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# Plastocyanin: Structural and Functional Analysis

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Plastocyanin is one of the best characterized of the photosynthetic electron transfer proteins. Since the determination of the structure of poplar plastocyanin in 1978, the structure of algal (*Scenedesmus*, *Enteromorpha*, *Chlamydomonas*) and plant (French bean) plastocyanins has been determined either by crystallographic or NMR methods, and the poplar structure has been refined to 1.33 Å resolution. Despite the sequence divergence among plastocyanins of algae and vascular plants (e.g., 62% sequence identity between the *Chlamydomonas* and poplar proteins), the three-dimensional structures are remarkably conserved (e.g., 0.76 Å rms deviation in the Cα positions between the *Chlamydomonas* and poplar proteins). Structural features include a distorted tetrahedral copper binding site at one end of an eight-stranded antiparallel β-barrel, a pronounced negative patch, and a flat hydrophobic surface. The copper site is optimized for its electron transfer function, and the negative and hydrophobic patches are proposed to be involved in recognition of physiological reaction partners. Chemical modification, cross-linking, and site-directed mutagenesis experiments have confirmed the importance of the negative and hydrophobic patches in binding interactions with cytochrome *f* and Photosystem I, and validated the model of two functionally significant electron transfer paths in plastocyanin. One putative electron transfer path is relatively short (~ 4 Å) and involves the solvent-exposed copper ligand His-87 in the hydrophobic patch, while the other is more lengthy (~ 12–15 Å) and involves the nearly conserved residue Tyr-83 in the negative patch.

**KEY WORDS:** Cytochrome *f*; Photosystem-I; blue-copper proteins; cytochrome $c_6$; electron transfer.

## INTRODUCTION

Plastocyanin is a small (97–104 amino acids) copper-binding protein whose function in oxygenic photsynthesis is the catalysis of electron transfer from cytochrome *f* in the $b_6f$ complex to $P700^+$ in Photosystem I. It is referred to as a "blue" copper protein on the basis of its spectroscopic properties ($\epsilon \sim 4700\,M^{-1}\,cm^{-1}$ at 600 nm). Other members of this class of small, blue copper proteins include azurin, pseudoazurin, and amicyanin (Table I) (reviewed by Adman, 1991). The structure of oxidized plastocyanin from poplar was first determined by Freeman and co-workers (Colman *et al.*, 1978). The protein is an eight-stranded, antiparallel β-barrel with a single copper atom liganded by the side chains of two histidines, a cysteine, and a methionine (Figs. 1A and 1B). In the most common representation of the structure, the copper site is at one end, often referred to as the "northern" end, of the 40 × 32 × 28 Å molecule. The surface at this end was noted to consist of exclusively hydrophobic residues, and the region is accordingly termed the "hydrophobic patch." A region of concentrated negative charge resulting from the deprotonated side chains of glutamyl and aspartyl residues is termed the "negative patch" and is, in the conventional representation of plastocyanin, located on the "east" side of the molecule. Two paths of electron transfer to and from the copper atom were identified in the original work (Colman *et al.*, 1978): an "adjacent" site via His-87 (the only solvent-exposed copper ligand), and a

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**Table I.** "Blue" Copper Proteins of Known Structure

| | Site of electron transfer function | $E_m$ | Known structures | | | Description of Cu(II) Site | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Bond lengths (Å) | | | Bond angles (°) | | |
| Azurin | Aerobic respiration in bacteria | 330 mV (pH 7.5) | Bacteria | | | Cu | Nδ1(46) | 2.08*[f] | Nδ1(46) | Cu | Nδ1(117) | 104 |
| | | | *P. aeruginosa* | Cu(II) (also Zn-subs, 2.1 Å[b]; apo, 1.85 Å[c]; mutants, 1.9 Å[d]; two pH's, 1.93 Å[e]) | 2.7 Å[a] | Cu | Nδ1(117) | 2.00 | Nδ1(46) | Cu | Sγ(112) | 135 |
| | | | | | | Cu | Sγ(112) | 2.14 | Nδ1(46) | Cu | Sδ(121) | 77 |
| | | | | | | Cu | Sδ(121) | 3.11 | Nδ1(117) | Cu | Sγ(112) | 119 |
| | | | *A. denitrificans* | Cu(II) | 1.8 Å[f] | Cu | O(45) | 3.12 | Nδ1(117) | Cu | Sδ(121) | 96 |
| | | | | Cu(I) (also apo, 1.9 Å[g]; Cd-subs, 2.3 Å[g]; mutants, 1.9 Å[h]) | 1.9 Å[g] | | | | Sγ(112) | Cu | Sδ(121) | 107 |
| | | | | | | | | | Nδ1(46) | Cu | O(45) | 74 |
| | | | | | | | | | Nδ1(117) | Cu | O(45) | 79 |
| | | | *P. denitrificans* | Cu(II) | 3.0 Å[i] | | | | Sγ(112) | Cu | O(45) | 103 |
| | | | | | | | | | Sδ(121) | Cu | O(45) | 147 |
| Pseudoazurin | Anaerobic respiration in bacteria | 270 mV (pH 7.0) | Bacteria | | | | | | | | | |
| | | | *A. faecalis* | Cu(II) | 1.55 Å[j] | Cu | Nδ1(40) | 2.16[j] | Nδ1(40) | Cu | Nδ1(81) | 100 |
| | | | | | | Cu | Nδ1(81) | 2.13 | Nδ1(40) | Cu | Sγ(78) | 136 |
| | | | | | | Cu | Sγ(78) | 2.16 | Nδ1(40) | Cu | Sδ(86) | 87 |
| | | | | | | Cu | Sδ(86) | 2.76 | Nδ1(81) | Cu | Sγ(78) | 112 |
| | | | | | | | | | Nδ1(81) | Cu | Sδ(86) | 112 |
| | | | | | | | | | Sγ(78) | Cu | Sδ(86) | 108 |
| Amicyanin | Respiratory chains of methylotrophic bacteria | 295 mV (pH 6.7) | Bacteria | | | | | | | | | |
| | | | *P. denitrificans* | Cu(II) (also apo, 1.8 Å[k]) | 2.0 Å[k] | Cu | Nδ1(53) | 1.95[k] | Nδ1(53) | Cu | Nδ1(95) | 109 |
| | | | | | | Cu | Nδ1(95) | 2.00 | Nδ1(53) | Cu | Sγ(92) | 135 |
| | | | | | | Cu | Sγ(92) | 2.15 | Nδ1(53) | Cu | Sδ(98) | 83 |
| | | | | | | Cu | Sδ(98) | 2.89 | Nδ1(95) | Cu | Sγ(92) | 109 |
| | | | | | | | | | Nδ1(95) | Cu | Sδ(98) | 104 |
| | | | | | | | | | Sγ(92) | Cu | Sδ(98) | 109 |
| Plastocyanin | Membranes of oxygenic photosynthetic organisms (plants, algae and cyanobacteria) | 370 mV† (pH 7.5) | Plants | | | | | | | | | |
| | | | Poplar | Cu(II) | 1.33 Å[l] | | | | | | | |
| | | | | Cu(I) (also apo, 1.8 Å[n]; Hg-subs, 1.9 Å[o]) | 1.7–2.15 Å (six pH's)[m] | Cu | Nδ1(37) | 1.91[l] | Nδ1(37) | Cu | Nδ1(87) | 97 |
| | | | | | | Cu | Nδ1(87) | 2.06 | Nδ1(37) | Cu | Sγ(84) | 132 |
| | | | French bean | Cu(I) | $^1$H-NMR[p] | Cu | Sγ(84) | 2.07 | Nδ1(37) | Cu | Sδ(92) | 88 |
| | | | Spinach | Cu(I) | $^1$H-NMR (partial structure)[q] | Cu | Sδ(92) | 2.82 | Nδ1(87) | Cu | Sγ(84) | 121 |
| | | | | | | | | | Nδ1(87) | Cu | Sδ(92) | 101 |
| | | | | | | | | | Sγ(84) | Cu | Sδ(92) | 110 |
| | | | Green algae | | | | | | | | | |
| | | | *E. prolifera* | Cu(II) | 1.85 Å[r] | | | | | | | |
| | | | *C. reinhardtii* | Cu(II) | 1.5 Å[s] | | | | | | | |
| | | | *S. obliquus* | Cu(I) | $^1$H-NMR[t] | | | | | | | |

† For eukaryotic plastocyanins; the value for *A. variabilis* plastocyanin at pH 7.5 is 340 mV (Jackman *et al.*, 1987, 1988).

* The bond lengths and bond angles given here for azurin are the mean values for two crystallographically independent molecules (Baker, 1988).

[a] Adman and Jensen, 1981; [b] Nar *et al.*, 1992a; [c] Nar *et al.*, 1992b; [d] Nar *et al.*, 1991; [e] Nar *et al.*, 1992c; [f] Baker, 1988; [g] Baker, 1991; [h] Romero *et al.*, 1993; [i] Korszun, 1987; [j] Petratos *et al.*, 1988; [k] Durley *et al.*, 1993; [l] Guss *et al.*, 1992; [m] Guss *et al.*, 1986; [n] Garrett *et al.*, 1984; [o] Church *et al.*, 1986; [p] Moore *et al.*, 1991; [q] Driscoll *et al.*, 1987; [r] Collyer *et al.*, 1990; [s] Redinbo *et al.*, 1993; [t] Moore *et al.*, 1988.

"remote" site via Tyr-83 on the east side of the molecule.

Since the determination of the structure of oxidized plastocyanin, structures of apoplastocyanin, mercury-substituted plastocyanin, and reduced plastocyanin at six pH values ranging from 3.8 to 7.8 have been determined (Garrett *et al.*, 1984; Church *et al.*, 1986; Guss *et al.*, 1986), and the structure of the oxidized poplar plastocyanin has been refined to 1.33 Å (Guss *et al.*, 1992). In addition, the structures of algal plastocyanins (*Scenedesmus obliquus*, *Enteromorpha prolifera*, and *Chlamydomonas reinhardtii*) and another plant plastocyanin (French bean) have been determined by either crystallographic (Collyer *et al.*, 1990; Redinbo *et al.*, 1993) or NMR (Chazin and Wright, 1988; Moore *et al.*, 1988, 1991) methods. Much of the detailed structural analysis of plastocyanin has been carried out by the groups of Guss and Freeman (papers cited above) and this review has therefore drawn heavily from their work.

Plastocyanin can be considered to have three main functions. First, it must maintain its copper-binding site so that the redox potential of the protein lies between that of its physiological reductant (cytochrome *f*) and oxidant ($P700^+$). Second, it must provide specific binding site(s) for its physiological reaction partners. And finally, it must provide the proper structural scaffolding for controlled electron transfer into and out of the copper center. This article will examine how the structure of plastocyanin maintains each of these functions. For additional aspects of plastocyanin chemistry and biology, the interested reader is directed to the reviews of Sykes (1985, 1991) on the electron transfer properties of plastocyanin, Church *et al.* (1987) which emphasizes structural properties, Adman (1991) on copper proteins, Haehnel (1984) on the function of plastocyanin, and Boulter *et al.* (1977) on the biochemistry and evolution of plastocyanin.

## 2. THE COPPER-BINDING SITE IN PLASTOCYANIN

### 2.1. Ligands and Geometry

The copper-binding site in plastocyanin is located in the "northern" portion of the molecule and consists of a single copper atom liganded by a cysteinyl sulfur (from Cys-84), a methioninyl sulfur (from Met-92), and two histidinyl nitrogens (from His-37 and His-87) (Fig. 1A). His-37, His-87, Cys-84, and Met-92 are all conserved in the plastocyanins of known sequence (reviewed by Sykes, 1991; Fig. 2). The copper-binding site is quasi-tetrahedral in shape, with bond lengths and bond angles as given in Table I. Since the distance from the Cu atom to $S^{\delta}$(Met-92) is quite long ($\sim$ 2.9 Å) and the Cu–$S^{\delta}$(Met-92) bond is not detected by EXAFS measurements, the involvement of Met-92 in binding the copper atom has been difficult to assess (Colman *et al.*, 1978; Sykes, 1985). Nevertheless, site-directed mutagenesis of Met-92 in plastocyanin suggests that it is indeed required for copper binding (Chang *et al.*, 1991). In contrast, Met-121 of azurin is not required for the formation of a blue copper site in azurin (Karlsson *et al.*, 1991; Murphy *et al.*, 1993). The copper-binding sites in the three crystal structures of oxidized plastocyanin are quite similar (Colman *et al.*, 1978; Collyer *et al.*, 1990; Guss *et al.*, 1992; Redinbo *et al.*, 1993), with the largest deviation being a 0.16 Å difference in the length of the Cu–$N^{\delta 1}$(His-87) bond in *E. prolifera* plastocyanin (2.17 Å) and *C. reinhardtii* plastocyanin (2.01 Å). This difference is well within the 0.25 Å coordinate error of the *C. reinhardtii* structure (Redinbo *et al.*, 1993).

The structure of the copper-binding site of oxidized poplar plastocyanin at pH 6.0 is almost identical to that of reduced poplar plastocyanin at pH 7.8, with the largest deviation being 0.15 Å in the length of the Cu–$N^{\delta 1}$(His-87) bond (Guss *et al.*, 1986). As the pH of the crystal is decreased, however, the geometry of the site undergoes a notable change (Guss *et al.*, 1986). The liganding nitrogen of His-87 of the reduced protein becomes protonated with decreasing pH and moves slightly up and away from the copper atom, whereas the copper atom moves down and away from His-87 so that it is eventually only trigonally coordinated by His-37, Cys-84, and Met-92. At pH values below 5.1, the copper atom and its three liganding atoms are essentially coplanar. At pH 3.8, the liganding atoms $N^{\delta 1}$(His-37), $S^{\gamma}$(Cys-84), and $S^{\delta}$(Met-92) are 2.11, 2.13, and 2.52 Å, respectively, from the copper atom, but the $N^{\delta 1}$(His-87) to copper distance has increased to 3.15 Å. Between pH 7.8 and 3.8 the copper-binding site of reduced plastocyanin can be considered to be intermediate between the quasi-tetrahedral (high pH) and trigonal (low pH) forms (Guss *et al.*, 1986). Since trigonal geometry stabilizes Cu(I) over Cu(II), the low pH forms of reduced plastocyanin (redox potential 430 mV at pH

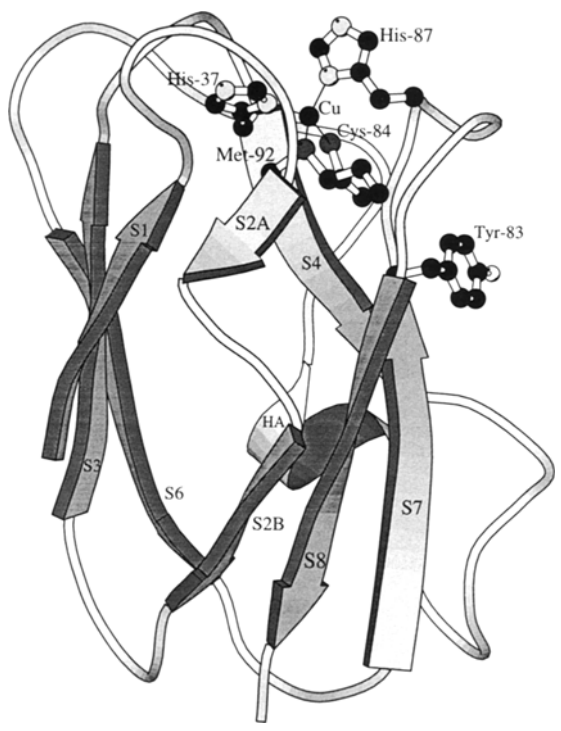



**Fig. 1A.** The structure of plastocyanin with emphasis on the copper-binding site. The molecule consists of two $\beta$-sheets made up of eight $\beta$-strands (S1 through S8) and one short region of $\alpha$-helix (HA). The copper-binding site is located at the "north" end of the molecule and consists of a single copper atom liganded in quasi-tetrahedral geometry by His-37, Cys-84, His-87, and Met-92. The side chain of Tyr-83, the putative "remote" site of electron transfer, lies within the negative patch on the "east" side of the molecule and is also shown. The structure shown is that of poplar plastocyanin (Guss *et al.*, 1992), although the other plastocyanins of known structure have almost identical folds and copper-binding sites (Chazin and Wright, 1988; Collyer *et al.*, 1990; Moore *et al.*, 1991; Redinbo *et al.*, 1993). The numbering of the strands is according to Guss and Freeman (1983) and does not included a $\beta$-strand 5. The carbon atoms and the copper atom are shown in black, the sulfur atoms in dark gray, the nitrogen atoms in light gray, and the oxygen atoms in white. This figure was created with the program MOLSCRIPT (Kraulis, 1991).

4.2 in isolation) are redox inactive with the inorganic complexes $Co(phen)_3^{3+}$ (370 mV) and $Fe(CN)_6^{3-}$ (410 mV) (Katoh *et al.*, 1962; Segal and Sykes, 1978; Sykes, 1985). The reduced protein nevertheless remains active in the thylakoid membrane with respect to the physiologically relevant reaction, $P700^+$ reduction, and is, in fact, most active at low pH (Takabe *et al.*, 1983, 1984).

Compared to hexaaquacopper $(Cu(H_2O)_6)^{2+/1+}$, redox potential 115 mV), the copper site of plastocyanin (370 mV) is optimized for its biological function (electron transfer from reduced cyt *f*, 360 mV, to

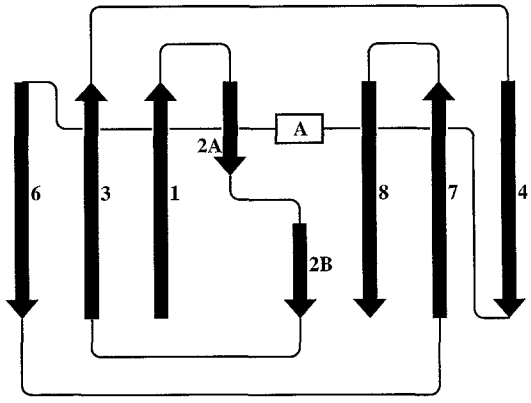



**Fig. 1B.** Schematic representation of the folding pattern in plastocyanin. $\beta$-strands 1, 2A, 3, and 6 make up $\beta$-sheet I, and $\beta$-strands 2B, 4, 7, and 8 make up $\beta$-sheet II. Helix A consists of a single turn of $\alpha$-helix.

$P700^+$, 520 mV), and is a consequence of the structure of the protein. The quasi-tetrahedral geometry of the copper site accommodates both the cuprous and cupric forms of copper with essentially no structural reorganization at high pH. In terms of "hard-acid-soft-base" theory, the site is favorable to both Cu(II) and Cu(I), since two of the four liganding atoms (the imidazole nitrogens) are intermediate bases, which are preferred by Cu(II), and the other two (the sulfurs) are soft bases, which are preferred by Cu(I) (Colman *et al.*, 1978). Several residues that are conserved in the known plastocyanin sequences (Fig. 2) lend structural stability to the copper site. The side chain of Phe-14 is in van der Waals contact with the side chain of Met-92 and, along with the main chain atoms between residues 12 and 13, helps to stabilize the position of this copper-binding site residue. The side chains of Leu-12 and Asn-31 are in van der Waals contact with the side chains of His-87 and His-37, respectively, and the side chain of Asn-38 provides a hydrogen-bond bridge between the peptide nitrogen of residue 85 and the carbonyl oxygen of residue 59, perhaps stabilizing the position of Cys-84. The copper-binding sites of the other "blue" copper proteins of known structure are also quasi-tetrahedral and, in the cases of pseudoazurin and amicyanin, are almost identical to that of plastocyanin (Table I). Azurin contains a fifth copper-binding ligand, the main chain carbonyl oxygen of Gly-35, that is 3.12 Å from the copper atom; this liganding oxygen provides azurin with a second long Cu-ligand bond (Baker, 1988). The Cu–O(Gly-35) interaction in azurin is detected by nuclear magnetic resonance spectroscopy (Ugurbil *et al.*, 1977), and its bond

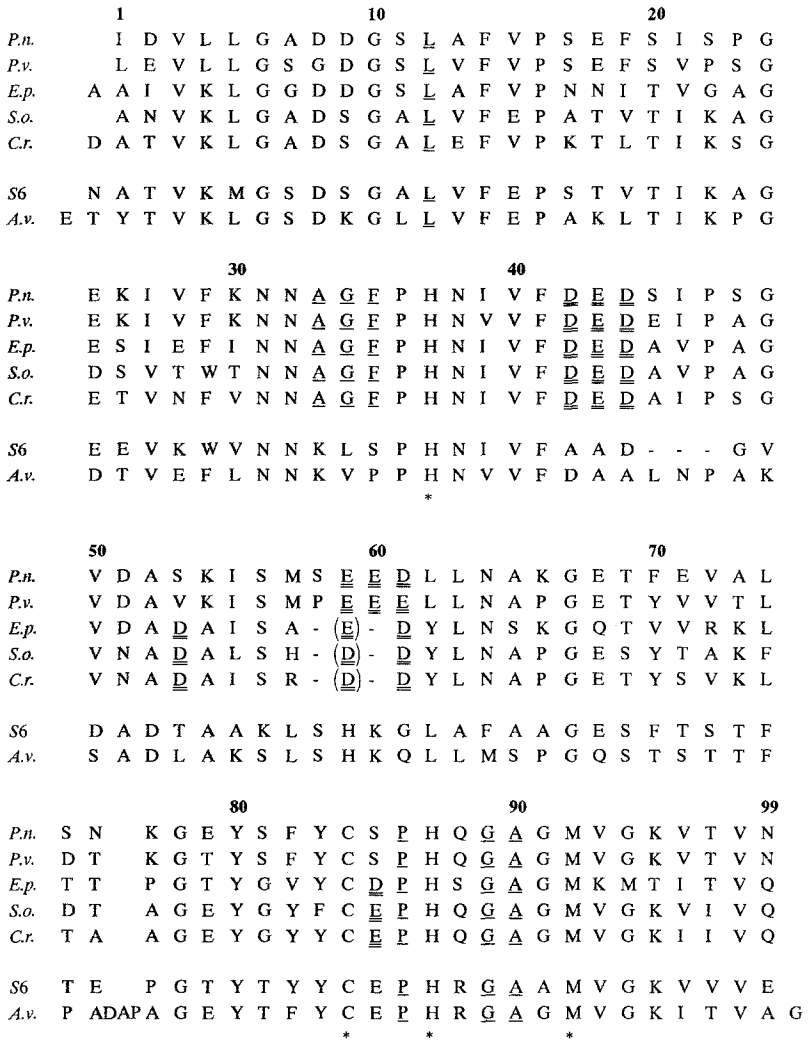
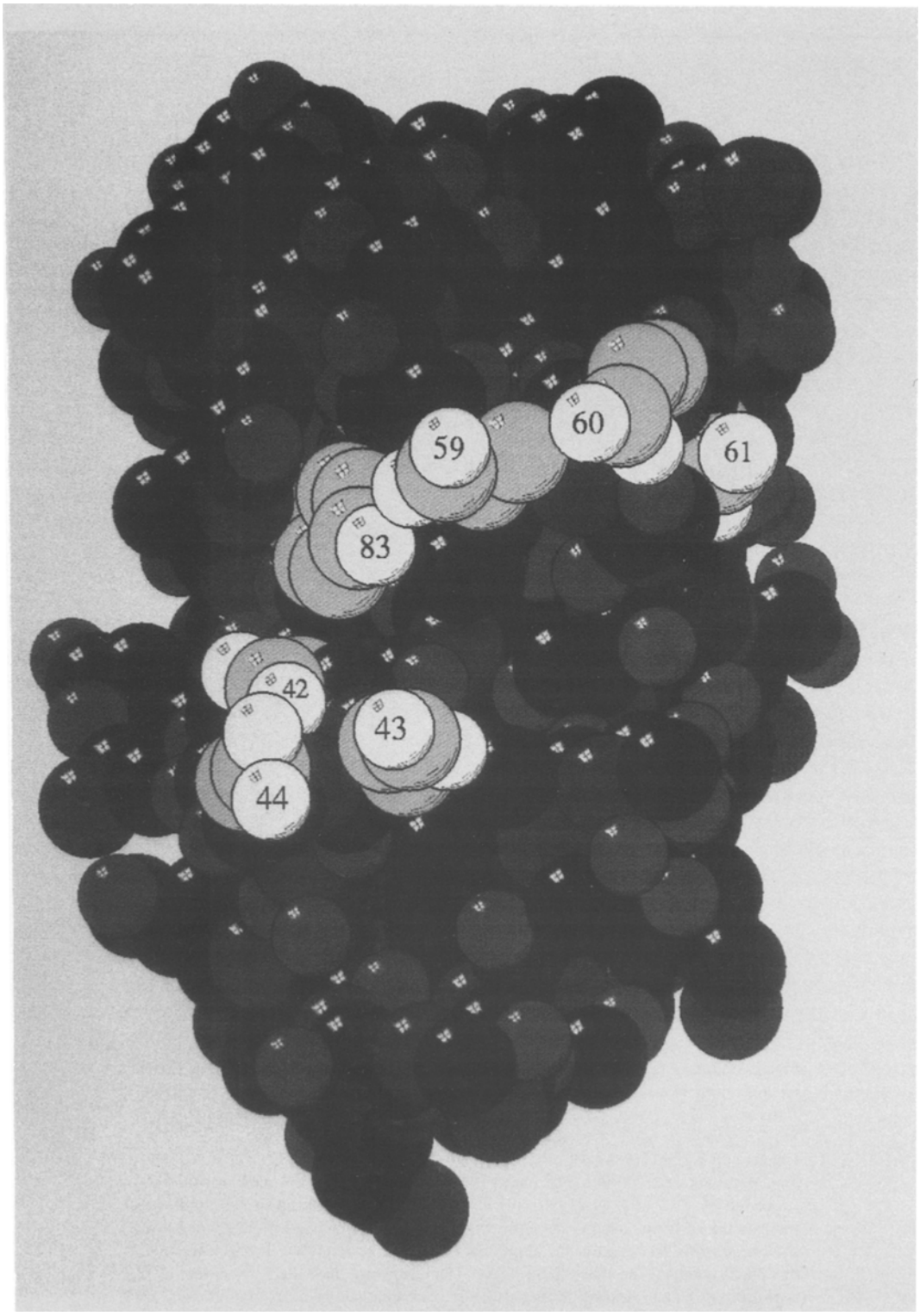



**Fig. 2.** Sequence alignment of the structurally characterized plastocyanins with the plastocyanins from cyanobacteria. *P.n., Populus nigra*; *P.v., Phaseolus vulgaris*; *E.p., Enteromorpha prolifera*; *C.r., Chlamydomonas reinhardtii*; *S.o., Scenedesmus obliquus*; S6, *Synechocystis* 6803; *A.v., Anabaena variabilis*. Sequences are from Aitken (1975), Chazin and Wright (1988), Moore *et al.* (1988); Briggs *et al.* (1990), Collyer *et al.* (1990) and Merchant *et al.* (1990). The numbering is according to the poplar sequence. As suggested by Collyer *et al.* (1990), the algal residue 59 is enclosed in parentheses to indicate that it is not structurally equivalent to residue 59 of poplar and French bean plastocyanin. In that region, the alignment of Collyer *et al.* (1990) has been retained; the positioning of the deleted residues is therefore not indended to represent an evolutionary event. Among the structurally characterized proteins, 39 residues are identical. Residues comprising the hydrophobic patch are underlined, those comprising the negative patch are double underlined, and the copper binding ligands are indicated by an asterisk. The cyanobacterial proteins have not been characterized by crystallographic or NMR methods; note the sequence divergence in the regions contributing to reaction partner binding sites.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length is considered to be at the limit for a weak axial Cu(II)–O bond, as defined by studies with small inorganic copper complexes (Gazo *et al.*, 1976).

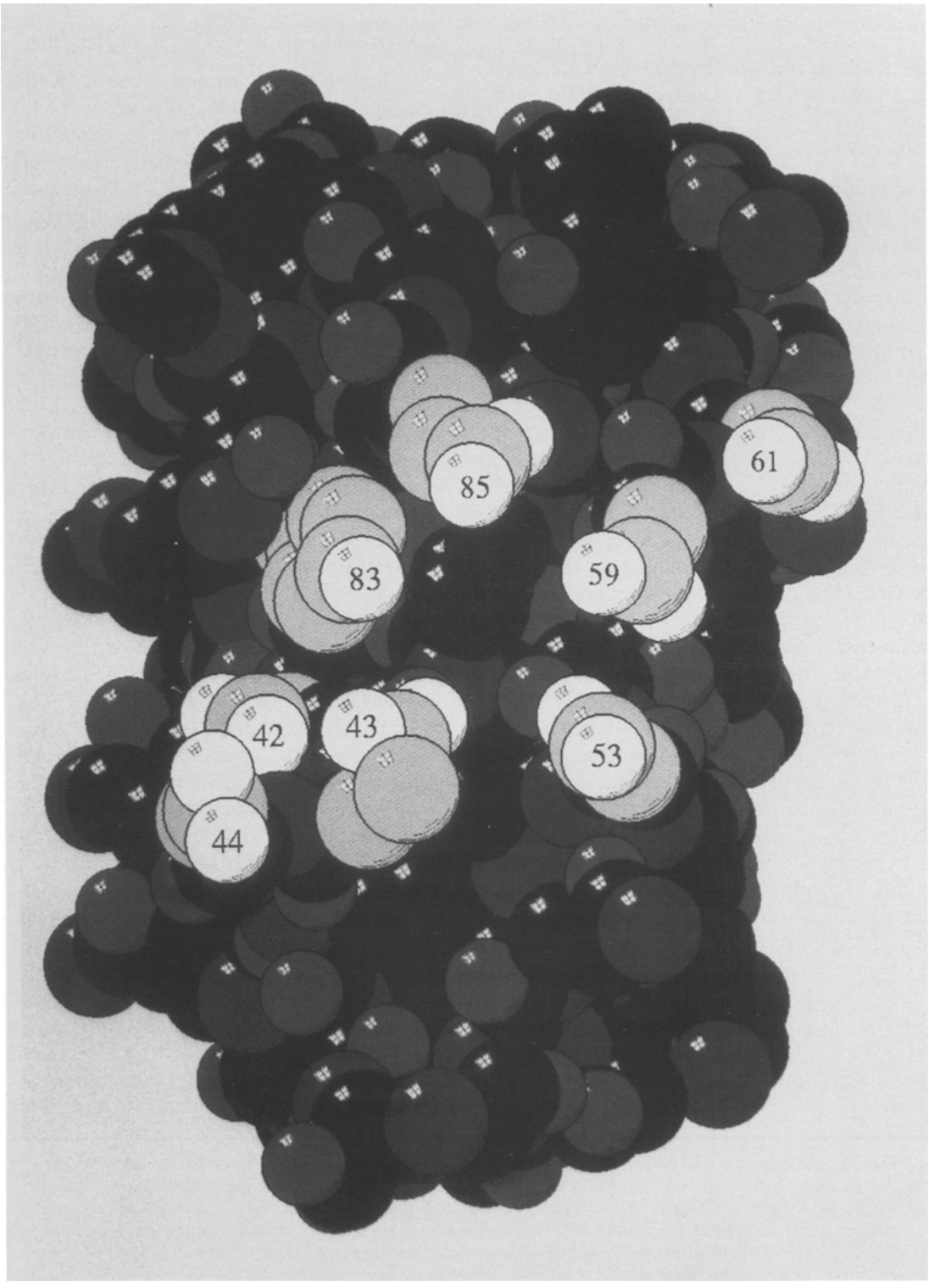
### 2.2. Apoplastocyanin

The determination of the structure of apoplastocyanin to 1.8 Å resolution (Garrett *et al.*, 1984) has shown that, in the crystalline state, the structure of the protein does not change upon removal of its copper atom. This result suggests that the positioning of the copper-binding residues is independent of the presence of the metal (Garrett *et al.*, 1984). In apoplastocyanin, the side chain of His-87 is rotated by 180° about its $C^{\beta}$–$C^{\gamma}$bond so that its copper-liganding atom $N^{\delta 1}$ is directed into the solvent. Garrett *et al.* (1984) have suggested that this residue may serve as a rotational "gate" for copper entry into the copper-binding site. Pro-36, which is adjacent to His-37 and is conserved in the known plastocyanin sequences, undergoes a slight conformational change, from $C^{\gamma}$-exo to $C^{\gamma}$-endo, upon the removal of copper, the substitution of mercury for copper, or with changes in oxidation state (Church *et al.*, 1987). These conformational differences are indicative of a region of flexibility around the copper site which could allow the addition of copper to the folded apoenzyme without relaxation of the tertiary structure of the molecule. Since copper-deficient plastocyanin is subject to rapid proteolytic degradation *in vivo* in the thylakoid lumen, whereas copper-containing plastocyanin is extremely stable (Merchant and Bogorad, 1986; Li and Merchant, 1992), the question of the mechanism by which apoplastocyanin may be recognized and targeted for proteolysis *in vivo* is raised. The present experimental evidence does not distinguish between the possibility that copper-deficient plastocyanin may be partially unfolded in solution under *in vivo* ionic conditions vs. the possibility that copper deficiency induces a protease that can degrade folded plastocyanin.

### 2.3. Orbital Interactions in the Copper Site

Type-I "blue" copper proteins exhibit both a strong absorption band at 600 nm, with a high extinction coefficient relative to free copper in solution, and a greatly reduced parallel hyperfine splitting (reviewed by Solomon and Lowery, 1993). The reduced parallel hyperfine splitting was originally thought to be the result of orbital mixing within the copper atom, but K- and L-edge absorption spectroscopy showed it to be the result of the general covalent nature of the copper site. The strong 600 nm absorption band with a high extinction coefficient is due to low-energy orbital charge transfer between the Cu $d_{x^2-y^2}$ orbital and the $S^{\gamma}$(Cys-84) $\pi$-orbital. This demonstrates the particularly strong covalent nature of the Cu–$S^{\gamma}$(Cys-84) bond. In hexaaquacopper, the orbital charge transfer is higher in energy and therefore has a lower extinction coefficient. The Cu $d_{x^2-y^2}$ orbital is implicated because it is the highest energy orbital and is therefore only half-occupied at the ground state. During biological electron transfer, the additional electron is stored in the $d_{x^2-y^2}$ orbital. Spectroscopic studies have also shown that the $d_{x^2-y^2}$ orbital of the copper atom essentially lies in the plane defined by the copper-liganding atoms $N^{\delta 1}$(His-37), $S^{\gamma}$(Cys-84), and $N^{\delta 1}$(His-87), and that the Cu–$S^{\gamma}$(Cys-84) bond bisects the lobes of this orbital. This strongly activates the Cu–$S^{\gamma}$(Cys-84) bond for electron transfer, lending support to the notion that electron transfer may occur via Cys-84 and Tyr-83 in plastocyanin. Han *et al.* (1991) have also suggested that the conserved coplanarity of the Cu and $S^{\gamma}$, $C^{\alpha}$, $C^{\beta}$, and N atoms of the (*n*)-cysteine ligand in "blue" copper proteins (i.e., Cys-84 in plastocyanin) is necessary to activate electron transfer through this residue to an adjacent, $(n-1)$-aromatic residue at a "remote" electron transfer site (i.e., Tyr-83). An exception is azurin, which contains coplanar Cu and $S^{\gamma}$, $C^{\alpha}$, $C^{\beta}$, and N(Cys-112) atoms but does not appear to transfer electrons via a putative "remote" electron transfer site at His-35

**Fig. 3A.** Space-filling model of the negative patch of poplar plastocyanin (Guss *et al.*, 1992). The side chains of Asp-42, Glu-43, and Asp-44 (conserved in the known eukaryotic plastocyanins; see Fig. 2) and Glu-59, Glu-60, and Asp-61 (present in some plant plastocyanins; see text and Fig. 2) make up this region of concentrated negative charge surrounding Tyr-83, which is involved in one of the two electron transfer paths in plastocyanin. This region of the molecule is also unique in shape, with residues 42–45 making up a type-I $\beta$-turn and residues 59-61 lying on a characteristic bulge not present in the algal and some plant plastocyanins (Fig. 4). Also see Fig. 3C for a side view of this surface. In the structure of poplar plastocyanin, Asp-61 is present in two conformations (Guss *et al.*, 1992); a single conformation has been chosen for this figure. In the highlighted residues, carbon atoms are shown in light gray and oxygen atoms are in white; in the remainder of the molecule, carbon atoms are shown in black and oxygen and nitrogen atoms are in two shades of dark gray (the nitrogen atoms are darker). The molecule in this figure has been rotated 130° about the vertical axis relative to Fig. 1A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(van de Kamp *et al.*, 1990). Perhaps coplanarity is simply a means of maintaining proper geometry about the copper site for the stabilization of both cuprous and cupric forms of copper.

## 3. THE NEGATIVE PATCH AND HYDROPHOBIC PATCH IN PLASTOCYANIN

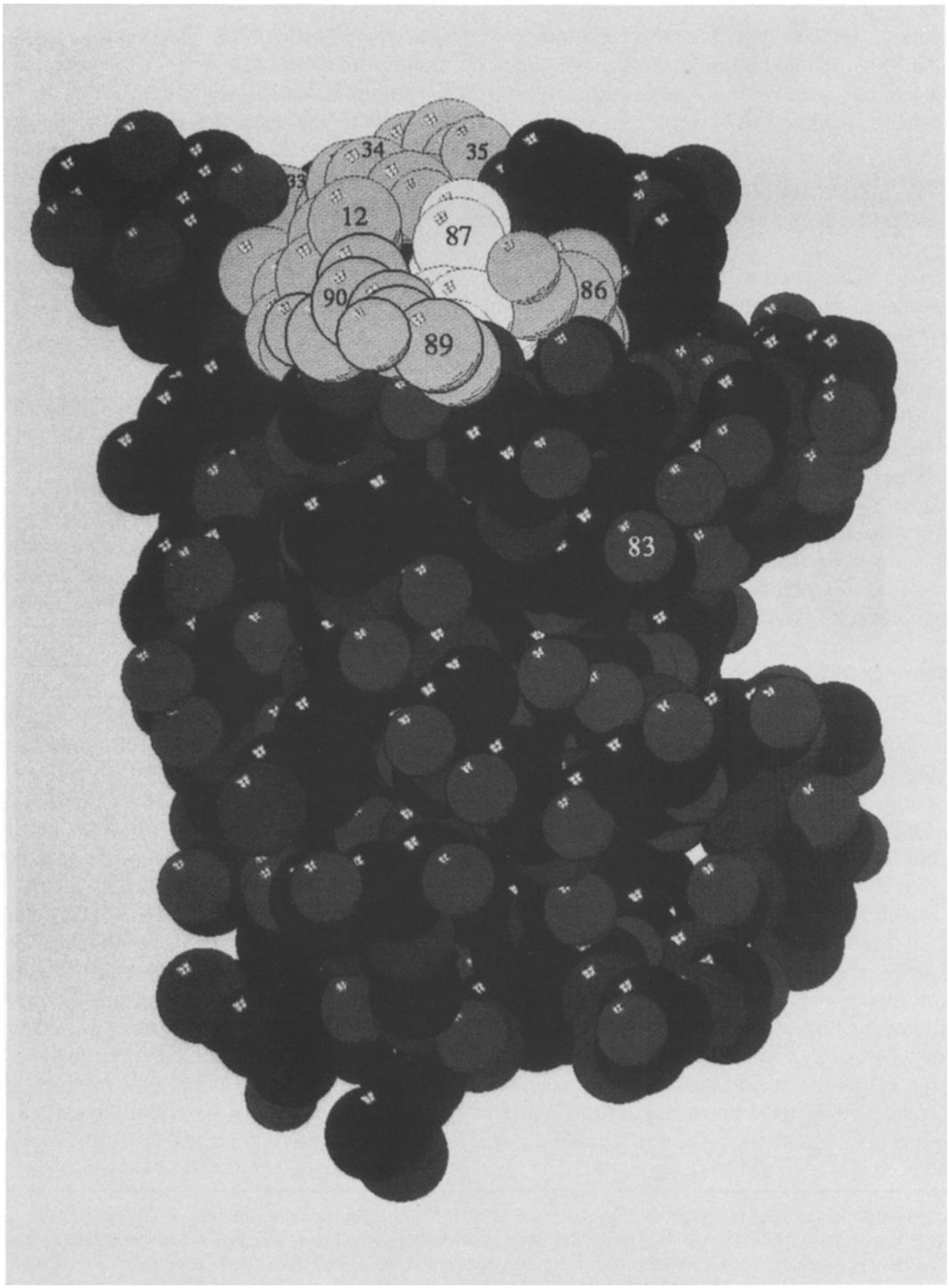
Based on the structure of the protein and the location in space of the conserved residues, Colman *et al.* (1978) suggested two possible routes of electron transfer in plastocyanin: one via His-87 at the "northern" end of the molecule, and another longer-distance path via Cys-84 and Tyr-83 at the "eastern" face (see Section 4 for further discussion). The conserved residues in the vicinity of His-87 and Tyr-83 form distinct hydrophobic and hydrophilic patches, respectively, on the surface of the molecule (Figs. 3A, B, C) and were therefore suggested to provide interaction sites for reaction with the physiological donor (cytochrome *f*) and acceptor ($P700^+$).

### 3.1. Structure and Characteristics of the Negative Patch

In the eukaryotic plastocyanins, the hydrophilic patch is negatively charged at physiological pH. The negative patch is located on the "east" side of the molecule and surrounds the nearly conserved residue Tyr-83 (Boulter *et al.*, 1977; Sykes, 1991; Figs. 1A, 3A, and 3B) that has been implicated in one of the two electron transfer pathways (Colman *et al.*, 1978; He *et al.*, 1991; Modi *et al.*, 1992a, b). The shape and charge distribution around the negative patch make it a unique region on the surface of the molecule in both plant and algal plastocyanins. There are no other prominent loops or concentrations of charge on any other region of the molecular surface. Three residues that are conserved in the known eukaryotic plastocyanin sequences, Asp-42, Asp-43, and Glu-44, lie in a Type-I $\beta$-turn on the "east" side of the molecule and make up part of the negative patch. Several other acidic residues reside on this side of the protein and complete the negative patch. In the plant plastocyanins, residues 59, 60, and 61 are either aspartic acids or glutamic acids (Figs. 2 and 3A). In the algal plastocyanins, the residues at positions 58 and 60 are deleted relative to the plant protein, resulting in a slight change in the shape of the negative patch in the algal protein (Collyer *et al.*, 1990; Figs. 3B and 4). Instead, the algal plastocyanins have two negatively charged residues, Asp-53 and either Asp or Glu-85, at positions in the sequence where there are no negative charges in the plant plastocyanins of known structure (Fig. 3B). Sequence analysis indicates that plastocyanin of parsley and barley are similar to the green algal forms in that two residues are deleted at the same position in the sequence relative to poplar and French bean, and either positions 53 and/or 85 contain residues with negatively charged side chains (Sykes, 1985; Nielsen and Gausing, 1987). The structures of parsley and barley plastocyanin have not been determined but are predicted to resemble the green algal proteins.

The structures of the cyanobacterial plastocyanins have not yet been determined by either crystallographic or NMR methods. Sequence analysis of the plastocyanins of *Anabaena variabilis* and *Synechocystis* 6803 indicates that residues constituting the negative patch in the eukaryotic plastocyanins are uncharged (Aitken, 1975; Briggs *et al.*, 1990). The negative patch thus appears to be a feature of only the eukaryotic proteins. Nevertheless, Tyr-83 is conserved, indicating that electron transfer via this remote site could occur in cyanobacteria, but without reaction partner recognition directed by negative charge. Of the other blue-copper proteins of known structure, only amicyanin appears to have a negative patch surrounding a potential distant site of electron transfer (Durley *et al.*, 1993).

**Fig. 3B**. Space-filling model of the negative patch of *C. reinhardtii* plastocyanin (Redinbo *et al.*, 1993). The side chains of Asp-42, Glu-43, Asp-44, Asp-53, Asp-59, Asp-61, and Glu-85 make up this region of concentrated negative charge surrounding Tyr-83. Residues 42–44 are analogous to the poplar structure in both shape and charge. Residue 61 is in a similar position relative to Tyr-83 in both poplar and *C. reinhardtii* plastocyanin, but in the latter molecule Glu-60 and a characteristic bulge present in the poplar structure are missing. Residue 59 is one methylene shorter than in the poplar structure (residue 59 is a Glu in poplar plastocyanin), but its approximate position is taken up by Glu-85 in *C. reinhardtii* plastocyanin. Thus, the distribution of negative charge around residues 59, 61, and 85 in *C. reinhardtii* plastocyanin is similar to the negative charge distribution around residues 59–61 in poplar plastocyanin. *C. reinhardtii* plastocyanin also contains an additional negatively charged residue, Asp-53, not found in the poplar structure (residues 53 and 85 are both serines in poplar plastocyanin). The shading of the atoms and orientation of the molecule is the same as that in Fig. 3A.


34
35
12
87
90
86
89
83

### 3.2. Binding Interactions at the Negative Patch

Although the negative patch had been implicated early on in interactions with both cytochrome *f* and P700, the evidence for cytochrome *f* interaction in the vicinity of Tyr-83 at the negative patch is much stronger. Beoku-Betts *et al.* (1985) showed that positively charged inorganic compounds that are specific for the Tyr-83 electron transfer site on plastocyanin are competitive inhibitors of plastocyanin-dependent oxidation of cytochrome *f*, which supports the assignment of cytochrome *f* binding to the negative patch near Tyr-83 and suggests further that there may be a positively charged functional region on cytochrome *f*. On the basis of chemical modification studies where ethylenediamine-modified negative patch carboxyl groups were found to inhibit cytochrome *f* oxidation, Anderson *et al.* (1987) suggested that the negative patch (including residues 42–45 and 59–61) of spinach plastocyanin was involved in an interaction with cytochrome *f*. Modification at 42–45 had no effect on $P700^+$ reduction, but interestingly, modification of residues 59–61 stimulated that reaction. In a cross-linked spinach plastocyanin–turnip cytochrome *f* complex, two sites of linkage were identified: Asp-44 of plastocyanin to Lys-187 of cytochrome *f*, and Glu-59 and/or Glu-60 to an unidentified site on cytochrome *f* (Morand *et al.*, 1989). This adduct was inactive in reducing Photosystem I. In conjunction with the results of Anderson *et al.* (1987), this work suggested an association of cytochrome *f* on the "east" side of plastocyanin via interaction with residues 42–45 and 59–61 of the negative patch, and an association of Photosystem I via interaction with residues 59–61. This latter interaction is not incompatible with an electron transfer path through His-87 for $P700^+$ reduction (see Sections 3.4 and 4.2). Takabe and Ishikawa (1989) found, on the other hand, that while a covalently linked plastocyanin–cytochrome *f* reduced an antenna-containing Photosystem I complex very poorly, it was capable of reducing $P700^+$ of a Photosystem I complex depleted of antenna chlorophylls (albeit more slowly compared to the electrostatic complex). They concluded also that intramolecular cytochrome *f* to plastocyanin electron transfer could occur in the adduct. Since the position of cross-links in the latter study was not determined, it is not useful to directly compare the work of Morand *et al.* (1989) and Takabe and Ishikawa (1989). It should be noted that cross-linking methods select only a subset of possible interactions (i.e., those where specific functional groups lie within range of the reagent). Thus, in some cases the resultant cross-linked product may not be the most optimally oriented with respect to function. In the case of the plastocyanin–cytochrome *f* complex, this has been demonstrated by Qin and Kostic (1993) who concluded that intermolecular electron transfer between cytochrome *f* and plastocyanin could not occur in the cross-linked complex.

Site-directed mutagenesis of Asp-42 to Asn does not alter the binding of plastocyanin to cytochrome *f* nor the intrinsic electron transfer rate (Modi *et al.*, 1992a). However, the change is not entirely analogous to the chemically modified form studied by Anderson *et al.* (1987), since ethylenediamine modification introduces a positive charge whereas the introduction of the amide group simply neutralizes the negative charge. Site-directed mutagenesis of Tyr-83 (to Phe and Leu), on the other hand, indicates that it is important for the plastocyanin–cytochrome *f* interaction as well as for electron transfer, and a hydrogen-bonding interaction (with the side chain hydroxyl group of Tyr-83 serving as an acceptor) has been suggested (He *et al.*, 1991; Modi *et al.*, 1992a). The study of cytochrome *f*–plastocyanin complexes by crystallographic methods and complementary mutagenesis studies of both plastocyanin and cytochrome *f* should further illuminate the binding interaction.

The structure of turnip cytochrome *f* (see Cramer *et al.* in this volume) reveals that Lys-187 resides in a positive patch (which includes Lys-181, Arg-184, and

**Fig. 3C.** Space-filling model of the hydrophobic patch of poplar plastocyanin (Guss *et al.*, 1992). Leu-12, Ala-33, Gly-34, Phe-35, Pro-86, Gly-89, and Ala-90, which are conserved in the known eukaryotic plastocyanins, are shown in light gray, and the copper-binding residue His-87, which is exposed to solvent and is involved in electron transfer adjacent to the copper atom, is shown in white. Pro-36 is also conserved in the known eukaryotic plastocyanins but is obstructed in this figure by residues 33–35. This region of the molecule is remarkably flat and is exclusively hydrophobic; the solvent-accessible surface area of these conserved residues is 525 $Å^2$. The oxygen atom of Tyr-83 is also labeled. This orientation of the molecule provides a side view of the residues that make up the negative patch surrounding Tyr-83 and further illustrates the unique shape of this surface. The shading scheme of the nonhighlighted residues is the same as that in Fig. 3A. The molecule in this figure has been rotated 75° about the vertical axis relative to Fig. 1A. Figures 3A, 3B, and 3C were created with the program MOLSCRIPT (Kraulis, 1991).

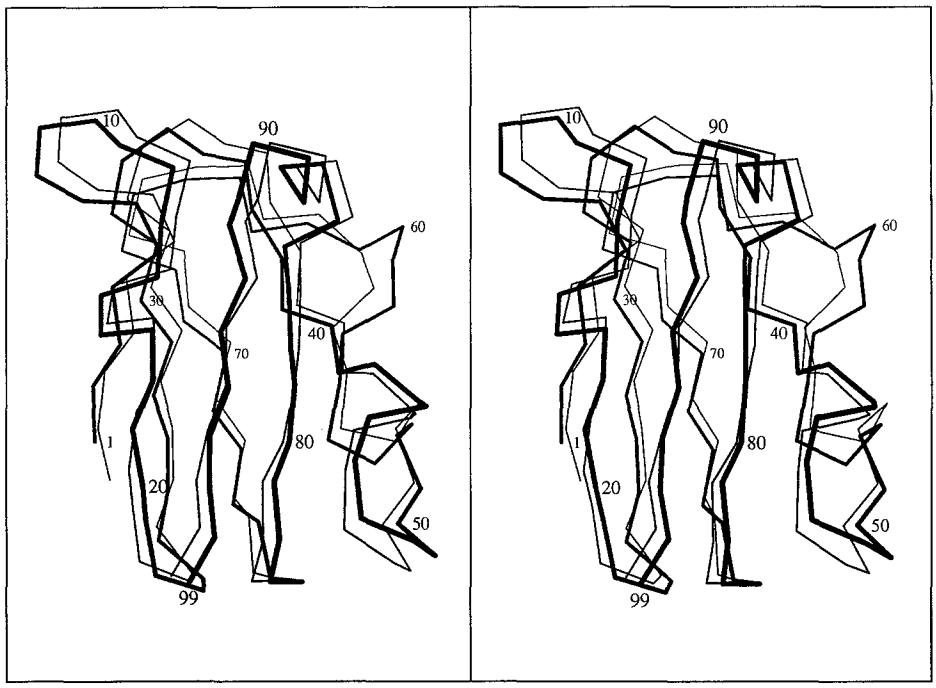



**Fig. 4.** Stereo pair of the Cα traces of poplar (dark lines; Guss *et al.*, 1992) and *Chlamydomonas reinhardtii* (light lines; Redinbo *et al.*, 1993) plastocyanin. Poplar and French bean plastocyanin contain a characteristic bulge near amino acid 60 owing to the presence of two additional amino acids (numbered 58 and 60) not found in the algal and some plant plastocyanins. *C. reinhardtii* plastocyanin (like some other algal and cyanobacterial plastocyanins) contains an additional amino acid at the N-terminus (numbered 0). Poplar and *C. reinhardtii* plastocyanin were chosen because they are the two highest-resolution crystal structures, 1.33 and 1.5 Å resolution, respectively. The superposition of these molecules was calculated using the method of Kabsch (1978); the rms deviation in Cα positions between the two structures (not including amino acids 58–60 in the poplar structure and amino acids 0 and 59 in the *C. reinhardtii* structure) is 0.76 Å. This figure was created with the program MOLSCRIPT (Kraulis, 1991).

Lys-185) in a small domain on the distal side of the heme cofactor (relative to the carboxyl terminus). This region of cytochrome *f* was one of several that were noted to have diverged significantly between plants and algae. In light of the divergent character of the negative patch of plastocyanin in plants compared to algae and cyanobacteria, a complementary divergence in cytochrome *f* is not unexpected (Widger, 1991). Other regions of cytochrome *f* that are implicated in the binding interaction with plastocyanin include an amino terminal, heme-containing proteolytic fragment (of about 90 residues) which is selected by an affinity column of bound plastocyanin, and residues Arg-88 and Arg-154 which are protected from chemical modifications in the presence of plastocyanin (Adam and Malkin, 1989).

Durell *et al.* (1990) and Roberts *et al.* (1991) have examined the electrostatic field potential on the surface of plastocyanin and have found that there exists a region of diffuse positive potential around the copper-binding site and the hydrophobic patch, and a region of concentrated negative potential around Tyr-83 and the negative patch. Roberts *et al.* also calculate a similar electrostatic field potential for cytochrome *c* and obtained, via the calculation of intermolecular electrostatic energies for different orientations of the two proteins, optimum precollisional orientations of plastocyanin and cytochrome *c* at specific distances. It will be of interest to see how similar experiments would align plastocyanin and cytochrome *f* for collision, especially in the absence of detailed structural knowledge of the electron transfer complex between them.

The use of chemical cross-linking methods to study the interaction of plastocyanin with Photosystem I (Wynn and Malkin, 1988; Hippler *et al.*, 1989) has revealed an association of plastocyanin with a 19–20-kDa polypeptide referred to as subunit

III (Bengis and Nelson, 1975) or the psaF gene product (Steppuhn *et al.*, 1988). Plastocyanin appears to be functionally associated with this subunit, since the half-time for reduction of flash-induced $P700^+$ by covalently bound plastocyanin (13 $\mu$sec) (Hippler *et al.*, 1989) was comparable to that in native membranes (11–12 $\mu$sec) (Haehnel *et al.*, 1989). The psaF gene product is positively charged and lumen localized with hydrophobic domains that are suggested to be involved in an interaction with the P700 apoprotein. An association of positive charges of Subunit III with the negatively charged residues (e.g., 59–61; see Section 3.1) of the negative patch has been hypothesized (Hippler *et al.*, 1989) but awaits experimental verification. Since modification of carboxyl groups inhibits cross-linking of plastocyanin to Photosystem I, the negative patch on plastocyanin has been proposed as the cross-linking site (Wynn *et al.*, 1988). If residues 59–61 are indeed involved in interaction with both physiological partners, a ternary complex (cytochrome *f*–plastocyanin-Photosystem I) may be ruled out. It would be useful therefore to test the covalent plastocyanin–Photosystem I complex for its ability to bind cytochrome *f*. Site-directed mutagenesis is also a promising approach to the determination of the role of residues 59–61 in the interaction of plastocyanin with cytochrome *f* and Photosystem I.

Recent genetic and biochemical studies have questioned the importance of the psaF gene product in the reaction between plastocyanin and Photosystem I, since psaF-minus cyanobacterial and algal strains retain photosynthetic activity (Chitnis *et al.*, 1991; Farah and Rochaix, personal communication), and purified Photosystem I particles that are depleted of the psaF gene product remain active (Hatakana *et al.*, 1993). However, since the gene product binds also to cytochrome $c_6$ (the alternative to plastocyanin in some organisms), its association with plastocyanin is unlikely to be fortuitous (Wynn *et al.*, 1989). Furthermore, the gene is conserved in the Photosystem I-containing photosynthetic organisms including those as divergent as *Cyanophora* where only a *c*-type cytochrome is used for electron transfer from cytochrome *f* to Photosystem I (Bryant, 1992). One possibility is that the cross-linking studies have revealed only one of two plastocyanin-binding sites on Photosystem I (Bottin and Mathis, 1987), and another possibility is that the psaF gene product stabilizes a plastocyanin–Photosystem I complex but is not essential for such an interaction. The lack of chemically reactive groups on the hydrophobic patch (see Section 3.3) may prevent the identification of an additional plastocyanin–Photosystem I interaction by chemical cross-linking methods. The methods of site-directed mutagenesis are more useful in the study of hydrophobic patch interactions (see Section 3.4).

### 3.3. Structure and Characteristics of the Hydrophobic Patch

The hydrophobic patch is located at the "north" end of the molecule and surrounds His-87, the only solvent-exposed copper ligand and a favorable site of electron transfer (Fig. 3C). The hydrophobic patch is made up of at least eight residues that are conserved in the eukaryotic plastocyanins: Leu-12, Ala-33, Gly-34, Phe-35, Pro-36, Pro-86, Gly-89, and Ala-90 (reviewed by Sykes, 1991). There are no charged or polar amino acids in this region of the molecule. Calculation of the solvent-accessible surface area (Connolly, 1983) of these conserved residues in the hydrophobic patch revealed that they make up approximately 550 $Å^2$ (525 $Å^2$ for poplar plastocyanin and 580 $Å^2$ for *C. reinhardtii* plastocyanin) of the molecular surface area. Residues 33 through 36 lie on a loop between $\beta$-strands 3 and 4 in the plastocyanins of known structure and provide a conserved region of hydrophobicity adjacent to the side chain of His-87. Position 66 is a proline in all the plastocyanins of known structure except poplar and *E. prolifera*, where it is a lysine (Sykes, 1991). Since residue 66 is approximately 14 Å from the solvent-exposed portion of His-87, a lysine at that position would be quite removed from the center of the hydrophobic patch and would contribute only insignificantly to the overall hydrophobic nature of this surface. The majority of the conserved residues in this region are small hydrophobic residues and, as a consequence, the hydrophobic patch is remarkably flat in shape (Fig. 3C). The side chain of the only large residue in this area, Phe-35, lies along the surface of the molecule without extending significantly into the solvent. The conservation of small hydrophobic residues suggests that the flatness of this surface is important for the interaction of plastocyanin with reaction partners that use the hydrophobic patch. In the cyanobacterial sequences, residue 33 is replaced by lysine and in one sequence residue 35 is polar (Fig. 2). The nature of the hydrophobic patch may therefore be different in the cyanobacterial proteins. All the blue-copper proteins of known structure contain a hydrophobic patch

surrounding their solvent-exposed copper-liganding histidine (Table I). In the case of amicyanin, an interaction of one of its reaction partners, methylamine dehydrogenase, at the hydrophobic patch surrounding the solvent-exposed His-95 copper ligand was recently revealed (Chen *et al.*, 1992).

### 3.4. Binding Interactions at the Hydrophobic Patch

The interaction of the hydrophobic patch of plastocyanin with the physiological reaction partners has not been studied extensively. One informative approach has involved the use of site-directed mutagenesis of a cloned plastocyanin-encoding sequence from spinach (Nordling *et al.*, 1990). Leu-12 was mutated to Asn and Glu in one study (Modi *et al.*, 1992a). The Asn-12 mutant showed a fourfold increase in affinity for cytochrome *f*, whereas the Glu-12 mutant exhibited fourfold weaker binding. This result is most simply explained if an interaction of cytochrome *f* with this surface of plastocyanin is proposed. In a separate study, Nordling *et al.* (1991) found that the Glu-12 mutant displayed much reduced affinity for Photosystem I particles and reduced electron transfer to $P700^+$. This work provides the best evidence for an interaction of plastocyanin with Photosystem I at the hydrophobic patch of plastocyanin. Taken together, these two studies (Nordling *et al.*, 1991; Modi *et al.*, 1992a) would, once again, argue against a ternary complex of plastocyanin with cytochrome *f* and Photosystem I, but the binding sites of cytochrome *f* and Photosystem I on plastocyanin are, nevertheless, distinct. For instance, a Tyr-83 to His mutant is unaffected in its affinity for Photosystem I, and the Glu-12 mutation affects more drastically the interaction of plastocyanin with Photosystem I than that with cytochrome *f* (Nordling *et al.*, 1991; Modi *et al.*, 1992a). Further, as mentioned previously, chemical modification of residues 42–45 affects only the reaction of plastocyanin with cytochrome *f* (Anderson *et al.*, 1987), and in one work, a plastocyanin–cytochrome *f* adduct remained somewhat functional in electron transfer to Photosystem I (Takabe and Ishikawa, 1989).

## 4. ELECTRON TRANSFER AND PLASTOCYANIN

On the basis of the physiological function of plastocyanin, Colman *et al.* (1978) rationalized the existence of two electron transfer paths in plastocyanin. Through the study of the reaction of plastocyanin with inorganic, redox-active complexes, Sykes and co-workers concluded that negatively and positively charged reactants (e.g., $Fe(CN)_6^{3-}$ and $Co(phen)_3^{3+}$) bind to two separate sites on plastocyanin (reviewed by Sykes, 1985, 1991). Cookson *et al.* (1980a, b) later demonstrated that these binding sites were separated by about 15 Å by monitoring specific proton resonances in plastocyanin with bound, redox-inactive chromium analogs of $Fe(CN)_6^{3-}$ and $Co(phen)_3^{3+}$. One site, now referred to as the adjacent site, was deduced to lie close to His-87 and the copper atom, and the other, now referred to as the remote site, was believed to lie in the region of Tyr-83. Accordingly, two distinct paths of electron transfer into and out of plastocyanin were indicated. The study of binding interactions with cytochrome *f* and Photosystem I (see Sections 3.2 and 3.4) strongly supports the utilization of two paths *in vivo*.

### 4.1. Theoretical Considerations

It is of interest to consider how the current theories about electron transfer in proteins describe the electron transfer routes in plastocyanin, especially the more lengthy of the two, from Tyr-83. General electron transfer theory defines the rate of electron transfer, according to the Dirac–Fermi golden rule, to be

$$k_{ET} = \left(\frac{2\pi}{\hbar}\right) T_{DA}^2 FC,$$

where $T_{DA}$ is the coupling matrix element between the donor and acceptor wave functions, FC is the Frank–Condon, or nuclear, factor, and $\hbar$ is Planck's constant (reviewed by Canters and van de Kamp, 1992). The dependence of $T_{DA}$ on the intervening medium is defined by

$$T_{DA}^2 = (T_{DA}^0)^2 f_M^2,$$

where $T_{DA}^0$ is the matrix coupling element when the donor and acceptor are in van der Waals contact, and $f_M$ is an attenuation factor that varies between 1 and 0 as the donor and acceptor move from van der Waals contact to infinitely far apart. There are currently two main theories of protein electron transfer, and they differ in how they define this attenuation factor, $f_M$ (Canters and van de Kamp, 1992). The first is the "organic glass" theory, developed by Dutton and co-workers based on photosynthetic reaction centers

(Moser *et al.*, 1992), in which the attenuation factor is defined to be dependent only on the distance between the donor and acceptor atoms and not on any discrete pathway:

$$f_M^2 = A \exp[-\beta(R - R_0)],$$

where $A$ is $10^{13}$ sec$^{-1}$, $\beta$ is 1.4 Å$^{-1}$, $R$ is the distance in space between the centers of the donor and acceptor atoms, and $R_0$ is 3.6 Å (these values of $A$ and $\beta$ have been chosen because they give optimum agreement with measured electron transfer rates) (Canters and van de Kamp, 1992). The second is the "pathway" model, developed by Beratan and Onuchic (Beratan *et al.*, 1991), in which the attenuation factor is based on the number of covalent, hydrogen-bonded and through-space interactions that make up a discrete pathway between the donor and acceptor atoms:

$$f_M^2 = A \exp(-\beta\sigma l),$$

where $A$ is $3 \times 10^{12}$ sec$^{-1}$, $\beta$ is 0.71 Å$^{-1}$, $\sigma$ is a function of the total number of covalent, hydrogen-bonded or through-space interactions along the pathway,[3] and $l$ is 1.4 Å (again, these values of $A$ and $\beta$ give optimum agreement with measured electron transfer rates) (Canters and van de Kamp, 1992). For electron transfer from the remote patch in plastocyanin, the two theories give similar results. The "organic glass" theory gives an attenuation factor of $8.9 \times 10^7$ sec$^{-1}$ using the through-space distance of 11.9 Å between the hydroxyl oxygen of Tyr-83 and the copper atom [for poplar plastocyanin (Guss *et al.*, 1992)]; the "pathway" model of Beratan and Onuchic gives an attenuation factor of $2.3 \times 10^7$ sec$^{-1}$ for a pathway of 12 covalent bonds from the hydroxyl oxygen of Tyr-83 to the copper atom. Each theory is essentially based on the attenuation of the coupling between the donor and acceptor atoms with increasing distance, although in the Dutton model it is "through-space" distance and in the Beratan and Onuchic model it is preferably "through-bond." Neither of these theories, however, takes into account the apparent activation of the Cu–N$^{\gamma}$(Cys-84) bond (see Section 2.3; Solomon and Lowery, 1993) that may stimulate electron transfer via Tyr-83 beyond what might be expected based on the distance between this residue and the copper atom, or the observed coplanarity of the Cu and S$^{\gamma}$, C$^{\alpha}$, C$^{\beta}$, and N(Cys-84) atoms in plastocyanin and other blue-copper proteins of known structure which may direct electron transfer through these atoms (Han *et al.*, 1991).

Electron transfer between plastocyanin and various substrates, including inorganic complexes, nonphysiological redox proteins (e.g., cytochrome *c*), and physiological reaction partners (e.g., cytochrome *f*), has been studied extensively. For studies dealing with inorganic complexes or cytochrome *c*, the interested reader is referred to the recent review by Sykes (1991) and the large body of work by Kostic's group (e.g., Zhou *et al.*, 1992; Zhou and Kostic, 1993).

### 4.2. Electron Transfer between Plastocyanin and Its Physiological Reaction Partners

The measurement of electron transfer between plastocyanin and cytochrome *f* of Photosystem I depends not only upon the intrinsic electron transfer rate between redox centers but also upon the formation of a protein–protein complex and the driving force dictated by the properties of the copper site. Thus, each variable has to be considered in evaluating the effects of site-specific changes in plastocyanin. This has been accomplished most elegantly for the cytochrome *f* to plastocyanin reaction in recent studies by He *et al.* (1991) and Modi *et al.* (1992a, b) where the binding interaction of the mutant vs. the wild-type protein was assessed by measurement of the association constant and the forward rate constant for association from the change in Soret band absorbance of cytochrome *f* on binding plastocyanin. The intrinsic electron transfer rate was calculated from the association constant, the forward rate constants for complex formation, and the measured forward rate constants for the overall electron transfer reaction. Structural integrity of the mutant proteins and the copper site was evaluated by application of a combination of various spectroscopic methods, including $^1$H-NMR, CD, UV-vis absorption, and EPR. These studies indicate that Tyr-83 is indeed part of the electron transfer path from Cu(I)-plastocyanin to Fe(III)-cytochrome *f*, since the intrinsic electron transfer rate of a Tyr-83 to Leu mutant protein is reduced greater than 10-fold compared to wild-type protein or a Tyr-83 to Phe mutant protein. In studies with cytochrome *c*, a similar result was obtained; however, the rate of electron transfer from this nonphysiological substrate was about 20-fold lower than that from cytochrome *f* (Modi *et al.*, 1992a). A more optimal cofactor to cofactor

[3] For a pathway that is entirely through-bond, $\sigma$ is simply the number of covalent bonds in the path.

distance/orientation is implied for the cytochrome *f*–plastocyanin pair compared to the cytochrome *c*–plastocyanin pair. Qin and Kostic (1992) have independently noted that the overall reaction of plastocyanin with cytochrome *f* is faster than with cytochrome *c*, but the question of binding vs. intrinsic electron transfer rate was not separated in that work.

Since the effect of Tyr-83 mutations implicates the remote site for cytochrome *f* oxidation, the adjacent site is associated with $P700^+$ reduction. Mutagenesis experiments at this site (His-87) may prove to be more difficult, since cofactor binding may affect the stability of the protein and influence its expression. However, binding interactions at the hydrophobic patch (Section 3.4) are consistent with this assignment.

## 5. CONCLUSIONS

### 5.1. Site-Directed Mutagenesis

The recent site-directed mutagenesis studies of plastocyanin have proven to be quite informative with respect to the role of specific residues in the binding and electron transfer functions of the protein. Undoubtedly, this approach will continue to be exploited. Structural analysis by crystallographic or NMR methods would be a useful complement to the biochemical and functional characterization of specific mutant proteins. The contribution of copper binding ligands to the properties of the copper site of plastocyanin has not been studied owing, perhaps, to the difficulty in expressing the copper-site mutants in *E. coli* (Chang *et al.*, 1991); in this regard, plastocyanin appears to be less stable than azurin. If alternative expression systems (e.g., Last and Gray, 1990; Quinn *et al.*, 1993) allow the synthesis and accumulation of the copper site mutants, questions relating to the copper site can also be addressed by site-directed mutagenesis.

### 5.2. Physiological Reaction Partners

Based on the present status of structural studies of the cytochrome $b_6f$ complex and the cyanobacterial Photosystem I (Martinez *et al.*, 1991; Cramer *et al.*, this volume; Krauss *et al.*, 1993), the functional and structural characterization of the plastocyanin–cytochrome *f* and plastocyanin–Photosystem I complexes are envisioned as future goals in this field. Since the cyanobacterial and algal experimental systems are the best suited for future mutagenesis experiments involving polypeptides of both complexes, structure determination of the cyanobacterial plastocyanins would not only be of interest because they appear to be structurally distinct from their eukaryotic counterparts, but would also be useful in the design and evaluation of mutagenesis experiments in the cyanobacterial systems.

### 5.3. Comparative Analysis with Cytochrome $c_6$

In the green algae and cyanobacteria, plastocyanin function can be replaced by a *c*-type cytochrome (cytochrome $c_6$) (Wood, 1978; Sandman *et al.*, 1983). In studying a variety of plastocyanins and cytochrome $c_6$ from cyanobacteria, Ho and Krogmann (1984) noted a co-variation in the pI of the two proteins isolated from the same organism, and accordingly suggested that the two evolutionarily unrelated proteins may have co-evolved in response to alterations/mutations in common reaction partners. Chemical cross-linking experiments reveal that cytochrome $c_6$, like plastocyanin, is associated with the psaF gene product in the Photosystem I complex (Wynn *et al.*, 1989), and de Silva *et al.* (1988) noted identical redox potentials for the protein pair from *S. obliquus* (380 mV) and *A. variabilis* (340 mV). The determination of the structures of cytochrome $c_6$ and plastocyanin from a single organism and the subsequent comparison of these proteins for similar reaction partner binding sites (e.g., hydrophobic or negative patches) would be of interest for both functional and structural studies.

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