Side-Chain Interactions in the Plastocyanin−Cytochrome *f* Complex†

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ABSTRACT: Cytochrome *f* and plastocyanin are redox partners in the photosynthetic electron-transfer chain. Electron transfer from cytochrome *f* to plastocyanin occurs in a specific short-lived complex. To obtain detailed information about the binding interface in this transient complex, the effects of binding on the backbone and side-chain protons of plastocyanin have been analyzed by mapping NMR chemical-shift changes. Cytochrome *f* was added to plastocyanin up to 0.3 M equiv, and the plastocyanin proton chemical shifts were measured. Out of approximately 500 proton resonances, 86% could be observed with this method. Nineteen percent demonstrate significant chemical-shift changes and these protons are located in the hydrophobic patch (including the copper ligands) and the acidic patches of plastocyanin, demonstrating that both areas are part of the interface in the complex. This is consistent with the recently determined structure of the complex [Ubbink, M., Ejdebäck, M., Karlsson, B. G., and Bendall, D. S. (1998) *Structure* 6, 323−335]. The largest chemical-shift changes are found around His87 in the hydrophobic patch, which indicates tight contacts and possibly water exclusion from this part of the protein interface. These results support the idea that electron transfer occurs via His87 to the copper in plastocyanin and suggest that the hydrophobic patch determines the specificity of the binding. The chemical-shift changes in the acidic patches are significant but small, suggesting that the acidic groups are involved in electrostatic interactions but remain solvent exposed. The existence of small differences between the present data and those used for the structure may imply that the redox state of the metals in both proteins slightly affects the structure of the complex. The chemical-shift mapping is performed on unlabeled proteins, making it an efficient way to analyze effects of mutations on the structure of the complex.

Many biological processes involve complex formation by highly specific interactions. Between proteins these interactions can be of a static or a transient nature, depending on the biological function of the complex. Static complexes are characterized by slow dissociation. Transient complexes are formed when a high turnover is required, as is often the case in electron-transfer processes. Electron transfer proteins are usually highly charged, with patches of negative or positive charges at the binding sites for their redox partners, suggesting that electrostatic forces are important at long-distance for preorienting and binding. Additional noncovalent forces are important at a closer distance between the partners to optimize the interactions and favor one particular configuration of the complex that allows efficient electron transfer. The transient complex between the redox proteins plastocyanin and cytochrome *f* is the subject of this study.

Both proteins are located in the thylakoid lumen of the chloroplast where they are part of the electron-transfer chain that links photosystem 2 to photosystem 1. Cytochrome *f* constitutes one subunit (33 kDa) of the cytochrome b_6f complex, and it is anchored in the thylakoid membrane via a single membrane-spanning helix placed near its C-terminus. In cruciferous plants, the large lumenal domain (28.2 kDa) can be isolated in soluble form and its crystal structure has been determined (*1*). Plastocyanin is a soluble protein (10.4 kDa) that acts as an electron carrier between cytochrome *f* and photosystem 1. It contains a single copper ion bound in a type 1 copper site (*2*). A number of structures of plant plastocyanins have been determined with X-ray diffraction and NMR[1] (*3*−*8*). Three prominent patches can be found on the surface of plastocyanin from higher eukaryotes, a flat hydrophobic patch and two acidic patches (residues 42−45 and 59−61) of exposed negatively charged amino acids (Figure 1A). In the electron-transfer chain, oxidized plastocyanin binds to the cytochrome b_6f complex, followed by electron transfer and dissociation of reduced plastocyanin. The interaction and electron transfer between plastocyanin mutants and cytochrome *f* has been the subject of both experimental and theoretical investigations. According to

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; TSP, sodium 3-(trimethylsilyl)-2,3-d_4-propionate.

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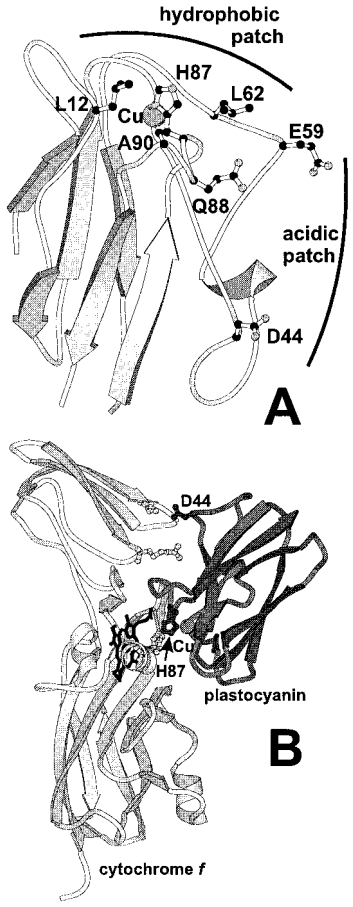

FIGURE 1: (A) Ribbon diagram of plastocyanin. Some relevant residues are shown in ball-and-stick. The copper is represented by a sphere. (B) The complex of plastocyanin (dark) and cytochrome *f* (light) (*21*), drawn from PDB entry 2PCF. Note that the orientation of plastocyanin is upside-down compared to panel A.

chemical cross-linking experiments (*9*) both acidic patches of plastocyanin are involved in complex formation, but the lack of electron transfer in the complex suggested the need of a protein rearrangement that would precede the electron-transfer reaction (*10*). In kinetic experiments using stopped-flow spectrophotometry on wild-type forms and mutants of plastocyanin (*11*−*13*) and cytochrome *f* (*14*), the importance of the charged patches in complex formation was further established, at least in vitro (*14*, *15*), although one report only stressed the importance of the larger acidic patch in plastocyanin (*16*). Theoretical docking models suggested that residues in both acidic patches on plastocyanin are important for the initial binding but not in the complex that is active in electron-transfer (*17*−*20*).

We have earlier reported on the structure of the complex between ^{15}N-labeled, Cd^{2+}-substituted spinach plastocyanin and the soluble domain of cytochrome *f* from turnip. The NMR chemical-shift changes of the H^N and N nuclei in ^{15}N-labeled plastocyanin were used to identify interacting residues and the interaction surfaces of plastocyanin were probed in this way (*21*). Furthermore, intermolecular paramagnetic effects, from the oxidized cytochrome *f* onto plastocyanin, were used in the determination of the relative orientation of the proteins in the complex. The results demonstrated a single orientation for plastocyanin, with both acidic and hydrophobic patches being part of the interface (Figure 1B). The close contact between the hydrophobic patch and the heme area on cytochrome *f*, with an Fe to Cu distance of ∼11 Å strongly suggests that this represents the complex that is active in electron transfer. In this study, the Cu^{2+} was replaced with Cd^{2+} to avoid unwanted effects of electron transfer between the proteins during the NMR experiments. The overall structure of the Cd^{2+}-substituted protein is very similar to the structure of the oxidized copper protein (*22*).

In the present work, the complex between unlabeled, reduced copper plastocyanin and the soluble domain of reduced cytochrome *f* has been studied by chemical-shift mapping. The chemical-shift changes of backbone as well as side-chain protons of plastocyanin upon complex formation were determined by two-dimensional NMR, providing a much more detailed picture of the effects of complex formation at the interface itself. The results support the finding that both acidic patches and the hydrophobic patch are part of the interface of the complex and they provide more information about the nature of the interactions in the hydrophobic and the acidic patches.

MATERIALS AND METHODS

NMR Samples. Spinach plastocyanin was expressed in *Escherichia coli* and purified essentially as described previously (*23*, *24*). The protein was reduced with a 2−3 M excess of sodium ascorbate and then dialyzed under nitrogen against 10 mM potassium phosphate, pH 6.00, by ultrafiltration in an Amicon minicell equipped with a 5K cutoff filter. The soluble domain of turnip cytochrome *f* was purchased in lyophilized form from Sigma and dissolved in 10 mM potassium phosphate, pH 6.00. The protein was reduced and washed in the same way as plastocyanin. Protein concentrations were calculated from the optical absorbance using $\epsilon_{597} = 4.7\ mM^{-1}\ cm^{-1}$ for oxidized plastocyanin and $\epsilon_{554} = 31.5\ mM^{-1}\ cm^{-1}$ for reduced cytochrome *f*. The NMR sample contained 1 mM reduced plastocyanin in 10 mM potassium phosphate, pH 6.00, 100 μM sodium ascorbate, 200 μM sodium 3-(trimethylsilyl)-2,2,3,3-d_4-propionate (TSP) and 10% D_2O. The pH was adjusted to 6.00 ± 0.05 and the solution degassed with argon. Reduced cytochrome *f* was added in successive steps to a molar amount of 10, 20, and 30%. After each addition, the pH was adjusted to 6.00 ± 0.05 and the solution degassed with argon before new spectra were acquired.

NMR Experiments. Two-dimensional TOCSY and NOESY spectra were recorded on an Avance DMX 600 MHz Bruker NMR spectrometer. Spectral widths were 8992.81 Hz, with 2048 points for t_2, 400−700 points for t_1, and 32 transients per increment. The mixing times were 100 ms and 40 ms in the NOESY and TOCSY spectra, respectively. Spectra were recorded at 300 and 310 K for free plastocyanin and the first titration point (10% cytochrome *f*) and at 310 K for the remaining (20 and 30% cytochrome *f*) titration points. The water signal was suppressed using WATERGATE (*25*). The total acquisition time was about 20 h for each titration point.

Data Processing and Analysis. Data processing was performed with AZARA (available from ftp://ftp.bio.cam.ac.uk/pub/azara/) and data analysis with Ansig (*26*, *27*). The spectra were referenced against the internal standard TSP. The proton assignments of spinach plastocyanin were taken from earlier experiments on this protein (Bergkvist, unpublished). The chemical-shift changes of the proton

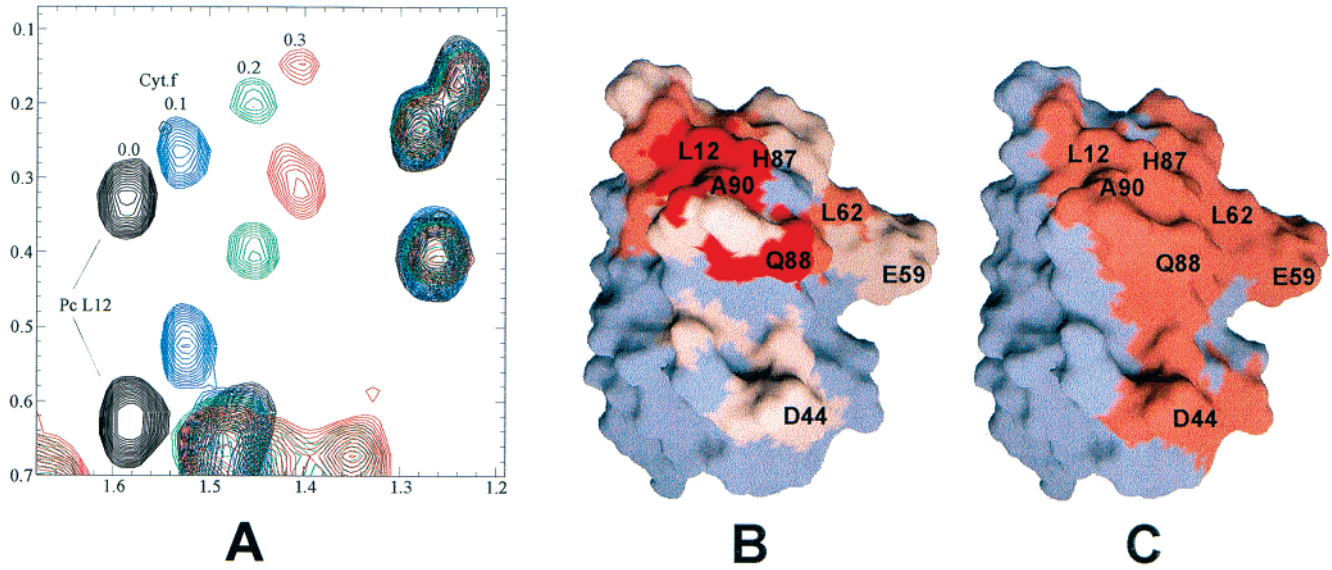

FIGURE 2: Chemical-shift changes for plastocyanin upon binding to cytochrome *f*. (A) Overlay of part of TOCSY spectra of plastocyanin with increasing amounts of cytochrome *f*. The shifting resonances represent cross-peaks between the plastocyanin Leu12 H^{γ} (1.58 ppm) and the Leu12 methyls (0.62 and 0.32 ppm). Black, plastocyanin; blue, cytochrome *f*/plastocyanin ratio of 0.1; green, cytochrome *f*/plastocyanin ratio of 0.2; red, cytochrome *f*/plastocyanin ratio of 0.3. (B) Surface representation of plastocyanin [NMR structure of spinach plastocyanin (A. Bergkvist, unpublished results), made with GRASP (*32*)] with each residue colored according to the largest chemical-shift difference observed for its protons in the presence and absence of 0.3 M equiv cytochrome *f*. Colorcoding according to Table 2. (C) Surface representation of plastocyanin with the residues in the interface of the complex of cytochrome *f* and plastocyanin (Figure 1B; ref *21*) shown in orange; others in blue. Residues that have heavy atom(s) within 4 Å of cytochrome *f* were defined as part of the interface.

signals were calculated by comparing the proton resonances in the spectra recorded for plastocyanin with and without 0.3 M equiv of cytochrome *f* added. Intermediate titration points were used to facilitate the assignment of the shifted peaks.

RESULTS

To determine the interactions in the complex between plastocyanin and cytochrome *f* (both reduced), two-dimensional ^{1}H NMR spectra of the complex were recorded and compared with those of free plastocyanin. Addition of cytochrome *f* to the NMR sample caused changes in the proton chemical shifts, as illustrated in Figure 2A. The magnitude of these changes increased with increasing amounts of added cytochrome *f*. The observed changes in the NMR spectra were all consistent with a fast exchange of bound and free plastocyanin on the NMR time scale. At the last titration point (0.3 M equiv of cytochrome *f*), resonances that originate from the cytochrome started to become visible. A small general line broadening was observed upon complex formation, due to the slow rotational correlation time of the complex.

The chemical-shift changes of H^{α}, H^{β}, and H^{γ} protons could mostly be determined from the cross-peaks generated by their intraresidual coupling to the H^N in TOCSY spectra. The chemical-shift changes of protons located further from the polypeptide backbone was generally determined from the side chain cross-peaks between H^{β}, H^{γ}, H^{δ}, or H^{ϵ} protons. Some H^{α}-resonances were located too close to the water signal to be observable in TOCSY spectra and they were observed in NOESY spectra instead. In this way we were able to monitor the chemical shifts of 86% of the 499 proton resonances of plastocyanin during the titration with cytochrome *f*. Chemical-shift changes of $\geq$0.03 ppm were considered to be significant and caused by interaction between the proteins. The chemical-shift changes of the residues that are significantly affected are shown in Table 1 and mapped onto a surface picture of plastocyanin in Figure 2B, using the colorcoding given in Table 2. Chemical-shift changes for all residues are supplied as Supporting Information, Table S1. In total, 96 (19%) proton resonances exhibiting significant chemical-shift changes were observed during the titration, while the majority of the proton signals (335; 67%) show no or very small chemical-shift changes ($<$0.03 ppm). For 68 (14%) of the proton resonances the changes in the chemical shift could not be determined, mainly due to overlap with other signals. The chemical-shift changes can be grouped into three regions of plastocyanin, the hydrophobic patch, the copper site, and the acidic patches.

Hydrophobic Patch. Protons of residues in the hydrophobic patch show the largest chemical-shift changes. All four loops that make up this region, are affected by binding, but the effects are strongest on the first loop (residues 6−13), in particular around Leu12. The side chain of Leu12 shows the largest chemical-shift changes and is located next to copper ligand His87. Extrapolated to a 1:1 molar ratio, the shift of Leu12 $H^{\delta 2}$ is more than 1 ppm. Also the so-called ligand loop (residues 84−92), which contains three of the four copper ligands, shows large changes around the His87 side chain, with the highest shift change for the H^{β} of Ala90. The chemical-shift changes in the other two loops are somewhat smaller and located on residues 32−36 and Leu62 and Pro66. The important observation that the largest chemical-shift changes are observed around the side chain of His87 suggests that this area makes close contact with cytochrome *f*, thus strongly supporting the view that electron transfer occurs from heme to copper occurs via His87. It means that the protons in this region experience a very different chemical environment in the complex, suggesting that water may be excluded from the interface. This would

Table 1: Plastocyanin Residues that Demonstrate Significant Chemical-Shift Changes[a] (ppm) upon binding to cytochrome *f*

residue	NH	Hα	Hβ	Hγ	Hδ	Hε	Hξ
Leu 5	**−0.03**	0.00	0.00, no	**−0.03**	**−0.03**, **−0.03**		
Gly 6	−0.02	**−0.08**, **0.03**					
Gly 7	0.02	**−0.05**, no					
Gly 8	**−0.03**	**−0.06**, no					
Asp 9	**−0.04**	−0.01	**−0.03**, **−0.05**				
Gly10	**−0.07**	**−0.09**, **−0.09**					
Ser 11	**−0.10**	**−0.11**	**−0.07**, **−0.07**				
Leu 12	**−0.13**	**−0.10**	**−0.14**, no	**−0.18**	**−0.18**, **−0.31**		
Ala 13	**−0.08**	0.02	**−0.05**				
Phe 14	**−0.03**	no	−0.02, **0.07**		−0.01	0.00	**−0.03**
Phe 19	0.01	0.02	**0.04**, 0.00		−0.01	0.00	−0.01
Asn 31	**0.03**	−0.02	−0.02, −0.01		0.01, −0.01		
Asn 32	0.00	**−0.04**	no, **−0.03**		**−0.04**, **0.03**		
Ala 33	−0.02	no	**−0.05**				
Gly 34	**−0.07**	**−0.04**, **−0.04**					
Phe 35	**−0.06**	**−0.03**	**−0.03**, −0.02		**−0.04**	−0.02	−0.02
Pro 36		**0.06**	**0.03**, no	0.02	−0.02, 0.01		
His 37	**−0.05**	**−0.03**	**−0.04**, −0.01		**−0.07**	**−0.11**	
Asn 38	0.02	0.00	0.00, **0.03**		0.00, **0.05**		
Asp 42	**0.03**	no	0.01, 0.01				
Glu 43	no	0.02	0.01, 0.02	−0.01, **−0.04**			
Asp 44	**0.06**	no	0.02, 0.01				
Ser 58	**0.03**	−0.01	0.02, 0.02				
Glu 59	−0.02	**0.03**	−0.01	0.02, 0.02			
Glu 60	**0.04**	**0.04**	0.02, **0.03**	0.01			
Asp 61	**0.03**	0.00	0.00, 0.00				
Leu 62	**0.03**	no	**0.04**, 0.00	**−0.12**	**0.05**, **−0.04**		
Leu 63	0.02	0.00	no, −0.01	**−0.03**	0.02, **0.04**		
Asn 64	−0.01	no	0.00, 0.00		**0.05**, −0.02		
Pro 66		**−0.03**	no, −0.02	**−0.03**, −0.01	**−0.04**, **−0.05**		
Lys 81	**−0.04**	0.02	0.00, −0.01	−0.01, −0.01	0.00	−0.02	
Cys 84	0.01	0.00	0.02, **−0.03**				
Ser 85	−0.02	no	**0.07**, −0.02				
His 87	**−0.03**	**−0.05**	−0.02, no		**−0.13**	**−0.14**	
Gln 88	**0.03**	**0.05**	**0.05**, −0.02	no		**−0.14**, **0.13**	
Gly 89	**0.04**	**0.06**, no					
Ala 90	0.00	no	**−0.19**				
Gly 91	−0.02	**−0.05**, −0.01					
Met 92	**−0.11**	**0.04**	0.00, **−0.10**	**−0.04**		**−0.05**	
Gly 94	**−0.03**	−0.01, no					

[a] Significant chemical-shift changes ($\Delta\delta \geq 0.03$ ppm) at 0.3 M equiv of cytochrome *f* are shown in bold. no = not observed.

Table 2: Classification of Proton Chemical-Shift Changes[a]

category	size (ppm)	residues color coding in Figure 2	no. of resonances
A	0.00−0.02	blue	335
B	0.03−0.06	pink	68
C	0.07−0.12	orange	18
D	≥0.13	red	10
E	not observed	*b*	68
total			499

[a] Chemical-shift changes are absolute values observed in the spectrum of plastocyanin in the presence of 0.3 M equiv cytochrome *f* complex as compared to plastocyanin in the absence of cytochrome *f*. [b] All residues have observed protons.

enable close contact with, for example, aromatic rings, which could cause the observed chemical shift changes.

Copper Site. Large chemical-shift changes are also observed for protons underneath the hydrophobic patch, around the copper atom. It appears that effects of binding to cytochrome *f* are being transmitted into the core of the protein through the copper, thus changing the chemical shifts of protons of the copper ligands (His37, Cys84, His87, and Met92) and other protons nearby (of residues Leu5, Phe14, and Leu63). This indicates that the copper site senses the binding event, despite being buried underneath the protein surface.

Acidic Patches. There are significant chemical-shift changes at both acidic patches (residues 42−44 and 59−61), and in protons nearby (NH protons of Ser58 and Lys81), but these are smaller than those in the hydrophobic patch. This indicates that electrostatic interactions between the two proteins exist in the complex, involving both the large and the small acidic patch, and that these interactions do not lead to large changes in the chemical environment of the involved residues. Perhaps transient salt bridges are being formed that are accessible to the solvent. These would cause only small chemical shifts changes because of averaging effects and efficient screening of changes in electrostatic environment by the water and small ions.

DISCUSSION

The structure of the complex between the two physiological redox partners cytochrome *f* and plastocyanin has recently been solved by NMR (*21*). Diamagnetic and paramagnetic chemical-shift changes in the amide protons and amide nitrogens of a ^{15}N-labeled plastocyanin, caused by binding to reduced and oxidized cytochrome *f*, were monitored and

used as input in restrained rigid body molecular dynamics calculations. In the structure of the complex, both electrostatic and hydrophobic interactions play a role in the contacts between plastocyanin and cytochrome *f*. In this study, the copper in plastocyanin had been substituted with cadmium(II), to avoid effects of electron exchange on the NMR spectra. Cadmium-substituted plastocyanin resembles Cu(II) plastocyanin more closely than the Cu(I) form (*22*). The ionic strength was 45 mM, resulting in a bound-to-free ratio for plastocyanin of about 0.3. Since the bound and free form are in fast exchange, the observed chemical shifts, as well as the relaxation rates (and thus the line widths), are weighted averages. Because of this averaged relaxation, it is advantageous to have a significant portion of plastocyanin in the free form.

In the current work, two-dimensional homonuclear NMR has been used to study the interaction between the two unlabeled proteins. The ionic strength was 10 mM and plastocyanin was in 3-fold excess over cytochrome *f*, resulting in the same bound-to-free ratio as in the previous study. However, in the present work, Cu(I)-plastocyanin was used with reduced cytochrome *f*. In this way, much information could be obtained about both the backbone and side-chain protons of plastocyanin in the complex. The results are consistent with the previously determined structure of the complex. They confirm that both the acidic patches as well as the hydrophobic patch are part of the interface of the complex, indicating that both electrostatic interactions and hydrophobic contacts are important in stabilization of the complex. This is illustrated in Figure 2. Panel B shows the surface residues of plastocyanin colored according to the highest chemical-shift change of their protons. Panel C shows plastocyanin in the same orientation, with all the residues colored in orange that are part of the interface of the complex (defined as having heavy atoms within 4 Å of cytochrome *f*). There is a good agreement between the areas with chemical-shift changes and those in the interface. The sizes of the chemical-shift changes suggest that the electrostatic contacts are solvent accessible and perhaps transient, while the hydrophobic contacts appear strong and specific, with exclusion of solvent water, in particular around copper-ligand His87. This supports the conclusion that electron transfer between the two proteins takes place via His87. This was strongly suggested by the structure of the complex, in which His87 and heme iron ligand Tyr1 of cytochrome *f* are at van der Waals distance, bringing iron and copper to within 11 Å of one another. The current work suggests that the hydrophobic patch is responsible for a well-defined, tight fit between the proteins, while the main function of the acidic patches is to increase the affinity, rather than the specificity, between the proteins via interactions with the basic patch of cytochrome *f*. The latter is in agreement with kinetic studies that show that the number of charges, and not their exact location on the surface, is determining the electron-transfer rate in vitro (*11*). In vivo experiments have given evidence for a complete lack of function of the basic residues on cytochrome *f* (*14*, *15*). It still unclear how these contrasting results can be reconciled. The resonance of Leu12 $H^{\delta 2}$ does not show an increased line width, despite a chemical-shift change of more than 600 Hz between bound and free form. This indicates that the lifetime of bound plastocyanin is much shorter than 0.75 ms, thus putting a lower limit to the exchange rate of 1300 s^{-1}.

Charge of the Copper. A detailed comparison between the previous (*21*) and the current study shows a few residues that have significantly different amide proton chemical-shifts changes (residues Gly7, Ser11, Leu12, Phe35, His37, and Asn38). The amide proton of Gly7 is hydrogen-bonded to the carboxygroup of Ser11. Ser11 and Leu12 are close to His87, Phe35 is close to copper ligand His37, and the backbone amide proton of Asn38 is hydrogen bonded to the S^{γ} of Cys84, another copper ligand. All these amides are thus closely linked to the copper site and their differences in chemical-shift changes may be related to the fact that Cd(II) was used in the previous and Cu(I) in the current work. Since the Cd(II)-substituted plastocyanin is very similar to the Cu(II) form (*22*), this would imply that there is a small difference between the complexes of oxidized and reduced plastocyanin with cytochrome *f*, as has been observed for other redox complexes (*28*−*30*). This is consistent with the observation that the copper site senses the binding, given the large chemical-shift changes of protons located around the copper site. However, it cannot be excluded that the differences between both studies are caused by a difference in the ionic strength (45 and 10 mM, respectively), rather than the charge of the metal in plastocyanin.

Plastocyanin in Complex with Cytochrome c. The nonphysiological complex between pea plastocyanin and horse cytochrome *c* has been characterized by NMR using equimolar amounts of unlabeled proteins (*31*). The small chemical-shift changes of less than 0.05 ppm as well as the absence of intermolecular paramagnetic shifts suggested an ensemble of structures in the complex that were in fast exchange. The largest effects were seen for residues around the acidic patches of plastocyanin. In the complex with cytochrome *f*, plastocyanin has a single, well-defined orientation at least part of the time, with the tightest contact at the hydrophobic patch. This indicates that the complexes are of a very different nature, despite the fact that both are transient. The nonphysiological plastocyanin:cytochrome *c* complex is dominated by electrostatic forces, while the physiological plastocyanin:cytochrome *f* complex also uses hydrophobic forces, creating a single, stable orientation that is optimal for electron transfer. This difference is borne out by the plastocyanin reduction rates (*12*), which are 10-fold higher with cytochrome *f* than with cytochrome *c*, despite a more favorable driving force for the latter. From these studies it is clear that NMR can play an important role in the structural characterization of redox complexes, even if the complex is large (39 kDa for cytochrome *f* and plastocyanin) and highly transient. The "interface mapping" technique that has been applied in the present study, requires only unlabeled proteins and is an easy method to screen for mutants that change the structure of the complex.

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SUPPORTING INFORMATION AVAILABLE

One table, Table S1, showing the ^1H NMR chemical-shift changes of all observed protons in plastocyanin. This material

is available free of charge via the Internet at http://pubs.acs.org

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