Importance of Protein Rearrangement in the Electron-Transfer Reaction between the Physiological Partners Cytochrome *f* and Plastocyanin†

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ABSTRACT: Cytochrome *f* from turnip and plastocyanin from French bean were noninvasively cross-linked in the presence of the carbodiimide EDC so that the exposed heme edge in the former protein abuts the acidic patch remote from the copper site in the latter [Morand, L. Z., Frame, M. K., Colvert, K. K., Johnson, D. A., Krogmann, D. W., & Davis, D. J. (1989) *Biochemistry 28*, 8039]. The molecular mass, reduction potentials, and UV–visible and ESR spectra of the covalent complex were consistent with the composition cyt/pc and with a lack of noticeable structural perturbations of the protein molecules. Isoelectric focusing showed the presence of *N*-acylurea groups, byproducts of the cross-linking reaction [Zhou, J. S., Brothers, H. M. II, Neddersen, J. P., Peerey, L. M., Cotton, T. M., & Kostić, N. M. (1992) *Bioconjugate Chem. 3*, 382]. Laser flash spectroscopy, with riboflavin semiquinone as the reductant, showed that the electron-transfer reaction within the covalent complex cyt(II)/pc(II) is either undetectably slow or reversible. The question was resolved by monitoring, during redox titrations, the ^{1}H NMR line widths of the heme methyl groups in free ferricytochrome *f* and in this protein cross-linked to plastocyanin. Line broadening showed that the intracomplex reaction does occur in the electrostatic complex cyt/pc; the lower limit of the rate constant, estimated from the line broadening, was consistent with the actual value of 2800 ± 300 s^{-1}, determined previously [Qin, L., & Kostić, N. M. (1992) *Biochemistry 31*, 5145]. These experiments verified the applicability of ^{1}H NMR spectroscopy to the study of electron-transfer reactions between cytochrome *f* and plastocyanin. Absence of noticeable line broadening during redox titrations of the covalent complex cyt/pc showed that the intracomplex electron-transfer reaction is undetectably slow; the upper limit is ca. 0.1 s^{-1}. Control experiments by stopped-flow spectrophotometry showed that *N*-acylurea groups do not appreciably alter the intrinsic electron-transfer reactivity of the proteins. Therefore it is the cross-links that abolish the intracomplex electron-transfer reaction in the covalent complex. Recent studies by others indicated that, in the noncovalent complex, ferrocytochrome *f* reduces cupriplastocyanin from the acidic patch. The present study indicates that this reaction between physiological partners requires conformational fluctuations of the noncovalent diprotein complex, which are impeded by cross-links.

Cytochrome *f* (designated cyt)[1] and plastocyanin (designated pc) are redox metalloproteins that mediate the flow of electrons from photosystem II to photosystem I in the dark phase of photosynthesis. After considerable study (Anderson et al., 1987; Beoku-Betts et al., 1983, 1985; Gross & Curtiss, 1991; He et al., 1991; Modi et al., 1992b; Morand et al., 1989; Niwa et al., 1980; Takabe et al., 1980, 1984; Tanaka et al., 1981; Takabe & Ishikawa, 1989; Wherland & Pecht, 1978; Wood, 1974), the electron-transfer reaction between these two metalloproteins remains incompletely understood. The main obstacle in this research is unavailability of the three-dimensional structure of cytochrome *f*. Structure of native plastocyanin and its derivatives in the crystalline state (Guss & Freeman, 1983; Guss et al., 1986; Garrett et al., 1984; Church et al., 1986; Collyer et al., 1990) and in solution (Moore et al., 1988a,b; Driscoll et al., 1987; Chazin & Wright, 1988; Chazin et al., 1988) and in both cupric and cuprous oxidation states is known in detail, but only amino acid sequences are known for cytochrome *f* from five higher plants (Hauska et al., 1988). Experiments with this protein are further complicated by its autoreduction (Garewal et al., 1974; Garewal & Wasserman, 1972; Tanaka et al., 1978) and aggregation (Gray, 1978). Fortunately, the protein from turnip, used in this study, does not aggregate.

Because of these difficulties and because three-dimensional structures of cytochrome *c* in the crystalline state and in solution, in both ferric and ferrous oxidation states, are known (Berghuis & Brayer, 1992; Bushnell et al., 1990; Gao et al., 1989), electron-transfer reactions of plastocyanin with this protein and its derivatives have been studied (Armstrong et al., 1986; Augustin et al., 1983; Bagby et al., 1990a,b; Brothers et al., 1993; Chapman et al., 1984; de Silva et al., 1992; Gross & Curtiss, 1991; King et al., 1985; Kostić, 1991, and references therein; Modi et al., 1992a; Pan et al., 1990; Peerey & Kostić, 1989; Roberts et al., 1991; Senthilathipan & Tollin, 1989; Takenaka & Takabe, 1984; Zhou & Kostić, 1991a,b, 1992a,b; Zhou et al., 1992) as much as its reaction with the true physiological partner, cytochrome *f*. Electron-transfer reactions between cytochrome *c* and plastocyanin are interesting in their own right, but caution is necessary if cytochrome *c* is considered a model or a replacement for cytochrome *f*. Although the two cytochromes are alike in several important respects, they differ in their capacity for docking with partners such as plastocyanin and in the rate constant of the electron-

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[1] Abbreviations: cyt, cytochrome *f*; cyt(II), ferrocytochrome *f*; cyt(III), ferricytochrome *f*; pc, plastocyanin; pc(I), cuproplastocyanin; pc(II), cupriplastocyanin; RF, riboflavin; RFH, riboflavin semiquinone; SDS, sodium dodecyl sulfate; NHE, normal hydrogen electrode; *A*, absorbance; ε, molar absorptivity; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; NMR, nuclear magnetic resonance; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid.

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transfer reaction within diprotein complexes that each of them forms with plastocyanin in solution (Qin & Kostić, 1992; Modi et al., 1992a).

Plastocyanin is the prototypical blue copper protein (Sykes, 1991a,b; Chapman, 1991). It is notable among small, well-characterized metalloproteins because it contains two distinct surface regions through which it can exchange electrons with redox partners. The negatively charged acidic patch is remote (14-19 Å) from the copper atom, and the electroneutral hydrophobic patch is proximate (3.9 Å) to this atom. (Different distances correspond to different amino acid residues in each patch.) The rate of electron transfer through proteins may be controlled by the donor–acceptor distance (Moser et al., 1992), or there may exist specific paths through bonds (Beratan et al., 1991; Wuttke et al., 1992; Betts et al., 1992). From either of these current points of view, plastocyanin possesses at least two channels, through the two surface regions, for electron transfer to and from the buried copper site. The choice between the two channels, and therefore between the two domains, is often attributed simply to the charge of the other reactant. Besides electrostatic interactions, whose treatment must go beyond the faimilar monopole–monopole interactions and include also the monopole–dipole and dipole–dipole interactions (Rush et al., 1987, 1988; Zhou & Kostić, 1991b, 1992a,b, 1993; Brothers et al., 1993), characteristics of the protein medium should also be considered. A recent theoretical analysis of paths through bonds indicated relatively strong coupling of the copper atom to the β-barrel strands to which it is ligated but not to other strands. Both the acidic and the hydrophobic patch lie on the ligated strands (Betts et al., 1992). Another theoretical analysis further distinguished the two channels corresponding to these patches (Christensen et al., 1990).

In both cytochrome *c* (Pettigrew & Moore, 1987; Moore & Pettigrew, 1990) and cytochrome *f* (Simpkin et al., 1989; Wiley et al., 1984) the exposed heme edge is surrounded by lysine residues. In the diprotein complexes cyt/pc that form in solution at low ionic strength, this positively charged (basic) patch in either cytochrome abuts the negatively charged (acidic) patch in plastocyanin (Morand et al., 1989; Bagby et al., 1990b; Roberts et al., 1991).

We use two general methods for the study of protein–protein orientation in reactions. In one method, the orientation in the transient diprotein complex during the bimolecular electron-transfer reaction is inferred from analysis of dipolar electrostatic interactions, which are anisotropic. In the other method, evidence for or against the initial docking orientation comes from comparison between unimolecular electron-transfer reactions within two persistent diprotein complexes—the electrostatic complex, which is flexible, and the covalent complex, in which the initial docking is reinforced by noninvasive cross-links between the two protein molecules. Both methods were applied to cytochrome *c* and plastocyanin (Rush et al., 1988; Peerey & Kostić, 1989; Peerey et al., 1991; Zhou & Kostić, 1992b; Brothers et al., 1993). These studies consistently indicated that ferrocytochrome *c* does not reduce cupriplastocyanin from the site of initial docking at the acidic patch, but from a different one. This latter site may be located elsewhere within the broad acidic patch or outside of it. There is growing evidence that reactions of plastocyanin with cytochrome *c* may be modulated by dynamic processes within the diprotein complexes involved (Zhou & Kostić, 1991b, 1992a,b, 1993; Koppenol & Margoliash, 1992; Modi et al., 1992a).

The reaction of plastocyanin with its physiological partner, cytochrome *f*, however, cannot be analyzed in terms of dipolar interactions because this analysis requires knowledge of three-dimensional structures of both proteins. In this study we examine the effects of covalent cross-linking of cytochrome *f* and plastocyanin on the unimolecular electron-transfer reaction within their complex. Since the two methods for the study of protein–protein orientations proved consistent in past application, application of the second method alone should be informative.

MATERIALS AND METHODS

Chemicals. Cytochrome *f* from turnip was obtained from Sigma Chemical Co. Its absorbance quotient A_{554}/A_{280} was 0.80. The total concentration of this protein was determined by complete reduction with ascorbic acid and measurement of its absorbance (ϵ_{554} = 27 700 M^{-1} cm^{-1}) with an IBM 9430 UV–vis spectrophotometer. Oxidations were done with $K_3[Fe(CN)_6]$. Either redox agent was removed by gel filtration. Because of autoreduction, the concentration of ferricytochrome *f* was usually ca. 75% of the total; the exact value was determined from the absorbance of the sample containing both the ferric and the ferrous forms of the protein and the total concentration of the sample. Plastocyanin from French bean was isolated and purified by standard procedures (Milne & Wells, 1970) until its absorbance quotient A_{597}/A_{280} became 1.20 or less. Riboflavin (RF) and all the chromatographic materials were obtained from Sigma Chemical Co. Other chemicals were of reagent grade. Distilled water was demineralized further by a Barnstead Nanopure apparatus.

Electrophoresis. Isoelectric focusing was done with a horizontal cell by Bio-Rad, Inc. Electrophoresis in SDS–polyacrylamide gels was done with a Protean II apparatus, also by Bio-Rad, Inc.

N-Acylurea Derivatives of Cytochrome f and of Plastocyanin. Solutions that contained 100 μM of the protein and 5.0 mM of the carbodiimide EDC in a 10 mM MOPS buffer at pH 6.5 were incubated for 16 h at 22 °C. The modified protein was desalted with a gel-filtration column P2 sized 1.5 × 25 cm, which had previously been equilibrated with a 1.0 mM phosphate buffer at pH 7.0.

Cross-Linking of Cytochrome f and Plastocyanin. Incubation was done according to a published procedure (Morand et al., 1989), but purification and assays were somewhat different from those in the published procedure. The cross-linking was stopped by fractionating the reaction mixture with a polyacrylamide column Bio-Gel P-2 sized 1.5 × 25 cm, which had previously been equilibrated with a 1.0 mM phosphate buffer at pH 7.0; this same buffer was the eluent. The diprotein complex cyt/pc was separated three times from the individual proteins, for completeness. In two identical separations with a Sephadex G75 gel-filtration column sized 1.5 × 40 cm, both equilibration and elution were done with a 1.0 mM phosphate buffer at pH 7.0. In one separation with a cellulose DEAE-52 anion-exchange column sized 1.5 × 30 cm, equilibration was done first with a more concentrated and then with a 1.0 mM phosphate buffer at pH 7.0, and elution was done with a gradient of 100–300 mM NaCl in a 1.0 mM phosphate buffer at pH 7.0. The eluent was collected in fractions and assayed by electrophoresis in SDS–polyacrylamide gels. Those fractions in which the covalent complex cyt/pc constituted more than 95% of the protein content were combined and used in kinetic experiments. The complex would be oxidized with $K_3[Fe(CN)_6]$, and the oxidant would be removed by gel filtration immediately before kinetic experiments by laser flash photolysis.

Reduction Potentials. Spectrophotometric titrations with $[Fe(CN)_6]^{3-/4-}$ were done according to the published procedure (Morand et al., 1989).

Stopped-Flow Spectrophotometry. An apparatus by Kinetic Instruments, Inc. had the mixing time of 2 ms. It was interfaced to a personal computer equipped with kinetic software by OLIS, Inc. The concentration of ferrocytochrome *f* or of its *N*-acylurea derivative was 0.20 μM, and the concentration of cupriplastocyanin or of its *N*-acylurea derivative was greater than 1.0 μM, so that first-order kinetic conditions always prevailed. Each pseudo-first-order rate constant was an average of 2–3 determinations, each of which was based on four runs (mixing experiments). The estimated error was ±5%. The temperature was 22 ± 1 °C. Oxidation of ferroheme was monitored at 554 and 422 nm.

Laser Flash Photolysis. The reaction mixtures were 5.0–10 μM in the covalent complex cyt(III)/pc(II), 80 μM in riboflavin, and 0.50 mM in EDTA. The solvent was a 1.0 mM sodium phosphate buffer at pH 7.0, whose ionic strength was adjusted with NaCl. Autoreduction of ferricytochrome *f* during kinetic experiments was negligibly small. Gently stirred solutions were deaerated by blowing wet, pure argon over the surface for ca. 1 h. A Phase-R DL 1100 laser with the dye LTD 425 was perpendicular to an optical bench, and changes in absorbance over time were analyzed by the aforementioned software. The temperature was 22 ± 1 °C. Reactions were started by a 0.4-μs laser pulse and followed by monitoring the transient absorbance of ferrocytochrome *f* at 554 nm. Each kinetic trace contained 500 points. Solutions were shaken after each pulse.

ESR Spectra. The covalent complex was fully oxidized into the cyt(III)/pc(II) state by treatment with an excess of $K_3[Fe(CN)_6]$ shortly before the measurements. The oxidant was removed by a Bio-Gel column P-2 sized 1.5 × 3.0 cm, which was previously equilibrated with a 1 mM phosphate buffer at pH 7.0. The spectra were recorded with a Bruker ER200D X-band spectrometer. The temperature was kept at 10 K with a continuous flow of liquid helium through the cryostat.

NMR Spectra. The samples were oxidized and the oxidant was removed, as for the ESR measurements. The solvent was a 1 mM phosphate buffer at pH 6.8. The proteins were dissolved in it and repeatedly lyophilized and dissolved in D_2O. The final volume was equal to the initial one, so that the (uncorrected) pH* value was close to the initial pH value. Partial autoreduction of ferricytochrome *f* proved unavoidable, but it was easily detected and taken into account. The total concentration of the cyt/pc covalent complex fell in the range from 0.40 to 1.0 mM. The spectra were recorded with a Bruker VXM-300 spectrometer; digital resolution was 1.2 Hz/point. The compound DSS was used as an internal reference and a standard for chemical shifts. The temperature was kept at 21 ± 0.1 °C. After averaging of signals, resonances proved sufficiently strong. In some experiments, ferrocytochrome *f* was titrated with a concentrated solution of cupriplastocyanin in order to minimize possible effects of dilution on the NMR spectrum. In other experiments, the covalent complex cyt(III)/pc(II) was titrated with cyt(II)/pc(I); both reacting complexes had a concentration of 0.40 mM. In yet other experiments, the covalent complex cyt(III)/pc(II) was titrated with a 20 mM solution of ascorbic acid.

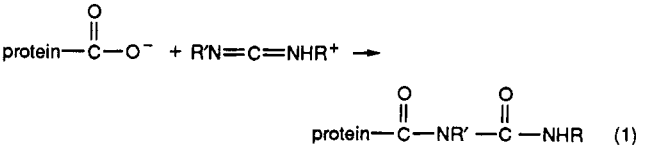
NMR Line Widths. Broad features underlying the resonances were removed from the baseline by a flattening subroutine in the program NMR1, obtained from New Methods Research, Inc. (Pearson, 1977). When the simulated baseline matched these broad features, it was subtracted from the spectrum. The full widths at half maximum of the paramagnetically shifted ^1H resonances in ferricytochrome *f* were estimated with the curve-fitting subroutine in the program NMR1. The resonances were represented wth Lorentzian functions. Line widths, relative intensities, and positions (chemical shifts) were allowed to vary; only in the case of very broad and appreciably overlapping resonances were their positions fixed, and these estimates are only approximately correct. The fitting was considered acceptable when the difference between the theoretical and experimental spectra was a horizontal line with only random noise.

RESULTS AND DISCUSSION

Characterization of the Covalent Complex cyt/pc. The apparent molecular masses of cytochrome *f*, plastocyanin, and their covalent complex, determined by electrophoresis in SDS–polyacrylamide gels, are 30, 11, and 40 kDa, respectively. Our findings confirm the 1:1 composition of the covalent complex (Morand et al., 1989). The UV–visible and ESR spectra of the heme and blue copper chromophores in the free proteins are not perceptibly altered in the diprotein complex. The reduction potentials of free ferricytochrome *f* (0.37 V) and of free cupriplastocyanin (0.38 V) are only slightly raised, to 0.38 and 0.39 V, respectively, in the covalent complex. This slight raise, which barely exceeds the error bounds of the experiments, can be attributed to neutralization of negatively charged groups on the protein surfaces; this effect has frequently been observed (Qin et al., 1991; Peerey & Kostić, 1989).

This study agrees with detailed previous studies of covalent complexes that plastocyanin forms with cytochrome *f* (Morand et al., 1989) and with cytochrome *c* (Peerey & Kostić, 1989; Peerey et al., 1991; Zhou et al., 1992) in showing that covalent cross-linking promoted by the carbodiimide EDC does not cause significant changes in the electronic structure of the metal sites and in the conformation of the proteins and that cross-linking captures the electrostatic complex existing in solution at low ionic strength. In this complex the positive patch around the exposed heme edge in cytochrome abuts the negative patch remote from the copper site in plastocyanin.

Isoelectric focusing, however, revealed that the complex is actually a mixture of species having different p*I* values. The lower the pH value of the reaction mixture, the greater both the heterogeneity and the yield of the covalent complex. We achieved a compromise between these two factors by cross-linking the proteins at pH 6.5. Carbodiimide can convert certain carboxylate anions into neutral *N*-acylurea groups according to eq 1 (Means & Feeney, 1971; Carraway & Koshland, 1972; Timkovich, 1977).



(1)

In previous studies of cytochrome *c* and plastocyanin in this laboratory, different *N*-acylurea derivatives of the covalent diprotein complex were chromatographically separated, characterized, and individually studied (Peerey & Kostić, 1989; Peerey et al., 1991; Brothers et al., 1993; Zhou et al., 1992; Zhou & Kostić, 1991a,b, 1992b). These experiments were affordable because cytochrome *c* is inexpensive. Different derivatives of the covalent diprotein complex proved to be

structurally similar to one another and to the electrostatic diprotein complex, but they proved to differ from one another in the number and distribution of *N*-acylurea groups. Most important, the *N*-acylurea derivatives behaved identically in intracomplex electron-transfer reactions. Because this study concerns the intracomplex reaction and because cytochrome *f* is expensive, chromatographic separation involving large quantities of the covalent complex was deemed unwarranted.

Question of the Unimolecular Reaction within the Covalent Diprotein Complex, Studied by Laser Flash Photolysis. Because flavin semiquinones are readily produced in situ and because they have proven useful in our previous studies of diprotein complexes (Peerey et al., 1991; Qin & Kostić, 1992; Brothers et al., 1993), we used them again. Because the diprotein complex is present in large excess over the semiquinone, complete reduction (of both the heme and the copper redox sites) is improbable. Because riboflavin semiquinone reacts faster with ferricytochrome *f* than with cupriplastocyanin, reduction of only the former is shown in eq 2. The

$$\text{cyt(III)/pc(II)} + \text{RFH} \xrightarrow{k} \text{cyt(II)/pc(II)} + \text{RF} + \text{H}^+ \quad (2)$$

$$\text{cyt(II)/pc(II)} \underset{k_{-et}}{\overset{k_{et}}{\rightleftarrows}} \text{cyt(III)/pc(I)} \quad (3)$$

question is whether this initial reduction is followed by the unimolecular reaction within the covalent complex, shown in eq 3. This unimolecular reaction is shown as an equilibrium because the redox potentials of the cross-linked proteins differ by only 0.01 V.

Typical kinetic traces in Figure 1 show the initial reduction of ferricytochrome *f* (eq 2) but seemingly do not show the subsequent unimolecular reaction (eq 3). These findings can be interpreted in two ways. A straightforward conclusion would be that the intracomplex electron exchange is too slow to be clearly observed. But the traces in Figure 1 are consistent with the rate law in eqs 4 and 5, which corresponds to a

$$k' = k[\text{cyt(III)/pc(II)}] \quad (4)$$

$$[\text{cyt(II)/pc(II)}] = 1/2[\text{RFH}]_0\left\{1 - \frac{2(k' - k_{-et})}{k' - 2k_{-et}}\exp(-k't) + \frac{k'}{k' - 2k_{-et}}\exp(-k_{-et}t)\right\} \quad (5)$$

reversible intracomplex reaction. The symbols mean the following: k and k' are the second-order and the pseudo-first-order rate constants for the initial reduction (eq 2); brackets indicate concentration; and subscript 0 indicates the initial concentration.

In our previous study of the electrostatic complex (Qin & Kostić, 1992), fitting of the experimental traces to eq 5 yielded $k_{et} = k_{-et} = 2800 \pm 300\ s^{-1}$. In this previous case, the plateau in the trace similar to that in Figure 1 did not mean the absence of the intracomplex reaction; the plateau was a consequence of reversibility of this reaction. This conclusion in our previous study was confirmed by direct evidence from spectrophotometric titrations and from stopped-flow kinetic measurements.

In the present study of the covalent complex, the plateau in the flash-photolysis trace (Figure 1) again presents the same possibilities. We resolve the question with the help of ^{1}H NMR spectroscopic experiments.

Unimolecular Reaction within the Electrostatic Diprotein Complex, Studied by NMR Spectroscopy. Analysis of NMR line widths (Rao, 1989) was successfully used to determine the rate constant for the "uphill" electron-transfer reaction between ferricytochrome *c* and cuproplastocyanin (King et al., 1985). We apply it here to electrostatic and covalent complexes between cytochrome *f* and plastocyanin.

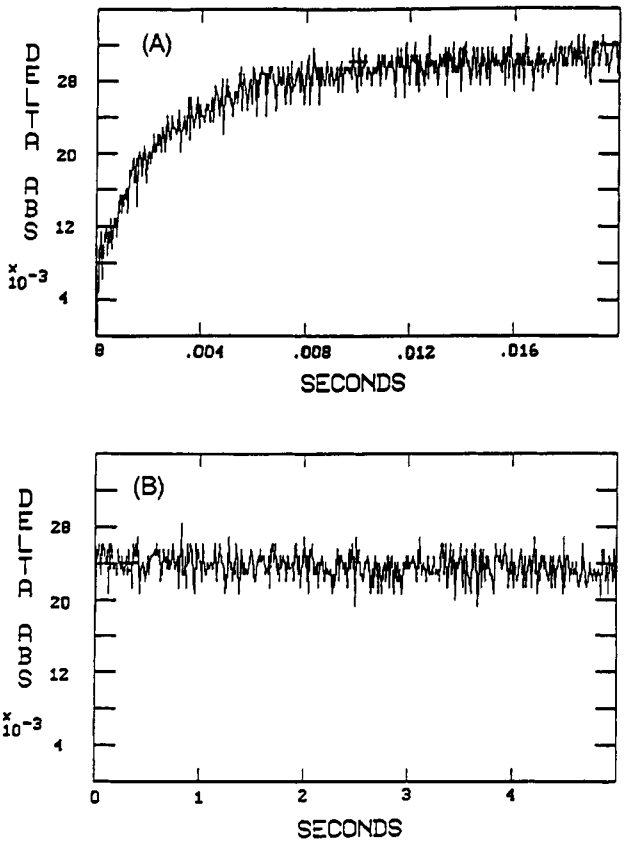


FIGURE 1: Absorbance at 554 nm of cytochrome *f* in a solution initially containing 6.0 μM covalent complex cyt(III)/pc(II), 70 μM riboflavin, and 500 μM EDTA in 1.0 mM sodium phosphate buffer at pH 7.0. (A) Bimolecular reduction of ferricytochrome *f* by riboflavin semiquinone generated by the laser flash. (B) Absence of the unimolecular electron-transfer reaction within the covalent complex cyt(II)/pc(II).

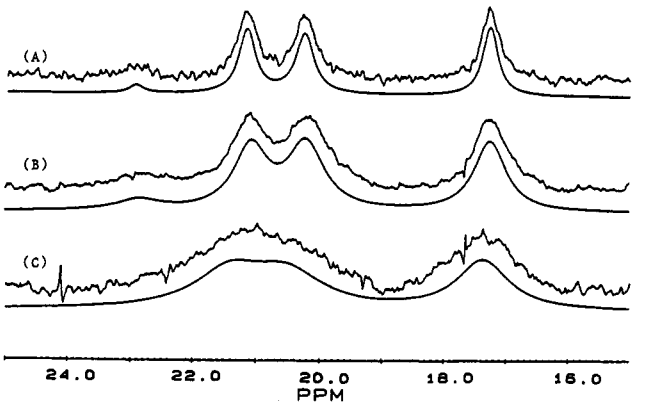


FIGURE 2: Proton NMR resonances (upper traces) and their fittings (lower traces) of the heme methyl groups in ferricytochrome *f*. The solvent was D_2O. (A) Solution in the phosphate buffer at pH* 6.8 that is 0.72 mM in ferrocytochrome *f* and 0.18 mM in ferricytochrome *f*. (B) Initial solution that was made 0.048 mM in cupriplastocyanin. (C) Initial solution that was made 0.20 mM in cupriplastocyanin.

The initial solution was 0.72 mM in ferrocytochrome *f*, which is diamagnetic, and 0.18 mM in ferricytochrome *f*, which is paramagnetic. As Figure 2A shows, the ^{1}H NMR spectrum of the ferric form contains three well-resolved resonances of heme methyl groups, which are clearly separated

Table I: Full Width at Half Maximum[a] of the ^{1}H NMR Resonances of the Heme Methyl Groups in Ferricytochrome *f*[b] in the Titration of Ferrocytochrome *f* by Cupriplastocyanin

molar ratio pc(II):cyt(II)	chemical shift 17.3 ppm	20.3 ppm	21.2 ppm
0	90	100	95
0.26	190	210	190
0.55	280	400	410

[a] Given in hertz; estimated error, ±10%. [b] Present already at the molar ratio of 0.

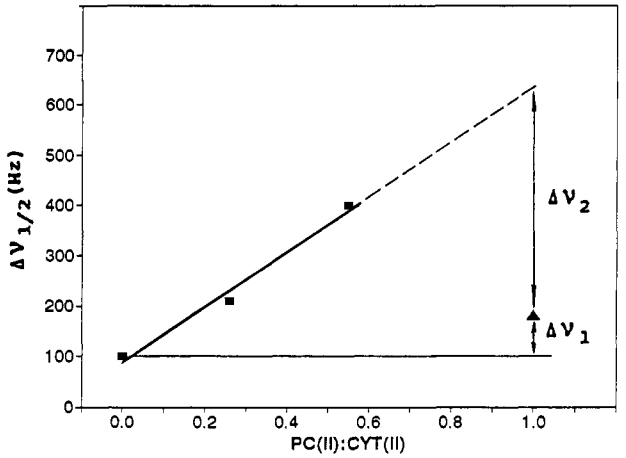


FIGURE 3: Full width at half maximum of the ^{1}H NMR resonance at 20.3 ppm in ferricytochrome *f* during the early stages of redox titration of ferrocytochrome *f* by cupriplastocyanin (■) and in the covalent complex cyt(III)/pc(II) (▲). The solution conditions are stated in the caption to Figure 2. The broadening increments $\Delta\nu_1$ and $\Delta\nu_2$ are explained in the main text.

from other resonances. The chemical shifts agree with those reported by others (Rigby et al., 1988). Broadening of all three resonances was monitored in the study of the electron-transfer reaction. The ferrous form has no resonances in this spectral region.

As Figure 2 and Table I show, the early stages of titration of ferrocytochrome *f* with cupriplastocyanin already bring about a dramatic broadening of the three resonances. Upon further titration toward the equivalence point, the resonances would become undetectably broad; in order to remain in the slow-exchange region (on the NMR time scale), only the early stages of the titration could be monitored.

Four possible causes of broadening should be considered: decrease in tumbling rate upon protein association, paramagnetic effect of the copper(II) ion, the equilibrium between free and associated protein molecules, and electron exchange. Covalent attachment of cupriplastocyanin to ferricytochrome *f* maximizes the first two factors because the cyt(III)/pc(II) complex is fully formed, with the protein ratio 1:1. Nonetheless, upon the formation of this covalent complex each methyl resonance is broadened by only 70-80 Hz, the increment $\Delta\nu_1$ in Figure 3. This moderate broadening can be ascribed to an increase in molecular mass and size, because of which the covalent complex tumbles more slowly than an individual protein molecule, and to the paramagnetic effect of the remote copper(II) atom (Moore & Pettigrew, 1990; Bertini et al. 1989). The previous NMR spectroscopic study (King et al., 1985) likewise showed that association with cupriplastocyanin has a relatively small effect on the width of the heme methyl resonances of cytochrome *c*. In the titration experiment this extent of broadening is greatly exceeded well before the equivalence point (the protein ratio 1:1) is reached. Since the difference in the methyl chemical shift between free ferricytochrome *f* and this protein in the covalent complex is only ca. 25 Hz, the association–dissociation equilibrium (the third factor) cannot cause broadening significantly greater than 25 Hz. For all of these reasons, the line broadening in the titration can be attributed for the most part to the fourth factor mentioned above—the electron exchange (eq 3).

Because the resonance at 20.3 ppm broadens the most during the titration, it should provide the closest estimate of the lower limit of the rate constant for electron exchange. Although the plot in Figure 3 is based on few data points, it can be cautiously extrapolated to the equivalence point of the titration. The broadening $\Delta\nu_2$ in excess of the line width in the covalent complex is ascribed to an average of the two opposite reactions in eq 3. Next we state and explain the assumptions on the basis of which the rate constants k_{et} and k_{-et} were estimated.

It is reasonable to assume that the diamagnetic ferrocytochrome *f* resembles its analog ferrocytochrome *c* in having the heme methyl resonances at chemical shifts smaller than 4 ppm (Keller & Wütrich, 1978). Then the difference between the positions of these resonances in the two oxidation states of cytochrome *f* is ca. 20 ppm or ca. 6000 Hz (at the resonance frequency of 300 MHz). Since $k_{et} = k_{-et} = 2800 \pm 300$ s^{-1} in the electrostatic complex (Qin & Kostić, 1992), the unimolecular reactions conveniently occur on the time scale of this particular NMR experiment.

To estimate the lower limit of these rate constants solely from the widths of NMR resonances, we first assume that early in the titration the reactions of interest are relatively slow on the time scale of the experiment, i.e., that the rate constants are less than ca. 6000 s^{-1}. This assumption is supported by the fact, evident in Figure 2, that the resonance positions (chemical shifts in parts per million) do not change appreciably. A lower limit for k_{et} and k_{-et} is $\pi\Delta\nu_2$. This estimated lower limit of 1500 s^{-1} is consistent with the actual value of 2800 ± 300 s^{-1}.

This agreement verifies the reliability of the NMR method for estimation of the rate constant for the unimolecular electron-transfer reactions in the complex of cytochrome *f* and plastocyanin. Although such estimates are at best semiquantitative, they are adequate for comparing electrostatic and covalent complexes between these two metalloproteins.

Absence of the Unimolecular Reaction within the Covalent Diprotein Complex. For the unimolecular reaction to be at all conceivable, the diprotein complex must exist in a "mixed-valent" state, i.e., cyt(II)/pc(II) or cyt(III)/pc(I). Figure 4A,B shows the typical NMR spectra; spectra of mixtures at other molar ratios are identical to the spectra shown. Evidently, resonances of the covalent complex cyt(III)/pc(II) are not broadened upon addition of the covalent complex cyt(II)/pc(I). This lack of broadening could result from either or both of the following causes. First, the intercomplex reaction to form a "mixed-valent" state may be too slow (on the NMR time scale). Second, a "mixed-valent" state may be formed, but the unimolecular reaction (eq 3) may be too slow to cause broadening. The first possibility is unlikely because the covalent complex of cytochrome *c* and plastocyanin does undergo the bimolecular (intercomplex) reaction that creates the "mixed-valent" state (Peerey et al., 1991). The rate constant for this reaction is 10^4–10^5 M^{-1} s^{-1}, and the relatively high concentrations in the NMR samples of the covalent complexes cyt(III)/pc(II) and cyt(II)/pc(I) containing cytochrome *f* would actually enhance the formation of the required "mixed-valent" species. For these reasons the second possibility, namely, the absence of the intracomplex reaction

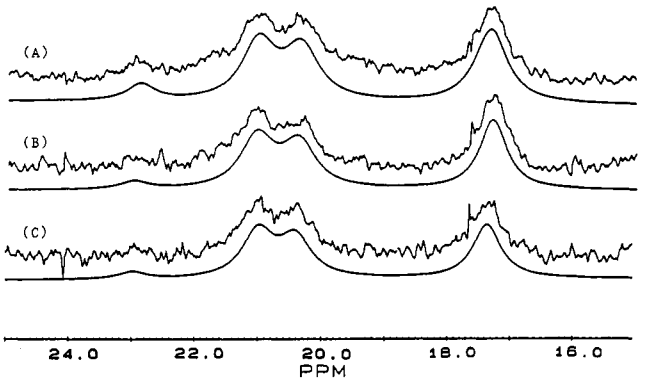


FIGURE 4: Proton NMR resonances (upper traces) and their fittings (lower traces) of the heme methyl groups in ferricytochrome *f* that is covalently cross-linked to plastocyanin. The solution conditions are stated in the caption to Figure 2. (A) Covalent complex cyt(III)/pc(II). (B) Mixture of the covalent complexes cyt(III)/pc(II) and cyt(II)/pc(I) in the molar ratio 1.0:0.35. (C) Mixture of the covalent complex cyt(III)/pc(II) and ascorbic acid in the molar ratio 1.0:0.29.

that equilibrates the oxidation states of the cross-linked proteins, is the more likely one.

The question of unreactivity was settled by experiments in which cyt(III)/pc(II) was treated with a subequivalent amount of ascorbic acid, a small reagent that reduces the cross-linked proteins and certainly generates the "mixed-valent" species required for the unimolecular reaction. Figure 4C shows a typical NMR spectrum; spectra of mixtures at other molar ratios are identical to the spectrum shown. Evidently, there still is no line broadening. We conclude that the covalent diprotein complex does not undergo electron exchange at an observable rate. The upper limit of the rate constant for the unimolecular reaction (eq 3) within the covalent complex is ca. 0.1 s^{-1}. Both the digital resolution in the NMR spectra (1.2 Hz/point) and the shape of the kinetic trace in Figure 1B (practically horizontal for well over 5 s) are consistent with this upper limit.

Effects of N-Acylurea Groups on Electron-Transfer Reaction and Association between Cytochrome f and Plastocyanin. The practical absence of the unimolecular reaction within the covalent complex may, in principle, be attributed to cross-links at the protein–protein interface or to *N*-acylurea groups elsewhere at the protein surfaces. Although our various studies showed *N*-acylurea groups to be structurally noninvasive and without effect on intracomplex electron-transfer reactions in diprotein systems similar to this one (Peerey & Kostić, 1989; Peerey et al., 1991; Brothers et al., 1993; Zhou et al., 1992; Zhou & Kostić, 1991a,b, 1992b), it is necessary to examine their possible effects on the reactivity of this particular pair of proteins. As eq 1 shows, formation of an *N*-acylurea group neutralizes a carboxylate anion and slightly changes the electrostatic properties of the protein molecules.

We studied, by stopped-flow spectrophotometry, the reaction in eq 6 in which either or both reactants were native proteins or their *N*-acylurea derivatives. There were four pairs of

$$\text{cyt(II)} + \text{pc(II)} \rightarrow \text{cyt(III)} + \text{pc(I)} \qquad (6)$$

reactants, and each was examined at two ionic strengths–1.0 mM and 1.00 M. The kinetic results in Tables II and III can be qualitatively interpreted as follows.

The reactions of native cupriplastocyanin with either native or modified ferrocytochromes *f* at low ionic strength occurred within the mixing time, ca. 2 ms. The reactions of modified cupriplastocyanin with the same two partners at low ionic strength occurred over a longer time period. As a typical kinetic plot in Figure 5 illustrates, these latter two reactions obey the second-order rate law. As Table II shows, modified plastocyanin is less reactive than the native plastocyanin toward both partners, whereas modified cytochrome *f* is approximately as reactive as the native cytochrome *f* toward native plastocyanin and slightly more reactive than native cytochrome *f* toward modified plastocyanin.

Table II: Unimolecular Rate Constants for the Reaction in Eq 3 and Bimolecular Rate Constants[a] for the Reaction in Eq 6, Both at the Ionic Strength of 1.0 mM and pH 7.0, Involving Native Cytochrome *f*, Native Plastocyanin, and Their N-Acylurea Derivatives

	native pc (s^{-1})	modified pc (M^{-1} s^{-1})
native cyt	2800[b]	1.0×10^6
modified cyt	>1000	2.6×10^6

[a] Major component. [b] Qin & Kostić (1992).

Table III: Bimolecular Rate Constants[a] for the Reaction in Eq 6 at the Ionic Strength of 1.00 M and pH 7.0, Involving Native Cytochrome *f*, Native Plastocyanin, and Their N-Acylurea Derivatives

	native pc (M^{-1} s^{-1})	modified pc (M^{-1} s^{-1})
native cyt	1.4×10^6	6.3×10^5
modified cyt	1.0×10^6	1.0×10^6

[a] Major component.

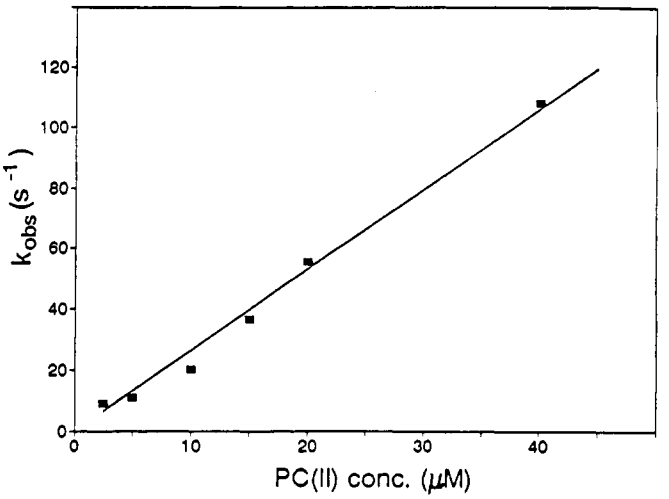


FIGURE 5: Stopped-flow spectrophotometric results, under pseudo-first-order conditions, for the electron-transfer reaction (eq 6) between *N*-acylurea derivatives of ferrocytochrome *f* (0.20 μM) and of cupriplastocyanin (variable concentration) in a 1.0 mM sodium phosphate buffer at pH 7.0.

We compared cross-linking yields with native and modified proteins. Modification of plastocyanin inhibits cross-linking of this protein with native cytochrome *f* by 60%, whereas modification of cytochrome *f* does not affect its cross-linking with native plastocyanin. All of these kinetic and cross-linking experiments confirm that the electron-transfer reaction is assisted by electrostatic association involving carboxylate side chains in plastocyanin but not carboxylate side chains in cytochrome *f*. Since modification of plastocyanin only inhibits, but does not abolish, the cross-linking reaction, not all of the side chains in the acidic patch seem to be converted into *N*-acylurea groups.

At the high ionic strength, at which electrostatic interactions are minimized, effects of *N*-acylurea groups on the protein reactivity are minimized as well. The reaction between the native proteins remains slightly faster than the reaction

involving one or both modified proteins, but the differences in Table III are small. More important than these small kinetic effects is the absence of any greater kinetic effects.

To conclude, these control experiments show that *N*-acylurea groups modify the electrostatic properties of the protein surface without appreciably altering the intrinsic electron-transfer reactivity of the proteins. The hindrance of the unimolecular reaction (eq 3) within the covalent complex cyt/pc cannot be attributed to secondary modification (eq 1). This unreactivity seems to be due to the cross-links themselves.

Protein–Protein Orientation for Electron Transfer. Ferrocytochrome *f*, like ferrocytochrome *c*, does not reduce the copper site when covalently attached to the acidic patch of cupriplastocyanin. Two recent studies found that ferrocytochrome *f* that is covalently cross-linked to the acidic patch in plastocyanin (as in the present study) cannot efficiently reduce the cation $P700^+$ of photosystem I (Takabe & Ishikawa, 1989; Morand et al., 1989). The present study explains this intriguing fact—covalent cross-linking abolishes the intracomplex electron-transfer reaction and thus prevents plastocyanin from mediating reduction of $P700^+$ by ferrocytochrome *f*. Kinetic experiments with site-directed mutants of plastocyanin indicate that either cytochrome docks in the general region of the acidic patch in the electron-transfer reactions (He et al., 1991; Modi et al., 1992a). All of these findings together indicate that the intracomplex electron-transfer step requires conformational fluctuation or minor rearrangement of the complex, which is impeded by the rigid cross-links. Effects of solution viscosity on the photoinduced electron-transfer reaction between zinc cytochrome *c* and cupriplastocyanin revealed a dynamic process with the rate constant of 2.2×10^{-5} s^{-1} (Zhou & Kostić, 1992a).

The latest study with mutants indicated that cytochrome *f* can interact even with residues in the hydrophobic patch in plastocyanin, which is proximate to the copper site (Modi et al., 1992b). A very recent X-ray crystallographic study showed that the blue copper protein amicyanin, which is closely similar to plastocyanin, turns its *hydrophobic* patch toward another redox protein even though their association depends at least in part on electrostatic attraction (Chen et al., 1992). The majority of evidence to date favors migration of cytochrome within the broad acidic patch, not outside of it, but this question has yet to be settled. We continue to study dynamic processes in metalloprotein complexes and the mechanisms by which these processes modulate electron-transfer reactions.

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