Complex of Plastocyanin and Cytochrome *c* Characterized by NMR Chemical Shift Analysis†

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Received December 30, 1996; Revised Manuscript Received March 13, 1997⊗

ABSTRACT: The complexes of horse ferrous and ferric cytochrome *c* with Cd-substituted pea plastocyanin have been characterized by nuclear magnetic resonance, in order to determine the binding sites and to study the effects of complex formation. Reproducible, small chemical shift changes (0.005−0.05 ppm) were observed for protons in both proteins upon formation of a 1:1 complex. The chemical shift changes depended on the ratio of free to bound protein, with a binding constant of $1.0 \pm 0.5 \times 10^5$ M^{-1}, indicating that they were caused by complex formation and that free and bound proteins were in fast exchange. Two-dimensional spectra of the complex of ferrocytochrome *c* and plastocyanin were screened systematically for chemical shift changes. For about 760 protons, or 70% of the assigned protons in the two proteins, the chemical shift in the complex could be established. In plastocyanin and cytochrome *c* 14% and 17% of the protons, respectively, showed a significant chemical shift change. These protons form two groups. The first consists of a limited number of surface-exposed side-chain protons. These map on the so-called east side of plastocyanin and the front side of cytochrome *c*. This group of chemical shift changes is interpreted as representing direct effects of binding, and the respective surfaces thus represent the binding sites. The second group includes backbone amide protons and a few aliphatic and aromatic protons in the hydrophobic core of each protein. The chemical shift changes of this group are interpreted as secondary, i.e., caused by very small structural changes which are transmitted deep into the core of the protein. Ferric cytochrome *c* caused the same chemical shift effects in plastocyanin as the ferrous form; no intermolecular paramagnetic effects were observed. The small size of the chemical shifts and the absence of intermolecular paramagnetic shifts and NOEs suggest that the complex consists of a dynamic ensemble of structures which are in fast exchange, rather than a single static complex. This study shows that small, reproducible chemical shifts can be used effectively to characterize protein complexes in detail.

Complexes of redox proteins have a fast turnover to achieve a rapid electron flow through biological redox chains. A high dissociation rate constant (k_{off}) is complemented by a high association rate constant (k_{on}) in order to obtain an acceptable binding constant. To increase the binding rate, complexes of redox proteins are often electrostatic in nature. Electrostatic attraction is a long-range force that can preorient approaching molecules and thus enhance the proportion of productive encounters. The complex of plastocyanin and cytochrome *c* is an example of an electrostatic complex. Plastocyanin is a 10.7 kDa copper protein, with negative residues at the so-called east side of the protein (Figure 1A), forming a large (residues 42−45) and a small acidic patch (59−61). Cytochrome *c* (12.5 kDa) has a ring of positive charges surrounding the heme edge, the part of the surface where the cofactor, a *c*-type heme, is most accessible (Figure 1B). Plastocyanin is part of the redox chain that links photosystem II to photosystem I and is located in the lumen of thylakoids in chloroplasts, and cytochrome *c* functions as an electron carrier in mitochondria. The complex of the proteins is therefore nonphysiological, yet the electron transfer rates are high. This makes it a good model system for the study of electron transfer between proteins.

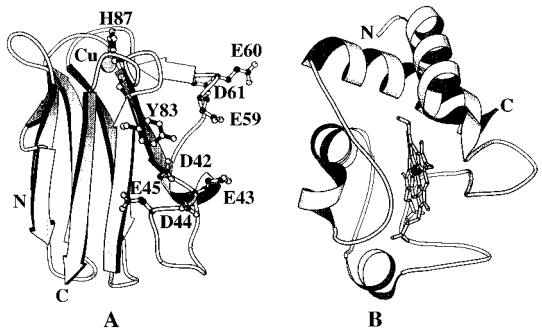

FIGURE 1: Plastocyanin and cytochrome *c*. Schematic representation of plastocyanin (A) and cytochrome *c* (B). Some relevant residues and the heme group are shown in ball-and-stick representation. N and C represent N- and C-termini, respectively. The figure was produced with Molscript (Kraulis, 1991).

Plastocyanin has two obvious sites for electron transfer from the cytochrome heme to the copper. Cytochrome *c* can react via the east side of plastocyanin, with electron transfer along Tyr83 and Cys84 to the copper, or at the northern side (the hydrophobic patch). His87, located in the hydrophobic patch, is a copper ligand and provides a very short pathway. The electronic coupling appears to be similar

† This work was supported by the Biotechnology and Biological Sciences Research Council.

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1997.

S0006-2960(96)03199-6 CCC: $14.00

for both pathways [see, for a discussion and references, Qin and Kostić (1996)], but molecular modeling based on the electrostatic potentials of the proteins indicates that binding should occur at the east side of plastocyanin (Roberts et al., 1991). Experimental work supports the conclusion that the binding is electrostatic in nature and occurs at the east side. The reaction rate is strongly dependent on the ionic strength (Rush et al., 1988; Meyer et al., 1993), and cytochrome *c* competes with Gd^{3+} ions for binding at the negative sites on plastocyanin (King et al., 1985). On the basis of an analysis of the ionic strength dependence of the reaction rate, Rush et al. (1988) proposed that the hydrophobic patch is used as the reaction site, but replacement of Tyr83 by Leu results in a decreased reaction rate, suggesting a role for Tyr83 in electron transfer (Modi et al., 1992a). Cross-linked forms of the complex show that a degree of mobility within the complex is necessary for efficient electron transfer (Peerey & Kostić, 1989). The rate of this surface diffusion has been determined ($k_{sd} = 2 \times 10^5$ s^{-1}) by an artificial increase of the electron transfer rate with a very high driving force (Zhou & Kostić, 1992a). An NMR[1] study and electrochemistry measurements support the idea that the complex is of a dynamic nature (Bagby et al., 1990a,b).

As with other reaction partners, cytochrome *c* binds to plastocyanin with the positive charges around the heme edge, which was shown by chemical modification (Augustin et al., 1983; Pan et al., 1990) and cross-linking studies (Geren et al., 1983; Zhou & Kostić, 1992b). NMR analysis of the complex showed chemical shift changes for protons that experience a contact shift in ferric cytochrome *c*. In particular, heme methyls 12^1 and 18^1 (IUPAC−IUB nomenclature; IUPAC−IUB, 1988; also called HM5 and HM8 or HMD and HMA, respectively) showed a considerable chemical shift change (King et al., 1985; Bagby et al., 1990b).

NMR is a very useful tool for the study of complexes of redox proteins because it can provide extensive, yet detailed information on the proteins in solution. Multidimensional NMR has made it possible to assign the ^{1}H spectra of proteins and provides the necessary resolution to analyze signals from individual protons. In this way each proton can act like a small probe that can give information about changes occurring in its immediate environment, e.g., complex formation. Thus, in principle, it should be possible to obtain a fairly complete picture of the interaction sites of the protein, with no or minimal changes in the proteins. Here, this concept has been applied to obtain information about the locations and sizes of the binding sites in the plastocyanin−cytochrome *c* complex. This complex was chosen because three-dimensional structures and NMR assignments are available, both proteins are stable and highly soluble, and the complex is relatively small (23 kDa). In order to be able to study the complexes of plastocyanin with both ferrous and ferric cytochrome *c*, without interference from electron transfer effects, the copper in plastocyanin was replaced by a cadmium ion. In a previous NMR study it has been shown that Cd−plastocyanin is a good substitute for the Cu−plastocyanin (Ubbink et al., 1996).

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

MATERIALS AND METHODS

Proteins. Expression and purification of pea plastocyanin and Cd incorporation were performed according to Ubbink et al. (1996). Horse heart cytochrome *c* (type VI) was purchased from Sigma-Aldrich, Poole, England, and purified further over a G100 Sephadex gel filtration column, followed by a CM52 cation-exchange column.

NMR Titration. A solution of ferrous cytochrome *c* (4.9 mM) in 10 mM potassium phosphate and 0.5 mM sodium ascorbate, pH 6.0, was added in small aliquots to a solution of 1.0 mM Cd−plastocyanin in the same buffer plus 100 μM sodium 3-(trimethylsilyl)propionate (TSP) and 10% D_2O. Before the titration and after each addition a one-dimensional ^{1}H NMR spectrum (8K, 1280 transients) was obtained on a 500 MHz Bruker AX spectrometer, using a NOESY pulse sequence with a 50 ms mixing time and a very short (3 μs), fixed t_1 period. During the relaxation delay (0.5 s) and the mixing time, the water signal was saturated. The spectral width was 16.12 ppm. At a cytochrome *c*:plastocyanin ratio of 1.2 the ascorbate concentration was increased to 1 mM to prevent oxidation of cytochrome *c*. A spectrum was also obtained for a 1.0 mM ferrocytochrome *c* solution, diluted from the stock in 10 mM potassium phosphate and 1.0 mM sodium ascorbate, pH 6.0, and with the addition of 10% D_2O and 100 μM TSP.

Chemical shift curves of plastocyanin protons were fitted with the equation (Kannt et al., 1996)

$$\Delta\delta = \frac{1}{2}\Delta\delta_\infty\left(A - \sqrt{A^2 - 4R}\right) \qquad (1)$$

$$A = 1 + R + \frac{PR + C}{PCK_a}$$

where R is the cytochrome *c*/plastocyanin ratio, $\Delta\delta$ is the chemical shift change, $\Delta\delta_\infty$ is the chemical shift change for $R \rightarrow \infty$, P and C are the total plastocyanin and cytochrome *c* concentrations, respectively, and K_a is the binding constant for a 1:1 complex. For cytochrome *c* protons, R is replaced in eq 1 by $1/R$ and $\Delta\delta_\infty$ is replaced by $\Delta\delta_0$, the chemical shift changes for cytochrome *c* protons for $R \rightarrow 0$.

A similar titration (up to a ratio of 1.2) but with 1.0 mM cytochrome *c* stock solution gave identical results, indicating that the total protein concentration does not affect the observed chemical shift changes.

Two-Dimensional NMR. To minimize chemical shift changes due to variations in experimental conditions, two-dimensional NMR spectra were obtained consecutively on a Cd−plastocyanin sample, on a 1:1 complex of plastocyanin and ferricytochrome *c*, and on the complex with reduced cytochrome *c*. Samples contained 1 mM plastocyanin in 90% buffer (10 mM potassium phosphate, ionic strength value 11 mM), 10% D_2O, and 100 μM TSP. The pH of the final sample was set to 6.00 (±0.02) with NaOH or HCl. After the NMR spectrum was obtained, ferricytochrome was added from a concentrated stock in the same buffer, thus limiting the dilution to 20% or less. The pH was corrected in the tube to 6.00 again. After the second set of NMR experiments, the cytochrome was reduced in the tube by addition of a crystal of sodium dithionite and 0.1 equiv of sodium ascorbate to maintain a reducing environment. This addition decreased the pH by 0.1−0.2 unit. It was corrected again to 6.00. The third set of NMR experiments was then

performed. Spectra of free cytochrome *c* were obtained with 1 mM solutions in the same buffer at the same pH. Spectra were obtained with three separate samples of the complex, using a single batch of Cu−plastocyanin, from which fresh Cd−plastocyanin was prepared for each sample separately and using two separate batches of cytochrome *c*. In this study pH 6 was chosen in order for the work to be comparable with earlier experiments performed in our laboratory (Modi et al., 1992a,b; Kannt et al., 1996) on the kinetics of plastocyanin and cytochrome *f*. In these, pH 6 was used because it is assumed to be a physiological pH value for the thylakoid lumen. Phosphate was chosen because of its convenience in NMR samples, even though its buffering capacity is relatively small at this pH. The effects of pH variations were checked by obtaining 2D NMR spectra of Cd−plastocyanin at various pH values. Between 6 and 7 few changes occur, not related to the changes observed upon complex formation.

All NMR spectra were obtained with a spectral width of 16.12 ppm, 4K points for t_2 and 500−800 points for t_1 (increment time 62 μs), with 32 or 64 transients per increment, on a 500 MHz Bruker AX spectrometer and at 300 K. The mixing time in NOESY and TOCSY experiments was 100 and 58 ms, respectively. In the TOCSY experiments, a spin lock was achieved with a DIPSI-2 pulse sequence (Shaka et al., 1988). The water signal was suppressed by low-power saturation during the relaxation delay (1 s) and the NOESY mixing time.

Data processing was performed using the Azara software package (W. Boucher, unpublished work; available by anonymous ftp from mole.bio.cam.ac.uk/pub/azara). Free induction decays were multiplied with a shifted sine square function (45° or 90°) and zero-filled once. The spectra were referenced against the internal reference (TSP). The program Ansig (Kraulis, 1989; Kraulis et al., 1994) was used to analyze the spectra. In Ansig, it is possible to overlay up to eight spectra and compare peak positions with cross hairs. These features are essential for the measurements of small chemical shift changes (>0.004 ppm). Peak picking was done manually by zooming in on the peak and taking into account the shape of the peak contours, rather than choosing the highest point of the peak. Both NOESY and TOCSY spectra were used to compare chemical shifts of protons in the free and bound proteins. COSY spectra were used for the assignments of ferrocytochrome *c* but not in the comparisons. The proton assignments for pea Cd−plastocyanin and horse heart ferrous and ferric cytochrome *c* at 10 mM potassium phosphate, pH 6.0 (90% H_2O/10% D_2O), and 300 K have been listed in assignment tables (Supporting Information). The assignments for Cd−plastocyanin were from Ubbink et al. (1996), and those for ferrous and ferric cytochrome *c* were largely based on data from Wand et al. (1989) and Feng et al. (1989), respectively.

Chemical shift changes were categorized according to their size (Table 1). The great majority of peaks showed the same chemical shift behavior for different samples, but a few changes were not reproducible. Phe14 $H^{\beta 2}$, His37 H^{α}, Asp42 $H^{\beta 2}$, and Asp44 NH and $H^{\beta 2}$ in plastocyanin proved very sensitive to sample conditions and showed small chemical shift differences (up to 0.02 ppm) for different samples. These were categorized as group E, along with the protons for which the chemical shift in the complex could not be determined because cross peaks were overlapping or not observed. The category has been listed for each proton in the assignment tables. Also, script files have been prepared (Supporting Information) in which the heteroatoms of plastocyanin and cytochrome *c* have been colored according to the chemical shift changes of the attached protons. The script files are for use in the viewing program RASMOL (Sayle & Milner-White, 1995). The coordinates for the script files for plastocyanin are from a model of the pea plastocyanin structure (Ubbink et al., 1996), based on the French bean plastocyanin structure (Moore et al., 1991). The coordinates for cytochrome *c* are from the crystal structure of the ferric form at low ionic strength (Sanishvili et al., 1995; PDB code 1crc) rather than the NMR structure of ferrocytochrome *c* (Qi et al., 1994; PDB code 1frc). The structures differ considerably; the rmsd between the two structures is 2.41 Å for the C^{α} atoms of residues 2−103. It was found that the crystal structure was in better agreement with a number of observed NOEs in several regions of the protein.

Table 1: Classification of Chemical Shift Changes[a]

category	size (ppm)	color coding[b]	no. of protons in plastocyanin	no. of protons in ferrocytochrome *c*
A	0.000−0.004	blue	324	317
B	0.005−0.009	pink	26	36
C	0.010−0.019	orange	16	27
D	≥0.020	red	9	9
E	not observed	white	154	147
total			529	536

[a] Chemical shift changes are absolute values observed in the 1:1 plastocyanin−cytochrome *c* complex as compared to the free proteins. [b] The color coding refers to Figure 4.

RESULTS

Titration of Plastocyanin with Cytochrome c. To investigate the complex of plastocyanin and cytochrome *c*, two-dimensional ^{1}H NMR spectra of the complex of Cd-substituted pea plastocyanin and horse heart ferrocytochrome *c* were compared with spectra from the separate proteins. Small chemical shift changes, of less than 0.05 ppm, were observed for a number of protons of both plastocyanin and cytochrome *c*, as illustrated in Figure 2. In order to establish whether the observed chemical shift differences can be ascribed to complex formation, a titration of plastocyanin with cytochrome *c* was performed. The one-dimensional spectrum was used to monitor the changes because this allows the most precise determination of the chemical shifts. Figure 3A depicts the chemical shift change for three plastocyanin protons as a function of the cytochrome *c*:plastocyanin ratio. For protons V39 $H^{\gamma 2}$ and Y80 H^{η} the chemical shift changes first increase with the cytochrome *c*:plastocyanin ratio and then level off. The chemical shift of I27 $H^{\gamma 2}$ remains unchanged during the titration. Figure 3B shows the chemical shift change for four cytochrome *c* protons. N31 NH, M80 $H^{\gamma 1}$, and E61 NH show chemical shift changes compared to free cytochrome *c* that are initially constant and then decrease with increasing cytochrome *c*:plastocyanin ratio. The chemical shift of M80 H^{ϵ} remains essentially unchanged during the titration (see the note in the caption in Figure 3). The chemical shift changes of the protons of both proteins suggest that they are caused by complex formation. The protons of cytochrome *c* show a behavior different from that of plastocyanin because the

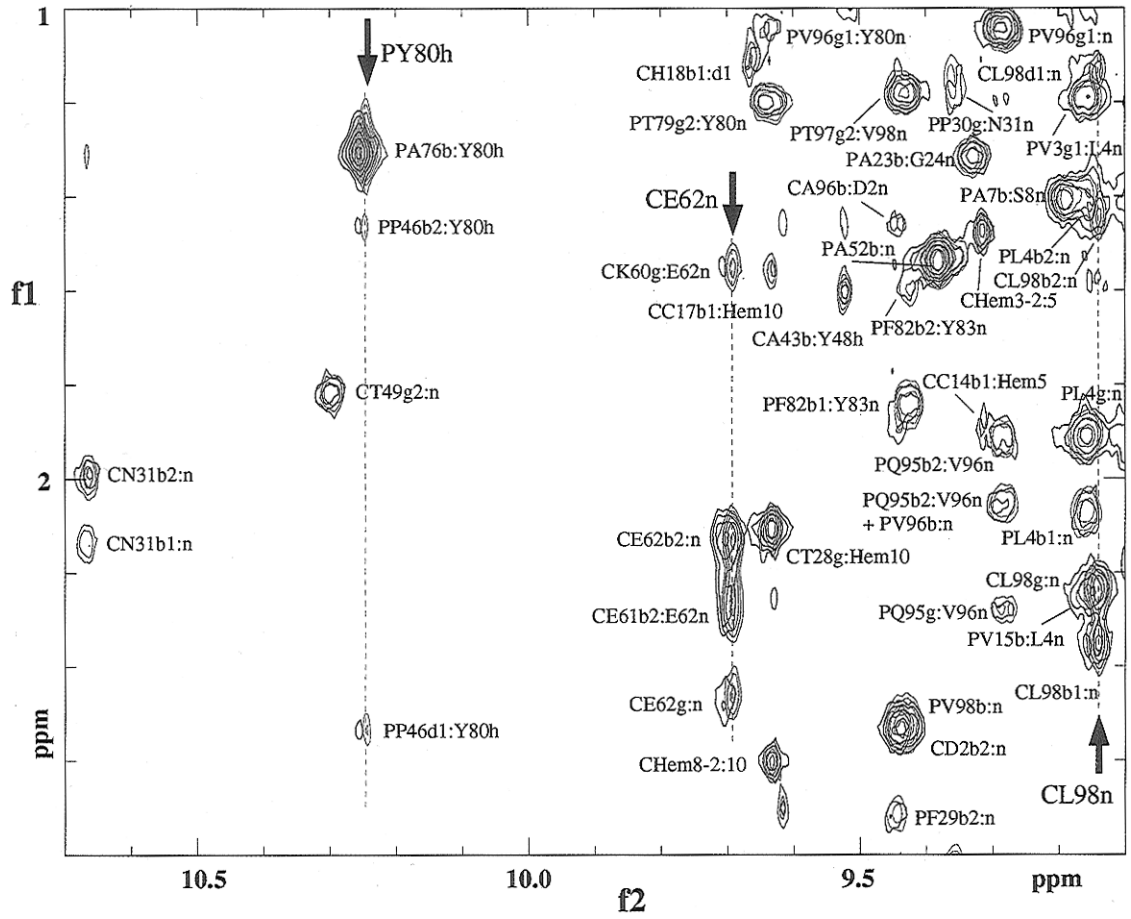

FIGURE 2: Spectral changes upon complex formation. Overlay of three NOESY spectra: red, ferrocytochrome *c*; blue, plastocyanin; black, 1:1 complex. The first capital letter in the assignments (P or C) refers to the protein (P, plastocyanin; C, cytochrome *c*), the second capital letter plus number to the residue, and the lower case letter plus number to the proton in f_1. The capital letter plus number and the lower case letter plus number after the colon refer to the f_2 residue and proton. The second residue designation is omitted for intraresidue cross peaks. Thus, PT97g2:V98n represents the cross peak between plastocyanin Thr97 $H^{\gamma 2}$ (f_1) and plastocyanin Val98 NH (f_2), and CHem8-2:10 represents the cross peak of cytochrome *c* heme protons 8^2 (f_1) and 10 (f_2). The arrows and dashed lines indicate f_2 protons that show a significant chemical shift change in the complex.

former protein is titrated into the latter. At the initial stages of the titration all cytochrome *c* is bound, while plastocyanin remains largely unbound, because it is present in excess. At the end of the titration the situation is nearly reversed. The spectra showed a small general line broadening, as expected for complex formation. For example, the resonance of V39 $H^{\gamma 2}$ demonstrated a decreasing T2 with increasing cytochrome *c*:plastocyanin ratio, from 56 to 39 ms, representing a 2.5 Hz line broadening at a ratio of 1.0 (results not shown). The chemical shift changes in Figure 3 have been fitted with a binding curve assuming a 1:1 complex, according to the formula given by Kannt et al. (1996), which takes the dilution effect into account (see Materials and Methods). The fit yields $K_a = 1.0 \pm 0.5 \times 10^5$ M^{-1} at 10 mM potassium phosphate, pH 6.0. This agrees well with the K_a determined by Meyer et al. (1993), which was 1.0×10^5 M^{-1} at 5 mM ionic strength. Others have reported 1.2×10^3 M^{-1} at 100 mM ionic strength (Modi et al., 1992b), 1.5×10^4 M^{-1} (King et al., 1985; Bagby et al., 1990a), and $>5 \times 10^6$ M^{-1} at 1 mM (Peery & Kostić, 1989). It should be realized that assessing the ionic strength is difficult for solutions with high concentrations of charged proteins and low concentrations of salt.

These titration results show that the small chemical shift changes observed for both proteins were caused by complex formation. From the fact that the chemical shifts changed according to the ratio of bound and free protein, it can be concluded that the dissociation rate (k_{off}) is fast compared to the chemical shift changes, that is, $k_{off} >> 60$ s^{-1}. King et al. (1985) have reported that k_{off} must be $> 10^3$ s^{-1}.

Complex of Plastocyanin and Ferrous Cytochrome c. In order to determine the sites of the proteins involved in complex formation, two-dimensional NMR spectra of the complex were systematically compared with the spectra of the free proteins. For this purpose, spectra of free Cd-substituted plastocyanin, of plastocyanin−ferricytochrome *c*, and of plastocyanin−ferrocytochrome *c* were always obtained consecutively on the same plastocyanin sample (see Materials and Methods for details). The concentrations were 1 mM for each protein, which implies that each protein molecule spent 91% of the time in the complex. All spectra were referenced internally with TSP, and comparisons were performed using the program Ansig (Kraulis, 1989; Kraulis et al., 1994). It was found that within Ansig comparisons of manually picked peaks in the high-resolution dimension are reproducible within 0.004 ppm. Chemical shift changes were classified according to the size of the change (Table 1). For plastocyanin 375 out of 529 assigned protons (71%) could be observed in the spectra of the 1:1 mixture; for ferrocytochrome *c* this was 389 out of 536 (73%).

The protons for which significant chemical shift changes (>0.004 ppm) were observed can be divided into two groups.

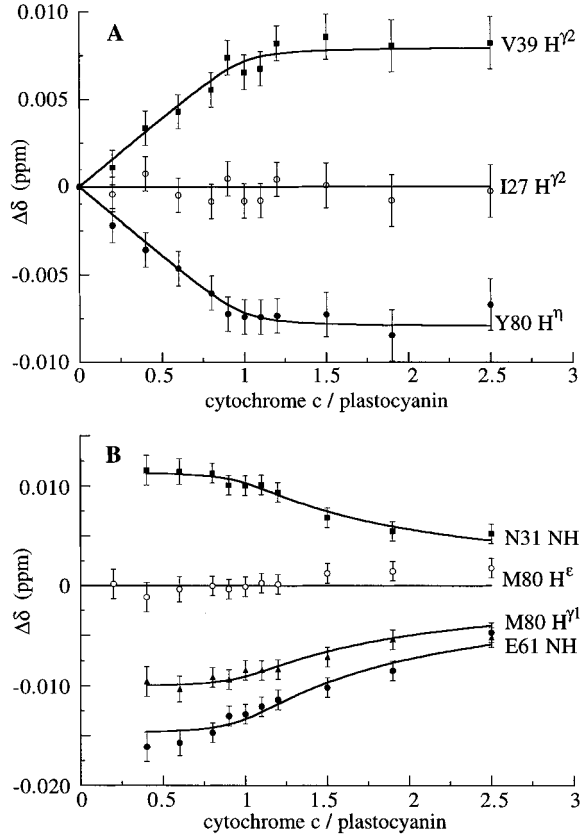

FIGURE 3: Titration of plastocyanin with cytochrome *c*. Chemical shift changes ($\Delta\delta$) are plotted as a function of the cytochrome *c* to plastocyanin ratio for three plastocyanin protons (A) and four cytochrome *c* protons (B). The data have been fitted assuming a 1:1 complex with $K_a = 1 \times 10^5$ M^{-1} (Materials and Methods, eq 1). The M80 H$^{\epsilon}$ proton in (B) shows a small increase in chemical shift for the highest three cytochrome *c*:plastocyanin ratios. The cause is unclear; it could be related to the addition of extra ascorbate. Since this appeared to be a general phenomenon, the other chemical shift changes shown in (B) have been corrected for these changes by subtracting the M80 H$^{\epsilon}$ chemical shift changes.

Group 1 consists of surface-exposed side-chain protons. Nearly all of these map onto one side of the protein, which suggests that these side chains are part of the binding site. Group 2 consists of a considerable number of backbone amide protons, a few protons that form hydrogen bonds to backbone carbonyls (such as some Tyr H$^{\eta}$ protons), and a small set of protons in the hydrophobic cores of the proteins. The largest changes for this group were observed in the same regions as the first group, while smaller changes were seen deep inside the protein, further away from the binding site.

The plastocyanin residues of which the surface-exposed side-chain protons showed chemical shift changes (group 1) have been colored according to the size of the change (see Table 1) in the surface representation of plastocyanin (Figure 4A). Figure 4B−D shows views of plastocyanin rotated in steps of −90° about the vertical axis, showing that the affected side chains are all located on the so-called east side of the protein, around the large and small acidic patches (residues V40, E43, I46, S56, P58, E59, and Q88) as well as at residues N64 and V93. Figure 4E illustrates the group 2 protons. It shows a trace of amide atoms of plastocyanin, in which each atom has been colored according to the chemical shift change of its proton in the same way as above. The biggest effects are seen in residues 40−60, which make up the large loop and small α-helix that form the east side of the protein. This is in agreement with the effects observed for the group 1 protons. However, other amide protons, not located in this area, also show chemical shift changes. It appears that effects of complex formation are transmitted from areas on the surface via the network of bonds and hydrogen bonds into the protein. These "secondary" effects have been clustered, using the information on hydrogen bonds in plastocyanin from the three published higher plant plastocyanin structures: poplar (Guss & Freeman, 1983; Guss et al., 1986, 1992), French bean (Moore et al., 1991), and parsley (Bagby et al., 1994). The effects in K81 NH, Y80 H$^{\eta}$, K77 NH, and G24 NH are linked most closely to the large acidic patch (D42−E45) and those of G94 NH, Q95 NH, and F82 H$^{\zeta}$ to V93. Extensive secondary effects are seen around the copper site, which is linked to the small acidic patch (E59−D61) via N38. This Asn residue is central in the hydrogen bond network (Guss & Freeman, 1983; Ubbink et al., 1996). Effects in this region are observed for both N38 H$^{\delta 2}$ protons, N38 NH and H$^{\alpha}$, H37 H$^{\delta 2}$, G34 NH and H$^{\alpha}$, H87 H$^{\epsilon 1}$, A90 NH, and Y70 H$^{\eta}$. The secondary effects are shown schematically in Figure 5A.

A similar picture was obtained for cytochrome *c*, but the effects were even more extensive in this protein. Affected surface-exposed side chains (group 1) are localized around the heme edge (K5, Q12, Q16, M80, and I81), as well as around K72 (N70, P71, and K72; Figure 4F−I). It is unclear whether three other surface regions, around residues K87 (region 1), T49 (region 2), and K25 (region 3), are involved in complex formation. In these regions secondary effects were clearly observed, but the relevant side chains could not be observed. Amide protons in region 1 (residues K86, K87, T89, E90, and R91) showed considerable chemical shift changes, but most of the lysine and arginine side-chain protons beyond the β-position could not be observed due to spectral overlap. Region 2 is located at the bottom of the heme crevice, around T49. The side chains of these residues are oriented toward the interior of the protein, where they form a network of hydrogen bonds involving the heme propionates and an internal water molecule [Bushnell et al., 1990; see Brayer and Murphy (1996) for a review on eukaryotic cytochrome *c* structures]. Chemical shift changes in this region were observed for F46 H$^{\delta 2}$, T49 NH and H$^{\gamma 1}$, D50 NH and H$^{\alpha}$, A51 NH, N52 NH, and K79 NH. The protons of the exposed side chain of K79 could not be observed in the spectrum of the complex due to overlap. In region 3, the loop from residues C17 to H33, chemical shift changes were observed for the side-chain protons H18 H$^{\delta 2}$, K22 H$^{\beta 2}$, N31 H$^{\delta 21}$, L32 H$^{\delta 2}$, and H33 H$^{\delta 2}$. These protons are partly or completely buried, and the changes therefore probably represent secondary effects. The part of this loop that is located next to the heme edge contains lysines 25 and 27. Again, the side chains of these residues could not be observed in the complex due to overlap. However, it appears from the effects in this loop that K25 and K27 are involved in the interaction and that the binding results in a small structural change, which is transmitted through the loop, as far as residue N103, which is situated directly behind the loop. In conclusion, the binding site includes the areas around the heme edge and N70 and probably also regions 1, 2, and 3, which would mean that the full front face of cytochrome *c* is involved in complex formation. This is shown schematically in Figure 5B; regions 1, 2, and 3 have been represented by a dashed border.

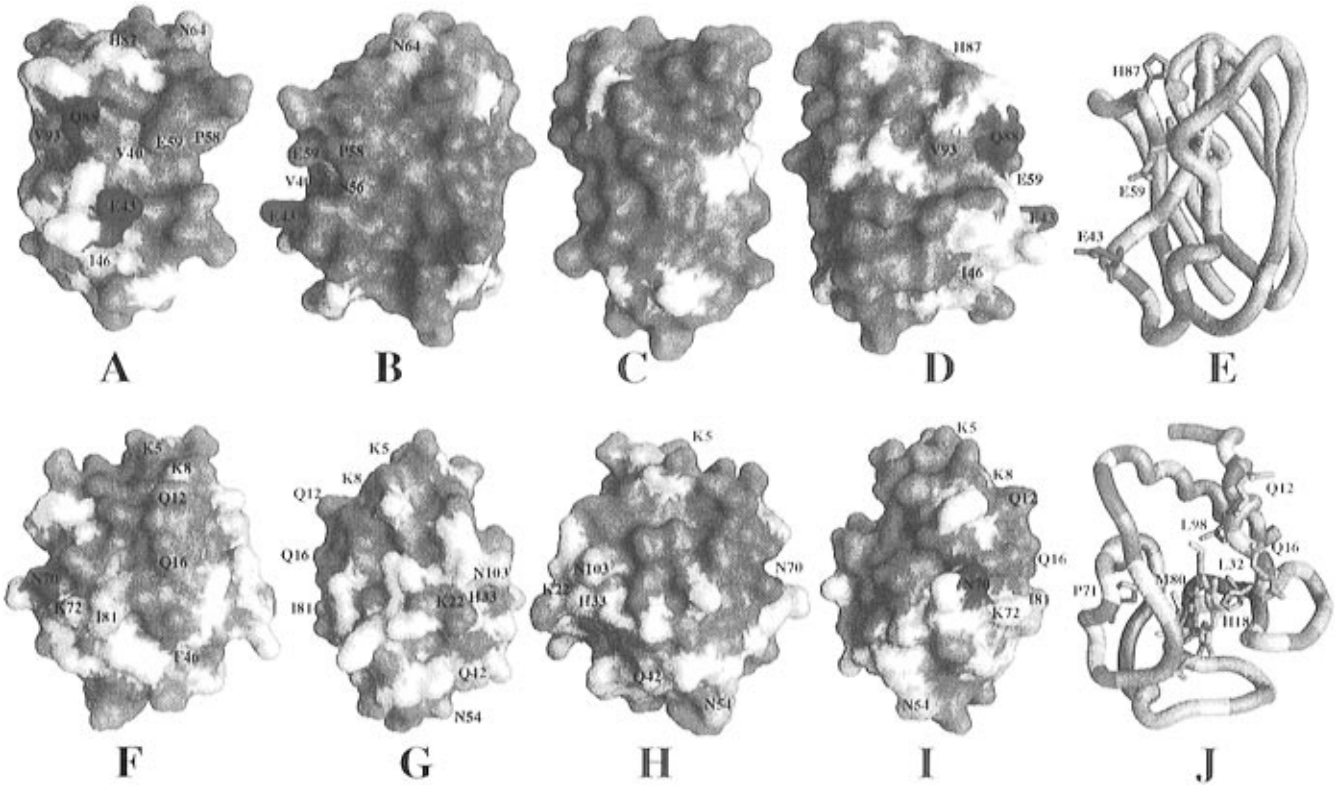

FIGURE 4: Chemical shift changes upon complex formation: (A−D) plastocyanin surface models; (F−I) cytochrome *c* surface models. Model A shows plastocyanin at the east side, and for models B−D model A has been rotated −90°, −180°, and −270° around the vertical axis. Model F shows cytochrome *c* at the heme edge, and models G−I have been obtained with the same rotations. Residues have been colored according to the largest chemical change observed among the side-chain protons, using the color coding in Table 1. Lysine residues that only showed a chemical shift change for H^{β} were only colored at the C^{β}, because for many lysines no data were available beyond the β-position. Models E and J show amide traces of plastocyanin and cytochrome *c*, respectively. The amides have been colored according to the same color coding as above. Some relevant residues and the heme are shown in stick representation.

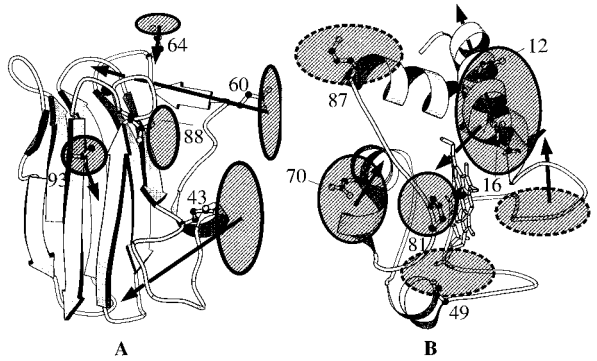

FIGURE 5: Schematic representation of the effects of complex formation. The hatched ovals indicate the regions in plastocyanin (A) and ferrocytochrome *c* (B) for which direct effects of complex formation are observed. The arrows indicate the suggested direction of transmittance of small structural changes into the rest of the protein (secondary effects). The dashed lines show regions for which the involvement in binding is inferred from secondary effects (see text).

The secondary effects observed in cytochrome *c* are illustrated in Figure 4J. The N-terminal α-helix showed chemical shift changes for the amide protons of C17, Q16, A15, C14, Q12, I9, and K7. Also Y97 NH and L98 NH showed chemical shift changes. Both residues make contact with the N-terminal helix via hydrophobic interactions with F10. Small chemical shift changes were seen for protons in the hydrophobic core of the protein: the heme methyls 2^1 and 3^2, L32 $H^{\delta 2}$, H18 $H^{\delta 2}$, T19 NH, V20 NH, and F36 NH. These may be linked to the effects of complex formation near the heme edge, via a small change in the position of the N-terminal helix, the 17−33 loop, and/or the heme group. Finally, chemical shift changes were observed for the amide protons of E61, E62, and G1 (which is acetylated). These three residues are on the back of the protein, far from the heme edge. E61 and E62 are located at the N-terminus of the α-helix which contains K72 at its C-terminus, and G1 is located near the start of the N-terminal helix. Both helices are directly affected at their C-termini by complex formation, which could be the reason for the effects on these three protons, e.g., as a consequence of the change in the α-helical dipole (Figure 5B).

The spectra were checked for the appearance of new NOE peaks. Few were found, none of which could be attributed to intermolecular interactions. A new NOE was observed between the amide protons of G23 (9.20 ppm) and G24 (7.85 ppm) in cytochrome *c*. In plastocyanin a new NOE was observed between E59 $H^{\gamma 2}$ (2.37 ppm) and E59 H^{α} (3.68 ppm). This could imply that upon complex formation the side chain flips. It is interesting to note that this side chain forms a hydrogen bond with Y83 H^{η} in poplar plastocyanin, which was observed in the structure determined at 1.33 Å resolution (Guss et al., 1992). The absence of intermolecular NOEs and the assignment of new NOEs must, however, be regarded with caution since the NOESY spectrum of the complex is crowded. The cross peaks in the spectra of the complex were broader than for the free proteins, due to the longer correlation time of the complex. No extra broadening for specific peaks was observed in the oxidized complex, indicating the absence of intermolecular paramagnetic relaxation. This is not surprising since low-spin Fe(III) has a

short electronic relaxation time and therefore is a relatively poor relaxing agent.

Two script files have been prepared in which the heteroatoms of plastocyanin and cytochrome *c*, respectively, have been colored according to the chemical shift changes of the attached protons, as described above. The script files are for use in the viewing program RASMOL (Sayle & Milner-White, 1995). They proved to be very helpful in the evaluation of the results. Two tables containing the assignments and chemical shift categories (as defined in Table 1) for the protons of plastocyanin and ferrocytochrome *c*, respectively, have been prepared as well. The script files and tables are available as Supporting Information.

Complex of Plastocyanin and Ferric Cytochrome c. The effects observed for Cd-substituted plastocyanin upon complex formation with ferric cytochrome *c* were essentially identical to those observed with the ferrous form of the cytochrome. The chemical shift changes for plastocyanin side-chain and backbone protons were all of the same sign and mostly of the same size (within error) in both complexes. Only a few of the plastocyanin chemical shift changes differed significantly between the two complexes: I46 NH (−0.023 and −0.030 ppm change in chemical shift in the complexes with ferro- and ferricytochrome *c*, respectively), Y70 H^{η} (−0.016 and −0.024 ppm), and Q88 $H^{\epsilon 22}$ (−0.007 and −0.014 ppm). This is quite a remarkable result, because it would be expected that the presence of the paramagnetic heme would cause additional (intermolecular) pseudocontact shifts on the plastocyanin protons. Such effects have been observed in the complexes of cytochrome *c*−cytochrome b_5 (Guiles et al., 1996) and plastocyanin−cytochrome *f* (M. Ubbink, unpublished results). The implication is discussed in the next section.

For ferricytochrome *c* the chemical shift analysis was limited to the amide protons and to the side-chain protons that were affected in ferrocytochrome *c*. The effects observed for ferricytochrome *c* were similar to those for the ferrous form. The chemical shift changes in the side chains of ferrocytochrome *c* were also present in the ferric form, as far as the resonances could be observed. The effects on the amide protons were even more extensive in the ferric form. This is particularly clear in the α-helices and the loop from C17 to H33, in which more amides showed chemical shift changes. The overall picture of the affected regions on cytochrome *c*, as described above, is confirmed by the results with the ferric protein. An assignment table and a RASMOL script file with the chemical shift changes for ferricytochrome *c* are available as Supporting Information.

DISCUSSION

Binding Sites. The results show that small chemical shift changes (0.005−0.05 ppm) can be used to probe protein−protein interactions, provided that experimental conditions are controlled as well as possible and that reproducibility is assured, by performing the same experiments on several separately prepared samples. Two advantages of this approach are, first, that the unmodified proteins can be used (except for the replacement of Cu by Cd in this study) and, second, that detailed information is obtained for the whole protein, rather than specific information about a single side chain as in mutagenesis studies. The many lysine side chains presented difficulties because of severe spectral overlap, not only in the complex but also in free cytochrome *c*. N-Acetimidylation could be used to make the lysines "visible" for NMR (Burch et al., 1990).

The chemical shift changes observed in the spectra of a 1:1 complex of plastocyanin and cytochrome *c* indicate which sides of each protein are involved in complex formation (Figure 4). For plastocyanin, this is the east side of the protein, which includes both acidic patches and Y83. This is in agreement with a number of studies using mutagenesis (Modi et al., 1992a), chemical modification (Augustin et al., 1983), cross-linking (Geren et al., 1983; Zhou & Kostić, 1992b), kinetics (Zhou & Kostić, 1993), modeling (Roberts et al., 1991), and NMR (King et al., 1985). For cytochrome *c* it is found that a large area around the heme edge is involved in the interaction with plastocyanin. It has been established in many studies that this region is the binding site for various physiological and nonphysiological redox partners. In this study, it is observed that the regions around K72 and the N-terminal α-helix are involved in complex formation, as judged from the chemical shift changes in the side chains in these areas. It is not quite clear whether the positive patch around K87 has a role in the binding of plastocyanin. It is noted that this is one of the anion binding sites [see Pielak et al. (1996) for a review]. In principle, phosphate could be competing with plastocyanin for this site. The binding constants for phosphate to horse ferrous and ferric cytochrome *c* have been reported as 476 and 1556 M^{-1} at pH 7 (Gopal et al., 1988). In the work reported here the phosphate concentration is 10 mM (1 mM cytochrome *c*) but the pH is 6.0, which renders most phosphate ions monovalent. This will decrease the affinity considerably. Many studies have analyzed the role of Phe82 in electron transfer [see Mauk (1991) and Caffrey and Cusanovich (1994) for reviews]. Our NMR spectra suggest that in free ferrocytochrome *c* the ring of F82 is largely buried, with strong NOEs from F81 H^{δ} to P71 $H^{\gamma 1}$ and heme methyl 7^1. Chemical shift changes are observed for F82 NH and the side-chain protons of M80 $H^{\beta 2}$ and $H^{\gamma 1}$ and I81 $H^{\gamma 2}$, but the F82 $H^{\beta 1}$, $H^{\beta 2}$, H^{δ}, and H^{ϵ} protons do not show chemical shift changes and the NOE cross peaks remain strong in the complex. F82 H^{ζ} could not be observed in the complex. These results suggest that the phenyl ring does not move significantly when ferrocytochrome *c* binds to plastocyanin.

Secondary Effects. A remarkable finding was that effects of complex formation were observed in the cores of both proteins, apparently transmitted from the surface into the protein via bonds and hydrogen bonds. For cytochrome *c*, other evidence exists that small structural changes occur upon complex formation with other proteins. Resonance Raman (Hildebrandt et al., 1990; Hildebrandt, 1996), NMR (Falk & Ångström, 1983), and circular dichroism and magnetic circular dichroism spectra (Michel et al., 1989; Garber & Margoliash, 1994) indicate changes in the vicinity of the heme upon binding to cytochrome *c* oxidase. These have been interpreted as an increase in heme mobility and greater exposure to solvent. Changes in the intensity of the Soret band (Weber et al., 1987) and shifts of heme methyl NMR resonances (Moench et al., 1992) of ferricytochrome *c* upon binding to cytochrome *c* peroxidase also demonstrate that complex formation affects the heme environment. Hydrogen-exchange studies have shown that the protection factor of slowly exchanging protons in ferricytochrome *c* is significantly changed by binding to cytochrome *c* peroxidase (Jeng

Table 2: Amide Proton Chemical Shift Changes and Protection Factors[a]

residue yeast/horse	protection	chemical shift change	residue yeast/horse	protection	chemical shift change
F10/F10	+	+	S65/M65	+	+
R13/K13	+	+	L68/L68	+	+
C14/C14	+	+	T69/E69	+	+
C17/C17	−	−	L94/L94	+	+
H18/H18	+	+	I95/I95	+	+
T19/T19	+	+	T96/A96	+	+
V20/V20	+	+	Y97/Y97	+	+
I35/L35	−	−	K99/K99	+	−
W59/W59	+	−	K100/K100	+	−

[a] Protection for yeast iso-1-ferricytochrome *c* amide protons in the complex with cytochrome *c* peroxidase (Yi et al., 1994) is compared with the occurrence of a chemical shift change for the same protons in horse heart ferricytochrome *c* upon binding to plastocyanin.

et al., 1994; Yi et al., 1994). Interestingly, a good agreement is found between the amide protons that show increased protection in this complex and those that change in chemical shift when ferricytochrome *c* is bound to plastocyanin. Out of 18 protons for which data have been obtained in both studies (Yi et al., 1994; this work) 13 show both significant protection and chemical shift change, 2 show insignificant protection and no chemical shift change, and only 3 show significant protection but no chemical shift change (Table 2). The protected protons are located in the N- and C-terminal α-helices and 70s α-helix, thus also at the back of the protein, which is very similar to the findings of the present study. More elaborate comparison is precluded by the fact that in the exchange studies only 26% of all amide protons exchange slowly enough to be observed. The results of the present study suggest that the effects on the amides at the sides and back of cytochrome *c* are secondary effects, because those residues do not show chemical shifts changes for their side-chain protons. This means that the complex partner is not bound to the back of cytochrome *c* for a significant amount of time but rather that the binding at the front causes effects that are sensed at the back of cytochrome *c*.

Cause of the Chemical Shift Changes. Three aspects of complex formation could cause the chemical shift changes that were observed, namely, side-chain rearrangement to optimize the fit, solvent exclusion at the interface, and the change in electrostatic potential. A combination of these factors might be involved, but the changed electrostatic potential may play the most important role, because similar effects are observed for various electrostatic protein−protein complexes, as illustrated for the cytochrome *c*−cytochrome *c* peroxidase complex above. This is supported by the resonance Raman results of cytochrome *c* adsorbed to a negative electrode, which show spectral changes similar to those of complex formation (Hildebrandt, 1996). Further, the changes in the heme methyl resonances in ferricytochrome *c* can be reproduced by addition of polyglutamate (Boswell et al., 1980). Also, the addition of 200 mM NaCl affects the NMR spectrum of ferrocytochrome *c* and causes chemical shift changes of more than 0.05 ppm in residues E61, V20, H33, and K86 (Feng & Englander, 1990), all of which are affected by plastocyanin binding as well. All these data suggest that the changes in the protein observed in complex formation are caused by the close presence of an oppositely charged molecule, rather than by specific binding. Hydrogen bonds in particular would be sensitive to the electrostatic potential, and the length of hydrogen bonds has a large effect on the chemical shift of the involved proton (Wishart et al., 1991; Ubbink et al., 1996), which would explain why amide protons appeared most sensitive to complex formation. It is unclear whether the group 2 protons, in the core of the protein far from the binding site, sense the opposite charges of the other protein directly or indirectly. In the latter case, incoming charges would affect the residues at or near the binding site, causing them to move slightly, and then these effects would be transmitted through covalent and hydrogen bonds into the core of the protein. The results of this study suggest that the effects are at least partly indirect, because they do not occur randomly within the protein core but, instead, appear to be linked to sites on the surface that take part in complex formation (Figure 5). It should be stressed that the structural changes that cause the effects observed in the present work and other studies mentioned above are small. The crystal structures of the complexes of cytochrome *c* peroxidase with horse and yeast cytochrome *c* (Pelletier & Kraut, 1992) indicated only small differences in the structure of bound compared to free cytochrome *c*. Apparently, spectroscopic techniques like resonance Raman and NMR allow the detection of subtle effects that may go unnoticed with X-ray diffraction.

The secondary effects are more extensive in the ferric form of cytochrome *c* than in the ferrous form. The chemical shifts of protons in ferricytochrome *c* consist of a diamagnetic and a paramagnetic contribution. Both can be affected by complex formation; any effects, direct or indirect, on the *g*-tensor will result in a change of the paramagnetic contribution to the chemical shift of a proton. This change is added to, or subtracted from, the diamagnetic chemical shift change. The extensive secondary effects may also be associated with the fact that ferricytochrome *c* shows larger conformational fluctuations than the ferrous form [see Schejter (1996) for a review].

The Complex as a Dynamic Ensemble of Structures. The results presented in Figure 4 suggest that cytochrome *c* and plastocyanin adopt more than a single orientation in the complex. It does not appear feasible for cytochrome *c* to interact with the acidic patches, V93, and N64 all at the same time. Rather, cytochrome *c* may have a number of orientations, which interact with partially different sites on plastocyanin, all at the east side. This view is supported by three other observations. The very small chemical shift changes and the absence of intermolecular NOEs suggest that no intimate contacts are present between the two proteins. Furthermore, no intermolecular pseudocontact shifts were observed in plastocyanin. It can be calculated that a proton near the heme edge, on the surface of cytochrome *c*, would experience a pseudocontact shift of ca. −1 ppm and a shift of −0.1 ppm at 13 Å from the surface. These shifts are many times larger than the chemical shift changes observed in this study and would not have gone unnoticed. The absence of intermolecular pseudocontact shifts on plastocyanin can be explained by assuming that the proteins adopt a multitude of different orientations in the complex which are in fast exchange. In this way paramagnetic contributions would average out, because they depend strongly on the orientation of the *g*-tensor relative to the proton. If cytochrome *c* would assume various orientations which are rotated around the normal of the plastocyanin surface at the

east side, the averaging effects would be strongest because the angle of the plastocyanin proton−heme iron vector with the g_z-component would vary greatly. This angle is the main factor determining the pseudocontact shift. Theoretical studies (Roberts et al., 1991; Northrup et al., 1988; Northrup, 1996) have indicated that cytochrome *c* can adopt multiple orientations in complexes with other proteins which are energetically equivalent. Roberts et al. found three families of favorable orientations for cytochrome *c* in the complex with plastocyanin, all on the east side. Experimental evidence exists showing that some degree of freedom in the plastocyanin−cytochrome *c* complex is essential for rapid electron transfer (Peerey & Kostić, 1989; Pan et al., 1990; Zhou & Kostić, 1992). In their study, Zhou and Kostić (1992) found a rearrangement rate constant of 2×10^5 s^{-1}. If it is assumed that the k_{off} is of the order of 10^3-10^4 s^{-1}, reorientations could occur both by full dissociation and reassociation of the proteins and within the encounter complex (surface diffusion). The dynamic view of the complex is also supported by the results of Bagby et al. (1990a,b). They showed that plastocyanin can bind to cytochrome *c* and still accommodate $Cr(NH_3)_6^{3+}$ ions, which bind at the east side of the protein. Also ternary interactions of the cytochrome *c*−plastocyanin complex with negatively charged electrodes suggest a dynamic interaction between the proteins.

All these observations support the idea that the plastocyanin−cytochrome *c* complex consists of a highly dynamic ensemble of structures, rather than a single, static structure. This may be characteristic of a nonphysiological redox complex. In a physiological complex the need for fast dissociation (rapid turnover) would have to be balanced by the need for specificity and fast electron transfer. Electron transfer requires a minimal interprotein jump in the electron transfer pathway which is accessible for a significant proportion of the time spent in the complex. This means that in a physiological complex the proteins would have to form a single, well-defined complex with optimized surface interactions (hydrophobic contacts, hydrogen bonding) for at least part of the time. Such a specific interaction would also increase the binding constant and thus the specificity. This appears to be the case in the complex of cytochrome *f* and plastocyanin, as judged from relatively large chemical shift changes and the presence of intermolecular paramagnetic effects (M. Ubbink, unpublished results). Work is in progress to characterize this complex.

SUPPORTING INFORMATION AVAILABLE

Three tables with ^{1}H NMR assignments and chemical shift categories for Cd−plastocyanin, ferrocytochrome *c*, and ferricytochrome *c*, respectively (14 pages). Three script files for the program RASMOL, in which the heteroatoms have been colored according to the chemical shift change of the attached proton(s), are available. The relevant coordinate files are supplied with the script files. The script files and coordinate files are available only in electronic form. Access and ordering information is given on any current masthead page.

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BI963199U