Photoinduced Electron-Transfer Reaction in a Ternary System Involving Zinc Cytochrome *c* and Plastocyanin. Interplay of Monopolar and Dipolar Electrostatic Interactions between Metalloproteins†

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ABSTRACT: A carbodiimide promotes noninvasive cross-linking between amino groups surrounding the exposed heme edge in zinc cytochrome *c* and carboxylic groups in the acidic patch in plastocyanin. Eight derivatives of the covalent complex Zncyt/pc(I), which have similar structures but different overall charges because of different numbers and locations of *N*-acylurea groups, are separated by cation-exchange chromatography. Kinetics of electron transfer from the diprotein complex in the triplet excited state, ^{3}Zncyt/pc(I), to free cupriplastocyanin at pH 7.0 and various ionic strengths is studied by laser flash spectroscopy. This reaction is purely bimolecular for all eight *N*-acylurea derivatives of the diprotein complex. The overall charges of the derivatives **1** and **2** at pH 7.0 are −2 and 0, respectively; both of them, however, have very large dipole moments of 410–480 D. The rate constants for their reactions with cupriplastocyanin, whose charge at pH 7.0 is −8 and whose dipole moment is 362 D, are determined over the range of ionic strengths from 2.5 mM to 3.00 M. The observed dependence of the rate constants on ionic strength cannot be explained in terms of net charges (monopole–monopole interactions) alone, but it can be fitted quantitatively with a theory that recognizes also monopole–dipole and dipole–dipole interactions [van Leeuwen, J. W. (1983) *Biochim. Biophys. Acta 743*, 408]. At ionic strengths up to ca. 10 mM monopole–monopole interactions predominate and Brønsted–Debye–Hückel theory applies. At higher ionic strengths the monopole–dipole and dipole–dipole interactions predominate, Brønsted–Debye–Hückel theory yields incorrect net charges, and all three types of electrostatic interactions must be recognized. Our previous study [Zhou, J. S., & Kostić, N. M. (1991) *J. Am. Chem. Soc. 113*, 7040] showed that cupriplastocyanin uses its acidic patch near Tyr 83 for interaction with free ^{3}Zncyt. Many fittings of the kinetic results to van Leeuwen theory, with reasonable parameters, are all consistent with the notion that cupriplastocyanin uses its hydrophobic patch near His 87 for interactions with ^{3}Zncyt in the complex ^{3}Zncyt/pc(I). The covalently attached cuproplastocyanin largely neutralizes the positively charged patch and largely shields the exposed heme edge in zinc cytochrome *c*. Therefore, the ^{3}Zncyt moiety of the diprotein complex is not significantly attracted to the acidic patch in free cupriplastocyanin, which is connected to the copper site by relatively inefficient electron-transfer paths. This moiety reacts at the hydrophobic patch in free cupriplastocyanin, the origin of efficient electron-transfer paths to the copper site.

Metalloproteins are involved in various biological oxidoreduction processes, and it is important to understand kinetics and mechanisms of their electron-transfer reactions (Hoffman et al., 1991; McLendon, 1991a,b; Therien et al., 1991; Beratan et al., 1991; Mauk, 1991; Cusanovich et al., 1988; Kostić, 1991). Respiratory and photosynthetic electron-transport chains are highly directional because reactions within redox enzymes and reactions between these enzymes and mobile electron carriers are highly selective. At a molecular level, this selectivity depends on protein–protein interactions and on electron-transfer paths between redox groups.

The heme protein cytochrome *c* (Pettigrew & Moore, 1987; Moore & Pettigrew, 1990) and the blue copper protein plastocyanin (Sykes, 1991a,b), which are designated cyt[1] and pc, are well suited to kinetic and mechanistic studies. Structures and other properties of these proteins are known in detail, and their reactions with various redox agents have been much studied. Since the two proteins carry large opposite charges and have large dipole moments, they permit study of electrostatic interactions that are involved in protein docking for electron-transfer reactions.

Plastocyanin has a broad negatively charged (acidic) patch around Tyr 83 remote (14–19 Å) from the copper atom and an electroneutral (hydrophobic) patch around His 87 proximate (3–9 Å) to the copper atom. The protein seems to use both patches in electron-transfer reactions, but their choice is usually attributed simply to the charge of the other reactant (Sykes, 1991a,b). The two patches differ, however, in more than electrostatic properties and distances from the copper site. Recent quantum-mechanical analysis showed that the electron-transfer paths through bonds to the copper site leading from the hydrophobic patch are more efficient than those leading from the acidic patch (Christensen et al., 1990).

Previous studies in this laboratory concerned electron-transfer reactions between plastocyanin as an acceptor and cytochrome *c*, cytochrome *f*, and triplet state of zinc cytochrome *c* as donors (Peerey & Kostić, 1989; Peerey et al., 1991; Zhou et al., 1992; Qin & Kostić, 1992; Zhou & Kostić, 1991a,b, 1992); they are shown in eqs 1–3.

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[1] Abbreviations: apopc, apoplastocyanin; CM, center of mass; cyt, cytochrome *c*; cyt(II), ferrocytochrome *c*; cyt(III), ferricytochrome *c*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonate; NHE, normal hydrogen electrode; pc, plastocyanin; pc(I), cuproplastocyanin; pc(II), cupriplastocyanin; Zncyt, zinc cytochrome *c*.

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$$\text{cyt(II)/pc(II)} \rightarrow \text{cyt(III)/pc(I)} \quad (1)$$

$$^3\text{Zncyt} + \text{pc(II)} \rightarrow \text{Zncyt}^+ + \text{pc(I)} \quad (2)$$

$$^3\text{Zncyt/pc(II)} \rightarrow \text{Zncyt}^+\text{/pc(I)} \quad (3)$$

In eq 1, cyt stands for cytochrome *f* as well as cytochrome *c*. The weak reductant (in the ground electronic state) ferrocytochrome *c* cannot, whereas the strong reductant (in the excited triplet state) zinc cytochrome *c* can, reduce copper(II) from the initial binding site within the acidic patch. Both reductants, however, migrate away from the initial binding site to a site providing shorter distance and better electronic coupling between the heme and copper redox groups (Peerey & Kostić, 1989; Peerey et al., 1991; Zhou & Kostić, 1991b, 1992). Since the two reductants have identical topographies and electrostatic properties, the reactive protein–protein orientation apparently depends not only on the docking interactions but also on the thermodynamic driving force for electron transfer or on the electronic state of the redox group(s). One goal of this study is to determine whether zinc cytochrome *c* still reacts at the acidic patch of cupriplastocyanin when the electrostatic attraction between these two proteins is greatly diminished. In other words, is the reactive protein–protein orientation governed by electrostatic attraction?

Most previous studies of electron-transfer reactions in solution concerned binary systems—noncovalent and covalent complexes or mixtures of two proteins. This study, however, concerns a ternary system—bimolecular reaction according to eq 4 between a diprotein complex and a single protein.

$$^3\text{Zncyt/pc(I)} + \text{pc(II)} \rightarrow \text{Zncyt}^+\text{/pc(I)} + \text{pc(I)} \quad (4)$$

Study of ternary systems is only beginning (Bagby et al., 1990; Peerey et al., 1991; Burrows et al., 1991), and analysis of such a system is another goal of this study. The positively charged patch in zinc cytochrome *c* is neutralized, and the exposed heme edge is largely covered, by covalent attachment of cuproplastocyanin (Zhou et al., 1992). This attachment ensures that the initial orientation of zinc cytochrome *c* and cupriplastocyanin in the ternary system (eq 4) will differ from the orientation in the binary system (eq 2). Since the attachment also nearly doubles the size of the reducing agent, the reaction in eq 4 cannot be considered quite analogous to the one in eq 2. Fortunately, a new analysis proved possible. Reactive orientations of cupriplastocyanin with respect to free zinc cytochrome *c* (eq 2) and with respect to this protein bound to cuproplastocyanin (eq 4) will be compared.

The complex Zncyt/pc, composed of two moieties with high opposite charges and large dipole moments, has a small overall charge and a very large dipole moment. This is a rare combination of properties in metalloproteins. The main goal of this study is to analyze electrostatic interactions that govern protein–protein reactions. Since the cross-links prevent dissociation of the diprotein complex, the reaction in eq 4 could be studied over a wide range of ionic strengths, and electrostatic interactions between the reactants could be treated quantitatively. This treatment shows an interplay of monopolar and dipolar interactions. Depending on the ionic strength of the medium, either of them can dominate the other and govern the reaction rate.

MATERIALS AND METHODS

Proteins. Chromatographic materials, monohydrogen and dihydrogen potassium phosphates, and the carbodiimide EDC were obtained from Sigma Chemical Co. Distilled water was demineralized to a resistance greater than 10 MΩ cm. Potassium phosphate buffer for kinetic experiments had pH 7.0 and ionic strength of 10 mM; ionic strength was raised by adding NaCl and lowered by dilution. Since the phosphate concentration is only 4 mM, association of alkali-metal cations and HPO_4^{2-} ions is negligible. The buffers for chromatography had pH 7.0 and specified concentrations. Ultrafiltration was done in Amicon cells, with YM-5 membranes, under pure nitrogen. Chromatography and ultrafiltration were done at 4 °C. The free-base form of horse heart cytochrome *c* (Sigma, type III) was prepared, purified, and reconstituted with zinc(II) by standard procedures, in the dark (Vanderkooi & Erecinska, 1975; Vanderkooi et al., 1976). Zinc cytochrome *c* was always handled in the dark. French bean plastocyanin was isolated by standard methods (Milne & Wells, 1970). It was purified repeatedly by gel filtration chromatography on Sephadex G-25 and G-75 columns with 50 mM acetate buffer at pH 6.0 and by anion-exchange chromatography on a Sephadex DEAE A-25 column with the same buffer, which was made 0.200 M in NaCl. The purification continued until the protein oxidized before anion-exchange chromatography with an excess of $K_3[Fe(CN)_6]$ had an absorbance quotient A_{278}/A_{597} of less than 1.20. Apoplastocyanin was prepared and purified by published procedures (McMillin et al., 1974).

The covalent complex Zncyt/pc was prepared by a published method (Peerey & Kostić, 1989). The reaction mixture was 80 μM in each protein and 1.0 mM in EDC; the solvent was a 5.0 mM MOPS buffer at pH 6.5. The incubation time was ca. 24 h at room temperature. The product Zncyt/pc was dialyzed by ultrafiltration and purified first on a Sephadex G-75 (50 mesh) column sized 1.0 × 75 cm, with an 85 mM phosphate buffer at pH 7.0 as an eluent. The product was reduced with ascorbic acid, dialyzed into a 5.0 mM phosphate buffer at pH 7.0, and purified next on a CM-52 column sized 2.5 × 30 cm. The first two fractions were eluted with the 5.0 mM buffer, and the following six required a shallow gradient from 8.5 to 40 mM phosphate buffer at pH 7.0.

Kinetics. Flash kinetic spectrophotometry (so-called flash photolysis) was done with a standard apparatus (Zhou & Kostić, 1991a,b). The sample solution in a 10-mm cuvette was thoroughly deaerated by gentle flushing with ultrapure argon, supplied by Air Products Co. A Phase-R DL 1100 laser contained a 50 μM solution of rhodamine 590 in methanol and delivered 0.4-μs pulses of excitation light. The monochromatic monitoring beam from a tungsten–halogen lamp was perpendicular to the excitation beam. The absorbance–time curves were analyzed with kinetic software from OLIS, Inc. Decay of the triplet state of zinc cytochrome *c*, ^{3}Zncyt, was monitored at 460 nm, where its transient absorbance reaches maximum. Formation and decay of the cation (radical) Zncyt^+ were monitored at 675 nm, where the difference in absorbance between it and the triplet state is greatest. Concentration of zinc cytochrome *c* and of Zncyt/pc was always 10.0 μM, and concentration of cupriplastocyanin was varied from 5.0 to 30.0 μM. Ionic strength at pH 7.0 was varied from 2.5 mM to 3.00 M. Temperature was kept at 25 ± 1 °C.

Calculation of Dipole Moments. Atomic coordinates for horse heart ferrocytochrome *c* and for French bean cupriplastocyanin were obtained from the crystal structures of the corresponding proteins from tuna heart (Takano & Dickerson, 1981) and poplar (Guss & Freeman, 1983) by replacing the relatively few nonhomologous amino acid residues. Atomic

coordinates for horse heart ferricytochrome *c* were also obtained from the crystal structure of this very protein (Bushnell et al., 1990). Replacement of iron(II) by zinc(II) does not perturb the conformation of cytochrome *c* and its association with other proteins (Vanderkooi & Erecinska, 1975; Vanderkooi et al., 1976; Moore et al., 1980). Since change in the oxidation state of iron does not appreciably change the protein dipole moment (Koppenol & Margoliash, 1982), neither should heme excitation. Therefore, the dipole moment of ^{3}Zncyt/pc was calculated on the basis of the atomic coordinates for the maximum–overlap model of the cyt(II)/pc(II) complex, obtained by molecular modeling and energy minimization (Roberts et al., 1991). Partial charges were assigned to all atoms in the separate proteins and in the diprotein complex (McCammon et al., 1979; Northrup et al., 1981). The net charge and magnitude and orientation of the dipole moment in all cases were calculated by an established method (Koppenol & Margoliash, 1982; Northrup et al., 1986). The iron(II) ion was replaced by a zinc(II) ion, and the oxidation state of the copper ion in plastocyanin was set at both I and II. It was reasonably assumed that at pH 7.0 all lysine and arginine side chains are protonated, that the terminal amino group is protonated in plastocyanin but not in cytochrome *c*, and that the coordinated sulfhydryl group in plastocyanin and all carboxylic groups are deprotonated. The effective radius of plastocyanin was taken to be 15.5 Å (Rush et al., 1988). The effective radius of the complex Zncyt/pc(I) was estimated at 20.6 Å by averaging the distances of 4000 solvent-accessible points to the center of mass; this value includes the radius of the water molecule (V. A. Roberts, private communication). The same result, 20.6 Å, was obtained by a formula that relates radius of a solvated protein and its molecular mass (Tanford, 1961).

Treatment of Electrostatic Interactions. Since the version of the Brønsted–Debye–Hückel theory that is commonly applied to metalloproteins (eq 5) implies that reaction partners

$$\ln k = \ln k_0 + \frac{2Z_1Z_2\alpha\mu^{1/2}}{1 + \beta R\mu^{1/2}} \quad (5)$$

have identical radii (Rush et al., 1988), in our simplified treatment both reactants in eq 4 were given the radius of 20.6 Å. k and k_0 are rate constants at ionic strengths of μ and 0; the parameters are $\alpha = 1.17$ in aqueous solution at 25 °C and $\beta = 0.329$ Å^{-1}. A more thorough treatment, which was formulated (van Leeuwen et al., 1981; van Leeuwen, 1983) and verified (Rush et al., 1987, 1988; Zhou & Kostić, 1991b) before this study, is described next. Bimolecular rate constant k for electron transfer between two dipolar proteins depends on ionic strength, defined in eq 6, according to eq 7. The

$$f(\kappa) = \frac{1 - e^{-2\kappa R_2}}{2\kappa R_2(1 + \kappa R_1)} \quad (6)$$

$$\ln k = \ln k_{inf} - [Z_1Z_2 + (ZP)(1 + \kappa R) + (PP)(1 + \kappa R)^2]\frac{e^2}{4\pi\epsilon_0\epsilon k_B T R}f(\kappa) \quad (7)$$

monopole–monopole interaction Z_1Z_2 is isotropic, but the monopole–dipole (eq 8) and dipole–dipole (eq 9) interactions depend on the location of the reaction sites on the protein surfaces with respect to the dipole vectors. In eqs 5–9 the subscripts 1 and 2 stand for cupriplastocyanin and the complex ^{3}Zncty/pc(I), respectively. The symbols have the following

$$(ZP) = (Z_1P_2\cos\theta_2 + Z_2P_1\cos\theta_1)/eR \quad (8)$$

$$(PP) = P_1P_2\cos\theta_1\cos\theta_2/(eR)^2 \quad (9)$$

meanings: Z is the net charge, P is the dipole moment, θ is the angle between the dipole vector and the vector from the center of mass (CM) to the reaction site on the surface (see Figures 1 and 2), $R = R_1 + R_2$ defines the relationship of the radii, ϵ_0 is the permittivity constant, ϵ is the static dielectric constant, k_B is the Boltzmann constant, and e is elementary charge. The rate constant at infinite ionic strength, k_{inf}, should be nearly equal to the rate constant at ionic strength of 3.00 M, which we determine experimentally. The reactive site in the complex Zncyt/pc(I) most likely lies at the protruding heme edge, whose atoms fall in the range $0 < \theta_2 < 20°$. For these reasons k_{inf} and θ_2 are treated as adjustable parameters in fitting of the kinetic results to eq 7. Estimated uncertainty in the parameters is the interval of their values over which the average difference between the best fitted and the determined values of k at all ionic strengths is 10% or less.

RESULTS

Nonredox Quenching of ^{3}Zncyt/pc(I). Each of the eight chromatographic fractions of this covalent complex showed exponential decay with the rate constant of 190 ± 10 s^{-1} at ionic strength of 10.0 mM. A typical trace is designated a in Figure 3. Fractions **1** and **2** were examined over the entire range of ionic strengths, from 2.5 mM to 3.00 M, and the rate constant proved to be independent of ionic strength. Noncovalent complexes ^{3}Zncyt/pc(I) and ^{3}Zncyt/apopc also have the rate constant of 190 ± 10 s^{-1}, but the free ^{3}Zncyt has the rate constant of 70–140 s^{-1}, according to different studies (Elias et al., 1988; Vos et al., 1987; Dixit et al., 1981, 1984; Horie et al., 1985; Zhou & Kostić, 1991a,b).

Redox Quenching of ^{3}Zncyt/pc(I) by Cupriplastocyanin. Decay of the triplet state ^{3}Zncyt/pc(I) in the presence of the quencher, cupriplastocyanin, remained exponential for all eight chromatographic fractions of the covalent complex and at all ionic strengths. Kinetic results are given in Figure 4 and Table I. The rate constants for decay of the triplet ^{3}Zncyt/pc(I) and for formation of the cation Zncyt$^+$/pc(I) were equal within the error bounds; typical results are shown in Figures 3 (trace b) and 5.

Dipole Moments. Applications of the same method (Northrup et al., 1986) to atomic coordinates of cytochromes *c* from tuna heart and from horse heart yielded magnitudes of 281 and 277 D, respectively, and only slightly different vector orientations. These calculations reproduced the published value of 281 D (Northrup et al., 1986). Our value of 362 D for French bean cupriplastocyanin, whose net charge is –8, agrees with the value of 358 D for the spinach protein (Rush et al., 1988). As Figure 1 shows, the dipole vector forms an angle of 80° with the vector from the center of mass (CM) to the copper atom. The positive end of the dipole vector penetrates the surface near the α carbon atom of Glu 2, and the negative end penetrates it near the oxygen atom of Val 41. The negatively charged (acidic) and the electroneutral (hydrophobic) patches on the protein surface are located at 10–60° and 73–110° with respect to the negative end of the dipole vector. Locations of important amino acid residues and of their clusters are given in Table II.

The net charge and the dipole moment of the covalent complex Zncyt/pc(I), assuming the absence of *N*-acylurea groups, are –3 and 456 D, respectively, if both heme propionate groups are deprotonated, or –2 and 474 D, respectively,

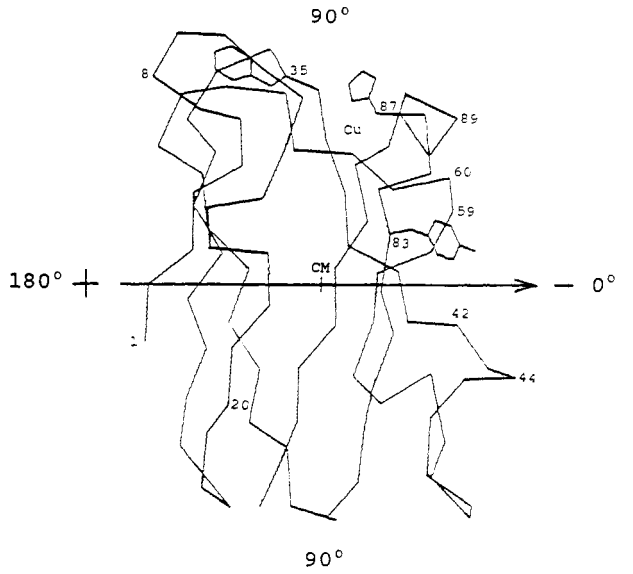

FIGURE 1: Chain of α carbon atoms (Guss & Freeman, 1983) and calculated dipole vector of cupriplastocyanin. The numerals represent selected amino acid residues, and angles θ_1 (see Table II) define positions with respect to the negative end (tip) of the dipole vector.

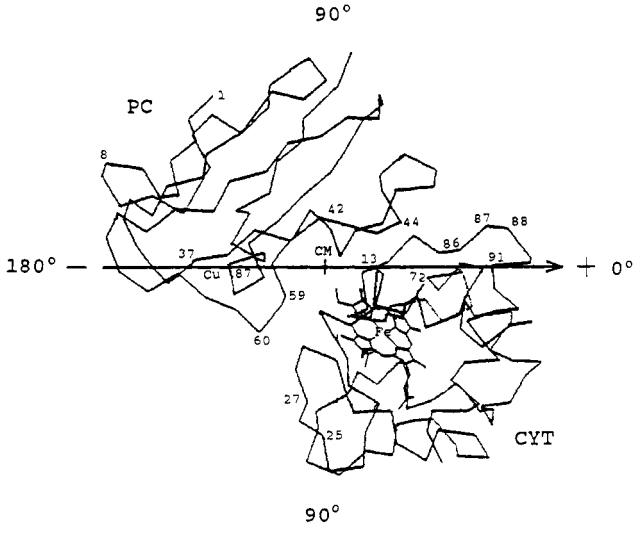

FIGURE 2: Chain of α carbon atoms and heme (Roberts et al., 1991) and calculated dipole vector of the complex between cytochrome *c* and plastocyanin. (In this study the Fe^{2+} ion is replaced by the Zn^{2+} ion in the heme.) The numerals represent selected amino acid residues, and angles θ_2 define positions with respect to the positive end (tip) of the dipole vector.

if one of them is protonated (Moore & Pettigrew, 1990). In the former case, which is shown in Figure 2, the positive end of the dipole vector penetrates the surface near the oxygen atom of Arg 91 in cytochrome *c*, and the negative end penetrates it near the ϵ_2 nitrogen atom of His 37 in plastocyanin. In the latter case, the corresponding points of penetration lie near the δ_1 carbon atom of Leu 68 and near the carboxylic carbon atom of Asn 32, respectively. The dipole orientation changes by only 8° when one of the propionate groups becomes protonated. A less rigorous calculation by the program Quanta, which is commercialized by Polygen, Inc., yielded a magnitude of 503 D and a vector orientation very similar to that defined above.

DISCUSSION

Covalent Complex Zncyt/pc. In the noncovalent (so-called electrostatic) complex cyt/pc the positively charged patch

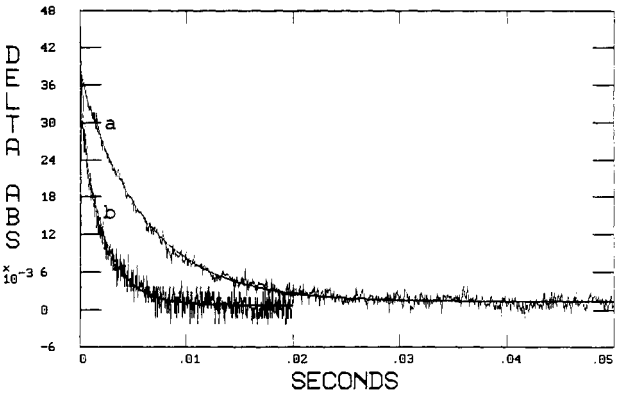

FIGURE 3: Decay of the triplet state ^{3}Zncyt/pc(I), monitored at 460 nm, in a solution containing 10.0 μM covalent complex Zncyt/pc(I) (*N*-acylurea derivative **5**) in phosphate buffer at pH 7.0, ionic strength of 10 mM, and 25 °C. The solid lines are single-exponential fits. (a) In the absence of cupriplastocyanin; (b) in the presence of 30 μM cupriplastocyanin.

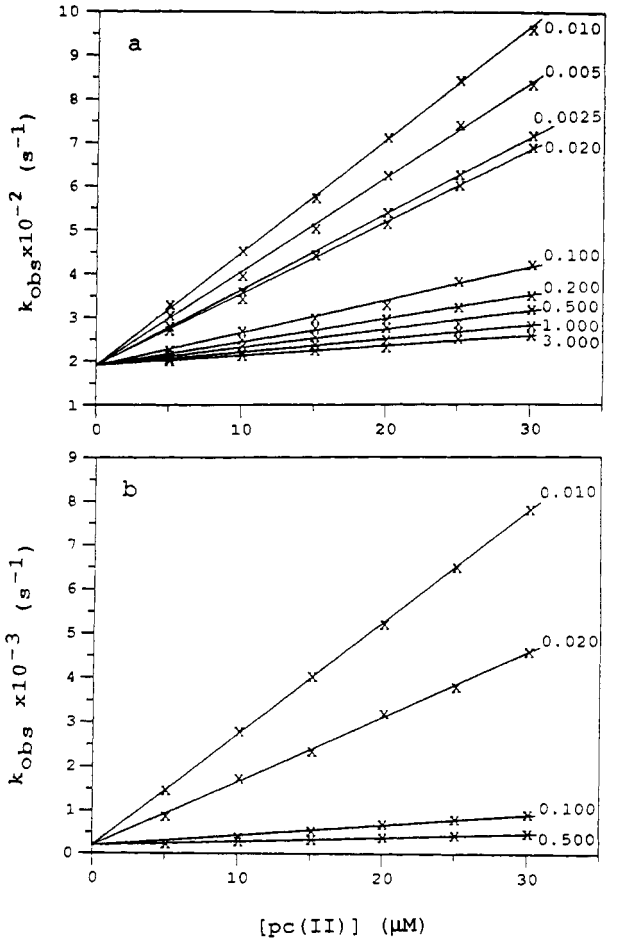

FIGURE 4: Rate of decay of the triplet state ^{3}Zncyt/pc(I), monitored at 460 nm, in a solution containing 10.0 μM covalent complex Zncyt/pc(I) and different concentrations of cupriplastocyanin in phosphate buffers of indicated ionic strengths, at pH 7.0 and 25 °C. (a) *N*-Acylurea derivative **1**; (b) *N*-acylurea derivative **5**.

around the exposed heme edge abuts the broad negatively charged patch remote from the copper atom (Augustin et al., 1983; Armstrong et al., 1986; Anderson et al., 1987; Burkey & Gross, 1981, 1982; Chapman et al., 1984; Geren et al., 1983; King et al., 1985; Bagby et al., 1990; Roberts et al., 1991). Much evidence shows that direct amide cross-links between lysine residues in cytochrome *c* and acidic residues in plastocyanin reinforce this very orientation (Geren et al., 1983; Peerey & Kostić, 1989; Peerey et al., 1991; Zhou et al.,

Table I: Dependence on Ionic Strength μ of the Rate Constant ($k \times 10^{-7}$, M^{-1} s^{-1})[a] for Reduction of Cupriplastocyanin at pH 7.0 and 25 °C

	μ (mM)								
reductant	2.5	5.0	10	20	100	200	500	1000	3000
^{3}Zncyt[b]	biexponential			saturation	13	4.0	1.4	0.94	0.67
^{3}Zncyt/pc(I) derivative[c]									
1	1.8	2.2	2.6	1.7	0.77	0.59	0.40	0.32	0.25
2	7.3	6.0	4.5	3.3	1.3	0.70	0.53	0.35	0.26
3			13	7.2	1.7		0.65		
4			16	11	2.1		0.81		
5			25	15	2.4		0.93		
6			31	18	3.5		0.99		
7			42	23	4.9		1.1		
8			57	41	7.0		1.4		

[a] Estimated error, ±10%. [b] Zhou and Kostić (1991a). [c] *N*-Acylurea derivatives of this covalent complex, numbered in the order of elution from CM 52 column.

Table II: Orientations of Selected Sites in French Bean Cupriplastocyanin with Respect to the Negative End of the Dipole Vector

patch	site	θ_1 (deg)
	Cu	80
hydrophobic	His 87, imidazole	73–80
	Phe 35, phenyl	107–110
	Pro 36, α-C	87
acidic	Tyr 83, phenyl	10–28
	cluster 42–45	18–42
	cluster 59–61	25–60

1992). All eight chromatographic derivatives of the covalent complex Zncyt/pc(I) have this orientation. Indeed, all of them have the same rate constant for the intracomplex reaction in eq 3 and the same rate constant for the natural decay of the triplet state; these rate constants are sensitive to protein–protein orientation. Besides efficiently promoting noninvasive cross-links, carbodiimide converts certain carboxylate anions into electroneutral *N*-acylurea groups (Timkovich, 1977). The chromatographic fractions of the covalent complex differ in location and number of these neutralized groups. Indeed, their mobility on the cation exchanger and their isoelectric points (see below) are consistent with this conclusion. Since the *N*-acylurea groups do not affect the properties of the triplet state and the intracomplex reaction in eq 3, and since these groups cannot be pinpointed by existing analytical methods (Zhou et al., 1992), their location is not pursued further at this time.

Nonredox Quenching of ^{3}Zncyt/pc(I). Since the copper atom is reduced in ^{3}Zncyt/pc(I) and absent in ^{3}Zncyt/apopc, the triplet decay with the rate constant of 190 ± 10 s^{-1} in both of these complexes cannot be due to electron transfer and to energy transfer. It is due to radiationless decay. Since this rate constant is close to the value of 100 ± 10 s^{-1} for free ^{3}Zncyt, the heme chromophore does not seem to be appreciably perturbed upon association with plastocyanin.

Kinetics of the Electron-Transfer Reaction. Quenching of the triplet state ^{3}Zncyt/pc(I) by cupriplastocyanin can be attributed solely to the electron-transfer reaction in eq 4 because the rate constants for the triplet decay, monitored at 460 nm, and for the cation formation, monitored at 675 nm, are equal. Indeed, our previous studies showed that energy transfer and other nonredox modes of quenching are negligible (Zhou & Kostić, 1991a). Since the plots in Figure 4 are strictly linear, the reaction in eq 4 is bimolecular at all ionic strengths. There is no evidence for a stable ternary precursor complex. In this regard the reaction in the ternary system (eq 4) differs from the reaction in the binary system. The latter reaction is bimolecular (eq 2) at intermediate and high ionic strengths and predominantly unimolecular (eq 3) at low ionic strength (Zhou & Kostić, 1991a). When the positively charged patch around the heme edge is neutralized and covered by the attachment of plastocyanin, cytochrome *c* no longer forms a stable precursor complex with another molecule of plastocyanin.

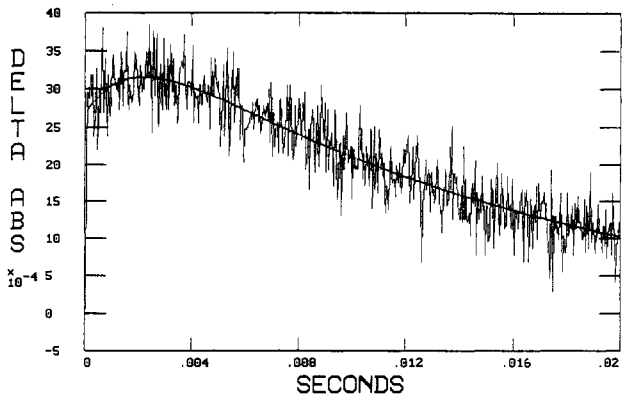

FIGURE 5: Appearance and disappearance of the cation radical Zncyt$^+$/pc(I), monitored at 675 nm, in a solution containing 10.0 μM covalent complex Zncyt/pc(I) (*N*-acylurea derivative **5**) and 30.0 μM cupriplastocyanin in phosphate buffer at pH 7.0, ionic strength of 10 mM, and 25 °C.

If, before the flash, the electron-exchange reaction in eq 10 had occurred, it would have altered the concentrations of the reactants in the reaction of interest (eq 4). We showed

$$\text{Zncyt/pc(I)} + \text{pc(II)} \rightarrow \text{Zncyt/pc(II)} + \text{pc(I)} \quad (10)$$

previously that excitation of a mixture of Zncyt/pc(II) and Zncyt/pc(I) complexes gives rise to biexponential decay of the triplet state; the exponential processes due to the faster, unimolecular quenching according to eq 3 and to the slower, bimolecular quenching according to eq 11 are fully separable

$$^3\text{Zncyt/pc(I)} + \text{Zncyt/pc(II)} \rightarrow \text{Zncyt}^+\text{/pc(I)} + \text{Zncyt/pc(I)} \quad (11)$$

in time (Zhou & Kostić, 1991b). Since the quenching observed in this study is strictly monoexponential, reactions in eqs 10 and 11 do not occur to a detectable extent. The rate constants in Table I should be correct.

Monopole–Monopole Interactions. The rate constants for the reaction in eq 4 (Table I) monotonically decrease with increasing ionic strength in the case of the *N*-acylurea derivatives (chromatographic fractions) **2–8** of Zncyt/pc(I). In this respect these derivatives qualitatively resemble the free zinc cytochrome *c* (Zhou & Kostić, 1991a). (Derivative **1**, which differs from the other seven and from free zinc cytochrome *c*, will be discussed separately below.) Since plastocyanin bears a negative charge, this dependence on ionic strength could be taken as evidence for the positive net charge of the derivatives **2–8**. Although Debye–Hückel theory does not apply rigorously to macromolecules (Marcus & Sutin, 1985), it is often used to estimate charges of small, globular proteins. Application of eq 5 to the results in Table I yielded the following estimates of Z_2 for the *N*-acylurea derivatives **2–8**, respectively, of the covalent complex Zncyt/pc(I): 1.8, 2.4, 2.6, 2.9, 2.9, 3.0, and 3.3. These values certainly are incorrect in the absolute sense, but their trend accounts for the smooth horizontal and vertical trends in Table I and agrees with the mobility of the derivatives on the cation-exchange column.

The isoelectric points for the derivatives **2–8**, respectively, are 7.1, 8.0, 8.2, 8.5, 9.0, 9.6, and >9.6 (Zhou et al., 1992). These pI values confirm that derivatives **3–8** of the covalent complex bear positive charges but show that derivative **2** is virtually electroneutral, all at pH 7.0. In this last case, simple consideration of monopole–monpole interactions and application of Brønsted–Debye–Hückel theory over a wide range of ionic strengths yielded a charge that is incorrect not only quantitatively, but qualitatively.

As Table I and Figure 4a show, the rate constant for derivative **1** first increases as ionic strength increases from 2.5 to 10 mM and then decreases as ionic strength increases further. Since Brønsted–Debye–Hückel theory applies best to conditions of low ionic strength, the estimate of $Z_2 \approx -2$, obtained from the rate constants at ionic strengths of 2.5, 5.0, and 10 mM, should be reasonable. This estimate agrees with the sum of net charges of zinc cytochrome *c* and cuproplastocyanin; amide cross-links do not affect this sum. Injudicious application of Brønsted–Debye–Hückel theory to rate constants at ionic strengths greater than 10 mM would yield the incorrect result $Z_2 = 1.4$. The isoelectric point of 6.2 confirms that derivative **1** bears a negative overall charge at pH 7.0.

Monopole–Dipole and Dipole–Dipole Interactions and the Protein Orientation for Electron Transfer. Although derivative **1** bears a negative overall charge, its reaction (eq 4) with the negatively charged plastocyanin is hindered by increasing ionic strength beyond 10 mM. Although derivative **2** is virtually electroneutral as a whole, its reaction markedly depends on ionic strength; this reaction, too, is hindered by the increase in ionic strength throughout the range. Neither of these findings can be explained in terms of net charges (monopole–monopole interactions) alone. Even though derivatives **1** and **2** are not positively charged, they behave like the positively charged derivatives **3–8** in their reactions with cupriplastocyanin (eq 4) at most or all ionic strengths.

These counterintuitive findings about the reaction in eq 4 can be explained in terms of monopole–monopole, monopole–dipole, and dipole–dipole interactions. If monopole–monopole interactions exist, they predominate at ionic strengths of 10 mM and lower. In the case of derivative **1** they hinder the reaction; in the case of derivative **2** they barely exist. If monopole–dipole and dipole–dipole interactions exist, they become dominant at intermediate and high ionic strengths. These interactions, which are together termed dipolar, reverse the dependence of the rate constant on ionic strength in the case of the negatively charged derivative **1**, cause this dependence in the case of the electroneutral derivative **2**, and contribute to this dependence, in the same sense in which monopole–monopole interactions do, in the case of the positively charged derivatives **3–8**. At very high ionic strength the charges are completely screened, and all electrostatic interactions become negligibly weak. Therefore, the rate constants for derivatives **1** and **2** become equal at ionic strengths of 1.00 and of 3.00 M even though these two derivatives have different net charges and probably somewhat different dipole moments. Evidently, the rate constants at the ionic strength of 3.00 M can be equated to k_{inf} without significant error, as we do in the quantitative treatment to be discussed next.

The dependencies shown in Table I can be analyzed in terms of eq 7, which includes all three aforementioned types of electrostatic interactions. Of the eight chromatographic fractions of the Zncyt/pc(I) complex, only the first two are suitable for quantitative treatment of dipolar interactions because only for them is the overall charge or the dipole moment known with some certainty. The first fraction probably does not contain *N*-acylurea groups; its overall charge of –2 at pH 7.0 indicates that, quite plausibly, the heme propionate group that is unprotonated because it is somewhat exposed in free cytochrome *c* becomes protonated when shielded by attachment of plastocyanin, and its dipole moment is 474 D. The overall charge of the second fraction at pH 7.0 is practically nil, but its dipole moment is uncertain because the location of *N*-acylurea groups is uncertain. Table I shows that the reaction in eq 4 for the other six fractions becomes increasingly dependent on ionic strength in the elution order of these fractions. Since, however, neither overall charges nor the dipole moments of these six *N*-acylurea derivatives of Zncyt/pc(I) can be estimated with any certainty, these derivatives were not quantitatively analyzed.

Table III: Fittings to Equation 7 of the Dependence on Ionic Strength of the Rate Constant for the Reaction in Equation 4 between *N*-Acylurea Derivative **1** of the Covalent Complex Zncyt/pc(I) (R_2 = 20.6 Å) and Cupriplastocyanin (R_1 = 15.5 Å, Z_1 = –8, and P_1 = 362 D)

Z_2	P_2 (D)	θ_2 (deg)	$k_{inf} \times 10^{-6}$ (M^{-1} s^{-1})	θ_1 (deg)
–3	456	0	2.0	84
			3.3	101
		10	2.0	83
			3.3	100
		20	2.0	80
			3.3	99
–2	474	0	2.0	92
		10		91
		20		88
		30		83
		10	3.1	106

Table III shows fittings to eq 7 of the rate constants for derivative **1** of Zncyt/pc(I) in the reaction with cupriplastocyanin (eq 4). The net charge (Z_2) and dipole moment (P_2) of this derivative are calculated independently of this kinetic analysis. An angle θ_2 for the diprotein complex is defined by the dipole vector and the vector from the center of mass (CM) to an atom at the protruding heme edge in zinc cytochrome *c* (see Figure 2). Different values of θ_2 correspond to different atoms within this reactive site. The rate constant at infinite ionic strength, k_{inf}, should be close to the rate constants at very high ionic strengths that we actually determined. Although this extrapolation should not introduce appreciable errors, we cautiously used two different values of k_{inf}. In all fittings in Table III except the last one the only variable parameter was θ_1. In the last fitting the variable parameters were both θ_1 and k_{inf}, and the latter fell neatly between the values of it that were fixed in previous fittings. Fittings with the values $Z_2 = -2$ and $P_2 = 474$ D were much better than those with the values $Z_2 = -3$ and $P_2 = 456$ D. This finding agrees with the result $Z_2 = -2$ of the Brønsted–Debye–Hückel treatment of the rate constants at ionic strengths of 10 mM and lower (see above). All of the fittings yielded consistent values of θ_1 for cupriplastocyanin, the angle between the dipole vector and the vector from the center of mass (CM) to the reaction site on the surface (see Figure 1). The average value from all of the fittings is $\theta_1 = 91 \pm 9°$. According to Figure 1 and Table II, it is consistent with the hydrophobic patch on the plastocyanin surface, which is proximate to the copper site. This value of θ_1 is inconsistent with the acidic patch, which is remote from the copper site. Figure 6a shows that these two patches can be clearly distinguished by the fitting procedure.

Since derivative **2** of Zncyt/pc(I) has an isoelectric point of 7.1 (Zhou et al., 1992), it is virtually electroneutral at pH

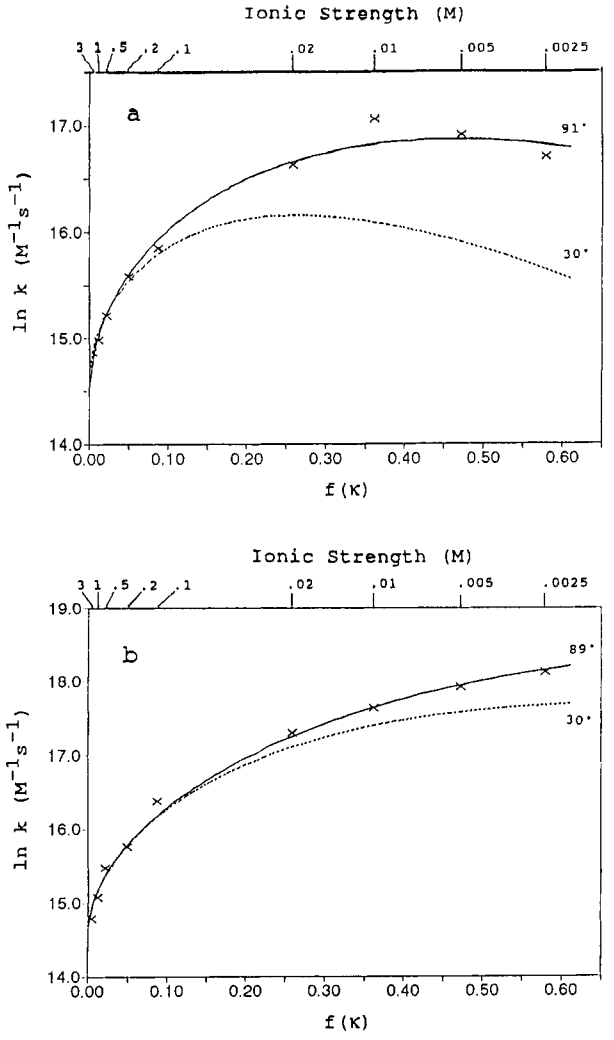

FIGURE 6: Dependence on ionic strength of the rate constant for the reaction in eq 4 at pH 7.0 and 25 °C. The error bounds are smaller than the crosses. The solid line is the best fit of the data to eq 7 with the specified value of θ_1; the dotted line corresponds to $\theta_1 = 30°$. (a) *N*-Acylurea derivative **1** of the covalent complex Zncyt/pc(I) with $\theta_1 = 91°$ (the parameters are given in Table III); (b) *N*-acylurea derivative **2** of the covalent complex Zncyt/pc(I) with $\theta_1 = 89$ (the parameters are given in Table IV).

Table IV: Fittings to Equation 7 of the Dependence on Ionic Strength of the Rate Constant for the Reaction in Equation 4 between *N*-Acylurea Derivative **2** of the Covalent Complex Zncyt/pc(I) (R_2 = 20.6 Å, Z_2 = 0) and Cupriplastocyanin (R_1 = 15.5 Å, Z_1 = −8, and P_1 = 362 D)

P_2 (D)	θ_2 (deg)	$k_{inf} \times 10^{-6}$ (M^{-1} s^{-1})	θ_1 (deg)
452	10	1.2	76
443		1.6	84
426		2.0	86
417		2.7	95
411		3.3	100
430	3	2.0	89

7.0; that is, $Z_2 \approx 0$. Two *N*-acylurea groups are required for overall electroneutrality of the complex. Since their locations can only be surmised, we did not calculate the dipole moment of the complex (P_2) but treated it, as well as θ_1, as a variable parameter in all fittings in Table IV. Adjustable parameters were the rate constant k_{inf} and the angle θ_2. As in the fittings in Table III, the values of these adjustable parameters were quite reasonable. The former was chosen on the basis of the actual rate constants at very high ionic strength (Table I), while the latter was set on the basis of the known protein structure. In the last fitting in Table IV only the rate constant k_{inf} was fixed while the other three parameters were allowed to vary. Again, all of the fittings yielded similar values of θ_1. Their average is $\theta_1 = 88 \pm 8°$, again consistent with the hydrophobic patch of free cupriplastocyanin (Table II). Again, Figure 6b shows that the kinetic results are consistent with the hydrophobic patch and inconsistent with the acidic patch.

The angles θ_1 and θ_2 define only bands on the protein surfaces. To define particular sites in the two reactants, the site in one of them has to be specified. Since there is a general agreement about the heme edge in cytochrome *c*, the reactive site in plastocyanin can be pinpointed. Although the angles θ_1 are obtained in fittings with multiple parameters, these results may be taken as evidence that cupriplastocyanin uses its hydrophobic (electroneutral) patch for the reaction in eq 4 with both *N*-acylurea derivatives **1** and **2** of the complex Zncyt/pc(I).

Comparison among the reactions of cupriplastocyanin as electron acceptor with ferrocytochrome *c*, ^{3}Zncyt, and ^{3}Zncyt/pc(I) as electron donors reveals interesting aspects of metalloprotein oxidoreduction reactions. The first two donors have the positively charged patch, and both of them bind somewhere within the broad acidic patch of cupriplastocyanin. Both of them then migrate away from the initial binding site to reduce the copper(II) atom (Rush et al., 1988; Peerey & Kostić, 1991; Peerey et al., 1991; Zhou & Kostić, 1991a,b, 1992). Since reinforcement of the noncovalent binary complex by covalent cross-links completely abolishes the reaction in eq 1 but only slows down the one in eq 3 (Peerey & Kostić, 1989; Peerey et al., 1991; Zhou & Kostić, 1991b), ferrocytochrome *c* seems to migrate farther than zinc cytochrome *c* from the initial binding site. The lower the reduction potential of the donor, the more the donor needs to find a favorable electron-transfer path to the acceptor. But neither of these two donors seems to migrate all of the way to the hydrophobic patch; the reactive orientation seems to be a compromise between the efficient electron-transfer paths, whose origins are away from the site of initial binding, and electrostatic attraction to this initial site. In the third donor, ^{3}Zncyt/pc(I), the heme edge is largely shielded and the positively charged patch around it is largely neutralized. The first property necessitates the search for an electron-transfer path to the copper(II) site that is more efficient than the paths originating at the acidic patch. The second property removes even the electrostatic reason for binding at the acidic patch. For both reasons, ^{3}Zncyt/pc(I) apparently reacts at the hydrophobic patch, the origin of the most efficient electron-transfer paths to the copper(II) atom. This comparative analysis will be verified in our future theoretical studies of electron-transfer paths in plastocyanin complexes with cytochrome *c* and zinc cytochrome *c* and in our experimental studies of electron-transfer reactions involving plastocyanin mutants.

Conclusions. Dipolar forces between proteins can be an important, perhaps even principal, factor in electrostatic interactions that govern molecular recognition and chemical reactions. While the orientation for *recognition* depends mostly on electrostatic, hydrophobic, steric, and other interactions between the protein molecules, the orientation for *redox reaction* depends on an interplay of these factors and electron-transfer paths. The interplay, in turn, depends on thermodynamic and electronic properties of the active sites.

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