Enforced Interaction of One Molecule of Plastocyanin with Two Molecules of Cytochrome *c* and an Electron-Transfer Reaction Involving the Hydrophobic Patch on the Plastocyanin Surface†

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ABSTRACT: Laser flash photolysis is used to study the photoinduced electron-transfer reaction cyt(III)//pc(II) + ^{3}Zncyt → cyt(III)//pc(I) + Zncyt$^+$ at pH 7.0 and 25 °C. In the covalent (symbol //) complex cyt(III)//pc(II) the acidic patch in cupriplastocyanin is directly cross-linked to the basic patch in ferricytochrome *c*. The triplet state of zinc cytochrome *c* reduces the pc(II) moiety, not the cyt(III) moiety, of the covalent complex. The reaction is strictly bimolecular in the entire range of ionic strength studied, from 1.25 mM to 1.00 M. The two reactants interact only transiently, in a collisional complex, and do not form a persistent complex cyt(III)//pc(II)/Zncyt. Because noncovalent (symbol /) association of three separate protein molecules is far less probable than association of the covalent complex and another protein molecule, we conclude that, without the aid of covalent cross-links, one molecule of plastocyanin will not form a ternary complex with two molecules of cytochrome *c*, cyt/pc/cyt. Dependence of the rate constant on ionic strength is analyzed in terms of van Leeuwen theory of electrostatic interactions, which recognizes the importance of dipole moments of the proteins. This analysis shows that ^{3}Zncyt reacts with the hydrophobic patch in the pc(II) moiety of the covalent complex cyt(III)//pc(II). At high ionic strength, at which electrostatic interactions are practically abolished, the blue copper site is reduced with approximately equal rates via the hydrophobic patch in the pc(II) moiety of the complex and via the acidic patch in free pc(II). This is evidence that the two distinct patches on the plastocyanin surface are comparable in their intrinsic "conductivity" for electrons coming to the copper site. Positively charged and electroneutral redox partners tend to react at the acidic patch (although not necessarily at the initial docking site in this broad patch) for electrostatic, not electronic, reasons. Earlier theoretical studies disagreed about the relative electronic conductivities of the two patches. This experimental study corroborates very recent theoretical studies that found the two patches to be comparable in the efficiency of electron transfer.

Respiratory and photosynthetic electron-transport chains are strictly directional because reactions among the metalloproteins and other redox agents involved are highly selective. At a molecular level, this selectivity depends on protein−protein interactions and on electron-transfer paths between redox groups. These interactions and paths are best studied with well-characterized, small proteins (Kostić, 1991; Hoffman et al., 1991; Mauk, 1991; McLendon, 1991a,b; McLendon & Hake, 1992; Therien et al., 1991; Winkler & Gray, 1992).

The heme protein cytochrome *c* (Pettigrew & Moore, 1987; Moore & Pettigrew, 1990) and the blue copper protein plastocyanin (Sykes, 1991a,b; Redinbo et al., 1994), designated cyt and pc,[1] are ideally suited to kinetic and mechanistic studies. Their three-dimensional structures in both oxidized and reduced states and in both crystal and solution are known in detail. Although their reactions with various redox agents and with each other have been studied, the mechanisms of these reactions are largely unknown.

Plastocyanin in chloroplasts carries electrons from the membrane-bound cytochrome *f* to the reaction center P700 of photosystem I. Plastocyanin has two distinct surface areas through which it can exchange electrons with other redox agents. The broad and negatively charged acidic patch is remote from the copper site, whereas the smaller and electroneutral hydrophobic patch is proximate to this site. The intriguing facts that plastocyanin has two physiological partners and also two patches on its surface have prompted various studies, some of which yielded contradictory results (Chapman, 1991). The current view is that plastocyanin accepts an electron from cytochrome *f* via the acidic patch and sends an electron to P700 via the hydrophobic patch (Modi et al., 1992a,b; Haehnel et al., 1994) but that it uses its acidic patch for recognition of positively-charged patches that both of these membrane-bound proteins have. Moreover, the protein−protein orientation that optimizes the *interactions* between the surfaces may differ from the orientation that favors the electron-transfer *reaction* between the active sites inside the respective proteins. Research in this laboratory showed that neither cytochrome *c* nor cytochrome *f* can reduce cupriplastocyanin (eq 1) in the initial docking orientation (Peerey & Kostić, 1989; Peerey et al.,

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[1] Abbreviations: apopc, apoplastocyanin; b_5, cytochrome b_5; b_5(II), ferrocytochrome b_5; b_5(III), ferricytochrome b_5; cm, center of mass; CD, circular dichroism; cyt, cytochrome *c*; cyt(II), ferrocytochrome *c*; cyt(III), ferricytochrome *c*; MCD, magnetic circular dichroism; pc, plastocyanin; pc(I), cuproplastocyanin; pc(II), cupriplastocyanin; Sncyt, tin(IV) cytochrome *c*; ^{3}Sncyt, triplet state of tin(IV) cytochrome *c*; Sncyt$^+$, tin(IV) cytochrome *c* cation radical; Zncyt, zinc cytochrome *c*; ^{3}Zncyt, triplet state of zinc cytochrome *c*; Zncyt$^+$, zinc cytochrome *c* cation radical.

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1991; Qin & Kostić, 1992, 1993). (The single slash mark represents electrostatic association; the double slash mark represents covalent cross-linking; and the Roman numerals are the oxidation states of iron and copper.) The thermal reaction in eq 1 is relatively slow, and it is initiated by external oxidation of cyt(II)/pc(I) or by external reduction of cyt(III)/pc(II). Although conclusive studies of the thermal reactions are possible and have been done (Peerey & Kostić, 1989; Peerey et al., 1991; Qin & Kostić, 1992, 1993), additional redox agents and their reactions complicate the kinetic analysis. Kinetics and mechanism of the necessary rearrangements of the cyt/pc complex have been studied with zinc cytochrome *c*, because the photoinduced reactions in eqs 2−7 are fast and do not require additional redox agents in solution (Zhou & Kostić, 1991a,b, 1992a,b, 1993a,b).

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

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

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

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

$$\text{Zncyt}^{+} + \text{pc(I)} \rightarrow \text{Zncyt} + \text{pc(II)} \quad (5)$$

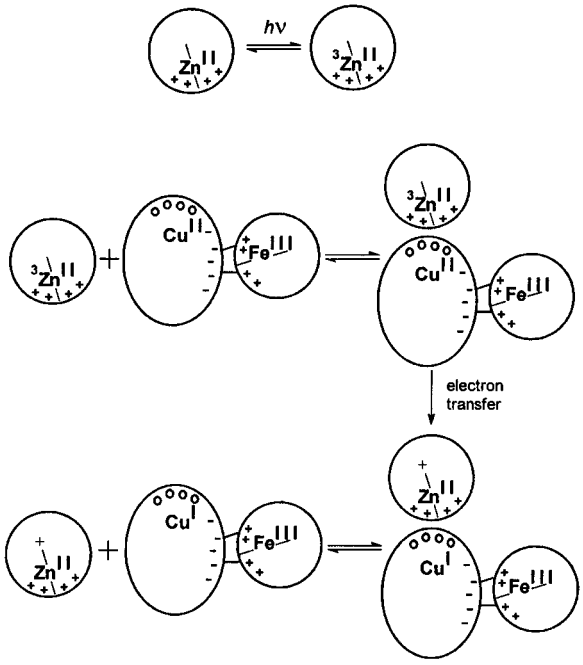
$$^{3}\text{Sncyt/pc(II)} \rightarrow \text{Sncyt}^{+}\text{/pc(I)} \quad (6)$$

$$^{3}\text{Zncyt}/b_5\text{(III)} \rightarrow \text{Zncyt}^{+}/b_5\text{(II)} \quad (7)$$

The reaction in eq 1, which has a small electromotive driving force, is truly an oxidoreduction—the rate-limiting step in it is electron transfer. The reactions in eqs 2, 4, 6, and 7, which have large driving forces, are gated. Because the electron transfer in these photoinduced reactions is faster than the protein rearrangement (a process independent of the electromotive force), the rearrangement becomes the rate-limiting step, the one actually observed in kinetic experiments. In some of the experiments (eq 7), plastocyanin was replaced as an electron acceptor by ferricytochrome b_5, a protein that has only one, acidic, patch on its surface (Qin & Kostić, 1994). The reactions in eqs 2 and 7 showed a very similar dependence on the solution viscosity and virtually identical rate constants for the rate-limiting rearrangement. Evidently, this rearrangement does not require multiple patches on the surface of the electron-accepting protein; the broad acidic patch is sufficient to allow for the protein rearrangement. This comparison shows that this dynamic process is a configurational fluctuation of the diprotein complex, a motion in which the two proteins remain docked in the same general orientation but wiggle with respect to each other. Finally, a theoretical analysis of electron-transfer paths between the heme and blue copper sites in various configurations of the cyt(II)/pc(II) electrostatic complex confirmed the experimental findings (Ullmann & Kostić, 1995). The configuration that optimizes the surface interactions does not optimize the heme−copper electronic coupling. Motions of the cytochrome *c* molecule within or near the acidic patch enhance the electronic coupling, and this improvement in inherent reactivity apparently drives the configurational fluctuation.

Previous studies answered some, but not nearly all, questions concerning the choice of surface patches that plastocyanin uses in reactions with other redox proteins.

Scheme 1: Photoinduced Electron-Transfer Reactions



Since both patches apparently are used, the question arises whether both of them can be used simultaneously, i.e., whether one molecule of plastocyanin can interact with two other protein molecules. The problem of ternary complexes of metalloproteins has been studied in this laboratory since 1991. Because, however, association of three molecules is statistically unlikely, in previous studies from this laboratory (Zhou & Kostić, 1992b; Brothers et al., 1993) covalent diprotein complexes were treated with single proteins (eqs 8−10). Noninvasive cross-links (designated with a double

$$\text{cyt(III)//pc(II)} + \text{cyt(II)} \rightarrow \text{cyt(III)//pc(I)} + \text{cyt(III)} \quad (8)$$

$$\text{cyt(II)//pc(II)} + \text{pc(II)} \rightarrow \text{cyt(III)//pc(II)} + \text{pc(I)} \quad (9)$$

$$^{3}\text{Zncyt//pc(I)} + \text{pc(II)} \rightarrow \text{Zncyt}^{+}\text{//pc(I)} + \text{pc(I)} \quad (10)$$

slash mark) ensured that a molecule of cytochrome *c* (with iron or zinc in the heme) remained attached to the acidic patch of plastocyanin during the bimolecular reaction of the covalent complex with another metalloprotein. Although these studies dealt with binary systems, their results shed some light on the elusive ternary complexes of metalloproteins. Very recently, Hoffman, Zhou, and co-workers obtained direct evidence for association of cytochrome *c* peroxidase with two molecules of cytochrome *c* (Stemp & Hoffman, 1993; Zhou & Hoffman, 1993, 1994). A ternary complex might be involved in the physiological function of these proteins.

Of the aforementioned reactions only the one in eq 8 pertains to the question whether a molecule of plastocyanin can simultaneously interact with two other protein molecules. This thermal reaction had to be initiated by external oxidation or reduction of the protein reactants, in a way that complicated the kinetic experiments and their interpretation. The present study concerns the reaction in eq 11, which was

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

initiated simply by a laser flash; see Scheme 1. Since the

acidic patch of plastocyanin is permanently occupied by ferricytochrome *c*, zinc cytochrome *c* cannot interact with this patch. The hydrophobic patch remains available, and the question is whether it is used for the electron-transfer reaction in eq 11.

MATERIALS AND METHODS

Proteins. Horse-heart cytochrome *c* was the so-called type VI from Sigma Chemical Co. The free-base form of this protein was prepared and reconstituted with zinc(II) by standard procedures as quickly as possible (Vanderkooi & Erecińska, 1975; Vanderkooi et al., 1976; Anni et al., 1995), and zinc cytochrome *c* was always handled in the dark. French-bean plastocyanin was isolated by standard methods (Milne & Wells, 1970) and purified repeatedly by gel-filtration chromatography on Sephadex G-25 and G-75 columns and by anion-exchange chromatography on a Sephadex DEAE A-25 column until the absorbence quotient A_{278}/A_{597} became less than 1.20.

The covalent complex cyt//pc was prepared by a published procedure (Zhou et al., 1992). Four fractions were separated on a CM-52 column, and their isoelectric points were determined with a Bio-Rad horizontal electrophoresis cell. Only the first fraction was used in this study. Copper was removed from the covalent complex by a method developed for plastocyanin alone (McMillin et al., 1974). To 0.40 mL of a buffered solution that was 1.0 mM in cyt//pc and 30 mM in ascorbic acid was added 3.60 mL of a 100 mM solution of thiourea in a 100 mM sodium phosphate buffer at pH 4.4. The resulting solution was dialyzed in a tubing against this same buffer at pH 4.4 under nitrogen, at 4 °C. After the copper was fully extracted, thiourea was removed by dialysis against a 100 mM sodium phosphate buffer at pH 7.0. The covalent complex containing apoplastocyanin is designated cyt//apopc.

All the experiments were done with distilled water that was further demineralized and purified to a resistance greater than 15 MΩ cm. All the common chemicals were of reagent grade. Circular dichroism spectra were recorded with a JASCO J-710 instrument, in the range from 200 to 470 nm.

Kinetics. Sodium phosphate buffer had pH of 7.0 and ionic strength of 5.00 mM. Ionic strength was raised up to 1.00 M by adding NaCl and lowered down to 1.25 mM by dilution. Laser flash photolysis on the microsecond scale was done with a standard apparatus (Zhou & Kostić, 1991a,b, 1992b, 1993a,b). The sample solution in a 10-mm cuvette was thoroughly deaerated by gentle flushing with ultrapure argon supplied by Air Products Co. Special precautions were taken at low ionic strengths. To avoid denaturation, the protein solution was slowly stirred while wet argon was blown over the surface.

A Phase-R (now Lumenex) DL 1100 laser contained a 50 μM solution of rhodamine 590 in methanol and delivered 0.4-μs pulses of excitation light. The monitoring beam from a tungsten−halogen lamp (Oriel 60065) passed through a filter monochromator (Oriel 7155) and was perpendicular to the excitation beam. The absorbance−time curves were obtained and analyzed with kinetic software from OLIS, Inc.

Disappearance of the triplet state of zinc cytochrome *c*, designated ^{3}Zncyt, was monitored at 460 nm, where the transient absorbance reaches a maximum. After each laser pulse 500 data points were collected, and each signal was

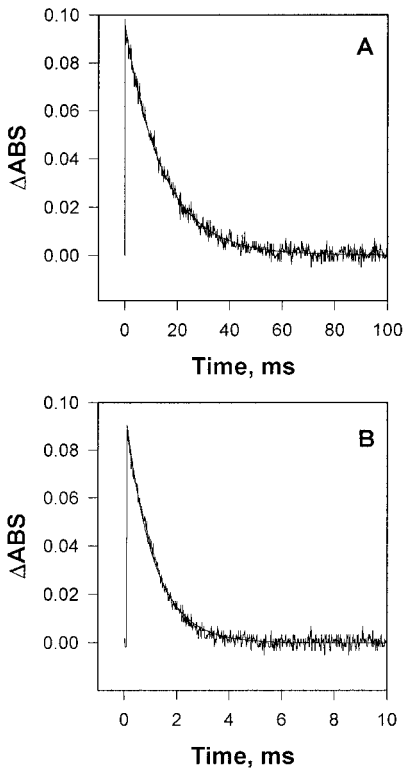


FIGURE 1: (A) Disappearance of the triplet state ^{3}Zncyt, monitored at 460 nm, in a solution containing 10 μM Zncyt in a sodium phosphate buffer at pH 7.0 and at ionic strength of 15 mM, at 25 °C. (B) As in part A, but with 15 mM covalent complex cyt(III)// pc(II). In each plot the solid line is a single-exponential fit.

an average of four pulses. Appearance and disappearance of the cation radical, designated Zncyt$^+$, were monitored at 675 nm, where the difference in absorbance between the cation radical and the triplet state is greatest.

The concentration of zinc cytochrome *c* was always 10.0 μM. The concentrations of ^{3}Zncyt and of Zncyt$^+$ depended on the excitation power. In this study the concentration of ^{3}Zncyt was 0.30−1.0 μM, and the concentration of the covalent complex cyt//pc was varied from 5.0 to 80 μM. At concentrations higher than 80 μM, the solution becomes difficult to deaerate and insufficiently transparent to the monitoring light beam. The concentration of the triplet state was always less than one-tenth the concentration of the quencher, so that pseudo-first-order conditions always prevailed. Each pseudo-first-order rate constant is an average result from at least four experiments, all at 25 °C. Second-order rate constants at different ionic strengths were obtained from the corresponding pseudo-first-order rate constants by least-squares fittings.

Calculation of Dipole Moments. Atomic coordinates for horse-heart cytochrome *c* were obtained from its crystal structure (Bushnell et al., 1990), and the iron(II) ion was replaced by a zinc(II) ion. Atomic coordinates for six conceivable configurations of the covalent complex cyt(III)// pc(II) are those obtained by molecular modeling and a thorough search for favorable electrostatic interactions of the corresponding electrostatic complex (Roberts et al., 1991). Tuna-heart cytochrome *c* was replaced by its horse-heart congener by overlapping the two crystal structures, and poplar plastocyanin was replaced by its bean cogener by overlapping the crystal structure of the former and the solution structure of the latter, determined by NMR spec-

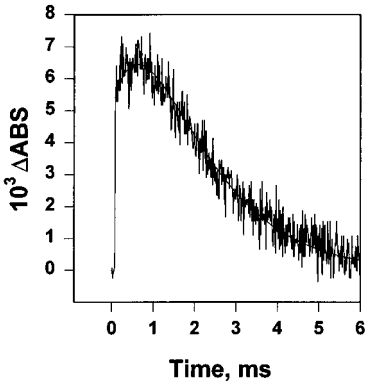


FIGURE 2: Appearance (eq 11) and disappearance (eq 18) of the cation radical Zncyt$^+$, monitored at 675 nm, in a solution specified in the caption to Figure 1. The solid line is a fit to both of these electron-transfer reactions.

troscopy (Moore et al., 1991). In both cases root mean square deviations of the atomic coordinates were minimized. The resulting six configurations of the diprotein complex were designated as in the original study (Roberts et al., 1991): maximum-overlap; maximum-overlap, rotated; northern equatorial; southern equatorial; maximum-charge; and minimum-distance. 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 and acetylated in cytochrome *c*, and that all carboxylic groups are deprotonated (Eltis et al., 1991; Zhou & Kostić, 1992b, 1993a). Partial charges were assigned to atoms (McCammon et al., 1979; Northrup et al., 1981). The net charge of the proteins and the magnitude and orientation of their dipole moments were calculated by an established method (Northrup et al., 1986).

Treatment of Electrostatic Interactions. The theory embodied in eq 12 recognizes not only net charges (Z) but also dipole moments (vectors **P** with magnitudes P) of the protein

$$\ln k = \ln k_{inf} - [Z_1Z_2 + Z\mathbf{P}(1 + \kappa R) + \mathbf{PP}(1 + \kappa R)^2]\frac{e^2}{4\pi\epsilon_0\epsilon k_B TR} f(\kappa) \quad (12)$$

molecules (van Leeuwen et al., 1981; van Leeuwen, 1983; Rush et al., 1987, 1988). The monopole−dipole (eq 13) and dipole−dipole (eq 14) interactions are anisotropic—they depend on the location of the reactive sites on the surfaces

$$Z\mathbf{P} = \frac{Z_1P_2\cos\theta_2 + Z_2P_1\cos\theta_1}{eR} \quad (13)$$

$$\mathbf{PP} = \frac{P_1P_2\cos\theta_1\cos\theta_2}{(eR)^2} \quad (14)$$

of the proteins with respect to the dipole vectors. The function of ionic strength is defined in eq 15. The new symbols in eqs 12−15 have the following meanings: θ is

$$f(\kappa) = \frac{1 - \exp(-2\kappa R)}{2\kappa R_2(1 + \kappa R_1)} \quad (15)$$

the angle between the dipole vector and the vector from the center of mass (cm) to the center of the reaction site on the surface; k_{inf} is the rate constant at infinite ionic strength; R is the sum of the radii of the two reacting proteins, $R = R_1 + R_2$; ϵ_0 is the permitivity constant; ϵ is the static dielectric constant; k_B is the Boltzmann constant; and e is elementary charge.

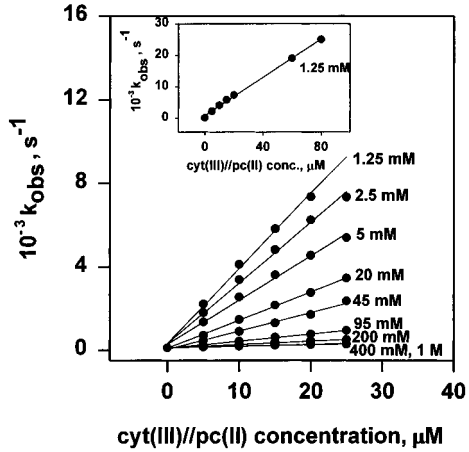


FIGURE 3: The observed rate constants for quenching of the triplet state ^{3}Zncyt by the covalent complex cyt(III)//pc(II) as in eq 11. The solutions contained 10 μM Zncyt and different concentrations of the quencher in sodium phosphate buffers at pH 7.0 and at the specified ionic strengths, at 25 °C. The rate constants in the inset were obtained over a particularly wide range of the quencher concentrations, at the lowest ionic strength.

In eqs 12−15, subscript 1 (Z_1, P_1, R_1, and θ_1) designates cyt//pc, and subscript 2 (Z_2, P_2, R_2, and θ_2) designates zinc cytochrome *c*. Estimated uncertainty in θ is the interval of its values over which the average difference between the best fitted and the experimentally determined values of k at all ionic strengths is 10% or less. Fitting to eq 12 was done with software Sigmaplot, marketed by Jandel Scientific.

RESULTS

Because all the kinetic experiments were done in sodium phosphate buffers at pH 7.0, only the ionic strength will be specified.

Nonredox Quenching of ^{3}Zncyt. In the absence of a quencher and in the presence of ferricytochrome *c*, the triplet state decays exponentially, with the rate constant of 110 ± 10 s^{-1}; see Figure 1A. This value is independent of ionic strength in the entire range studied, from 1.25 mM to 1.00 M. In the presence of 10 μM cyt(III)//apopc, in a 2.5 mM buffer, the decay is still exponential, with the rate constant of 180 ± 10 s^{-1}. In experiments with monitoring at 675 nm, the cation radical Zncyt$^+$ was not observed.

Redox Quenching of ^{3}Zncyt. In the presence of cyt(III)//pc(II) the decay of the triplet state became much faster but remained exponential; see Figure 1B. The cation radical Zncyt$^+$ was clearly evident; see Figure 2. The pseudo-first-order rate constant is linearly proportional to the concentration of the quencher, cyt(III)//pc(II), at all ionic strengths; see Figure 3. The second-order rate constants, obtained from the slopes of the plots in Figure 3, decreased as the ionic strength increased; see Table 1.

Circular Dichroism Spectra. Molecular ellipticities (in degrees) at the wavelengths (in nm), recorded with solutions in a 2.5 mM buffer at 25 °C, were as follows. For the electrostatic complex cyt(III)/pc(II): −3.65, 17.76, and −9.63 deg at 222, 406, and 420 nm, respectively. For the covalent complex cyt(III)//pc(II): −2.57, 16.00, and −9.63 deg at 221, 407, and 420 nm, respectively.

Table 1: Dependence on Ionic Strength of the Second-Order Rate Constants[a] for Oxidative Quenching of ^{3}Zncyt by Cupriplastocyanin That Is Free[b] and Cross-Linked to Ferricytochrome *c*

	$10^{-6}k$/M^{-1} s^{-1} at 25 °C and pH 7.0			$10^{-6}k$/M^{-1} s^{-1} at 25 °C and pH 7.0	
μ/mM	pc(II), eq 4	cyt(III)//pc(II), eq 11	μ/mM	pc(II), eq 4	cyt(III)//pc(II), eq 11
1.25		380	80	150	
2.5		290	95		33
5.0		220	100	130	
20		130	200	40	16
40	700		400		8.5
45		84	500	14	
50	310		1000	9.4	7.5
60	230				

[a] Estimated error, ±10%. [b] Zhou and Kostić (1993a).

Dipole Moments. The dipole moment of zinc cytochrome *c* in which one of the propionic groups in the heme is deprotonated (Moore & Pettigrew, 1990) is 250 D. The dipole vector forms an angle of 30° with the vector from the center of mass to the iron atom. The positive end of the dipole moment penetrates the protein surface near the carbonyl carbon atom of Ile81, and the negative end penetrates it near the δ_2 carbon atom of Phe36. The exposed heme edge is located at 20−40° with respect to the positive end of the dipole moment (Zhou & Kostić, 1993a). Very similar results were obtained by different calculational methods (Koppenol et al., 1991).

The covalent complex cyt(III)//pc(II) in the so-called maximum-overlap configuration and with both propionic acid groups in the heme deprotonated has a calculated net charge of −1.5 at pH 7.0 and a dipole moment of 490 D. The negative end of the dipole vector penetrates the protein surface near the γ-carbon atom of His37 in the plastocyanin moiety, and the positive end penetrates the surface near ring B of the heme in the cytochrome *c* moiety; see Figure 4.

DISCUSSION

Zinc Cytochrome c. Replacement of iron(II) by zinc(II) does not perturb the conformation of cytochrome *c* and its association with other proteins (Vanderkooi & Erecińska, 1975; Vanderkooi et al., 1976; Moore et al., 1980; Anni et al., 1995). This replacement renders the triplet state of the heme long-lived and suitable for kinetic studies. The net charge of the protein at pH 7.0 is +7 if one of the propionic groups in the heme is deprotonated (Moore & Pettigrew, 1990; Mathews, 1985). Since the change in the oxidation state of iron does not significantly alter the dipole moment of native cytochrome *c* (Koppenol & Margoliash, 1982), neither should the electronic excitation of the heme. Therefore, the dipole moment of ^{3}Zncyt was calculated with the atomic coordinates of ferrocytochrome *c*.

The Covalent Complex Cyt(III)//Pc(II). Because ferricytochrome *c* and cupriplastocyanin bear opposite net charges, and because they contain oppositely-charged surface patches, these two proteins associate in solution at low ionic strength. Much evidence showed that the positively-charged patch around the exposed heme edge docks against the broad negatively-charged patch in plastocyanin (King et al., 1985; Bagby et al., 1990; Roberts et al., 1991). This configuration is noninvasively reinforced by covalent cross-linking in the presence of a carbodiimide (Geren et al., 1983). Besides

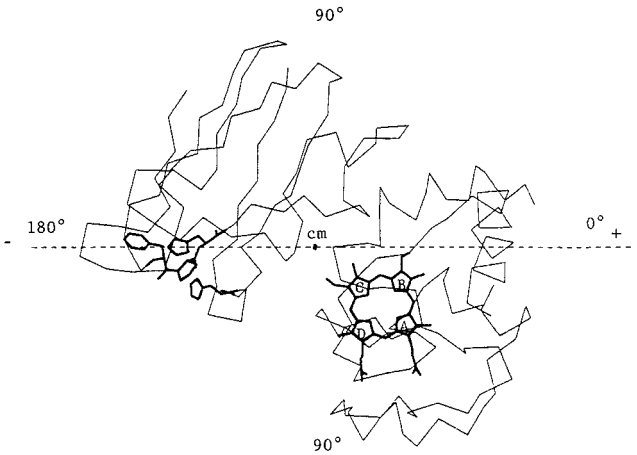


FIGURE 4: The backbones (α-carbon atoms) of cytochrome *c* and plastocyanin in the so-called maximum-overlap configuration of the covalent complex cyt(III)//pc(II), according to the atomic coordinates from Roberts et al. (1991). The heme group of the cytochrome *c* moiety and the Phe35, Pro36, His37, and His87 of the plastocyanin moiety are highlighted. The calculated dipole moment is shown as a dashed line. The angle θ_1 is defined with respect to the positive end of the dipole moment; four reference values of θ_1 are shown.

efficiently cross-linking the proteins, the carbodiimide converts certain carboxylate anions into neutral *N*-acylurea groups (Means & Feeney, 1971; Carraway & Koshland, 1972; Timkovich, 1977). Cation-exchange chromatography therefore yields several derivatives of the covalent complex cyt(III)//pc(II) (Peerey & Kostić, 1989; Peerey et al., 1991; Zhou et al., 1992). Analysis of chromatographic mobility and of UV−vis, EPR, CD, MCD, resonance Raman, and surface-enhanced resonance Raman spectra of these derivatives showed that they differ somewhat in the distribution of surface charge but not in the protein−protein orientation (Zhou et al., 1992). The new experiments show a good match, in both the UV and visible regions, between the CD spectra of the electrostatic and covalent complexes. This evidence further supports the conclusion that the two complexes have the same general configuration, i.e., that cross-links reinforce the electrostatic complex that forms spontaneously in solution at low ionic strength. Since a thorough theoretical analysis (Roberts et al., 1991) found the so-called maximum-overlap configuration to be optimal, it is reasonable to assign this configuration also to the covalent complex.

Each covalent cross-link abolishes a unit of positive charge (of a lysine residue) in cytochrome *c* and a unit of negative charge (of a carboxylate group) in plastocyanin. In the absence of *N*-acylurea groups, therefore, the net charge at pH 7.0 of the cyt(III)//pc(II) complex should be 0 or −1, the sum of the charges of the constituent proteins. These charges are +8 or +7 for ferricytochrome *c*, if one or both propionic groups are deprotonated, and −8 for cupriplastocyanin. Kinetic experiments were done with the first fraction from the chromatographic column, which has the p*I* value of 6.2 and which evidently bears a small negative charge at pH 7.0. This result agrees with the expected charge of −1 in the absence of *N*-acylurea groups. It is virtually impossible to know whether this net charge is caused by deprotonation of both propionic acid groups in the heme or by perturbations of some side chains and consequent charges in the pK_a values upon protein association. For practical

reasons we had to assume the former in our calculations of the dipole moment. Even if this untestable assumption were incorrect, the error in the dipole moment would be relatively small.

Nonredox Quenching of ^{3}Zncyt. Our rate constant of 100 ± 10 s^{-1} for the natural decay of the triplet state of zinc cytochrome *c* falls in the middle of a narrow range, 70−140 s^{-1}, spanned by the values reported by others (Elias et al., 1988; Vos et al., 1987; Dixit et al., 1981, 1984; Horie et al., 1985). The slight variation in the rate constant, perhaps caused by small differences in the protein preparation and experimental conditions, does not affect the following kinetic arguments and conclusions. The decay rate (i.e., the lifetime) of the triplet state is unaffected by the presence of ferricytochrome *c*. Because two molecules of cytochrome *c* (the zinc and the ferric forms) bear large positive charges, collision of the two, and consequent quenching, are unlikely.

Although the covalent complex cyt(III)//apopc has a charge of −3 at pH 7.0, it does not appreciably quench the triplet state; the value of 180 ± 10 s^{-1} is less than 2 times greater than the rate constant for natural decay. Since the cation radical Zncyt$^+$ was not detected, the slight quenching is not caused by electron transfer. It is probably due to enhancement of the radiationless decay upon electrostatic association of ^{3}Zncyt with cyt(III)//apopc. Since, however, the exposed heme edge is covered by apoplastocyanin in the diprotein complex, association does not result in a detectable electron-transfer reaction. Very similar enhancements of radiationless decay were found earlier in this laboratory (Zhou & Kostić, 1992b). Clearly, redox quenching of ^{3}Zncyt by ferricytochrome *c* is too slow to compete with the natural decay.

Redox Quenching of ^{3}Zncyt by Cyt(III)//Pc(II). The experiments discussed in the preceding subsection prove that the reaction in eq 16 does not occur to an observable extent. Previous studies in this laboratory showed that neither does the reaction in eq 17 (Peerey & Kostić, 1989; Peerey et al., 1991). Evidently, it is the cupriplastocyanin moiety of the covalent complex that quenches ^{3}Zncyt, as shown in eq 11. This "forward" electron transfer and the "back" electron transfer, shown in eq 18, occur simultaneously. The kinetic profile of the cation Zncyt$^+$, Figure 2, is affected by both of these reactions, and the solid line is a fitting to both of them (Zhou & Kostić, 1991a). The same rate constant for the forward reaction (eq 11) is obtained from the disappearance of ^{3}Zncyt (Figure 1) and from the appearance of Zncyt$^+$ (Figure 2, after the initial sharp increase in absorbance owing to the flash and the ^{3}Zncyt). The time scale in Figure 2 is somewhat shorter than that in Figure 1B because Zncyt$^+$ is consumed by the back-reaction, shown in eq 18. In the absence of this back-reaction the two figures could be given with the same time scale.

$$\text{cyt(III)//pc(II)} + {}^3\text{Zncyt} \nrightarrow \text{cyt(II)//pc(II)} + \text{Zncyt}^+ \quad (16)$$

$$\text{cyt(II)//pc(II)} \nrightarrow \text{cyt(III)//pc(I)} \quad (17)$$

$$\text{cyt(III)//pc(I)} + \text{Zncyt}^+ \rightarrow \text{cyt(III)//pc(II)} + \text{Zncyt} \quad (18)$$

Kinetics of the Electron-Transfer Reaction. The linear plots in Figure 3 show that the reaction in eq 11 is bimolecular. As the inset in Figure 3 shows, no curvature is seen even at the very low ionic strength and at the relatively high concentration of the covalent complex cyt(III)//pc(II). Although the two reactants in eq 11 must collide in order to react, their association is only transient. There is no saturation in the plots, i.e., no kinetic evidence for a persistent binary complex cyt(III)//pc(II)/Zncyt. This complex would resemble a ternary, noncovalent complex of plastocyanin with two molecules of cytochrome *c*.

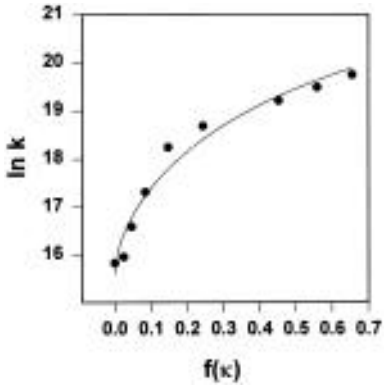


FIGURE 5: Dependence on ionic strength of the second-order rate constant for the reaction in eq 11; the numerical values are given in Table 1. The error margins are smaller than the symbols. The solid line is the best fit to eq 12, with the parameter $\theta_1 = 160°$.

The reaction between ^{3}Zncyt and free cupriplastocyanin at low ionic strength does involve the persistent binary complex, shown in eq 2 (Zhou & Kostić, 1991a, 1992a, 1993b). A molecule of cytochrome *c* covalently attached to the acidic patch shields this patch and almost neutralizes the negative charge of plastocyanin. Both of these effects hinder association of cyt(III)//pc(II) with zinc cytochrome *c*.

Electrostatic Interactions and Protein Orientation. As Table 1 shows, the rate constant for the reaction in eq 11 decreases 50-fold as the ionic strength increases from 1.25 mM to 1.00 M. As eq 12 shows, at very low ionic strength (as the quantity κ approaches zero), the monopole−monopole forces dominate the electrostatic interactions. The net charge of Zncyt was kept at $Z_2 = +7$. Fitting of the rate constants at the ionic strengths of 1.25, 2.5, and 5.0 mM yielded an estimate of $Z_1 = -1.3 \pm 0.2$ for the net charge of the covalent complex cyt(III)//pc(II). This approximate result is consistent with the measured isoelectric point of 6.2 for this complex.

The complete set of nine rate constants in Table 1 could be fitted to eq 12 assuming that the covalent complet cyt(III)//pc(II) has either the maximum-overlap or southern-equatorial configuration. Fittings to the other four configurations (Roberts et al., 1991) were unsuccessful in various degrees. These two configurations have similar structures and very similar electrostatic stabilization energies (Roberts et al., 1991). Fitting to the former configuration was better than that to the latter, and this better fitting is shown in Figure 5. The following parameters were known: $P_1 = 498$ D, $R_1 = 20.6$ Å, and $Z_1 = -1.5$ for cyt(III)//pc(II); and $P_2 = 250$ D, $R_2 = 15.5$ Å, $Z_2 = +7.0$, and $\theta_2 = 30°$ for zinc cytochrome *c*. The only variable parameter was θ_1, the angle between the dipole moment of cyt(III)//pc(II) and the vector from the center of mass to the center of the reaction site on the surface of this diprotein complex; see Figure 4. The fitting in Figure 5 yielded the result $\theta_1 = 160°$ with respect to the positive (or 20° with respect to the negative) end of the dipole moment. Fitting to the southern-equatorial configuration yielded the result $\theta_1 = 168°$. The centers of

the rings in Phe35, Pro36, and His37 in the plastocyanin moiety have the respective θ_1 values of 170°, 160°, and 160°. Clearly, the reaction site in the cyt(III)//pc(II) complex is the hydrophobic patch of the plastocyanin moiety.[2] The same conclusion is reached regardless of the exact configuration of the covalent complex. These two configurations are similar, as mentioned above. When the acidic patch is blocked, even the reactant such as zinc cytochrome *c*, which bears a high positive charge, reacts at the hydrophobic patch.

This conclusion was obtained by applying the established theory in eq 12 to the reliable rate constants in Table 1. Clearly, this is chemical evidence for the spatial orientation of the proteins reacting according to eq 11. Given the complexity of the system under investigation, no evidence can be direct and conclusive and neither is ours.

Electron-Transfer Reactivity at the Hydrophobic Patch in Plastocyanin. A number of studies addressed the docking interactions and redox reactions of transition-metal complexes and of other "small" reagents with the acidic and hydrophobic patches in plastocyanin (Sykes, 1985, 1991a,b). Relatively little, however, is known about the involvement of the hydrophobic patch in reactions between plastocyanin and other redox proteins. An early study (Rush et al., 1988) led to a conclusion that ferrocytochrome *c* could react at the hydrophobic patch but not at the acidic patch. When computational findings about protein *docking* were applied to the interprotein electron-transfer *reaction*, it seemed that ferrocytochrome *c* reacts from the optimal docking site in the acidic patch (Roberts et al., 1991). Studies from this laboratory cited above showed, however, that the triplet state of zinc cytochrome *c*, and possibly also ferrocytochrome *c*, reduces the blue copper site from the broad acidic patch or from its periphery toward the hydrophobic patch, but not from the initial docking site within this broad patch.

A couple of theoretical studies have recently addressed the question of electron transfer via the two patches on the plastocyanin surface. In a semiquantitative analysis of tunneling paths, the two patches were not explicitly compared (Betts et al., 1992). Extended Hückel calculations of superexchange pathways indicated that the electron-transfer "channel" via Tyr83, in the acidic patch, is almost as efficient as the channel via His87, in the hydrophobic patch (Christensen et al., 1990, 1991). On the basis of tunneling distances through space, the hydrophobic channel is expected to be approximately 1500 times more efficient than the acidic one, whereas on the basis of discrete paths through bonds the acidic channel is expected to be approximately 3000 times more efficient than the hydrophobic one (Lowery et al., 1993). Experiments with small redox reagents, however, led to a conclusion that the two channels are approximately equally efficient, with the respective rates of electron transfer differing not more than approximately 10-fold (Sykes, 1985, 1991b; Christensen et al., 1991). The apparent contradiction between the theoretical and experimental estimates disappears when the anisotropy of electronic coupling between the copper atom and its amino acid ligands in plastocyanin is taken into account (Lowery et al., 1993; Ullmann & Kostić, 1995). The present kinetic study provides experimental evidence for this new theoretical notion.

Because zinc cytochrome *c* and cupriplastocyanin associate at low ionic strength, the electron-transfer reaction between them is predominantly unimolecular, as in eq 2 (Zhou & Kostić, 1991a,b, 1992a, 1993b). This is why there are no second-order rate constants at ionic strengths lower than 40 mM in the pc(II) column in Table 1. At intermediate ionic strengths, at which the monopole−monopole interactions are weak, the covalent complex, which bears a small net negative charge, is only about 3−5 times less effective in quenching the triplet state of zinc cytochrome *c* than free cupriplastocyanin, which bears a large negative charge. At very high ionic strengths, at which the electrostatic interactions are practically abolished, the two quenchers are approximately equally effective in their reactions with ^{3}Zncyt, shown in eqs 4 and 11. Under these conditions the reactivity is governed by intrinsic electron "conductivity" of the two quenchers. Evidently, the acidic patch (in free cupriplastocyanin, eq 4) and the hydrophobic patch (in the covalent complex, eq 11) are similar in the efficiency with which they transmit electrons into the blue copper site.

This conclusion from the comparison of the free plastocyanin and the covalent complex can reasonably be applied to plastocyanin per se. The reactions in eqs 1−7 involve the acidic patch (although not the initial docking site in that broad patch) because of the electrostatic attractions, not because of the existence of particularly favorable paths for electron transfer between the acidic patch and the copper site.

The plots in Figure 3 are linear even at an ionic strength as low as 1.25 mM and at a quencher concentration as high as 80 μM. The absence of curvature shows the absence of persistent association of the reactants in eq 11. This electron-transfer reaction, therefore, involves only the transient encounter, as shown in Scheme 1. The highest pseudo-first-order rate constant measurable was 2.5×10^4 s^{-1}, and this value is only a lower limit to the rate constant for the intracomplex reaction designated "electron transfer" in Scheme 1.

Plastocyanin versus Cytochrome c Peroxidase. There is no evidence for persistent association between the two reactants in eq 11 and Scheme 1. Since a molecule of plastocyanin evidently cannot "hold" two molecules of cytochrome *c* (one containing iron and the other zinc) even when one of these two is held by cross-links, we conclude that three separate molecules will not form a genuine ternary complex in solution under the conditions used in this study. Plastocyanin has only one (albeit broad) acidic patch, and this protein is too small to allow simultaneous binding of two other protein molecules comparable to it in size. There is, however, evidence that a molecule of cytochrome *c* peroxidase binds two molecules of cytochrome *c* (Kang et al., 1977; Stemp & Hoffman, 1993; Zhou & Hoffman, 1993, 1994), although this notion is still controversial (Pelletier & Kraut, 1992). Since a molecule of cytochrome *c* peroxidase is relatively large and possesses at least two surface domains with an appreciable negative electrostatic potential (Northrup et al., 1988), the electrostatic attraction for two molecules of cytochrome *c* and the electrostatic and steric repulsions between these two molecules may be balanced in a way that allows the existence of a ternary complex.

[2] Fitting to eq 12 of the rate constants for the reaction in eq 4 was done (Zhou & Kostić, 1993a) with the following known parameters for cupriplastocyanin: $P_1 = 362$ D, $R_1 = 15.5$ Å, and $Z_1 = -8$; these values, of course, differ from those used in the present study. The parameters for zinc cytochrome *c* were the same as in this study. The reaction site in free cupriplastocyanin had the value $\theta_1 = 36°$, which corresponds to the acidic patch (Zhou & Kostić, 1993a).

Biological electron-transport chains likely involve association of more than two redox proteins, and most redox enzymes consist of multiple subunits. A systematic investigation of ternary complexes (Stemp & Hoffman, 1993; Zhou & Hoffman, 1993, 1994) and of systems that resemble them (Zhou & Kostić, 1992b; Brothers et al., 1993) will contribute to a better understanding of the biological processes and of chemical electron-transfer mechanisms.

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REFERENCES

Anni, H., Vanderkooi, J. M., & Mayne, L. (1995) *Biochemistry 34*, 5744.
Bagby, S., Driscoll, P. C., Goodall, K. G., Redfield, C., & Hill, H. A. O. (1990) *Eur. J. Biochem. 188*, 413.
Betts, J. N., Beratan, D. N., & Onuchic, J. N. (1992) *J. Am. Chem. Soc. 114*, 4043.
Brothers, H. M., II, Zhou, J. S., & Kostić, N. M. (1993) *J. Inorg. Organomet. Polym. 3*, 59.
Bushnell, G. W., Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 214*, 585.
Carraway, K. L., & Koshland, D. E. (1972) *Methods Enzymol. 25*, 616.
Chapman, S. K. (1991) *Perspect. Bioinorg. Chem. 1*, 95.
Christensen, H. E. M., Conrad, L. S., Mikkelsen, K. V., Nielsen, M. K., & Ulstrup, J. (1990) *Inorg. Chem. 29*, 2808.
Christensen, H. E. M., Conrad, L. S., Ulstrup, J., & Mikkelsen, K. V. (1991) *Met. Ions Biol. Syst. 27*, 57.
Dixit, B. P. S. N., Moy, V. T., & Vanderkooi, J. M. (1984) *Biochemistry 23*, 2103.
Dixit, S. N., Waring, A. J., & Vanderkooi, J. M. (1981) *FEBS Lett. 125*, 86.
Elias, H., Chou, M. H., & Winkler, J. R. (1988) *J. Am. Chem. Soc. 110*, 429.
Eltis, L. D., Herbert, R. G., Barker, P. D., Mauk, A. G., & Northrup, S. H. (1991) *Biochemistry 30*, 3663.
Geren, L. M., Stonehuerner, J., Davis, D. J., & Millett, F. (1983) *Biochim. Biophys. Acta 724*, 62.
Haehnel, W., Jansen, T., Gause, K., Klösgen, R. B., Stahl, B., Michl, D., Huvermann, B., Karas, M., & Herrmann, R. G. (1994) *EMBO J. 13*, 1028.
Hoffman, B. M., Natan, M. J., Nocek, J. M., & Wallin, S. A. (1991) *Struct. Bond. 75*, 86.
Horie, T., Maniara, G., & Vanderkooi, J. M. (1985) *FEBS Lett. 177*, 287.
Kang, C. H., Ferguson-Miller, S., & Margoliash, E. (1977) *J. Biol. Chem. 252*, 919.
King, G. C., Binstead, R. A., & Wright, P. E. (1985) *Biochim. Biophys. Acta 806*, 262.
Koppenol, W. H., & Margoliash, E. (1982) *J. Biol. Chem. 257*, 4426.
Koppenol, W. H., Rush, J. D., Mills, J. D., & Margoliash, E. (1991) *Mol. Biol. Evol. 8*, 545.
Kostić, N. M. (1991) *Met. Ions Biol. Syst. 27*, 129.
Lowery, M. D., Guckert, J. A., Gebhard, M. S., & Solomon, E. I. (1993) *J. Am. Chem. Soc. 115*, 3012.
Mathews, F. S. (1985) *Prog. Biophys. Mol. Biol. 45*, 1.
Mauk, A. G. (1991) *Struct. Bond. 75*, 131.
McCammon, J. A., Wolynes, P. G., & Karplus, M. (1979) *Biochemistry 18*, 927.
McLendon, G. (1991a) *Struct. Bond. 75*, 160.
McLendon, G. (1991b) *Met. Ions Biol. Syst. 27*, 183.
McLendon, G., & Hake, R. (1992) *Chem. Rev. 92*, 481.
McMillin, D. R., Rosenberg, R. C., & Gray, H. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4760.
Means, G. E., & Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden-Day, San Francisco, CA, Sections 7-2 and A-4.
Milne, P. R., & Wells, J. R. E. (1970) *J. Biol. Chem. 245*, 1566.
Modi, S., He, S., Gray, J. C., & Bendall, D. S. (1992a) *Biochim. Biophys. Acta 1101*, 64.
Modi, S., Nordling, M., Lundberg, L. G., Hansson, Ö, & Bendall, D. S. (1992b) *Biochim. Biophys. Acta 1102*, 85.
Moore, G. R., & Pettigrew, G. W. (1990) *Cytochromes c: Evolutionary, Structural and Physiochemical Aspects*, Springer-Verlag, Berlin.
Moore, G., Williams, R. J. P., Chien, J. C. W., & Dickinson, L. C. (1980) *J. Inorg. Biochem. 12*, 1.
Moore, J. M., Lepre, C. A., Gippert, G. P., Chazin, W. J., Case, D. A., & Wright, P. E. (1991) *J. Mol. Biol. 221*, 533.
Northrup, S. H., Pear, M. R., Morgan, J. D., McCammon, J. A., & Karplus, M. (1981) *J. Mol. Biol. 153*, 1087.
Northrup, S. H., Reynolds, J. C. L., Miller, C. M., Forrest, K. J., & Boles, J. O. (1986) *J. Am. Chem. Soc. 108*, 8162.
Northrup, S. H., Boles, J. O., & Reynolds, J. C. L. (1988) *Science 241*, 67.
Peerey, L. M., & Kostić, N. M. (1989) *Biochemistry 28*, 1861.
Peerey, L. M., Brothers, H. M., II, Hazzard, J. T., & Kostić, N. M. (1991) *Biochemistry 30*, 9297.
Pelletier, H., & Kraut, J. (1992) *Science 258*, 1748.
Pettigrew, G. W., & Moore, G. R. (1987) *Cytochromes c: Biological Aspects*, Springer-Verlag, Berlin.
Qin, L., & Kostić, N. M. (1992) *Biochemistry 31*, 5145.
Qin, L., & Kostić, N. M. (1993) *Biochemistry 32*, 6073.
Qin, L., & Kostić, N. M. (1994) *Biochemistry 33*, 12592.
Redinbo, M. R., Yeates, T. O., & Merchant, S. (1994) *J. Bioenerg. Biomembr. 26*, 49.
Roberts, V. A., Freeman, H. C., Getzoff, E. D., Olson, A. J., & Tainer, J. A. (1991) *J. Biol. Chem. 266*, 13431.
Rush, J. D., Lan, J., & Koppenol, W. H. (1987) *J. Am. Chem. Soc. 109*, 2679.
Rush, J. D., Levine, F., & Koppenol, W. H. (1988) *Biochemistry 27*, 5876.
Stemp, E. D. A., & Hoffman, B. M. (1993) *Biochemistry 32*, 10848.
Sykes, A. G. (1985) *Chem. Soc. Rev. 14*, 283.
Sykes, A. G. (1991a) *Adv. Inorg. Chem. 36*, 377.
Sykes, A. G. (1991b) *Struct. Bond. 75*, 177.
Therien, M. J., Chang, J., Raphael, A. L., Bowler, B. E., & Gray, H. B. (1991) *Struct. Bond. 75*, 110.
Timkovich, R. (1977) *Anal. Biochem. 79*, 135.
Ullmann, G. M., & Kostić, N. M. (1995) *J. Am. Chem. Soc. 117*, 4766.
Vanderkooi, J. M., & Erecińska, M. (1975) *Eur. J. Biochem. 60*, 199.
Vanderkooi, J. M., Adar, F., & Erecińska, M. (1976) *Eur. J. Biochem. 64*, 381.
van Leeuwen, J. W. (1983) *Biochim. Biophys. Acta 743*, 408.
van Leeuwen, J. W., Mofers, F. J. M., & Veerman, E. C. I. (1981) *Biochim. Biophys. Acta 635*, 434.
Vos, K., Lavalette, D., & Visser, A. J. W. G. (1987) *Eur. J. Biochem. 169*, 269.
Winkler, J. R., & Gray, H. B. (1992) *Chem. Rev. 92*, 369.
Zhou, J. S., & Kostić, N. M. (1991a) *J. Am. Chem. Soc. 113*, 6067.
Zhou, J. S., & Kostić, N. M. (1991b) *J. Am. Chem. Soc. 113*, 7040.
Zhou, J. S., & Kostić, N. M. (1992a) *J. Am. Chem. Soc. 114*, 3562.
Zhou, J. S., & Kostić, N. M. (1992b) *Biochemistry 31*, 7543.
Zhou, J. S., & Hoffman, B. M. (1993) *J. Am. Chem. Soc. 115*, 11008.
Zhou, J. S., & Kostić, N. M. (1993a) *Biochemistry 32*, 4539.
Zhou, J. S., & Kostić, N. M. (1993b) *J. Am. Chem. Soc. 115*, 10796.
Zhou, J. S., & Hoffman, B. M. (1994) *Science 265*, 1693.
Zhou, J. S., Brothers, H. M., II, Neddersen, J. P., Cotton, T. M., Peerey, L. M., & Kostić, N. M. (1992) *Bioconjugate Chem. 3*, 382.

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