The 1.5-Å Crystal Structure of Plastocyanin from the Green Alga *Chlamydomonas reinhardtii*[†,‡]

Matthew R. Redinbo, Duilio Cascio, Marie K. Choukair, Danny Rice, Sabeeha Merchant, and Todd O. Yeates[*]

Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California at Los Angeles, 405 Hilgard Avenue, Los Angeles, California 90024

Received May 18, 1993; Revised Manuscript Received July 19, 1993[⊛]

ABSTRACT: The crystal structure of plastocyanin from the green alga *Chlamydomonas reinhardtii* has been determined at 1.5-Å resolution with a crystallographic *R* factor of 16.8%. Plastocyanin is a small (98 amino acids), blue copper-binding protein that catalyzes the transfer of electrons in oxygenic photosynthesis from cytochrome *f* in the quinol oxidase complex to $P700^+$ in photosystem I. *Chlamydomonas reinhardtii* plastocyanin is an eight-stranded, antiparallel β-barrel with a single copper atom coordinated in quasi-tetrahedral geometry by two imidazole nitrogens (from His-37 and His-87), a cysteine sulfur (from Cys-84), and a methionine sulfur (from Met-92). The molecule contains a region of negative charge surrounding Tyr-83 (the putative distant site of electron transfer) and an exclusively hydrophobic region surrounding His-87; these regions are thought to be involved in the recognition of reaction partners for the purpose of directing electron transfer. *Chlamydomonas reinhardtii* plastocyanin is similar to the other plastocyanins of known structure, particularly the green algal plastocyanins from *Enteromorpha prolifera* and *Scenedesmus obliquus*. A potential "through-bond" path of electron transfer has been identified in the protein that involves the side chain of Tyr-83, the main-chain atoms between residues 83 and 84, the side chain of Cys-84, the copper atom, and the side chain of His-87.

Plastocyanin is a small (97–99 amino acids), redox-active copper protein whose biochemical function in oxygenic photosynthesis is the catalysis of electron transfer from reduced cyt[1] *f* of the quinol oxidase complex to $P700^+$ in photosystem I. Of the blue copper proteins, plastocyanin is probably the best characterized at the structural level [see a review by Sykes (1991)]. The protein folds into an eight-stranded, β-sandwich cylinder (Colman *et al.*, 1978; Chazin & Wright, 1988; Collyer *et al.*, 1990; Moore *et al.*, 1991). The copper-containing active site, although not solvent-exposed, is close to the surface of the molecule and is coordinated to a surface-exposed histidinyl (His-87[2]) imidazole. The three other coordinating groups are a cysteinyl (Cys-84) thiolate, a methioninyl (Met-92) thioether, and another histidinyl (His-37) imidazole. The geometry of the active site is an irregular or distorted tetrahedron. This distortion is presumably imposed by the folding of the polypeptide and, by stabilizing the Cu(I) form, is responsible for the relatively high midpoint potential (~370 mV) of the protein (Garrett *et al.*, 1986).

In addition to the redox chemistry at its active site, specific interactions of plastocyanin with electron donors and acceptors are also relevant to its biochemical function. These interactions have been proposed to occur at one or both of two sites [reviewed by Sykes (1991)]. One site is near His-87 and is referred to as the hydrophobic patch, and the other is a region of high negative charge density and is referred to as the negative patch[3] (Farver *et al.*, 1982). Since the distance from the Cu atom to the surface of the hydrophobic patch is shorter than the distance to the surface of the negative patch, theoretical considerations favor the participation of the hydrophobic patch and His-87 in the electron-transfer path. Nevertheless, electron-transfer measurements in conjunction with chemical modification studies suggest that electrostatic effects strongly influence the interactions of plastocyanin with donors and acceptors, and the negative patch has been implicated in this interaction (Beoku-Betts *et al.*, 1985; Anderson *et al.*, 1987; Morand *et al.*, 1989; Qin & Kostic, 1992). The involvement of the negative patch is underscored by a recent study where mutagenesis of Tyr-83 at the center of the negative patch was shown to affect the rate of electron transfer from either the physiological substrate (cyt *f*) or a nonphysiological substrate (cyt *c*) (He *et al.*, 1991; Modi *et al.*, 1992). However, electron transfer out of plastocyanin may use a different route that involves interaction at the hydrophobic patch (Nordling *et al.*, 1991).

Under conditions of copper deficiency, many algae and cyanobacteria use a *c*-type cytochrome as an effective physiological substitute for the copper-containing plastocyanin (Kunert *et al.*, 1976; Wood, 1978). These organisms contain genetic information for both proteins, but choose between the synthesis of one or the other depending upon the availability of copper in the growth medium. In a survey of plastocyanins

[†] This work was supported in part by the United States Department of Agriculture/National Research Initiative Competitive Grants Program (91-37306-6348 to S.M.), by USPHS grant GM31299 and NSF award DNB-9158602 to T.O.Y., and by the Committee on Research of the Academic Senate of the Los Angeles Division of the University of California. M.R.R. was supported by a National Institutes of Health National Research Service Award (GM07185-18), and S.M. was supported by a National Institutes of Health Research Career Development Award (GM00594).

[‡] The atomic coordinates have been deposited with the Protein Data Bank, Brookhaven National Laboratories, Upton, NY, and have been assigned the filename 2PLT.

[*] Author to whom correspondence should be addressed.

[⊛] Abstract published in *Advance ACS Abstracts*, September 15, 1993.

[1] Abbreviations: cyt, cytochrome; NMR, nuclear magnetic resonance.

[2] We maintain the amino acid numbering scheme of Collyer *et al.* (1990), with the N-terminal residue numbered 0 and residues 58 and 60 missing from the sequence, to simplify the comparison of structures.

[3] We use the term "negative patch" instead of "acidic patch" because it more accurately describes the nature of that region of the molecular surface at physiological pH.

0006-2960/93/0432-10560$04.00/0

and cytochromes c_6 from a variety of cyanobacteria and algae, Ho and Krogmann (1984) noted that the isoelectric points of the two proteins (reflecting the content of charged residues) were closer for the two evolutionarily unrelated proteins within a single organism than between plastocyanins from different organisms. They suggested that this resulted from convergent evolution of plastocyanin and cyt c_6 in each organism in response to a change in a common interacting reaction partner. Comparative chemical cross-linking studies and primary sequence analysis of cytochromes *f* from numerous organisms for variation in charge content support this suggestion (Wynn & Malkin, 1988; Wynn *et al.*, 1989; Morand *et al.*, 1989; Widger, 1991).

To compare the interactions of cyt c_6 and plastocyanin with their physiological donors and acceptors, we have undertaken a structural study of plastocyanin and cyt c_6 from a single organism. The green alga *Chlamydomonas reinhardtii* was chosen as the experimental system, since (i) its photosynthetic apparatus is well-characterized at the biochemical and genetic levels and (ii) the isolation and expression of *C. reinhardtii* plastocyanin, cyt *f*, and cyt c_6 encoding genes have been described (Hill *et al.*, 1991; Buschlen *et al.*, 1992; Quinn *et al.*, 1993; J. Quinn and S. Merchant, unpublished results). These features allow site-directed mutagenesis of each reaction partner from a single organism and will facilitate the characterization of these mutants in a physiologically relevant background. In this work, we report the structure of *C. reinhardtii* plastocyanin at 1.5-Å resolution. The structure was determined from a crystal specimen displaying "perfect" hemihedral twinning by utilizing a novel method of detwinning the observed crystallographic intensities. This method is described in detail elsewhere (Redinbo & Yeates, 1993).

EXPERIMENTAL PROCEDURES

Purification. All of the experimental work was performed with a single preparation of plastocyanin from 90 L ($\sim 1 \times 10^7$ cells/mL) of photoheterotrophically grown wild-type cells of *Chlamydomonas reinhardtii*. The previously described procedure for purification (Merchant & Bogorad, 1986), described below in brief, was appropriately scaled up. Collected cells were suspended in approximately 1 L of 10 mM sodium phosphate (pH 7.0) and subjected to two slow freeze–thaw cycles (−70 to 0 °C). Broken cell debris was removed by centrifugation (13000*g*, 30 min). Ammonium sulfate was added to the plastocyanin-containing supernatant (38 g per 100 mL), and the mixture was stirred overnight at 4 °C. Precipitated proteins were removed by centrifugation (25000*g*, 20 min). After the addition of potassium ferricyanide (to 1 mM), the supernatant (920 mL) was clarified by filtration through Whatman 1 paper and loaded on a Sepharose 4B column (120 mL) equilibrated with 1.9 M ammonium sulfate in Tris–KCl buffer. Plastocyanin retained on the column was eluted isocratically in the same solution except that it also contained 1 mM potassium ferricyanide. Fractions containing plastocyanin (A_{600nm} between 0.075 and 0.3) were pooled, dialyzed against Tris–KCl buffer with two changes, and loaded on a Whatman DE-52 column (20 mL) equilibrated in Tris–KCl buffer. Plastocyanin was eluted from the ion exchange column with a 10–300 mM KCl gradient in the same solution. Collected fractions were oxidized by the addition of potassium ferricyanide, and those with A_{597nm} between 0.17 and 0.52 were pooled, concentrated with the use of a Centricon 3 filter, and further purified on a Sephadex G-50 column (60 × 1.5 cm) followed by ultrafiltration on a Centricon 30 filter to remove high molecular weight impurities. Analysis of 1 μg of the final preparation by silver staining following separation on SDS-containing polyacrylamide gels revealed no impurities.

Table I: Data Collection and Processing

	specimen A	specimen B
unit cell constants	$a = b = 61.8$ Å, $c = 25.2$ Å $\alpha = \beta = 90°$, $\gamma = 120°$	
crystal dimensions (mm)	2.0 × 0.4 × 0.4	0.7 × 0.6 × 0.5
twinning fraction	1/2	0.046
space group	$P3_2$ (actual) $P6_2$ (apparent)	$P3_2$
resolution (Å)	15.0–1.58	15.0–1.50
no. recorded observations	20432	46606
no. unique reflections[a]	6793	15938
completeness	91.0%	92.2%
R_{merge}[b]		
$\bar{3}$	7.06%	4.08%
$6/m$	6.41%	46.6%

[a] Specimen A was reduced in the asymmetric unit of reciprocal space corresponding to Laue symmetry $6/m$ and therefore has approximately one-half as many reflections as specimen B. [b] $R_{merge} = \{\sum_r^N [\sum_i^{n(r)} |I_{i,r} - \langle I_r \rangle|] / \sum_r^N [n(r) \langle I_r \rangle]\}$, where N is the number of unique reflections, $I_{i,r}$ is the ith measurement of the rth reflection, $\langle I_r \rangle$ is the average of the rth reflection, and $n(r)$ is the number of measurements of the rth reflection in the data set.

Crystallization and Data Collection. Crystals were grown using the hanging drop vapor diffusion method. The reservoir contained 2.7 M ammonium sulfate, 0.1 M $CoCl_2$, 0.1 M $CaCl_2$, and 0.08 M sodium phosphate buffer (pH 6.6); the drop contained 2 μL of protein (12.6 mg/mL) and 2 μL of the reservoir solution. The crystals grew in 3–5 days at 22 °C and generally were bundles of hexagonal rods with the intense blue color characteristic of oxidized plastocyanin.

Data were collected from a large single-crystal specimen (specimen A) on a Rigaku R-AXIS-IIc imaging plate using Cu Kα radiation from a rotating anode source. The data collection and processing statistics are given in Table I. The unit cell was determined to be hexagonal with $a = b = 61.8$ Å and $c = 25.2$ Å by a combination of precession photography and autoindexing software on the R-AXIS. The data were reduced in Laue group $6/m$, and reflections with $(I/\sigma) > 1$ were accepted. However, the unit cell was found to be too small to accommodate six protein molecules (as required by apparent Laue group $6/m$), according to the packing limits described by Matthews (1968). Further analysis of intensity statistics[4] suggested that the true Laue group was $\bar{3}$ and that the crystal specimen was twinned by hemihedry with a twinning fraction of one-half (Redinbo & Yeates, 1993). Hemihedral twinning refers to the existence of two twin domains in the crystal specimen. In this case, the twinning operation that relates the two twin domains was a 2-fold axis of rotation parallel to the *c*-axis. The twinning was shown to be "perfect" (i.e., twinning fraction of exactly one-half) for all practical purposes by the observation that the diffraction data could be reduced as well in $6/m$ as in $\bar{3}$ (Table I). Perfect twinning leads to higher apparant symmetry; the apparent space group was $P6_2$ (or $P6_4$), while the true space group was $P3_2$ (or $P3_1$). The twinning problem was overcome by a novel method of detwinning the observed data using the positioned molecular replacement search model to obtain estimates of the true crystallographic intensities (Redinbo & Yeates, 1993).

An additional data set was collected from a crystal specimen displaying only partial hemihedral twinning (specimen B). This crystal specimen was grown after the structure deter-

[4] A general analysis of intensity statistics by Stanley (1972) provides the following specific test for "perfect" hemihedral twinning: the expected value of $\langle I^2 \rangle / \langle I \rangle^2$ for a thin resolution shell of acentric data is 2.0 for the case of no twinning and 1.5 for the case of perfect twinning. For the data described here, $\langle I^2 \rangle / \langle I \rangle^2 = 1.57 \pm 0.03$ using acentric data from 1.7- to 1.8-Å resolution.

mination from the perfectly twinned data was complete. Similar crystallization conditions were used, except that 0.2 M $CaCl_2$ replaced 0.1 M $CaCl_2$ and 0.1 M $CoCl_2$. Crystal specimen B took approximately 10 days to grow and was the only crystal specimen obtained with a low twinning fraction from either set of crystallization conditions. The merging R factor in Laue group $6/m$ for data from crystal specimen B was unacceptably high (Table I) and ruled out the possibility of perfect twinning for this specimen. The data were therefore reduced to the asymmetric unit of reciprocal space corresponding to Laue symmetry $\bar{3}$. Again, reflections with $(I/\sigma) > 1$ were accepted. These data were detwinned in a straightforward fashion (Buerger, 1960), according to a calculated twinning fraction of 0.046 (data not shown), and were used to finalize the refinement of the structure obtained with the perfectly twinned data and to extend the resolution to 1.5 Å.

Molecular Replacement and Structural Refinement. The 1.85-Å crystal structure of *Enteromorpha prolifera* plastocyanin (62% sequence identity) was used as the molecular replacement search model (Collyer *et al.*, 1990). Thirty-seven nonidentical amino acid side chains were truncated to alanine, leaving a total of 95 non-hydrogen atoms missing from the model (87% of the model was OK). One conformation was chosen for side chains present in multiple conformations. The search for a molecular replacement solution was carried out using 15- to 2.5-Å data from crystal specimen A. A rotation function (Crowther, 1972) showed 6-fold redundancy since the data have apparent 6-fold symmetry. The model was placed in a box (50 × 50 × 50 Å) for the rotation function and a Patterson radius cutoff of 15 Å was used. Within the unique region of the rotation function, there were two distinct peaks with heights of 4.8σ and 4.2σ. Intensity-based rigid-body refinement (Yeates & Rini, 1990; Brünger, 1992) unambiguously identified the top rotation function peak as the correct orientation (the error function for the 4.8σ peak fell to one-half the error function of the 4.2σ peak) and resulted in a 3.7° rotation relative to the rotation function solution. The data were then expanded according to the true Laue group $\bar{3}$, and a translation function was run using X-PLOR (Brünger, 1992) in space groups $P3_1$ and $P3_2$. Space group $P3_2$ gave the top translation function peak (T = 0.3919, δ = 0.0843); space group $P3_1$ gave no significant translation function peaks (T = 0.2236, δ = 0.0028).[5] The R factor of the top translation function solution in $P3_2$ was 0.397. This R factor is deceptively high because the twinning had not been accounted for at this stage of structure determination. One round of rigid-body refinement was performed prior to model building, and it reduced the R factor only slightly, to 0.393, indicating that the rotation and translation function solutions were accurate.

An initial $2F_o - F_c$ electron density map revealed clear density for all main-chain atoms and for a majority of the side-chain atoms present in the search model. Electron density was also seen for nonidentical side chains truncated from the search model. Model building was accomplished using the program FRODO (Jones, 1978) and was accompanied by rounds of rigid-body refinement and positional refinement using X-PLOR. The molecular parameters described by Engh and Huber (1991) were used in structural refinement. Diffraction data from 15 to 2.5 Å were used during model building. Atoms and residues were built into five-residue omit maps. The first round of model building was performed prior to any atomic refinement. Of the 37 nonidentical side chains, all but two surface lysines were built into the model at this stage. After model building was complete, data to 1.58 Å were slowly added in 0.1-Å resolution shells, and rounds of positional and restrained and unrestrained temperature factor refinement were performed using X-PLOR. The Cu atom was assigned full occupancy, using the scattering form factor of an oxidized copper atom. The geometry of the copper-binding site was not constrained during structure determination. Two cycles of refinement by simulated annealing were also performed (Brünger, 1991). The first cycle reduced the R factor from 0.269 to 0.249; the second cycle was less effective, reducing the R factor from 0.196 to only 0.195. Water molecules were built into $F_o - F_c$ difference maps at sites with electron density greater than 4σ above background and with potential hydrogen bond donors or acceptors within 3.6 Å. Nineteen well-resolved solvent sites were added to the model, as well as one large cation tentatively identified as calcium. The twinned R factor (Redinbo & Yeates, 1993) at this stage was 0.156, taking into account contributions from both twin domains.

The model was then refined against the data from crystal specimen B, which was only partially twinned. This extended the resolution to 1.5 Å and allowed the positioning of one of the missing surface lysines (Lys-95) into a simulated-annealing omit map [in which a selected region of the molecule is omitted from the phase calculation and the remainder of the model is refined using simulated annealing prior to map calculation (Hodel *et al.*, 1992)]. The second surface lysine (Lys-22) remained unresolved. Additional rounds of positional and temperature factor refinement were performed, as well as one more round of simulated annealing. Minor structural adjustments were made using electron density maps. The quality of the electron density maps was improved by the 2-fold increase in the amount of data available from crystal specimen B (Figure 1). A second conformer of the side chain of Arg-57 (beyond the N^{ϵ} atom) was built into a simulated-annealing omit map. The occupancies of the two conformers were estimated at 0.7 and 0.3 on the basis of the strength of electron density. The temperature factors of six exposed side chains refined to unusually high values (>35 Å^2), but did not have obvious discrete alternate conformations in electron density maps. We calculated partial occupancies for these residues using a least-squares fit between observed and calculated structure factors. Subsequent refinements were carried out with these fixed occupancies (Table II). Sixty well-resolved water molecules were added to the model, bringing the total number of solvent sites to 79. The occupancies of 17 water molecules with high temperature factors (>45 Å^2) but with persistent electron density were reduced to one-half.

RESULTS AND DISCUSSION

The structure of plastocyanin from *Chlamydomonas reinhardtii* has been determined and refined to a crystallographic R factor[6] of 0.168 at 1.5-Å resolution. The structure contains 727 protein atoms, one copper atom, one large cation, tentatively identified as calcium, and 79 water molecules. The side chain of one surface lysine (Lys-22) was not resolved and is not present in the model. The rms deviations from ideality in bond lengths and bond angles are 0.020 Å and 1.88°, respectively. The estimated error in atomic positions based on a Luzatti plot is between 0.25 and 0.30 Å (Luzatti, 1952). Assessment of the final structural model using the PRO-

[5] T, the top translation function score, is a weighted linear correlation between the observed and calculated normalized structure factors (Brünger, 1992). δ represents the difference between the top translation function score and the next highest score.

[6] $R \equiv (\sum_h |F_{calc}(h) - F_{obs}(h)|/\sum_h F_{obs}(h))$.

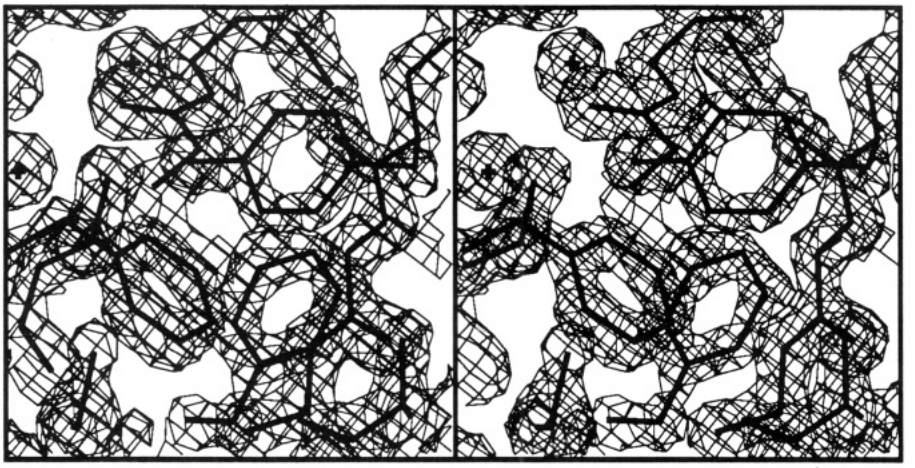

FIGURE 1: Electron density map of the final model of plastocyanin. Three aromatic residues, Phe-14, Phe-29, and Tyr-82, are shown, as are two water molecules, HOH-102 and HOH-110. HOH-110 is an internal water molecule that lies 2.7 Å from the carboxyl oxygen of Tyr-82. The side chain of Val-3 can also be seen in front of Phe-14. This is a simulated-annealing omit map (see text) with Phe-14, Phe-29, and Tyr-82 omitted from the phase calculation; the map is contoured at 2σ.

Table II: Residues in Multiple Conformations or with Partial Occupancies

residue[a]	first disordered atom	occupancy	mean *B* factor (Å^2) of affected atoms
Arg-57	C^{ζ}	0.70	31.4
		0.30	27.5
Glu-43*	C^{γ}	0.72	25.5
Asp-44	C^{β}	0.71	24.4
Asp-61*	C^{γ}	0.86	25.2
Glu-85*	C^{γ}	0.56	21.7
Gln-88	C^{β}	0.65	28.3
Lys-95	C^{δ}	0.61	26.4

[a] Residues marked with an asterisk (*) are also disordered in the crystal structure of *E. prolifera* plastocyanin. The occupancies and corresponding temperature factors must be interpreted with caution since the two values are too highly anticorrelated to be determined simultaneously with precision (see text).

CHECK program package, which evaluates the stereochemical quality of the structure, revealed that it had excellent geometry, exceeding the expected parameters for a protein at 1.5-Å resolution in every case but one (the standard deviation of main-chain hydrogen-bond energy was slightly higher than the expected value) (Morris *et al.*, 1992). An additional check of structural accuracy based on expected patterns of nonbonded contacts between different atom types (Colovos & Yeates, 1993) did not identify any regions with improbable conformations.

Chlamydomonas reinhardtii plastocyanin has the same overall fold as the other plastocyanin molecules of known structure (Figure 2) [Colman *et al.*, 1978; Chazin & Wright, 1988; Collyer *et al.*, 1990; Moore *et al.*, 1991; see also Guss *et al.* (1986, 1992) and Driscoll *et al.* (1987)]. *C. reinhardtii* plastocyanin is most similar structurally to the other green algae plastocyanins, from *E. prolifera* and *S. obliquus*, in that they are all missing the characteristic bulge present in poplar plastocyanin at positions 58–60. The rms deviation, calculated using the method of Kabsch (1978), in the Cα positions between *C. reinhardtii* and *E. prolifera* plastocyanin (used as the molecular replacement search model) is 0.49 Å and between *C. reinhardtii* and poplar plastocyanin (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. The largest difference between the *C. reinhardtii* and *E. prolifera* structures is 1.2 Å in the Cα positions of residue 26 (Thr-26 in *C. reinhardtii* plastocyanin and Ser-26 in *E. prolifera*

FIGURE 2: Structure of *Chlamydomonas reinhardtii* plastocyanin showing the overall fold and copper site. The structure is made up of two β-sheets, composed of eight β-strands (S1–S8), and one short region of α-helix (HA). β-sheet I is made up of β-strands S2A, S1, S3, and S6, and β-sheet II is made up of β-strands S2B, S8, S7, and S4. The single oxidized copper atom is liganded in quasi-tetrahedral geometry by His-37, His-87, Cys-84, and Met-92. This figure was created using the program MOLSCRIPT (Kraulis, 1991).

plastocyanin). The main-chain atoms of this residue lie next to the side-chain atoms of amino acid 0, which is an alanine in *E. prolifera* plastocyanin and an aspartic acid in *C. reinhardtii* plastocyanin. The additional space taken up by the aspartic acid side chain accounts for the movement of Thr-26 in *C. reinhardtii* plastocyanin relative to the *E. prolifera* structure. The molecule is made up of two β-sheets (I and II) comprising eight β-strands (1, 2A, 2B, 3, 4, 6, 7, and 8)[7] in a canonical antiparallel β-sheet Greek key motif (Richardson, 1981). β-sheet I is composed of strands 1, 2A, 3, and 6, and β-sheet II is composed of strands 2B, 4, 7, and 8. The

[7] The strands are also numbered according to the method of Collyer *et al.* (1990) and do not include a β-strand 5.

Table III: Comparison of the Copper-Binding Sites

	C. reinhardtii	*E. prolifera*	poplar[a]
bond lengths (Å)			
Cu–$N^{\delta 1}$(H37)	2.02	1.89	1.91
Cu–$N^{\delta 1}$(H87)	2.01	2.17	2.06
Cu–S^{γ}(C84)	2.11	2.12	2.07
Cu–S^{δ}(M92)	2.89	2.92	2.82
bond angles (deg)			
S^{γ}(C84)–Cu–S^{δ}(M92)	107	108	110
S^{γ}(C84)–Cu–$N^{\delta 1}$(H37)	131	125	132
S^{γ}(C84)–Cu–$N^{\delta 1}$(H87)	122	120	121
S^{δ}(M92)–Cu–$N^{\delta 1}$(H37)	88	90	88
S^{δ}(M92)–Cu–$N^{\delta 1}$(H87)	100	102	101
$N^{\delta 1}$(H37)–Cu–$N^{\delta 1}$(H87)	100	104	97

[a] Oxidized poplar plastocyanin (Guss *et al.*, 1992).

structure contains a single turn of α-helix (helix A, from residues 52–56) that follows β-strand 4. The structure also contains six hydrogen-bonded β-turns (Chou & Fasman, 1977): two type I β-turns (residues 7–10 and 42–45), three type II β-turns (residues 22–25, 47–50, and 65–68), and one type III β-turn (residues 87–90).

The average temperature factors for main-chain atoms, side-chain atoms, and water oxygens are 13.7 17.6, and 29.4 Å^2, respectively. The side chain of Arg-57 adopts two discrete conformations. Three of the six residues with disordered side chains, Glu-43, Asp-61, and Glu-85, are conserved or nearly conserved between *C. reinhardtii* and *E. prolifera* plastocyanin (Table II) (Glu-85 is an aspartic acid in the *E. prolifera* protein) and were reported in two conformations in the *E. prolifera* plastocyanin structure (Collyer *et al.*, 1990). These side chains remain disordered in the *C. reinhardtii* plastocyanin structure, which indicates that there is some degree of conservation of motion in these side chains between different plastocyanin molecules, as has been noted previously (Moore *et al.*, 1991).

The copper-binding site is located in the loops above the two β-sheets at the "north" end of the molecule (Figure 2). A single oxidized copper atom is coordinated by the S^{γ} atom of Cys-84, and S^{δ} atom of Met-92, an the $N^{\delta 1}$ atoms of His-37 and His-87. The temperature factor of the Cu atom is 17.6 Å^2, and the mean temperature factors for the liganding side chains are 11.7 (Cys-84), 12.6 (Met-92), 12.2 (His-37), and 13.7 Å^2 (His-87). The temperature factor of the Cu atom is greater than the mean temperature factors for the liganding side chains in all the previous plastocyanin crystal structures but one: the 1.33-Å crystal structure of poplar plastocyanin in which corrections for anomalous scattering by the copper atom were made (Guss *et al.*, 1992). The bond lengths and bond angles of the copper site are similar to those of the previous plastocyanin crystal structures (Table III) (Collyer *et al.*, 1990; Guss *et al.*, 1992). The differences in the copper sites can be accounted for in the approximate error in atomic positions, which was estimated to be between 0.25 and 0.30 Å for the structure reported here. The greatest differences between the copper-binding sites are 0.16 Å in the Cu–$N^{\delta 1}$-(His-87) bond length between the *C. reinhardtii* and *E. prolifera* structures and 6° in the S^{γ}(Cys-84)–Cu–$N^{\delta 1}$(His-37) bond angle between the *C. reinhardtii* and the poplar structures.

The negative patch and hydrophobic patch are regions on the surface of the molecule thought to be involved in the recognition of reaction partners for the purpose of directing electron transfer. The negative patch surrounds Tyr-83, which is the putative distant site of electron transfer to plastocyanin. In *C. reinhardtii* plastocyanin, the negative patch is composed of seven amino acids, Asp-42, Glu-43, Asp-44, Asp-53, Asp-59, Asp-61, and Glu-85, that cluster on one side of the molecule around helix A and the β-turn from residues 42–45 (Figure 3). Two polar amino acids, Asn-51 and Gln-88, also lie in this area, and there are no hydrophobic or nonpolar amino acids nearby. Asp-42, Glu-43, Asp-44, and Asp-61 are all conserved or nearly conserved in the known plastocyanin structures (residue 61 is a glutamic acid in French bean plastocyanin); Asp-53, Asp-59, and Glu-85 are conserved in the algal plastocyanin structures (Sykes, 1991; Collyer *et al.*, 1990; Merchant *et al.*, 1990). Helix A and the type I β-turn from

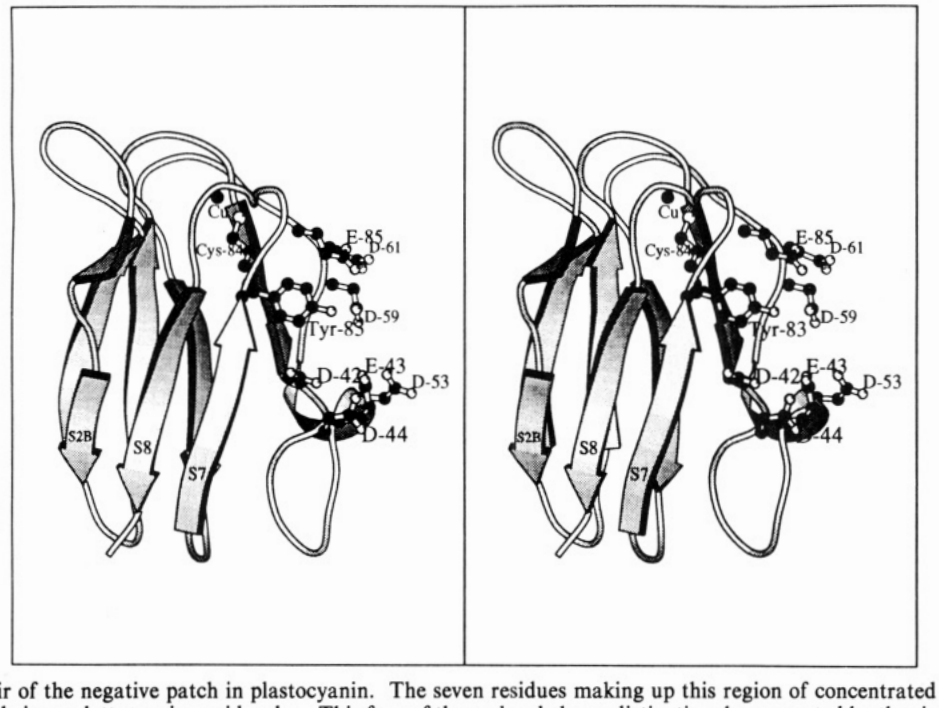

FIGURE 3: Stereopair of the negative patch in plastocyanin. The seven residues making up this region of concentrated negative charge are shown, labeled with their one-letter amino acid codes. This face of the molecule has a distinctive shape created by the single region of α-helix and the type I β-turn from amino acids 42–45. Tyr-83, the putative distant site of electron transfer, is also shown, as is Cys-84, the most likely path of electron transfer to the copper atom (the S^{γ} atom of Cys-84 is shown in white). The molecule in this figure has been rotated 60° about the vertical axis relative to that shown in Figure 2. This figure was created with the program MOLSCRIPT (Kraulis, 1991).

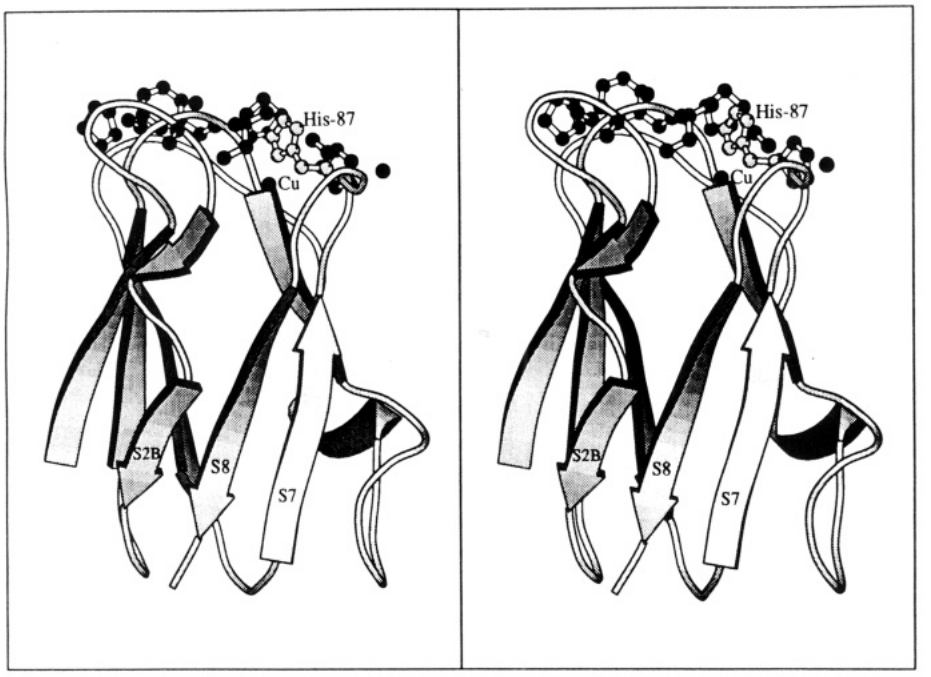

FIGURE 4: Stereopair of the hydrophobic patch in plastocyanin. The side chains of eight amino acids that make up this exclusively hydrophobic region are shown, as are the Cα positions of two glycine residues. There are no polar amino acids nearby, which makes approximately 290 Å^2 of the molecular surface hydrophobic. His-87, on a favorable path of electron transfer, is located in the middle of this region and is solvent-accessible. The molecule in this figure has been rotated 30° about the vertical axis relative to that shown in Figure 2. This figure was created with the program MOLSCRIPT (Kraulis, 1991).

42–45 give this face of the molecule a distinctive shape which, combined with the concentration of negative charge, makes it a unique region of the molecular surface and attractive for molecular recognition. An area of negative charge has been described on the surface of amicyanin, a blue copper-binding protein that also catalyzes an electron-transfer reaction between two reaction partners (Durley *et al.*, 1993). The hydrophobic patch covers the northern portion of the molecule and surrounds His-87, one of the copper ligands and a favorable path of electron transfer. In *C. reinhardtii* plastocyanin, the hydrophobic patch is composed of eight hydrophobic amino acids, Ala-11, Leu-12, Ala-33, Phe-35, Pro-36, Pro-66, Pro-86, and Ala-90, and two glycines, 34 and 89 (Figure 4). There are no polar or hydrophilic amino acids nearby, which leaves approximately 290 Å^2 of the molecular surface area purely hydrophobic. The majority of these amino acids is conserved between the known plastocyanin structures. Residue 11 is a serine in the *E. prolifera*, poplar, and French bean plastocyanins, and residue 66 is a lysine in the *E. prolifera* and poplar plastocyanins (Collyer *et al.*, 1990; Guss & Freeman, 1983; Moore *et al.*, 1991). Residue 66 is approximately 14 Å from His-87, and a lysine side chain in that position would extend into the solvent and have an effect on the overall hydrophobicity of the region. Hydrophobic patches have also been noted on the surfaces of the blue copper proteins amicyanin (Durley *et al.*, 1993), azurin (Nar *et al.*, 1991), pseudoazurin (Petratos *et al.*, 1988), and the cucumber basic blue protein (Guss *et al.*, 1988). The importance of the hydrophobic patch in mediating electron transfer has been demonstrated by van de Kamp *et al.* (1990) using site-directed mutagenesis studies of azurin.

An internal water molecule lies 4.0 Å beneath the surface of the protein near the aromatic oxygen of Tyr-82 (Figure 5). It is hydrogen-bonded to the hydroxyl group of Tyr-82 (2.7 Å away) and to the backbone carbonyl oxygen of Pro-16 (2.9 Å away). It provides a hydrogen-bonding bridge between these two atoms, which are 4.4 Å apart and not near any other hydrogen-bond donors or acceptors. The solvent site is highly ordered, with a temperature factor of 13.3 Å^2 and full occupancy. This is the first time an internal water molecule has been located near residue 82 in plastocyanin. There is no

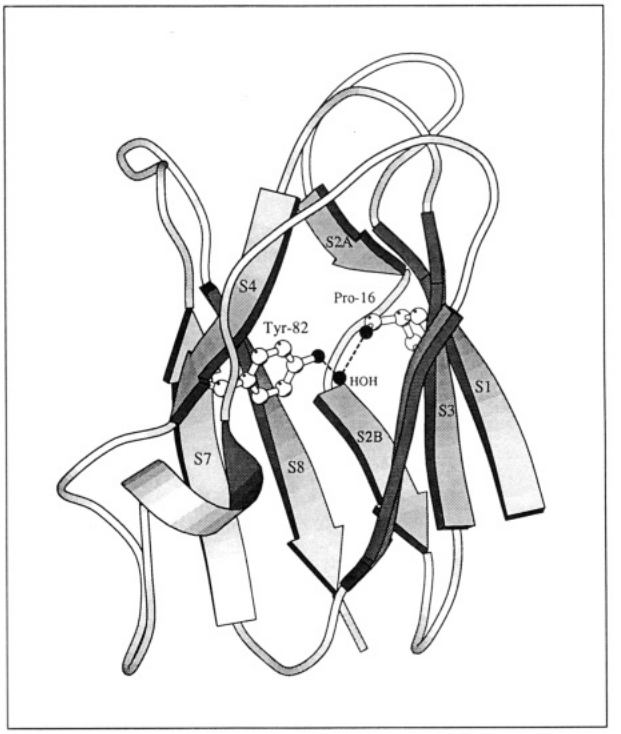

FIGURE 5: An internal water molecule in *C. reinhardtii* plastocyanin. This water molecule is buried 4.0 Å beneath the surface of the molecule and provides a hydrogen-bonding bridge between the ring oxygen of Tyr-82 (2.7 Å away) and the carbonyl oxygen of Pro-16 (2.9 Å away). Residue 82 lies at the C-terminal end of β-strand 7, and residue 16 lies in the loop between β-strands 2A and 2B. The point on the surface of the molecule that is closest to this internal solvent site is the region between β-strand 8 and the N-terminus of β-strand 2B. There are no clear channels to this site that would allow for the rapid exchange of solvent molecules. The molecule in this figure has been rotated 160° about the vertical axis relative to that shown in Figure 2. This figure was created with the program MOLSCRIPT (Kraulis, 1991).

indication of an expansion of the molecule around this site relative to the *E. prolifera* structure to accommodate the internal water molecule. Two internal waters have been described in poplar plastocyanin near amino acids 21, 25, and 27 (Guss *et al.*, 1992). Internal waters have also been identified in amicyanin and pseudoazurin, although not in the same location as the internal water described here (Durley *et al.*, 1993; Petratos *et al.*, 1988). Poplar and *E. prolifera* plastocyanins both contain nonpolar residues at position 82 (phenylalanine and valine, respectively) that require no hydrogen bonds. The carbonyl oxygen of residue 16 is in a similar position in *C. reinhardtii*, *E. prolifera*, and poplar plastocyanins, but it forms no hydrogen bonds in the latter two structures (Collyer *et al.*, 1990; Guss & Freeman, 1983). *Scenedesmus obliquus* plastocyanin (determined by ^{1}H NMR methods) contains a tyrosine residue at position 82, but no internal water was reported in this structure (Moore *et al.*, 1988). Residue 82 is also involved in a complementary mutation in plastocyanin. Complementary mutations are the least commonly observed form of accommodating amino acid changes in the interior of β-sheet proteins (Lesk & Clothia, 1982). In the *E. prolifera* structure, Val-82 and Met-94 occupy the region in the interior of the molecule that is taken up by the Tyr-82 side chain in *C. reinhardtii* plastocyanin. In order to accommodate this aromatic ring, residue 94 is a glycine in *C. reinhardtii* plastocyanin. This complementary mutation has been noted previously for poplar and *E. prolifera* plastocyanins (Collyer *et al.*, 1990).

A single large cation was placed in the first sphere of hydration of the structure. It is liganded by the backbone amide nitrogen of Lys-17, a carboxylate oxygen of Asp-61 from a symmetry-related molecule, and two water oxygens. The bond lengths are 3.4, 3.3, 2.9, and 3.5 Å, respectively. It has prominent spherical electron density and is tentatively identified as calcium because calcium chloride was present in the crystallization of both specimens used to solve the structure. In the structure of *E. prolifera* plastocyanin, there is a water molecule in a similar location, 3.05 Å from a carboxylate oxygen of Asp-61. The IONS program, which locates probable metal-binding sites by locating regions of contrasting hydrophobicity (Yamashita *et al.*, 1990), did not identify this site or the copper site as probable metal ion binding sites. Interestingly, the site identified by the program as the most probable for metal binding was the negative patch, particularly residues 42–44.

The possible paths of electron transfer through the protein have been analyzed using the program PATHWAYS II (Regan, 1993). The program identifies a network of σ and lone-pair orbitals in the protein and, using a pathway search algorithm, locates the optimum electron-transfer path between an initial and final orbital by maximizing the electronic coupling matrix element, T_{DA} (Onuchic *et al.*, 1992). General electron-transfer theory defines T_{DA} to be proportional to the product of the electronic decay elements of the orbital interactions that make up the pathway, and it defines the rate of electron transfer (k_{ET}) to be proportional to the square of T_{DA}[8] [reviewed in Onuchic *et al.* (1992); see also Marcus and Sutin (1985)]. The electron path can proceed via covalent, hydrogen-bonded, and through-space interactions. The search strategy used in PATHWAYS II seeks to maximize T_{DA} using simple approximations of the electronic decay factors that make up the pathway (Onuchic *et al.*, 1992). The paths of electron transfer from Tyr-83 (in the negative patch) to the copper atom and from the copper atom to His-87 (in the hydrophobic patch) in *C. reinhardtii* plastocyanin were analyzed (Figure 6). The initial orbital in the path from Tyr-83 was a lone-pair orbital on the hydroxyl oxygen of Tyr-83, and the final orbital in the path to His-87 was the lone-pair orbital on the N$^{\epsilon 2}$ atom of His-87. The optimum path identified by the program from Tyr-83 to the copper atom proceeds through the side chain of Tyr-83 (C$^{\zeta}$, C$^{\epsilon 1}$, C$^{\delta 1}$, C$^{\gamma}$, and C$^{\beta}$), the backbone Tyr-83 and Cys-84, the side chain of Cys-84, and the copper atom. This path is 15.3 Å long and is entirely through-bond; the distance in space between the initial and final orbitals is 11.9 Å. The optimum path identified by the program from the copper atom to His-87 involves the copper atom and the side chain of His-87 (N$^{\delta 1}$, C$^{\epsilon 1}$, and N$^{\epsilon 2}$). This path is 4.0 Å long. The products of the electronic decay elements of the path are 2.16×10^{-1} for the path between the copper atom and His-87 and 2.17×10^{-3} for the path between the copper atom and Tyr-83. The electronic coupling elements for the two paths are proportional to these decay factors, so that the coupling factor for the path to His-87 is greater by a factor of 100. A similar preference for the path to His-87 has been observed by Beratan *et al.* (1991) for poplar plastocyanin.

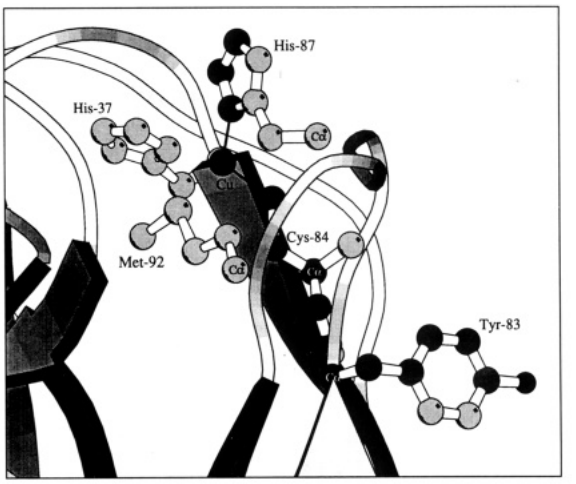

FIGURE 6: Potential paths of electron transfer in plastocyanin, as identified by the program PATHWAYS II (Regan, 1993). The atoms implicated in electron transfer are shown in black; all other atoms are in light gray. A potential path of electron transfer from Tyr-83 to the copper atom involves the side chain of Tyr-83, the main-chain atoms between residues 83 and 84, the side chain of Cys-84, and the copper atom. A potential path of electron transfer from the copper atom to His-87 involves the side chain of His-87. The side chains of His-37 and Met-92, which ligand the copper atom along with Cys-84 and His-87, are also shown. The molecule in this figure is in approximately the same orientation as that shown in Figure 3. This figure was created with the program MOLSCRIPT (Kraulis, 1991).

The structure determination of *C. reinhardtii* cytochrome c_6, which substitutes for plastocyanin under conditions of copper deficiency (Kunert *et al.*, 1976; Wood, 1978), is in progress in our laboratory. Plastocyanin and cytochrome c_6 from this organism are presumably quite different structurally [*c*-type cytochromes are α-helical (Johnson *et al.*, 1990)]. It will be of interest to examine the structural basis of their functional complementarity, and we hope to address several questions by comparing the two structures. For example, are there regions on the surface of cytochrome c_6 that are similar in charge, hydrophobicity, and shape to the negative and hydrophobic patches on plastocyanin? Are there other regions of distinctive molecular shape that cytochrome c_6 and plastocyanin share? Do the potential electron-transfer paths in cytochrome c_6 and plastocyanin have similar lengths and electronic coupling? A detailed comparison of the structural

[8] According to the Dirac–Fermi golden rule, $k_{ET} = (2\pi/\hbar)T_{DA}^2$ [FC], where FC is the Franck–Condon, or nuclear, factor.

characteristics of these proteins may allow us to gain more complete information on how they interact with the reaction partners they have in common. *Chlamydomonas reinhardtii* is also amenable to genetic manipulation, which allows the structure–function relationship of these proteins to be studied *in vivo*. The structure of plastocyanin reported here facilitates the design of specific mutational studies in this system.

REFERENCES

Anderson, G. P., Sanderson, D. G., Lee, C. H., Durell, S., Anderson, L. B., & Gross, E. L. (1987) *Biochim. Biophys. Acta 894*, 386–398.

Beoku-Betts, D., Chapman, S. K., Knox, C. V., & Sykes, A. G. (1985) *Inorg. Chem. 24*, 1677–1681.

Beratan, D. N., Betts, J. N., & Onuchic, J. N. (1991) *Science 252*, 1285–1288.

Brünger, A. T. (1991) *Annu. Rev. Phys. Chem. 42*, 197–223.

Brünger, A. T. (1992) *X-PLOR Manual*, Version 3.0., Yale University, New Haven, CT.

Buerger, M. J. (1960) *Crystal Structure Analysis*, John Wiley & Sons, New York.

Buschlen, S., Choquet, Y., Kuras, R., & Wollman, F.-A. (1991) *FEBS Lett. 284*, 257–262.

Chazin, W. J., & Wright, P. E. (1988) *J. Mol. Biol. 202*, 623–636.

Chou, P. Y., & Fasman, G. D. (1977) *J. Mol. Biol. 115*, 135–175.

Collyer, C. A., Guss, J. M., Sugimura, Y., Yoshizaki, F., & Freeman, H. C. (1990) *J. Mol. Biol. 211*, 617–632.

Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshow, J. A. M., & Venkatappa, M. P. (1978) *Nature 272*, 319–324.

Colovos, C., & Yeates, T. O. (1993) *Protein Sci. 2*, 1511–1519.

Crowther, R. A. (1972) *The Molecular Replacement Method* (Rossmann, M. G., Ed.) pp 173–178, Gordon & Breach, New York.

Driscoll, P. C., Hill, H. A. O., & Redfield, C. (1987) *Eur. J. Biochem. 170*, 279–292.

Durley, R., Chen, L., Lim, L. W., Mathews, F. S., & Davidson, V. L. (1993) *Protein Sci. 2*, 739–752.

Engh, R. A., & Huber, R. (1991) *Acta Crystallogr. A47*, 392–400.

Farver, O., Shahak, Y., & Pecht, I. (1982) *Biochemistry 21*, 1885–1890.

Garrett, T. P. J., Clingeleffer, D. J., Guss, J. M., Rogers, S. J., & Freeman, H. C. (1984) *J. Biol. Chem. 259*, 2822–2825.

Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521–563.

Guss, J. M., Harrowell, P. R., Murata, M., Norris, V. A., & Freeman, H. C. (1986) *J. Mol. Biol. 192*, 361–387.

Guss, J. M., Merritt, E. A., Phizackerley, R. P., Hedman, B., Murata, M., Hodgson, K. O., & Freeman, H. C. (1988) *Science 241*, 806–811.

Guss, J. M., Bartunik, H. D., & Freeman, H. C. (1992) *Acta Crystallogr. B48*, 790–811.

He, S., Modi, S., Bendall, D. S., & Gray, J. C. (1991) *EMBO J. 10*, 4011–4016.

Hill, K. L., Li, H. H., Singer, J., & Merchant, S. (1991) *J. Biol. Chem. 266*, 15060–15067.

Ho, K. K., & Krogmann, D. W. (1984) *Biochim. Biophys. Acta 766*, 310–316.

Hodel, A., Kim, S.-H., & Brünger, A. T. (1992) *Acta Crystallogr. A48*, 851–858.

Johnson, M. S., Sutcliffe, M. J., & Blundell, T. L. (1990) *J. Mol. Evol. 30*, 43–59.

Jones, T. A. (1978) *J. Appl. Crystallogr. 11*, 268–272.

Kabsch, W. (1978) *Acta Crystallogr. A34*, 827–828.

Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946–950.

Kunert, K.-J., Bohme, H., & Boger, P. (1976) *Biochim. Biophys. Acta 449*, 541–553.

Lesk, A. M., & Clothia, C. (1982) *J. Mol. Biol. 160*, 325–342.

Luzatti, P. V. (1952) *Acta Crystallgor. 5*, 802–810.

Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265–322.

Matthews, B. W. (1968) *J. Mol. Biol. 33*, 491–497.

Merchant, S., & Bogorad, L. (1986) *Mol. Cell. Biol. 6*, 462–469.

Merchant, S., Hill, K., Kim, J. H., Thompson, J., Zaitlin, D., & Bogorad, L. (1990) *J. Biol. Chem. 265*, 12372–12379.

Modi, S., He, S., Gray, J. C., & Bendall, D. S. (1992) *Biochim. Biophys. Acta 1101*, 64–68.

Moore, J. M., Chazin, W. J., Powls, R., & Wright, P. E. (1988) *Biochemistry 27*, 7806–7816.

Moore, J. M., Lepre, C. A., Gippert, G. P., Chazin, W. J., Case, D. A., & Wright, P. E. (1991) *J. Mol. Biol. 221*, 533–555.

Morand, L. Z., Frame, M. Z., Colvert, K. K., Johnson, D. A., Krogmann, D. W., & Davis, D. J. (1989) *Biochemistry 28*, 8039–8047.

Morris, A. L., MacArthur, M. W., Hutchinson, E. G., & Thornton, J. M. (1992) *Proteins 12*, 345–364.

Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1991) *J. Mol. Biol. 218*, 427–447.

Nordling, M., Sigfridsson, K., Young, S., Lundberg, L. G., & Hansson, O. (1991) *FEBS Lett. 291*, 327–330.

Onuchic, J. N., Beratan, D. N., Winkler, J. R., & Gray, H. B. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 349–377.

Petratos, K., Dauter, Z., & Wilson, K. S. (1988) *Acta Crystallogr. B44*, 628–636.

Qin, L., & Kostic, N. M. (1992) *Biochemistry 31*, 5145–5150.

Quinn, J., Li, H. H., Singer, J., Morimoto, B., Mets, L., Kindle, K., & Merchant, S. (1993) *J. Biol. Chem. 268*, 7832–7841.

Redinbo, M. R., & Yeates, T. O. (1993) *Acta Crystallogr. D49*, 375–380.

Regan, J. J. (1993) *Pathways II* software v2.0, Jeffrey J. Regan, San Diego, CA.

Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167–339.

Stanley, E. (1972) *J. Appl. Crystallogr. 5*, 191–194.

Sykes, A. G. (1991) *Struct. Bonding 75*, 175–224.

van de Kamp, M., Silverstrini, M. C., Brunori, M., Van Beeumen, J., Hali, F. C., & Canters, G. W. (1990) *Eur. J. Biochem. 194*, 109–118.

Widger, W. R. (1991) *Photosynth. Res. 30*, 71–84.

Wood, P. M. (1978) *Eur. J. Biochem. 87*, 9–19.

Wynn, R. M., & Malkin, R. (1988) *Biochemistry 27*, 5863–5869.

Wynn, R. M., Omaha, J., & Malkin, R. (1989) *Biochemistry 28*, 5554–5560.

Yamashita, M. M., Wesson, L., Eisenman, G., & Eisenberg, D. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5648–5652.

Yeates, T. O., & Rini, J. M. (1990) *Acta Crystallogr. A46*, 352–359.