵ-oxygen/Trp^{13} ring face interaction, by contrast, would be electrostatically unfavorable. The distorted tetrahedral geometry of the copper binding site of CST is consistent with the rhombic nature of its EPR spectrum and the band at 450 nm in its visible absorption spectrum (Lu et al., 1993; Andrew et al., 1994). Figure 3 illustrates the copper and its ligands superimposed on 1.6 Å electron density for this region. The bond distances and angles for *C. sativus* stellacyanin and a number of other type I copper proteins are listed in Table 1.

Discussion

Overall protein fold

The overall fold of *C. sativus* stellacyanin is similar to that of CBP, and the hypothesis that stellacyanins have a "pytocyanin-like" fold as evidenced by the position and structural role of the disulfide bond at the northern end of the molecule is confirmed (Guss et al., 1988; Fields et al., 1991). The protein backbone atomic positions are conserved between the two molecules except in regions of amino acid insertion and in part of the variable strand (CST residues 62–72). As seen in the sequence alignment in Figure 1, there is both an N-terminal and C-terminal extension of three residues in CST. If CST is a cell wall protein, as the presence of the extensin-like domain suggests (Nersissian et al., 1996), the function of the C-terminal residues may be to space the copper binding domain away from the cell wall. In addition, a five-residue insertion between residues Phe^{13} and Trp^{18} (in CBP) must be accommodated

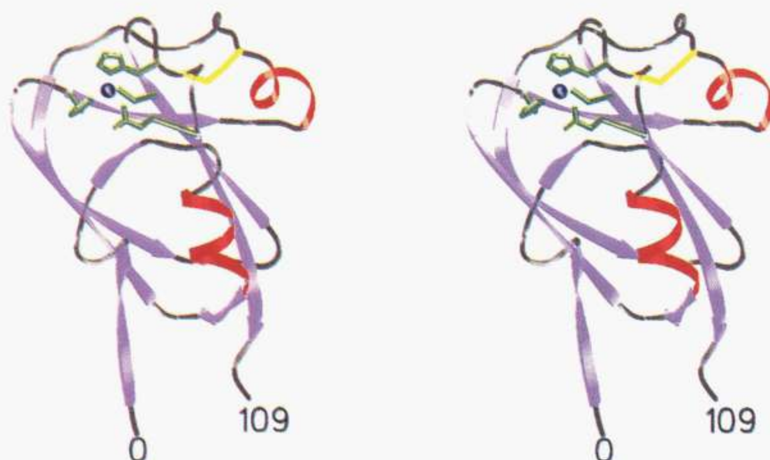

Fig. 2. The cucumber stellacyanin backbone and copper binding site. The copper ion is shown as a blue sphere, copper ligands are in green, and the disulfide bond is in yellow. The protein fold is composed of eight β-strands (purple with arrows) and two α-helices (red) as determined by the graphical program SETOR (Evans, 1993). The anatomy of a cupredoxin has been described (Guss & Freeman, 1983; Adman, 1991). The copper is bound at the "northern" end of the molecule, and is coordinated by an "upstream" (N-terminal) and "downstream" (C-terminal) His ligand. The "backside" of the molecule (away from the viewer in this figure) contains the "variable" strand (residues C-terminal to the disulfide bond and N-terminal to β-strand #6).

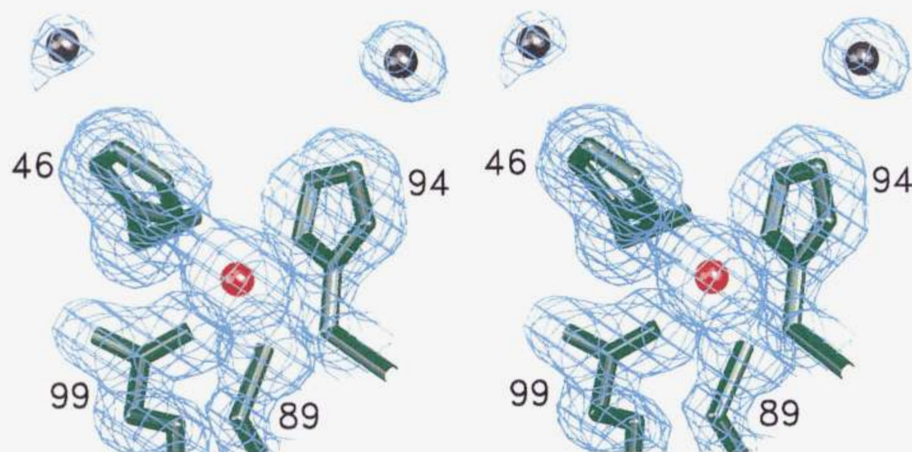

Fig. 3. Cucumber stellacyanin copper binding site superimposed on 1.6 Å electron density. The electron density map is of the form (2Fo-Fc)α_c contoured at 1 σ. The metal ion is coordinated by a nearly tetrahedral arrangement of the Cys(His)$_2$Gln ligands, with bond lengths averaging 2.09 Å. The unusual axial Gln ligand (Gln[99]) has been modeled with its side chain ϵ-oxygen forming the bond with copper. Two water molecules are shown as gray orbs hydrogen bonded to the copper-distal imidazole nitrogens of the histidine ligands.

in the CST structure. The accommodations of CST in this region create a larger loop (residues 14–19) plus an α-helix (residues 20–28) that is absent in CBP (Guss et al., 1996).

The variable strand (Adman, 1991) adopts differing motifs in the mononuclear blue copper proteins. Azurin contains a back "flap" (Baker, 1988), while plastocyanin contains an "acidic patch" (Guss et al., 1992) thought to be responsible for interaction with its electron donor cytochrome *f*. CST contains a partially disordered string of residues of sequence Phe[62]-Val-Asn-Ser-Asp-Asn-Asp-Val-Glu-Arg-Thr-Ser[73]. The analogous string of residues in cucumber basic protein is hydrogen bonded to the adjacent strand in the molecule (Guss et al., 1996), but this string of residues is beyond hydrogen bonding distance from this interior strand in cucumber stellacyanin, possibly due to the influence of His[49], which is on the adjacent interior strand. His[49] has two conformations. The positions of this "variable" strand in the two molecules differ by roughly 8 Å. Also, the types of amino acid residues that are found on the backside of the molecule differ. CST has nine acidic and two basic residues, and CBP has one acidic and four basic residues. Superposition of CBP and CST can be viewed in the electronic appendix.

Copper binding site

The crystal structure of an engineered azurin mutant, Met121Gln, which mimics some of the properties of the copper binding site in CST was reported previously (Romero et al., 1993). While the Cu-ligand bond distances are nearly the same in CST and the

Table 1. *Comparison of copper site geometry in cucumber stellacyanin with other crystallographically characterized cupredoxins: plastocyanin (PCY) (Guss et al., 1992), azurin (AZ) (Baker, 1988), and its M121Q mutant (AZM121Q) (for two crystallographically independent molecules per asymmetric unit) (Romero et al., 1993), pseudoazurin (PAZ) (Petratos et al. 1988), and amicyanin (AMC) (Durley et al., 1993)*[a]

	PCY	*AZ1*	*AZ2*	*PAZ*	*AMC*	*CBP*	*CST*	*AZM121Q*	
				Cu-ligand bond lengths					
Cu-N(His1)	1.91	2.08	2.09	2.16	1.95	1.93	1.96	1.91	1.96
Cu-S(Cys)	2.07	2.12	2.17	2.16	2.15	2.16	2.18	2.13	2.11
Cu-N(His2)	2.06	2.01	1.99	2.13	2.00	1.95	2.04	2.06	2.03
Cu-S(Met)	2.82	3.12	3.10	2.76	2.89	2.61			
Cu-O(Gln)							2.21	2.25	2.28
				Ligand-Cu-ligand bond angles (deg)					
N(His1)-Cu-(Cys)	132	135	135	136	135	138	134	141	133
N(His1)-Cu-N(His2)	97	101	108	100	109	99	101	97	105
N(His1)-Cu-S(Met)	88	79	75	87	83	83			
N(His1)-Cu-O(Gln)							94	87	91
S(Cys)-Cu-N(His2)	121	122	116	112	109	110	118	116	117
S(Cys)-Cu-S(Met)	110	109	105	108	109	111			
S(Cys)-Cu-O(Gln)							101	106	103
N(His2)-Cu-S(Met)	101	94	98	112	104	112			
N(His2)-Cu-O(Gln)							102	98	97

[a]His1 and His2 refer to the up- and downstream histidine ligands, respectively.

azurin mutant, there are four notable differences between the two proteins: (a) the copper binding site in CST is much more solvent exposed. (b) The copper displacement from the equatorial plane in CST is greater than that for Met121Gln azurin (0.33 Å vs. 0.26 Å). (c) The fifth axial ligand, a carbonyl oxygen from Gly^{45} in Met121Gln azurin, is at an average distance of 3.37 Å from the copper, while the analogous carbonyl oxygen from Ala^{45} in CST is 3.97 Å away. (d) Met121Gln azurin does not exhibit the pH-dependent reversible changes in EPR and visible absorption spectra observed in stellacyanins (Nersissian et al., 1996).

The copper binding site in CST is stabilized by two hydrogen bonds formed between residues immediately adjacent to the His^{46} and Cys^{89} ligands. The side chain of Asn^{47} hydrogen bonds with a backbone amide proton from Thr^{90} as well as the γ-oxygen atom of that residue. The unusual Gln ligand residue (Gln^{99}) is found sandwiched between two apolar side chains, two residues apart (Trp^{13} and Val^{15}). This feature is commonly seen for the axial Met copper ligands in other cupredoxins (Adman, 1991). However, the degree of solvent exposure of the copper binding site in CST is quite unlike those of the other cupredoxins. The copper resides only about 3 Å beneath the surface of the protein and, hence, is much more exposed to solvent than in poplar plastocyanin and azurin from *Alcaligenes denitrificans*, where it is buried by 5 Å or more (Baker, 1988).

The upstream His (His^{46} in CST) in other blue copper proteins is found to be in a buried nonpolar environment, and it forms a hydrogen bond with a side chain or main chain atom from an adjacent strand. In the case of the downstream His (His^{94} in CST) in other cupredoxins, the edge of the imidazole ring protrudes from a hydrophobic face. In *C. sativus* stellacyanin, however, the situation is different because the copper binding site is much more open to solvent. Both His ligands protrude from the protein surface, which is composed of both nonpolar (Val^{15}, Pro^{42}, Ala^{43}, Ala^{45}, Ala^{59}, Cys^{60}, Phe^{62}, Val^{63}, Val^{91}, Gly^{92}, Cys^{95}) and polar (Ser^{14}, Ser^{17}, Ser^{18}, Asn^{44}, Asn^{61}, Asn^{68}, Arg^{71}, Thr^{93}, Ser^{96}, Asn^{97}) residues. Figure 4 shows a space-filling model of CST, azurin, and plastocyanin in the same orientation relative to the $(His)_2Cys$ triad, highlighting the relative degrees of solvent exposure for the His ligands in these three molecules. Water molecules form strong hydrogen bonds with the distal imidazole nitrogens of both the up- and downstream CST His ligands (2.64 Å for His^{46} and 2.76 Å for His^{94}). The highly solvent-exposed nature of the copper binding site of *C. sativus* stellacyanin offers explanation for the unusually fast electron transfer rates observed for *Rhus vernicifera* stellacyanin as well as its ability to undergo oxidation and reduction directly with electrodes without the assistance of mediators (Wherland et al., 1975; Holwerda et al., 1976; Mauk et al., 1980; Tollin et al., 1986).

The Cys^{89} sulfur atom exists in a tetrahedral geometry, interacting with the copper, the β-carbon of Cys^{89}, and two backbone amide hydrogen atoms donated from residues Val^{91} and Asn^{47}. CST is only the second cupredoxin to have two of these NH–S bonds (azurin is the other). All the other cupredoxins have Pro at the Val^{91} position. This extra NH–S bond coupled with the unusually short axial ligand distance probably contribute to CST's unusual spectroscopic characteristics. In the azurin structure, a carbonyl oxygen is located *trans* to the Met ligand; the copper geometry must be described as five- rather than four-coordinate (Baker, 1988). In the case of CST, the carbonyl oxygen closest to the copper atom, analogous to the copper carbonyl ligand in azurin, comes from Ala^{45} at a distance of 3.97 Å. The geometry, with a C^{45}-O^{45}-Cu angle of 123.3°, and the environment, which is predominantly nonpolar, are both favorable for an oxygen–copper interaction. The distance from the copper ion is too large, however, for such an interaction to be very strong.

Comparison with plastocyanin and azurin

Figure 5 shows a superposition of the CST, azurin (Baker, 1988) and plastocyanin (Guss et al., 1992) copper ligands, highlighting their similarities and differences. The electronic appendix contains the full plastocyanin and azurin molecules rotated onto the CST coordinate reference frame based on the $(His)_2Cys$ ligand superposition. The $(His)_2Cys$ ligand side chains of azurin and plastocyanin superimpose on those of cucumber stellacyanin with a RMSD of 0.18 and 0.24 Å, respectively. α-Carbon atoms of plastocyanin

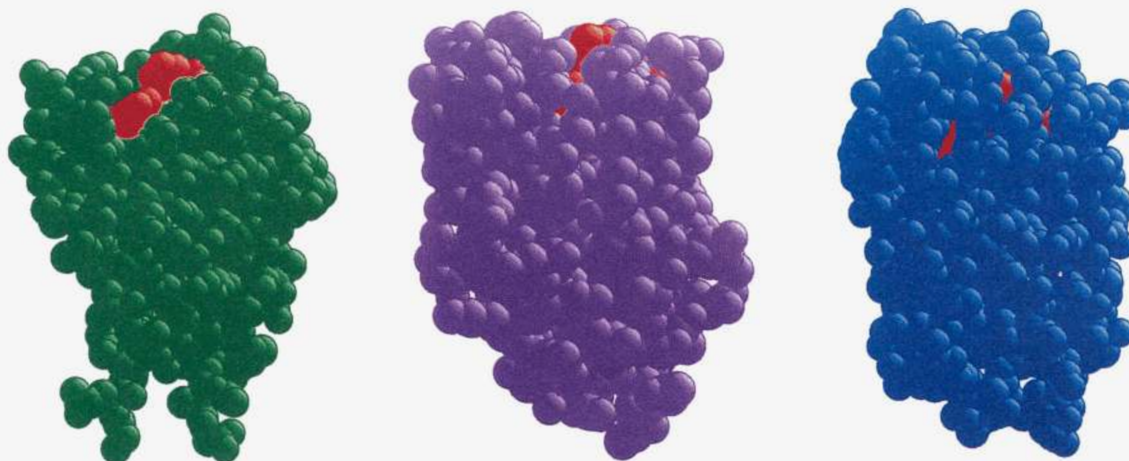

Fig. 4. Degree of solvent exposure of up- and downstream His ligands (red) in left) cucumber stellacyanin, center) poplar plastocyanin, and right) azurin from *Alcaligenes denitrificans*. The plastocyanin and azurin molecules are shown in an orientation equivalent to that of cucumber stellacyanin based on the structural alignment procedure described in Materials and methods. The upstream His ligand is on the left and the downstream His ligand is on the right in all three molecules.

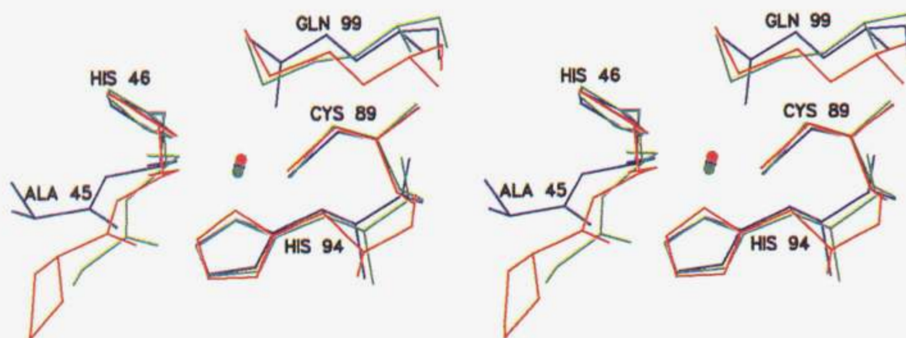

Fig. 5. Superposition of cucumber stellacyanin (blue), azurin (green), and plastocyanin (red) copper ligands. Structural alignment was accomplished as described in Materials and methods by superimposing the Cys and His ligands. Ligand numbering corresponds to that found in CST.

and azurin superimpose on CST backbone atoms with RMSDs of 1.47 Å (66 target pairs) and 2.86 Å (91 target pairs), respectively. The largest differences in the copper binding residues in Figure 5 are observed in the position of the axial liganding atoms, the thioether sulfur of the methionine side chain in plastocyanin and azurin, and the oxygen of the glutamine side chain in cucumber stellacyanin. In CST, the oxygen of the glutamine side chain extends much closer to the copper ion, dipping an average distance of 0.78 Å closer to the trigonal plane than the thioether sulfurs of plastocyanin and azurin. Azurin's fifth axial ligand, a carbonyl oxygen from the residue immediately N-terminal to the upstream His ligand (His^{46} in CST) makes the closest approach to the trigonal plane from the opposite direction, coming 0.24 Å closer than the analogous carbonyl oxygen from plastocyanin and 0.71 Å closer than that from CST. Although the copper binding sites of CST, PCY, and AZ are different as described above, their overall architecture is strikingly similar, even though CST has less than 16% overall sequence identity with either protein.

Surface features and possible electron transfer pathways

The physiological role of *C. sativus* stellacyanin (and of the other stellacyanins) and phytocyanins such as CBP remains unknown. Some immunochemical evidence implicates spinach plantacyanin (and by inference, CBP) as being associated with PS-II particles in chloroplasts, leading to the suggestion that it may be involved in the evolution of O_2 (Nersissian & Nalbandyan, 1990). In cucumber stellacyanin, a chimeric protein is formed from two structurally and functionally distinct protein domains, a type I copper electron transfer center and an extensin-like cell wall structural element (Nersissian et al., 1996). The presence of the extensin-like domain suggests that stellacyanin may have a function other than that of a diffusible electron transfer protein, conceivably participating in redox reactions localized at the plant cell wall, which occur in response to wounding or infection of the plant.

Whether cucumber stellacyanin is diffusible or fixed in the cell wall, we may look for potential electron transfer pathways in CST and to compare them to those known or inferred in other cupredoxins. The most likely pathway for electron transfer is via one of the solvent accessible His ligands (the "northern" pathway). In addition, the backside of the molecule (which contains the variable strand) in CST is a cup-shaped depression lined with negatively charged amino acid side chains, forming an acidic patch reminiscent of plastocyanin. Figure 6 illustrates this region of the protein. The distribution of negative charges is similar to that of plastocyanin, although CST has one more negative charge, and the distribution encircles a solvent exposed His residue (His^{49}) with two conformations.

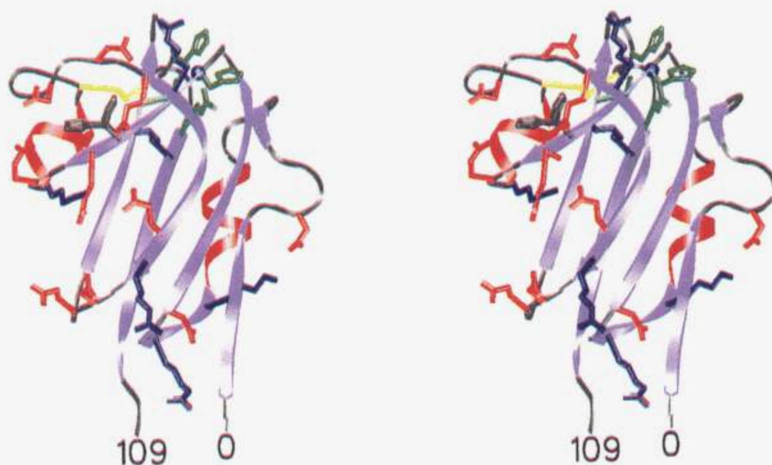

Fig. 6. Backbone of the cucumber stellacyanin molecule highlighting the variable region to show a potential docking site for a biological partner. All negatively charged amino acids (Asp/Glu) are shown in red, and all positively charged amino acids (Arg/Lys) are in blue. The histidine with two conformations (see text) is in dark gray.

His[49] is a candidate as a site for electron transfer because of its position at the center of this acidic ring of charge, with only two intervening residues between it and copper ligand His[46]. A second potential electron transfer pathway from the copper atom to His[49] is possible via the Cys[89] copper ligand, through a hydrogen bond formed between the carbonyl oxygen of Val[88] and the backbone nitrogen of His[49] (see electronic appendix). When CST and plastocyanin are structurally aligned, His[49] is close to Tyr[83] of plastocyanin, with the NE2 atom of one of the rotamers of CST His[49] only 2.5 Å from the solvent-exposed OH atom of PCY Tyr[83]. PCY Tyr[83] has been implicated in electron transfer (the "remote pathway") with PS-I (Farver et al., 1982; Haehnel et al., 1994) and has also been shown to be functionally involved in the oxidation of cytochrome *f* (Modi et al., 1992). Tyrosines analogous to PCY Tyr[83] (in terms of solvent accessibility and general location in the three-dimensional structures) are conserved in cupredoxins, including CST Tyr[86], STC Tyr[85], and CBP Tyr[76]. CST Tyr[85] and CBP Tyr[76] are solvent accessible and, therefore, could also serve as "remote pathway" electron transfer sites. When CST and azurin from *Alcaligenes denitrificans* are structurally aligned, His[49] occupies the analogous position in space (although it sits on a different β-strand) to His[83], a residue conserved in all azurins and implicated in electron transfer in several studies (Farver & Pecht, 1981; Cho et al., 1984; Margalit et al., 1984) (see electronic appendix).

Experiments on electron transfer in STC also suggest that CST His[49] participates in a remote pathway of electron transfer. Farver et al. (1987) reduced STC with Cr(II) leaving the Cr(III) product bound to the protein at the site of electron transfer—Asp[49]. Notice in Figure 1 that STC Asp[49] aligns with CST His[49]. We expect that STC and CST are structurally similar, based on the alignment of their sequences. Thus, both STC Asp[49] and CST His[49] are accessible to solvent, and are both potential sites of electron transfer. It should be noted, however, the residues responsible for the negative ring of charge in CST are hydrophobic or positively charged in the STC sequence. The implication is that the specificity of interaction between the two proteins and their putative electron transfer donors/acceptors is different.

Although cucumber stellacyanin is assumed to exist as a monomer in solution, lattice packing indicates that a dimer interface is formed about a twofold axis of rotation centered on Ile[76], and that it is held together by both electrostatic and hydrophobic interactions. The association of two monomers in the crystal lattice to form the dimer results in the formation of a negatively charged depression. It is attractive to speculate that such a surface, coupled with the accessibility of the symmetrically related His[49] residues, could function in the docking of putative electron donors or acceptors. Ultimately, further biochemical studies are needed to elucidate both the localization and function of stellacyanins in general and cucumber stellacyanin in particular.

Table 2. *Crystallographic data for cucumber stellacyanin*[a]

Space group	$P3_121$
Unit cell dimensions (Å)	$a = b = 59.18$, $c = 74.21$, $a = b = 90°$, $g = 120°$
Asymmetric unit	1 stellacyanin molecule
Temperature (°C)	20
Wavelength (Å)	1.54
Crystal-to-plate distance (mm)	75
Oscillation range (°)	2
No. of observations	82,930
No. of unique reflections	18,488
Completeness (%)[b]	90.9 (53.5[c])
Resolution limit (Å)1.60	
R_{sym} (%)[d]	7.3 (33.2[c])

[a]5 μL of protein solution at ~40 mg/mL in 10 mM acetate buffer pH 5.5 was mixed with 2 μL of reservoir solution (solution C4, ammonium sulfate Grid Screen, Hampton Research) containing 100 mM Hepes pH 7.0, 2.4 M ammonium sulfate, and allowed to equilibrate at 4 °C. Large, block-like prisms (0.5 × 0.5 × 0.3 mm) grew within two weeks in space group $P3_121$ with unit cell parameters $a = b = 59.2$ Å, $c = 74.2$ Å. Three-dimensional diffraction data were collected to 1.6 Å resolution using a Rigaku RAXIS IIc imaging plate detector. The X-ray source was a Rigaku RU-200 generator with focusing mirrors running at 50 kV, 100 mA. Crystals were rotated about ϕ, and oscillation images were collected every 2°. The data were reduced using the program DENZO (Otwinowski, 1993) to 1.6 Å (90.9% complete) with an R_{sym} (on I) of 7.3%. Data in the highest resolution shell (1.66–1.60 Å) were 53.5% complete.

[b]Of all reflections to 1.60 Å.

[d]These numbers denote completeness in the highest resolution shells (1.66–1.60 Å).

[d]R_{sym} = conventional discrepancy R-factor for scaling symmetry-related intensities.

Materials and methods

Cucumber stellacyanin was cloned, expressed, and spectroscopically characterized as previously described (Nersissian et al., 1996). Briefly, the protein precursor consists of four sequence domains: (I) a 23-amino acid hydrophobic N-terminal signal peptide, (II) a 109-amino acid copper binding domain, (III) a 26 amino acid hydroxyproline- and serine-rich peptide characteristic of motifs found in the extensin family, and (IV) a 22-amino acid hydrophobic C-terminal extension. Domains I, III, and IV were removed using PCR. The glycosylation sites (a single N-linked site via Asn[109] and multiple O-linked sites via several hydroxyproline residues of domain III) were, thus, engineered out of the molecule in this process. The copper binding domain (domain II) of the non-glycosylated protein was expressed in *E. coli*, refolded, and metallated. The spectroscopic characteristics of the protein were found to be virtually identical to those of *Rhus vernicifera* stellacyanin (Nersissian et al., 1996).

Crystals of the non-glycosylated copper binding domain of CST were obtained by the hanging drop vapor diffusion method and contain one molecule per asymmetric unit. High resolution crystallographic data were collected to 1.6 Å resolution on a rotating anode X-ray generator equipped with an imaging plate detector. Table 2 summarizes crystallization methods and data collection parameters and statistics. The 1.8 Å resolution refined model of cucumber basic protein (coordinates kindly provided by Dr. Hans Freeman, Sydney, Australia) was employed as the search model in cross rotation and translation functions. Where amino acid residues were in common in both CBP and CST as shown in Figure 1, their side chains were retained in the molecular replacement searches. The remaining amino acid side chains were truncated to alanine in CBP. Rotation and translation functions were carried out with the XPLOR program package (Brünger, 1988) using 15–3.0 Å data and varying radii of integration. The rotation searches gave an unambiguous solution after Patterson Correlation (PC) refinement. Translation searches revealed the correct enantiomer to be space group $P3_121$. The initial R-value was 44.1% (R_{free} = 51.6%) after rigid body refinement (10–2.5 Å data).

Model building was undertaken using all data (no σ cutoff) during 14 rounds of crystallographic refinement in XPLOR. A "round" of refinement is defined as sequential utilization of positional, simulated annealing, and isotropic temperature factor refinement routines followed by visual inspection of electron density maps coupled with manual model building (when necessary), using the molecular graphics program FRODO (Jones, 1978). No stereochemical constraints were imposed on the coordination geometry of the active site copper ion during refinement. As refinement in XPLOR continued, higher resolution data shells were systematically added to a final resolution of 1.6 Å. Model atom positions were verified by the examination of conventional and simulated annealing omit maps (Hodel et al., 1992).

Further refinement was subsequently performed using the program SHELXL-93 (Sheldrick & Schneider, 1995). Thirteen rounds were undertaken with the starting model being the product of the XPLOR refinement. One hundred nine water molecules were incorporated into the model during this process, with a final R-value of 0.195 (R_{free} = 0.237) for all 18,488 reflections in the 50–1.6 Å shell. Luzzati and SIGMAA analyses were calculated using all data.

Structural alignments with azurin from *Alcaligenes denitrificans* (PDB2AZA) (Baker, 1988), poplar plastocyanin (PDB1PLC) (Guss et al., 1992), and cucumber basic protein (PDB2CBP) (Guss et al., 1996) were accomplished using a modified version of the program ALIGN (Satow et al., 1986). The azurin, plastocyanin, and cucumber basic protein $(His)_2Cys$ ligand side chains were superimposed on the cucumber stellacyanin $(His)_2Cys$ ligand side chains. The resulting rotation and translation vectors were applied to all the atoms of azurin, plastocyanin, and cucumber basic protein molecules, respectively, to put them on the same coordinate basis as cucumber stellacyanin. Figures were created with the program SETOR (Evans, 1993). Atomic coordinates and observed structure factors have been deposited with the Protein Data Bank with entry codes 1JER and R1JERSF respectively.

Acknowledgments

Discussions with Drs. H. Freeman, D. Cascio, M. Weiss, W. Patterson, and E. Adman are gratefully acknowledged. We thank Dr. H. Freeman for generously making available high resolution atomic coordinates of CBP for use in this work. This research was supported in part by an appointment (to P.J.H.) to the Alexander Hollaender Distinguished Postdoctoral Fellowship Program, sponsored by the U.S. Department of Energy, Office of Health and Environmental Research and administered by the Oak Ridge Institute for Science and Education, and by postdoctoral fellowships (to A.M.N.) from the European Molecular Biology Organization and from the Alexander von Humboldt Foundation. Work at UCLA was also funded by National Institute of Health Grant GM-28222 (J.S.V.) and Grant GM-31299 (D.E.).

References

Adman ET. 1985. Structure and function of small blue copper proteins. In: Harrison P, ed. *Metalloproteins*. London: Macmillan. pp 1–42.

Adman ET. 1991. Copper protein structures. *Adv Prot Chem 42*:145–197.

Andrew CR, Yeom H, Valentine JS, Karlsson BG, Bonander N, Pouderoyen G, Canters GW, Loehr TM, Sanders-Loehr J. 1994. Raman spectroscopy as an indicator of Cu-S bond length in type 1 and type 2 copper cysteinate proteins. *J Am Chem Soc 116*:11489–11498.

Baker EN. 1988. Structure of azurin from *Alcaligenes denitrificans*: Refinement at 1.8A resolution and comparison of the two crystallographically independent molecules. *J Mol Biol 203*:1071–1095.

Bergman C, Gandvick E-K, Nyman PO, Strid L. 1977. The amino acid sequence of stellacyanin from the lacquer tree. *Biochem Biophys Res Commun 77*:1052–1059.

Brünger AT. 1988. Crystallographic refinement by simulated annealing. In: Isaacs NW, Taylor MR, eds. *Crystallographic Computing 4: Techniques and New Technologies*. Oxford: Clarendon Press. pp 126–140.

Canters GW, Van de Kamp M. 1992. Protein-mediated electron transfer. *Curr Opin Struct Biol 2*:859–869.

Cho KC, Blair DF, Banerjee U, Hopfield JJ, Gray HB, Pecht I, Chan SI. 1984. NMR spectroscopic identification of a hexacyanochromate(III) binding site on *Pseudomonas* azurin. *Biochemistry 23*:1858–1862.

Durley R, Chen L, Lim LW, Mathews FS, Davidson VL. 1993. Crystal structure analysis of amicyanin and apoamicyanin from *Paracoccus denitricans* at 2.0 A and 1.8 A resolution. *Protein Sci 2*:739–752.

Evans SV. 1993. SETOR: Hardware lighted three-dimensional solid model representations of macromolecules. *J Mol Graphics 11*:134–138.

Farver O, Licht A, Pecht I. 1987. Electron transfer pathways in stellacyanin: A possible homology with plastocyanin. *Biochemistry 26*:7317–7321.

Farver O, Pecht I. 1981. Identification of an electron transfer locus in plastocyanin by chromium(II) affinity labeling. *Proc Natl Acad Sci USA 78*:4190–4193.

Farver O, Shahak Y, Pecht I. 1982. Electron uptake and delivery sites on plastocyanin in its reactions with the photosynthetic electron transport system. *Biochemistry 21*:1885–1890.

Fields BA, Guss JM, Freeman HC. 1991. Three-dimensional model for stellacyanin, a "blue" copper-protein. *J Mol Biol 222*:1053–1065.

Guckert JA, Lowery MD, Solomon EI. 1995. Electronic structure of the reduced blue copper active site: Contributions to reduction potentials and geometry. *J Am Chem Soc 117*:2817–2844.

Guss JM, Bartunik HD, Freeman HC. 1992. Accuracy and precision in protein structure analysis—Restrained least-squares refinement of the structure of poplar plastocyanin at 1.33 Å resolution. *Acta Cryst B48*:790–811.

Guss JM, Freeman HC. 1983. Structure of oxidized poplar plastocyanin at 1.6 Å resolution. *J Mol Biol 169*:521–563.

Guss JM, Merritt, EA, Phizackerley RP, Freeman, HC. 1996. The structure of a phytocyanin, the basic blue protein from cucumber, refined at 1.8 Å resolution. *J Mol Biol*. Forthcoming.

Guss JM, Merritt EA, Phizackerley RP, Hedman B, Murata M, Hodgson KO, Freeman HC. 1988. Phase determination by multiple-wavelength x-ray diffraction: Crystal structure of a basic "blue" copper protein from cucumber. *Science 241*:806–811.

Haehnel W, Jansen T, Gause K, Klösgen RB, Stahl B, Michl D, Huvermann B, Karas M, Herrmann RG. 1994. Electron transfer from plastocyanin to photosystem I. *EMBO J 13*:1028–1038.

Hodel A, Kim SH, Brunger AT. 1992. Model bias in macromolecular crystal structures. *Acta Cryst A48*:851–858.

Holwerda RA, Wherland S, Gray HB. 1976. Electron transfer reactions of copper proteins. *Annu Rev Biophys Bioeng 5*:363–396.

Ikeda O, Sakurai T. 1994. Electron transfer reaction of stellacyanin at a bare glassy carbon electrode. *Eur J Biochem 219*:813–819.

Jones TA. 1978. A graphics model building and refinement system for macromolecules. *J Appl Crystallogr 11*:268–272.

Lu Y, LaCroix LB, Lowery MD, Solomon EI, Bender CJ, Peisach J, Roe JA, Gralla EB, Valentine JS. 1993. Construction of a "blue" copper site at the native zinc site of yeast copper-zinc superoxide dismutase. *J Am Chem Soc 115*:5907–5918.

Luzzati V, 1952. Traitment statistique des erreurs dans la determination des structures cristallines. *Acta Cryst 5*:802–810.

Malmström BG. 1994. Rack-induced bonding in blue-copper proteins. *Eur J Biochem 223*:711–718.

Margalit R, Kostic NM, Che CM, Blair DF, Chiang HJ, Pecht I, Shelton JB, Shelton JR, Schroeder WA, Gray HB. 1984. Preparation and characterization of pentaammineruthenium-(histidine-83)azurin: Thermodynamics of intramolecular electron transfer from ruthenium to copper. *Proc Natl Acad Sci USA 81*:6554–6558.

Mauk AG, Scott RA, Gray HB. 1980. Distances of electron transfer to and from metalloproteins redox sites in reactions with inorganic complexes. *J Am Chem Soc 102*:4360–4363.

Mims WB, Davis JL, Peisach J. 1984. The accessibility of type I Cu(II) centers in laccase, azurin, and stellacyanin to exchangeable hydrogen and ambient water. *Biophys J 45*:755–766.

Modi S, Nordling M, Lundberg LG, Hansson O, Bendall DS. 1992. Reactivity of cytochromes *c* and *f* with mutant forms of spinach plastocyanin. *Biochim Biophys Acta 1102*:85–90.

Murata M, Beggs, GS, Lambrou F, Leslie B, Simpson RJ, Freeman HC, Morgan FJ. 1982. Amino acid sequence of a basic blue protein from cucumber seedlings. *Proc Natl Acad Sci USA 79*:6434–6437.

Nersissian AM, Mehrabian ZB, Nalbandyan RM, Hart PJ, Fraczkiewicz G, Czernuszewicz RS, Bender CJ, Peisach J, Herrmann RG, Valentine JSS. 1996. Cloning, expression, and spectroscopic characterization of *Cucumis sativus* stellacyanin in its non-glycosylated Form. *Protein Sci 5*:2184–2192.

Nersissian AM, Nalbandyan RM. 1990. Particles of photosystem 2 contain plantacyanin. *Biochem Biophys Res Commun 168*:232–239.

Obokata J, Mikami K, Hayashida N, Nakamura M, Sugiura M. 1993. Molecular heterogeneity of photosystem I. *psaD, psaE, psaF, psaH* and *psaL* are all present in isoforms in *Nicotiana* spp. *Plant Physiol 102*:1259–1267.

Otwinowski Z. 1993. Oscillation data reduction program. In: Proceedings of the CCP4 study weekend: Data collection and processing. Compiled by Sawyer L, Issacs N, and Bailey S. SERC Laboratory, England. pp 56–62.

Peisach J, Levine WG, Blumberg WE. 1967. Structural properties of stellacyanin, a copper mucoprotein from *Rhus vernicifera*, the Japanese lac tree. *J Biol Chem 242*:2847–2858.

Peisach J, Powers L, Blumberg WE, Chance B. 1982. Stellacyanin. Studies of the metal-binding site using x-ray absorption spectroscopy. *Biophys J 38*:277–285.

Petratos K, Dauter Z, Wilson KS. 1988. Refinement of the structure of pseudoazurin from *Alcaligenes faecalis* S-6 at 1.55 Å resolution. *Acta Cryst B44*:628–636.

Ramachandran GN, Sasiskharam V. 1968. Conformation of polypeptides and proteins. *Adv Protein Chem 23*:283–437.

Read RJ. 1986. Improved fourier coefficients for maps using phases from partial structures with errors. *Acta Cryst A42*:140–149.

Romero A, Hoitink CWG, Nar H, Huber R, Messerschmidt A, Canters GW. 1993. X-ray analysis and spectroscopic characterization of M121Q azurin. A copper site model for stellacyanin. *J Mol Biol 229*:1007–1021.

Satow Y, Cohen GH, Padlan EA, Davies DR. 1986. Phosphocholine binding immunoglobulin Fab McPC603. An x-ray diffraction study at 2.7 Å. *J Mol Biol 190*:593–604.

Sheldrick GM, Schneider TR. 1996. SHELXL: High-resolution refinement. *Methods Enzymol.* Forthcoming.

Solomon EI, Baldwin MJ, Lowery MD. 1992. Electronic structures of active sites in copper proteins: Contributions to reactivity. *Chem Rev 92*:521–542.

Solomon EI, Lowery MD. 1993. Electronic structure contributions to function in bioinorganic chemistry. *Science 259*:1575–1581.

Strange RW, Reinhammar B, Murphy LM, Hasnain SS. 1995. Structural and spectroscopic studies of the copper site of stellacyanin. *Biochemistry 34*:220–231.

Thomann H, Bernardo M, Baldwin MJ, Lowery MD, Solomon EI. 1991. Pulsed endor study of the native and high pH perturbed forms of the blue copper site in stellacyanin. *J Am Chem Soc 113*:5911–5913.

Tollin G, Meyer TE, Cheddar G, Getzoff ED, Cusanovich MA. 1986. Transient kinetics of blue copper proteins by free flavin and flavodoxin semiquinones. *Biochemistry 25*:3363–3370.

Wherland S, Farver O, Pecht I. 1988. Three-dimensional model of stellacyanin and its implications for electron transfer reactivity. *J Mol Biol 204*:407–415.

Wherland S, Holwerda RA, Rosenberg RC, Gray HB. 1975. Kinetic studies of the reduction of blue copper proteins by Fe(EDTA)2-. *J Am Chem Soc 97*:5260–5262.

Wuttke DS, Gray HB. 1993. Protein engineering as a tool for understanding electron transfer. *Curr Opin Struct Biol 3*:555–563.