Unimolecular and Bimolecular Oxidoreduction Reactions Involving Diprotein Complexes of Cytochrome *c* and Plastocyanin. Dependence of Electron-Transfer Reactivity on Charge and Orientation of the Docked Metalloproteins†

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Received February 21, 1991; Revised Manuscript Received July 11, 1991

ABSTRACT: Cytochrome *c* and plastocyanin form an electrostatic complex, which can be reinforced by amide bonds in the presence of a carbodiimide. Besides this cross-linking, carbodiimide also converts carboxylate side chains into neutral *N*-acylurea groups. Four derivatives of the covalent diprotein complex, which differ in the degree of this charge neutralization, are separated by cation-exchange chromatography. Electron-transfer reactions at different ionic strengths involving the electrostatic complex and the four derivatives of the covalent complex are studied by laser flash photolysis with flavin semiquinones as reducing agents. The reactivity of the associated proteins toward external reductants cannot be predicted simply on the basis of this reactivity of the separate proteins. Qualitative analysis of the dependence on ionic strength of the reactions between FMN semiquinone and the covalent derivatives indicates sites at which this reductant interacts with the cross-linked proteins. The surprisingly small steric shielding of the protein redox sites in the covalent complex, as deduced from the reactions at high ionic strength, may indicate that the proteins have multiple reaction domains on their surfaces or that the complex is dynamical or both. The intracomplex (unimolecular) electron-transfer reaction is fast in the electrostatic complex (k_{et} = 1300 ± 200 s^{-1}) but undetectably slow in each of the four derivatives of the covalent complex (k_{et} < 0.2 s^{-1}). This reaction apparently requires a migration of cytochrome *c* from the acidic domain in plastocyanin, which is far from the copper redox site, to the hydrophobic domain, which is near this site. This migration is possible in the electrostatic complex, which is flexible, but impossible in the covalent complex, which contains rigid cross-links.

Oxidoreduction reactions between metalloproteins are interesting from both biological and mechanistic points of view. Respiratory and photosynthetic electron-transport chains are highly directional because reactions within the redox enzymes and reactions between these enzymes and mobile electron carriers are highly selective. The molecular basis of this selectivity can be understood only if the various factors that govern interprotein electron-transfer rates are understood (Sigel & Sigel, 1991). The donor–acceptor distance and orientation, the protein matter in the transfer pathway, the thermodynamic driving force, structural rearrangement coupled with electron transfer, and perhaps some other factors need to be separated from one another and analyzed experimentally and theoretically. These studies require well-characterized metalloproteins that form stable complexes, so that in the overall interprotein reaction the electron-transfer process of interest can be separated from diffusion processes (Kostić, 1991).

The heme protein cytochrome *c* (Moore et al., 1984; Cusanovich et al., 1987) and the blue copper protein plastocyanin (Sykes, 1985) are well suited to these kinetic and mechanistic studies; they are designated cyt and pc.[1] Their structures are known in detail, their properties have been thoroughly examined by spectroscopic and electrochemical methods, and their reactions with various redox agents have been studied by kinetic methods. Plastocyanin in chloroplasts carries electrons from the membrane-bound cytochrome *f* to the reaction center P700 of photosystem I. Kinetics of electron transfer between plastocyanin and cytochrome *f* has been studied (Wood, 1974; Niwa et al., 1980; Tanaka et al., 1981; Beoku-Betts et al., 1983, 1985; Anderson et al., 1987), but because cytochrome *f* is a large and incompletely characterized protein, cytochrome *c* has often been used instead (Wood, 1974; Wherland & Pecht, 1978; Augustin et al., 1983; Chapman et al., 1984; King et al., 1985; Armstrong et al., 1986; Rush et al., 1988; Bagby et al., 1990a,b). Both of these cytochromes react according to eq 1. (The numerals in parentheses are the oxidation states

$$\text{cyt}(2+) + \text{pc}(2+) \rightarrow \text{cyt}(3+) + \text{pc}(1+) \qquad (1)$$

of iron and copper.) Although cytochrome *f* reacts faster, it and cytochrome *c* give similar values of the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (King et al., 1985). Both cytochromes use a positively charged domain on the surface, near the exposed heme edge, for binding to plastocyanin (Beoku-Betts et al., 1983, 1985; Augustin et al., 1983; Chapman et al., 1984; King et al., 1985; Armstrong et al., 1986; Wiley et al., 1984; Takabe et al., 1984; Takenaka & Takabe, 1984; Takabe & Ishikawa, 1989). The plastocyanin from French bean (which is studied here), the one from poplar (whose structure is known in detail), and the one from spinach are very similar to one another (Ulrich & Markley, 1978; Driscoll, 1987; Chazin et al., 1988; Colman et al., 1978; Freeman, 1981; Guss & Freeman, 1983).

† This work was supported by the U.S. Department of Energy, Division of Chemical Sciences, Office of Basic Energy Sciences under Contract W-7405-Eng-82 (N.M.K.) and by NIH Grant DK15057 (G.T.).

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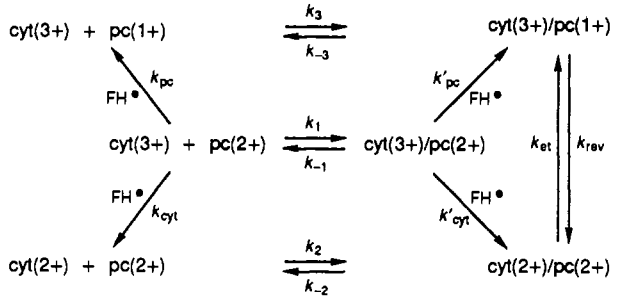
[1] Abbreviations: cyt(3+), ferricytochrome *c*; cyt(2+), ferrocytochrome *c*; pc(1+), cuproplastocyanin; pc(2+), cupriplastocyanin; cyt/pc, either the electrostatic or the covalent diprotein complex with the oxidation states as indicated; NHE, normal hydrogen electrode.

0006-2960/91/0430-9297$02.50/0

Scheme I



Two of us previously investigated, by pulse radiolysis and stopped-flow spectrophotometry, electron-transfer reactions of the electrostatic and the covalent complexes between cytochrome *c* and plastocyanin (Peerey & Kostić, 1989). The complexes of both type are designated cyt/pc. The present study deals further with the intracomplex (unimolecular) reaction in eq 2 and, for the first time, with external (bimolecular) reductions and oxidations of the electrostatic and covalent cyt/pc complexes by flavin semiquinones and by the cyt/pc complexes themselves. This bimolecular reactivity should depend on the accessibility to the external reagent of the electron-transfer sites in the associated proteins, which in turn depends on the charge of the proteins and the reagent and on the configuration and dynamics of the protein complexes. In the absence of crystal structures for complexes of redox proteins, such indirect information is valuable for their characterization and for evaluation of structures derived from docking simulations and theoretical calculations.

$$\text{cyt(2+)/pc(2+)} \xrightarrow{k_{et}} \text{cyt(3+)/pc(1+)} \qquad (2)$$

Experimental Procedures

The purification of the two proteins, spectrophotometric determination of their concentrations, preparation of the electrostatic cyt/pc complex, cross-linking of the proteins with a carbodiimide, and chromatographic separation of four major derivatives of the covalent cyt/pc complex (numbered in the order of elution from the CM 52 column) were done as before (Peerey & Kostić, 1989; Geren et al., 1983). Isoelectric points were determined in focusing experiments with a Pharmacia PhastSystem. The kinetic experiments with laser flash photolysis also were done as before (Ahmad et al., 1982; Simondsen & Tollin, 1983). The resolution of the kinetic apparatus was 0.3–1.0 μs. The flavin (quinone) mononucleotide, designated FMN, and the lumiflavin quinone, designated LF, were purified by size-exclusion chromatography on a Bio-Gel P2 column sized 1.0 × 75 cm, lyophilized, and dissolved in the phosphate buffer at pH 7.0. The final buffer was 0.07 mM flavin quinone and 0.5 mM EDTA; its ionic strength was 10 mM (for the concentration of 4.1 mM) or 90 mM (for the concentration of 49 mM); the latter buffer was adjusted with NaCl to 500 mM. The buffers were deaerated with argon, and the protein solutions were kept under this gas. All solutions containing flavins were kept in the dark. The laser dyes BBQ (in the mixture of equal volumes of toluene and ethanol) and LD425 (in methanol) have emission maxima at 420 and 431 nm, respectively. The absorption maxima of lumiflavin and FMN are at 442 and 446 nm, respectively. Most reactions of the diprotein complexes were followed by monitoring a large change in the heme absorbance ($\Delta\epsilon$ = 21 100 M^{-1} cm^{-1}) at 550 nm. Some reactions were also followed by monitoring the smaller change in the copper absorbance ($\Delta\epsilon$ = 2400 M^{-1} cm^{-1}) at 557 nm, an isosbestic point for ferrocytochrome *c* and ferricytochrome *c*. Partitioning of flavin semiquinones between associated proteins was estimated from the increase in the heme absorbance and decrease in the copper absorbance at a time after the pulse when these changes were greatest. In each experiment five traces were averaged, and each trace was a sum of three different signals, obtained with successive laser pulses. The amount of reduced proteins calculated from different traces was reproducible to ±20%. The range of ferricytochrome *c* and cupriplastocyanin concentrations was 15–60 μM. Because the diprotein complex was in pseudo-first-order excess over the flavin semiquinones, simultaneous reduction of both proteins was improbable (Tollin et al., 1986b). Reactions of the separate proteins were monitored at 575 nm (for cytochrome *c*) and at 595 nm (for plastocyanin) over three to four half-lives (500 points), and second-order rate constants were determined from three to five concentrations of the proteins. The reported margins of error were determined in repeated measurements, not simply from regression analysis. The unreported margins of error are approximately ±15%. Representative kinetic plots are shown in Figure 1.

Table I: Flavin Properties

property	lumiflavin (LF)	flavin mononucleotide (FMN)
R at N^{10}	CH_3	$CH_2(HCOH)_3CH_2OPO_3^{2-}$
net charge at pH = 7.0	0	−2
$E°$ for F/FH• at pH = 7.0 (mV) vs NHE	−231[a]	−238[b]

[a] From Draper and Ingraham (1970). [b] From Draper and Ingraham (1968).

Overview of the Reactions

Some of the reactions that can occur in these kinetic experiments are summarized in Scheme I. (Other reactions are discussed under Bimolecular Intercomplex Reaction.)

The flavin semiquinones, generally designated FH•, were generated by EDTA reduction of the corresponding flavin quinones, designated F, in the triplet state (Tollin et al., 1986b). These well-known reductants have several advantages over the aquated electron in the study of the reactions in Scheme I. Substituents at the N^{10} atom in the isoalloxazine ring cause predictable differences in steric bulk and charge. The flash photolysis experiments require smaller volumes of solutions, and thus permit higher protein concentrations, than the pulse radiolysis experiments. The properties of the two flavins used in this study are given in Table I.

At the beginning of each experiment both proteins are in the oxidized state. The flavin semiquinones can reduce both the separate and the associated proteins; the respective bimolecular rate constants are *k* and *k'*. The comparisons between k'_{cyt} and k_{cyt} and between k'_{pc} and k_{pc} can show the effects of electrostatic association (in the case of the electrostatic complex) and of cross-linking (in the case of the covalent complex) on the protein reactivity toward the external reductants.

The overall charges of ferricytochrome *c* and of cupriplastocyanin at pH 7.0 are +7 and −8, respectively. Because these charges are high and because the association constant K_a varies little when the oxidation states of the proteins change by one unit each (McLendon et al., 1988), the approximation in eq 3 is justified. The reduction potentials for pc(2+/1+)

$$K_a = \frac{k_2}{k_{-2}} \approx \frac{k_1}{k_{-1}} \qquad (3)$$

and for cyt(3+/2+) are 360 and 260 mV vs NHE, respectively. When cuproplastocyanin is formed (in the reactions

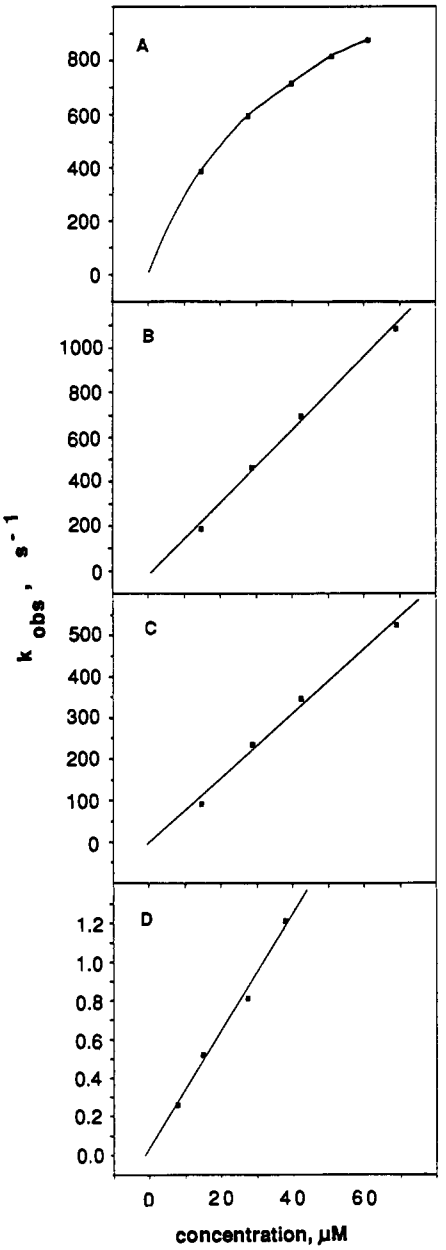


FIGURE 1: Kinetics of various electron-transfer reactions involving FMN semiquinone and different complexes of cytochrome *c* and plastocyanin at ionic strength of 10 mM, pH 7.00. (A) Unimolecular reaction k_{et} within the electrostatic complex cyt(2+)/pc(2+), monitored at 550 nm. (B) Bimolecular reaction k'_{cyt} of the covalent complex cyt(3+)/pc(2+), derivative 1, monitored at 550 nm. (C) Bimolecular reaction k'_{pc} of the covalent complex cyt(3+)/pc(2+), derivative 1, monitored at 557 nm. (D) Bimolecular reaction between molecules of the covalent complex, derivative 2, monitored at 550 nm.

k_{pc} and k'_{pc}), it should mostly remain in the reduced state because the reaction eq 4 and the reaction k_{rev} are thermo-

$$\text{cyt(3+)} + \text{pc(1+)} \not\rightleftharpoons \text{cyt(2+)} + \text{pc(2+)} \qquad (4)$$

dynamically unfavorable by ca. 100 mV. When, however, ferrocytochrome *c* is formed (in the reactions k_{cyt} and k'_{cyt}), it should be reoxidized by cupriplastocyanin because the net reaction in eq 1 and the reaction k_{et} are thermodynamically favorable by ca. 100 mV. Because cupriplastocyanin is present in excess over ferrocytochrome *c*, the bimolecular reaction between them occurs under pseudo-first-order conditions. The values in Table IV are derived from exact solutions for a mechanism consisting of a second-order reaction followed by a first-order reacton; the observed rate constants and concentrations of the electrostatic complex cyt/pc were fitted with the program SATFIT 3M (Simondsen & Tollin, 1983). The reactions k_3 and k_{-3}, involving the successor complex cyt(3+)/pc(1+), generally are not observed (Cusanovich et al., 1987).

When the complex cyt(2+)/pc(2+) is very stable, either because of the low ionic strength or because of covalent cross-linking, the rate constant for the unimolecular (intracomplex) electron transfer should be independent of concentration, and eq 5 should apply.

$$k_{obs} = k_{et} \qquad (5)$$

Diprotein Complexes cyt/pc

The proteins associate with each other via the positively charged domain encompassing several lysine residues near the exposed heme edge in cytochrome *c* and via the negatively charged domain encompassing the acidic residues 42–45 and 59–61 in plastocyanin (Augustin et al., 1983; Chapman et al., 1984; King et al., 1985; Armstrong et al., 1986; Bagby et al., 1990b; Roberts et al., 1991); the numbering is for the spinach protein. The physiological partners cytochrome *f* and plastocyanin associate via these same domains (Beoku-Betts et al., 1983, 1985; Anderson et al., 1987; Wiley et al., 1984; Takabe et al., 1984; Takenaka & Takabe, 1984). Amide cross-links between plastocyanin and either cytochrome most likely involve the same amino acid carboxylic groups that are responsible for the electrostatic docking (Geren et al., 1983; Davis & Hough, 1983; Morand et al., 1989). This notion is generally accepted (Mauk & Mauk, 1989, and references cited therein) but difficult to prove. Peptide mapping proved inadequate for unambiguous location of the amide cross-links induced by carbodiimide (Takabe & Ishikawa, 1989; Mauk & Mauk, 1989, and references cited therein), and radiolabeling is inapplicable to this task because carbodiimide atoms are not incorporated into the cross-links. The great decrease in the cross-linking yield as ionic strength is raised (Geren et al., 1983) would be difficult to explain if the cross-links were not formed between the residues near the heme edge in cytochrome *c* and those in the acidic patch in plastocyanin.

The number and the location of the cross-links can be determined only approximately. Studies of chemically modified spinach plastocyanin implicated four carboxyl groups in the side chains 42–45 and 59–61 (Anderson et al., 1987; Burkey & Gross, 1981, 1982), but it is unlikely that all four of them are simultaneously cross-linked to cytochrome *c*. Kinetic studies of the reaction in eq 1 between chemically modified cytochrome *c* and native plastocyanin implicated the following lysine residues in cytochrome *c*: residues 13 and 27 as the most important; residues 25, 86, and 87 as less important; and residues 7 and 27 as the least important (Augustin et al., 1983; Armstrong et al., 1986; Rush et al., 1988). It is unlikely that all of them, especially the less important ones, are involved in cross-linking. It is reasonable to assume that the complex contains one or two amide cross-links and that they involve the following residues: aspartate residues 43 and 45 and glutamate residues 44, 46, 60, 61, and 62 in the bean plastocyanin (note the shift by one from the spinach sequence) and lysine residues 13, 25, 27, 86, and 87 in the horse cytochrome *c*.

The number of cross-links can be estimated from the reduction potentials. Cross-linking lowers the reduction potential of cytochrome *c* by ca. 10 mV and raises that of plastocyanin by ca. 25 mV (Peerey & Kostić, 1989). This small "divergence" is probably due mainly to the neutralization of the cationic side chain(s) in the former and of the anionic side chain(s) in the latter. Formation of single amide bonds between one amino group of $H_2NCH_2CH_2NH_2$ and different

Table II: Bimolecular Rate Constants for the Reduction by Flavin Semiquinones of Ferricytochrome *c* and of Cupriplastocyanin[a]

		$k \times 10^{-7}$ (M^{-1} s^{-1})		
reductant	oxidant	μ = 10 mM	μ = 90 mM	μ = 500 mM
LFH•	cyt(3+)	8.7 ± 0.5	5.7 ● 0.4	4.5 ± 1
	pc(2+)	2.0 ± 0.2	2.8 ± 0.6	3.3 ± 0.5
FMNH•	cyt(3+)	16 ± 3	6.1 ± 1	1.1 ± 0.3
	pc(2+)	0.40 ± 0.1	1.4 ± 0.1	1.7 ± 0.2

[a] Phosphate buffer at pH = 7.0, three ionic strengths.

carboxylate groups in the anionic domain (residues 42–45 and 59–61) raises the reduction potential of spinach plastocyanin by 15–20 mV (Anderson et al., 1987). Somewhat smaller increases were reported in an earlier study (Takabe et al., 1984). Because the remaining amino group in ethylenediamine is protonated, this modification converts an anionic residue into a cationic one. The cross-linking with cytochrome *c*, however, converts an anionic residue in plastocyanin into a neutral one, so the reduction potential of plastocyanin probably increases by less than 15–20 mV per amide bond. The observed increase of ca. 25 mV corresponds to approximately two cross-links, on the average, in the four derivatives of the covalent cyt/pc complex. Because the aforementioned lysines in cytochrome *c* and aspartates and glutamates in plastocyanin form distinct domains, the structure of the diprotein complex is defined even though the cross-links are not precisely located.

Beyond our previous spectroscopic, electrochemical, and chromatographic experiments (Peerey & Kostić, 1989), our new UV–vis, circular dichroism, and magnetic circular dichroism measurements (Zhou, J. S., Brothers, H. M. II, Peerey, L. M., & Kostić, N. M., submitted for publication) showed no appreciable structural perturbations of the proteins as they form the electrostatic and covalent complexes cyt/pc. These new measurements showed no appreciable differences between the electrostatic and covalent complexes and among the four derivatives of the covalent complex, obtained by ion-exchange chromatography. Particularly informative are our determinations of the heme exposure (Schlauder & Kassner, 1979; Fiechtner & Kassner, 1978), which decreases from 24% in the free cytochrome *c* to 4 ± 1% in all the derivatives of the covalent cyt/pc complex. These derivatives evidently have similar or identical structures, with the acidic domain and the heme edge cross-linked to each other. The carbodiimide can convert a carboxylate anion to a neutral *N*-acylurea derivative (Carraway & Koshland, 1972), and these conversions were found with cytochrome *c* (Timkovich, 1977). We verified these results and also found similar conversions with plastocyanin. The four derivatives of the covalent complex evidently do not differ in structure but differ in overall charge because they contain different numbers of neutralized carboxylate groups (Zhou, J. S., Brothers, H. M. II, Peerey, L. M., & Kostić, N. M., submitted for publication). Mobility of the four bands on the cation exchanger CM 52 is consistent with small differences in charge. Indeed, isoelectric points of the four derivatives of the covalent complex (in the elution order) are 6.2, 6.8, 7.1, and 8.2.

Bimolecular Reactions k_{cyt} and k_{pc}

Reactivity of the separate metalloproteins toward the two flavin semiquinones is summarized in Table II; these findings agree with previous studies of these reactions (Meyer et al., 1983, 1984; Tollin et al., 1986a,b, 1987; Ahmad et al., 1981) and add to them. Reduction potential of cytochrome *c* depends only slightly on ionic strength (Gopal et al., 1988), and presumably so does the potential of plastocyanin. Since interatomic distances in protein crystals grown from concentrated solutions of salts (X-ray crystallography) are consistent with distances in dissolved protein molecules (NMR spectroscopy), the protein structures are not significantly affected by changes in ionic strength. Differences between the rate constants for different reductants and at different ionic strengths are relatively small, but revealing. The anionic FMN semiquinone at low ionic strength reacts faster with the cationic cytochrome *c* than with the anionic plastocyanin, as expected. But so does lumiflavin semiquinone, although the difference between the proteins now is smaller. If this neutral reductant behaves as if it carried some negative charge, it may be because partial localization of the unpaired electron in it (Ahmad et al., 1981; Ehrenberg et al., 1967; Guzzo & Tollin, 1964) gives it a zwitterionic character. This interesting effect is more pronounced with cytochrome *c* most likely because the phosphate anions in the buffer can bind to the cationic residues in this protein (Gopal et al., 1988).

The reactions of FMN semiquinone with the two proteins depend in different degrees on ionic strength, but such a dependence alone cannot reveal the precise electron-transfer site on a protein surface. In the case of charged proteins, effects of ionic strength must be considered skeptically. The trends in Table II are consistent with the finding that almost the entire surface of cytochrome *c* is covered with a positive electrostatic potential. Although this potential was examined in the protein from tuna (Weber & Tollin, 1985), the protein from horse, used in this study, probably is similar. The hydrophobic domain in plastocyanin, presumably the electron-transfer site for the flavin semiquinones, is distant from a negative electrostatic potential (Koppenol & Margoliash, 1982; Rush et al., 1988). Anionic metal complexes, too, bind at this latter domain; it includes His 87, the only solvent-exposed ligand to the copper atom (Cookson et al., 1980a,b; Handford et al., 1980; Sykes, 1985).

At high ionic strength, when charges become unimportant, the two proteins are equally reactive toward a given reductant even though plastocyanin has a somewhat higher reduction potential than cytochrome *c*. Because steric factors become important, the smaller reductant is somewhat more reactive than the larger one toward both proteins.

Bimolecular Reactions k'_{cyt} and k'_{pc}

One of the goals in this study is to determine how association of the two redox proteins, in both electrostatic and covalent complexes, affects the reactivity of their prosthetic groups. The findings in Table II may lead one to expect preferential reduction of ferricytochrome *c* in the complexes cyt(3+)/pc(2+) by FMN semiquinone at low ionic strength. But absorbances of each protein in the electrostatic complex, measured after the laser pulse, show otherwise. Reduction by FMN semiquinone is virtually nonselective, and reduction by lumiflavin semiquinone is partially selective in the unexpected direction—the yields of the electrostatic complexes cyt(2+)/pc(2+) and cyt(3+)/pc(1+) are approximately 1:3. Evidently, the reactivity of the protein complex cannot be predicted simply on the basis of the reactivity of the separate proteins. One kinetic advantage of free cytochrome *c* over free plastocyanin—the accessible heme edge—no longer exists in the complex because this edge is partially shielded. The other advantage—positive charge—no longer exists either because it is nearly neutralized, both locally and globally, upon the protein association. In fact, plastocyanin may now have an advantage because its reduction potential is higher than that of cytochrome *c*.

Because the bimolecular reductions by flavin semiquinones of the electrostatic complex cyt(3+)/pc(2+) could not be fully

Table III: Bimolecular Rate Constants for the Reduction by FMN Semiquinone of Covalently Cross-Linked Ferricytochrome *c* and Cupriplastocyanin[a]

cyt(3+)/pc(2+) derivative[b]	$k'_{cyt} \times 10^{-7}$ (M^{-1} s^{-1})			$k'_{pc} \times 10^{-7}$ (M^{-1} s^{-1})		
	μ = 10 mM	μ = 90 mM	μ = 500 mM	μ = 10 mM	μ = 90 mM	μ = 500 mM
1	1.6	1.2	1.0	0.78	0.73	1.1
2	7.2	3.4	0.68	3.7	3.4	1.2
3	8.3	6.6	1.2	7.3	5.4	1.4
4	10	9.7	1.5	7.5	9.7	3.5

[a] Phosphate buffer at pH = 7.0, three ionic strengths. [b] Order of elution from the cation exchanger CM 52.

separated from the subsequent unimolecular reaction k_{et}, the rate constants k'_{cyt} and k'_{pc} for this complex could not be determined correctly. But the bimolecular reductions of the covalent complex were fully separable because the unimolecular reaction within it was absent (see Unimolecular Intracomplex Reaction k_{et}). The rate constants for the four derivatives of the covalent complex cyt(3+)/pc(2+) are given in Table III. The reductant of choice was FMN semiquinone because it attacks the two proteins more nonselectively than does lumiflavin semiquinone.

The rate constants in Table III at low and intermediate ionic strengths generally differ less than 10-fold from the corresponding rate constants in Table II, and those at high ionic strength are virtually identical. This interesting finding and similar findings in recent kinetic studies of covalent diprotein complexes (Takabe & Ishikawa, 1989; Peerey & Kostić, 1989; Geren et al., 1983; Erman et al., 1987; Hazzard et al., 1988) and of stable electrostatic complexes between cytochrome *c* and other redox proteins (Hazzard et al., 1986, 1987, 1991) indicate one or both of the following. First, the reagent can attack a protein at multiple sites with similar efficiency, so that shielding of a portion of the protein surface, even of the partially exposed redox group, does not abolish the reaction. Second, the cross-linked protein molecules and the complex as a whole are dynamic and flexible, so that the preferred surface sites remain accessible to the external reagent. Only in the case of *iso*-1 cytochrome *c* and cytochrome *c* peroxidase from yeast does electrostatic association cause a substantial (10-fold) decrease in the rate constant for external reduction by flavin semiquinones (Hazzard et al., 1987). Multiple reaction sites and protein dynamics should be considered when theoretical models for diprotein complexes, obtained by computer graphics and by optimization of protein–protein interactions, are evaluated in terms of the bimolecular reactivity of associated proteins.

A diprotein complex may even form a larger aggregate with an external reagent. A recent NMR study showed that electrostatic association with cytochrome *c* does not mask any residue in plastocyanin from the paramagnetic probe $[Cr(NH_3)_6]^{3+}$, probably because the two proteins and the metal complex form a dynamic ternary complex (Bagby et al., 1990). The "front" side of the cytochrome *c* molecule, surrounding the heme edge, probably is large enough to accommodate both plastocyanin and FMN semiquinone in a ternary complex. Because plastocyanin apparently uses its negatively charged (acidic) domain for the binding of cytochrome *c* and its neutral (hydrophobic) domain for binding of FMN semiquinone (see below), the ternary complex involving FMN semiquinone should be sterically even more favorable than the ternary complex involving $[Cr(NH_3)_6]^{3+}$.

Comparisons among the corresponding rate constants k' (in Table III) and k (in Table II) for the same protein show more quantitatively the effects of cross-linking on the reactivity toward FMN semiquinone. (These trends in rate constants are presented graphically in Figure 1 in the supplementary material.) At low ionic strength the presence of cupriplastocyanin somewhat inhibits the reduction of ferricytochrome *c*, while the presence of ferricytochrome *c* somewhat assists the reduction of cupriplastocyanin. At high ionic strength the cross-linked proteins are approximately as reactive as the free proteins toward FMN semiquinone; rate constants agree with those obtained before (Meyer et al., 1983; Tollin et al., 1986). Both of these findings will be discussed next for the four derivatives of the covalent complex. Because the complex is nearly electroneutral, the effects of ionic strength can be interpreted with more confidence than in the case of separate proteins (Alberty & Hammes, 1958).

Although any two comparable rate constants in Table III may differ little from each other, their tendencies are clear. First, the "vertical" trends: At low ionic strength, when electrostatic interactions are important, both k'_{cyt} and k'_{pc} increase in the same order in which the four derivatives are eluted from the cation exchanger CM 52. All these three trends are consistent with an increasing extent of carboxylate neutralization in covalent cyt/pc derivatives 1–4. Because reactivity of both constituent proteins is affected, both of them contain *N*-acylurea derivatives of carboxylate groups. At high ionic strength, however, the covalent derivatives barely differ from one another in reactivity because their different charges no longer matter, and their structures are similar.

Second, the "horizontal" trends: Both reactions k'_{cyt} and k'_{pc} of covalent derivative 1 are virtually independent of ionic strength. Evidently, FMN semiquinone interacts with electroneutral sites in both proteins. Because there are only one or two cross-links in the covalent complex (see Diprotein Complexes cyt/pc), this neutralization is mostly caused by pairing of oppositely charged residues in the two proteins, not by extensive cross-linking. Both reactions of the remaining three covalent derivatives depend more on ionic strength, an indication that FMN semiquinone feels a positive electrostatic potential at the interaction sites in both proteins. This electrostatic potential of the cytochrome *c* moiety is smaller in the covalent complex than in the free protein because of the charge neutralization upon cross-linking. Even the plastocyanin moiety shows a slight positive electrostatic potential. The negative domain is neutralized by charge pairing, and the hydrophobic domain, at which the anionic reductant interacts with the protein, lies fairly close to the cationic residues in cytochrome *c*. The reaction k'_{pc} of derivative 4, only one case out of eight, depends on ionic strength in a way that is not readily explainable.

According to a recent survey (Mauk & Mauk, 1989, and references cited therein), little or no attention has been paid to the heterogeneity of the covalent complexes formed by cross-linking of redox proteins with carbodiimides, with the rare exception of the cyt/pc complex. Such heterogeneity was noted also with cross-linked cytochrome *c* and cytochrome *c* peroxidase (Erman et al., 1987). The present study shows that heterogeneity may be caused by chemical modification of side chains that does not result in cross-linking and not necessarily by structural factors such as different orientations of the proteins within the covalent complex.

Unimolecular Intracomplex Reaction k_{et}

The bimolecular (external) reaction k'_{cyt} yields cyt(2+)/pc(2+). Electron transfer from ferricytochrome *c* to cupriplastocyanin is thermodynamically favorable, and it can occur unimolecularly (within the complex) or bimolecularly (between the complexes). The former reaction, which is favored at low

Table IV: Unimolecular Electron-Transfer Reaction within the Electrostatic Complex of Ferrocytochrome *c* and Cupriplastocyanin[a]

monitored chromophore	reductant	$K_a \times 10^{-4}$ (M^{-1})	$k_{et} \times 10^{-3}$ (s^{-1})
cyt(2+) → cyt(3+)	FMNH•	5.1 ± 0.1	1.2 ± 0.1
	LFH•	3.8 ± 0.5	1.3 ± 0.2
pc(2+) → pc(1+)	FMNH•	6.2 ± 0.5	1.5 ± 0.2
	LFH•	4.9 ± 0.8	1.5 ± 0.3

[a] Phosphate buffer at pH = 7.0, μ = 10 mM.

concentrations of the complex, is discussed here; the latter reaction is discussed in under Bimolecular Intercomplex Reaction.

The Electrostatic Complex. The findings are given in Table IV. The association constant K_a and the rate constant k_{et} were determined by fitting the dependence of k_{obs} on the cupriplastocyanin concentration to the minimal kinetic mechanism consisting of a rapid preequilibrium followed by electron transfer. Identical values, within the error bounds, were obtained when the reaction was initiated with two different flavin semiquinones and when it was monitored at the electron donor and at the electron acceptor. The absorbance changes showed that the metalloproteins react in a stoichiometric ratio of 1:1 and that the reaction is complete. Because the absorbance and the absorbance change are greater for the heme than for the copper chromophore, the best kinetic results, $K_a = (5.0 \pm 1.0) \times 10^4$ M^{-1} and $k_{et} = (1.3 \pm 0.2) \times 10^3$ s^{-1}, reflect the former experiments more than the latter. The previous (Peerey & Kostič, 1989) and the present studies yielded k_{et} values that are identical (within the error bounds) and independent of ionic strength. The K_a values from the two studies are similar, but their dependence on ionic strength makes comparison difficult. Electrostatic complexes containing plastocyanin and $[Ru(bpy)_3]^{2+}$-labeled derivatives of cytochrome *c* show a range of k_{et} values that bracket our value (Pan et al., 1990). The adduct in which the heme-edge domain of cytochrome *c* is matched with the acidic domain of plastocyanin is unlikely to be the "dead-end" product. It may be the reactive precursor complex, or it may rapidly rearrange into the reactive configuration. These two mechanisms cannot be distinguished by kinetic methods but can be distinguished by studying the effect of cross-linking on the unimolecular electron-transfer reaction.

The Covalent Complex. Reduction of cyt(3+)/pc(2+) by FMN semiquinone yields both cyt(2+)/pc(2+) and cyt(3+)/pc(1+), as explained under Overview of the Reactions and Bimolecular Reactions k'_{cyt} and k'_{pc}. None of the four derivatives of the covalent cyt(2+)/pc(2+) complex in dilute solution (below 20 μM) shows absorbance changes attributable to intramolecular electron transfer over a period of at least 1.0 s. Hence, an estimated upper limit for the rate constant $k_{et} < 0.2$ s^{-1}. The physiological partners cytochrome *f* and plastocyanin behave similarly. Their cross-linking with carbodiimide yields the covalent complex—perhaps a mixture of derivatives—in which the intracomplex electron-transfer reaction is undetectable (Morand et al., 1989) or extremely slow (Takabe & Ishikawa, 1989). Both of these findings are consistent with our upper limit on the rate constant.

The Rearrangement. The unimolecular reaction within the covalent complex cyt(2+)/pc(2+) does not occur because the cross-links apparently impede the rearrangement of the protein molecules from an unreactive to a reactive configuration. It is very likely that cytochrome *c* initially binds, and is cross-linked to, the acidic domain in plastocyanin, whose various residues are 14–19 Å distant from the copper atom (King et al., 1985; Bagby et al., 1990b; Geren et al., 1983). Our findings indicate that, in the electrostatic complex, cytochrome *c* migrates to the hydrophobic domain, which is only 3–9 Å distant from the copper atom. This migration not only may shorten the donor–acceptor distance but also may alter the mutual orientation of the donor and the acceptor and the protein medium between them. Indeed, very recent extended Hückel calculations of exchange integrals for electron transfer through bonds showed that reduction of copper should occur much more efficiently from the hydrophobic than from the acidic site (Christensen et al., 1990). According to a kinetic analysis of electron transfer from excited states of octahedral metal complexes to cupriplastocyanin, this reaction is 10 times faster when the metal complex is at the proximate (hydrophobic) site than when it is at the remote (acidic) site (Brunschwig et al., 1985). The difference in reactivity between the electrostatic (1300 ± 200 s^{-1}) and covalent (less than 0.2 s^{-1}) cyt/pc complexes is much greater than 10-fold. Although the excited-state and the ground-state electron-transfer processes are not completely analogous, it is reasonable to conclude that plastocyanin reacts differently with another metalloprotein (cytochrome *c*) and with a metal complex. This difference should be kept in mind when biological electron-transfer mechanisms are studied with highly charged inorganic redox agents.

According to a recent analysis (Rush et al., 1988), an agent that is strongly bound at the acidic domain of plastocyanin or that is a strong reductant may transfer an electron to the copper site even from this remote domain. Cytochrome *c* supposedly is not such an agent because its electrostatic association with plastocyanin is weak, so it transfers an electron to the copper site from the proximate hydrophobic domain (Rush et al., 1988). Since, however, even covalent binding of cytochrome *c* at the remote domain does not ensure an intracomplex electron-transfer reaction, the mere strength of association is not a decisive factor for redox reactivity. (In the unlikely case that cross-links do not connect the heme edge and the acidic domain, this analysis would not apply.) The importance of the reducing strength of plastocyanin's partner can be assessed if the native cytochrome *c* ($E° = 0.26$ V) is replaced by its zinc derivative, because the triplet excited state of zinc protoporphyrin IX is a strong reductant ($E° = -0.88$ V) (Zhou & Kostič, 1991a,b). This and other studies (Rush et al., 1988; Bagby et al., 1990b; Takabe & Ishikawa, 1989; Peerey & Kostič, 1989; Hazzard et al., 1991) support the notion of rearrangement, and another study (Bagby et al., 1990a) is consistent with it. In the case of cytochrome *c* and cytochrome *c* peroxidase, rearrangement of the electrostatic diprotein complex was inferred both from theoretical calculations of Brownian dynamics (Northrup et al., 1987a,b, 1988) and from dependence on ionic strength of the rate constant for intracomplex electron transfer (Hazzard et al., 1987, 1988a,b).

Bimolecular Intercomplex Reaction

Although ferrocytochrome *c* does not reduce the cupriplastocyanin molecule to which it is cross-linked, it does reduce the cupriplastocyanin moiety in another diprotein complex according to eq 6. (The one unspecified oxidation state is irrelevant to the reaction.) These bimolecular reactions were initiated as before, by external reduction of cyt(3+)/pc(2+) with FMN semiquinone, but the concentrations of the diprotein complex were higher, and the monitoring time was longer, than in the study of the intracomplex reactions (see Unimolecular

$$\text{cyt(2+)/pc(2+)} + \text{cyt/pc(2+)} \rightarrow \text{cyt(3+)/pc(2+)} + \text{cyt/pc(1+)} \quad (6)$$

Table V: Bimolecular Rate Constants for Electron Transfer from Ferrocytochrome *c* to Cupriplastocyanin in Covalent Diprotein Complexes[a]

cyt/pc derivative[b]	$k \times 10^{-4}$ (M^{-1} s^{-1})		
	μ = 10 mM	μ = 90 mM	μ = 500 mM
1	1.1	0.64	0.62
2	3.1	3.1	0.53
3	7.7	4.6	3.9
4	64	19	9.9

[a] Phosphate buffer at pH = 7.0, three ionic strengths. [b] Order of elution from the cation exchanger CM 52.

Intracomplex Reaction k_{et}). The rate constants in Table V are much smaller than the rate constant for the reaction between the native proteins (eq 1), which is 1.5×10^6 M^{-1} s^{-1} at an ionic strength of 0.10 M (Wood, 1974; Augustin et al., 1983; Chapman et al., 1984). The rate constants for the four covalent derivatives span 16–60-fold ranges. Although the cross-linked proteins do not appreciably shield each other from the relatively small agent FMN semiquinone, they do shield each other from another molecule of the diprotein complex. The rate constant for the intercomplex reaction changes only 2–6-fold (for different derivatives), as expected for an approach between the oppositely charged moieties in the two complexes. This inhibition is small because the charged domains of the proteins partially neutralize each other and the overall charges of the proteins nearly cancel each other.

Conclusions

Cross-linking of cytochrome *c* and plastocyanin with a carbodiimide yields the covalent complex, whose carboxy-modified derivatives are easily separable by ion-exchange chromatography. The cross-linked proteins remain redox-active, and their bimolecular reactivity is affected mostly by their neutralization of each other's charges and not by their shielding of each other's redox sites. Because the tight cross-links prevent rearrangement of the complex into a reactive configuration, the intracomplex electron-transfer reaction is too slow to measure by conventional methods. Because the salt bridges, perhaps combined with hydrophobic interactions, do not prevent the required rearrangement, the unimolecular electron-transfer reaction within the electrostatic complex is fast. These studies show that electron-transfer between proteins and within multisubunit proteins and redox enzymes can be highly anisotropic and that the same redox proteins may bind to each other and react with each other in different orientations.

Supplementary Material Available

Tables I–IV, giving kinetic data for Tables II–V in the text, and Figure 1, showing the dependence on ionic strength of the bimolecular rate constants for reduction by FMN semiquinone of cytochrome *c*, plastocyanin, and derivatives of the diprotein complex (5 pages). Ordering information is given on any current masthead page.

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