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Interactions and electron-transfer reactions between metalloproteins

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Interactions and electron-transfer reactions between metalloproteins

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Signature was redacted for privacy.

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For the Major Program

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For the Graduate College

To my parents Miroslav and Nada, and to my brother Dušan

Посвећено мојим родитељима Мирославу и Нади, и мом брату Душану

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CHAPTER 1. GENERAL INTRODUCTION

General Overview

Electron-transfer reactions are essential for life. They provide a way to harvest the energy of the Sun and present the energy to living beings by transforming the chemical energy into heat or work. Electron-transfer reactions, though are the simplest of chemical reactions since they do not involve making or breaking of bonds, are still poorly understood.

How metalloproteins, as electron carriers in different biological processes, recognize, interact, and react with each other began to emerge in recent years.[1-4] A pair of proteins can form multiple complexes in solution and orientation that is optimal for recognition need not be optimal for electron transfer.[5-8] Though the number of structures of electron-transfer proteins being solved in last two decades have increased significantly, the structures of protein complexes are still scarce.[9,10]

We use theory and experiments to develop a physical picture of all the factors that are responsible for recognition, interaction, and reactivity of electron-transfer proteins. The heme protein cytochrome *c*[11-13] and the blue-copper protein plastocyanin,[14-16] designated cyt and pc, are well suited for kinetic and mechanistic studies since their three-dimensional structures are known. The structure of the binding complex, in which proteins associate, and reactive complex, in which proteins react, are not known. This laid the ground work for our research.

We study the effects of ionic strength, viscosity, site-directed mutations, reaction free energy, and pH on the association and reactivity of cytochrome *c* and plastocyanin protein complex. This thesis represents what we have discovered about the interactions and reactivity of these two electron-transfer proteins.

Dissertation Organization

This dissertation consists of systematic studies to understand the factors influencing association and reactivity of cytochrome *c* and plastocyanin. Chapter 2 explores the effects of site-directed mutations and viscosity on the rate-limiting rearrangement and identifies the reactive configuration of the cytochrome *c*/plastocyanin complex. The reactive configuration is in between the acidic and the hydrophobic patch of plastocyanin. Chapter 3 surveys the reactivity of the zinc cytochrome *c* cation radical. The reorganization energy and the rate constant for electron self-exchange of the cation radical show that the iron and zinc forms of the same protein, cytochrome *c*, differ markedly in their properties as redox agents. In chapter 4 we present the extended proton-linkage model to treat the protein association. We use interplay of experiments and calculations to obtain the protonation constants for amino acid side chains in plastocyanin and cytochrome *c*. Chapter 5 deals with the effects of pH on protein rearrangement. The rearrangement is independent of pH giving us information about the protonation states of amino acid residues in the complex of plastocyanin and cytochrome *c*.

Chapters 2-5 represent papers that either have been or will be published in peer refereed journals. The dissertation ends with Chapter 6, which is a presentation of the overall conclusions.

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CHAPTER 2. EFFECTS OF MUTATIONS IN PLASTOCYANIN ON THE KINETICS OF THE PROTEIN REARRANGEMENT GATING THE ELECTRON-TRANSFER REACTION WITH ZINC CYTOCHROME *C*. ANALYSIS OF THE REARRANGEMENT PATHWAY

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All kinetic experiments, interpretation of results, and molecular modeling were done by the primary author except for kinetic experiments with changing viscosity.

Abstract

We study, by flash kinetic spectrophotometry on the microsecond time scale, the effects of ionic strength and viscosity on the kinetics of oxidative quenching of the triplet state of zinc cytochrome *c*, ^{3}Zncyt, by the wild-type form and the following nine mutants of cupriplastocyanin: Leu12Glu, Leu12Asn, Phe35Tyr, Gln88Glu, Tyr83Phe, Tyr83His, Asp42Asn, Glu43Asn, and the double mutant Glu59Lys/Glu60Gln. The unimolecular rate constants for the quenching reactions within the persistent diprotein complex, which predominates at low ionic strength, and within the transient diprotein complex, which is involved at higher ionic strength, are equal irrespective of the mutation. Evidently, the two complexes are the same. In both reactions the rate-limiting step is rearrangement of the diprotein complex from a configuration optimal for docking to the one optimal for the subsequent electron-transfer step, which is fast. We investigate the effects of plastocyanin mutations on this rearrangement, which gates the overall electron-transfer reaction.

Conversion of the carboxylate anions into amide groups in the lower acidic cluster (residues nos. 42 and 43), replacement of Tyr 83 with other aromatic residues, and mutations in the hydrophobic patch in plastocyanin do not significantly affect the rearrangement. Conversion of a pair of carboxylate anions into a cationic and a neutral residue in the upper acidic cluster (residues nos. 59 and 60) impedes the rearrangement. Creation of an anion at the position no. 88, between the upper acidic cluster and the hydrophobic patch, facilitates the rearrangement. All of these kinetic effects and noneffects of mutations consistently indicate that in the protein rearrangement the basic patch of zinc cytochrome *c* may move from a position between the two acidic clusters to a position at or near the upper acidic cluster. The rate constant for the rearrangement smoothly decreases as the solution viscosity increases, irrespective of the mutation. Fittings of this dependence to the modified Kramers's equation and to an empirical equation show that zinc cytochrome *c* follows the same trajectory on the surfaces of all the plastocyanin mutants, but that the obstacles along the way vary as mutations alter the electrostatic potential.

Introduction

Electron-transfer reactions of metalloproteins are involved in photosynthesis, respiration, and many other biological processes. Chemical research into molecular mechanisms of these important reactions is best done with well-characterized proteins and their pairs (Hoffman et al., 1991; Mauk, 1991; Pelletier & Kraut, 1992; Chen et al., 1992, 1994; McLendon, 1991a,b; McLendon & Hake, 1992; Zhou & Hoffman, 1994; Zhou et al., 1995; Therien et al., 1991; Winkler & Gray, 1992; Kostić, 1991). The heme protein cytochrome *c* (Pettigrew & Moore, 1987; Moore & Pettigrew, 1990; Scott & Mauk, 1996)

and the blue copper protein plastocyanin (Redinbo et al., 1994; Gross, 1993; Sykes, 1991a,b), designated cyt and pc,[1] are well suited to quantitative studies because their three-dimensional structures in both oxidized and reduced states and in both crystal and solution are precisely known.

A pair of metalloproteins can associate in multiple configurations (Wendoloski et al., 1987; Northrup et al., 1988; Rodgers et al., 1988; Burch et al., 1990; Wallin et al., 1991; Roberts et al., 1991; Nocek et al., 1991; Willie et al., 1992; McLendon et al., 1993; Harris et al., 1993; Mauk et al., 1994; Zhou & Hoffman, 1994). A configuration that optimizes binding need not optimize the subsequent electron-transfer reaction. The rate of this reaction within the complex may be controlled by the rate of some structural change; in this case the redox reaction is said to be gated (Hoffman & Ratner, 1987, 1988; Brunschwig & Sutin, 1989; Hoffman et al., 1990; Nocek et al., 1991; Feitelson & McLendon, 1991; Walker & Tollin, 1992; Sullivan et al., 1992). The phenomenon of gating is common with proteins and is found in various biochemical processes.

In the chemical equations below the slash mark represents protein association, i.e., the diprotein complex. The Roman numerals are the oxidation states of iron and copper. In zinc cytochrome *c* the oxidation state of zinc is always II, and an electron is given and accepted by the porphyrin ring.

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[1] Abbreviations: cyt, cytochrome *c*; cyt(III), ferricytochrome *c* ; cyt(II), ferrocytohrome *c*; pc, plastocyanin; pc(II), cupriplastocyanin; pc(I), cuproplastocyanin; Zncyt, zinc cytochrome *c*; ^{3}Zncyt, triplet (excited) state of zinc cytochrome *c*; Zncyt$^+$, cation radical of zinc cytochrome *c*.

Because zinc cytochrome *c* and the wild-type cupriplastocyanin bear respective net charges of +6 and -8 at pH 7.0, and because they contain oppositely-charged surface patches, these two proteins associate in solution at low ionic strength. Much evidence shows that in the complexes cyt/pc the basic (positive) patch around the exposed heme edge abuts the broad acidic (negative) patch in plastocyanin (King et al., 1985; Bagby et al., 1990; Roberts et al., 1991; Zhou et al., 1992).

Studies in our laboratories and by others of the unimolecular reaction in eq 1 (Peerey & Kostić, 1989; Peerey et al., 1991; Meyer et al., 1993) and of the bimolecular reaction in eq 2 (Modi et al, 1992a) showed that ferrocytochrome *c* reduces cupriplastocyanin from the acidic patch, but not from the initial binding site within this large patch. Similar conclusions were reached in studies of reactions analogous to those in eqs 1 and 2, but involving ferrocytochrome *f* instead of ferrocytochrome *c* (Qin & Kostić, 1992, 1993; Modi et al., 1992b).

Kinetic studies of thermal reactions, which involve the proteins in their ground electronic states, are relatively complicated. The reaction in eq 1 has to be initiated by external reduction of the complex cyt(III)/pc(II), and the reaction in eq 2 involves both protein association and subsequent electron transfer. Replacement of iron(II) with zinc(II) in the heme does not significantly perturb the surface of cytochrome *c* and its interactions with other proteins (Ye et al., 1996; Angiolillo & Vanderkooi, 1995; Anni et al., 1995). Use of zinc cytochrome *c* in the studies of photoinduced reactions, those in eqs 3-6, obviates the need for external reducing agents and permits detailed studies of the most interesting step in the reactions — electron transfer within the diprotein complex (Zhou & Kostić, 1991a,b,

1992a,b,c, 1993a,b; Qin & Kostić, 1994, 1996; Kostić, 1996; Ivković-Jensen & Kostić, 1996). The reactions in eqs 3 and 5, in which the triplet state of the porphyrin is the electron donor, are termed forward reactions. Those in eqs 4 and 6, in which the cation radical of the porphyrin is the electron acceptor, are termed back reactions. The thermal reactions in eqs 1 and 2 have the driving force of only ca. 0.10 eV and are true redox reactions; the rate-limiting step in them is electron transfer. Raising the driving force assists the electron transfer but does not affect the structural dynamics of the proteins. The photoinduced forward reactions in eqs 3 and 5 have the driving force of ca. 1.2 eV; in them the protein rearrangement is the

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

$$\text{cyt(II)} + \text{pc(II)} \longrightarrow \text{cyt(III)} + \text{pc(I)} \quad (2)$$

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

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

$$^{3}\text{Zncyt} + \text{pc(II)} \longrightarrow \text{Zncyt}^{+} + \text{pc(I)} \quad (5)$$

$$\text{Zncyt}^{+} + \text{pc(I)} \longrightarrow \text{Zncyt} + \text{pc(II)} \quad (6)$$

rate-limiting step, the one actually observed in kinetic experiments. In conclusion, the reactions in eqs 3 and 5 are gated.

Kinetic studies (Zhou & Kostić, 1992a, 1993b; Qin & Kostić, 1994; Ivković-Jensen & Kostić, 1996) began to reveal the interplay between the structural rearrangement and the electron transfer. The gating process seems to be configurational fluctuation of the diprotein complex, during which the two proteins remain docked in the same general orientation but slide on each other's surface or wiggle with respect to each other. A theoretical analysis by an established method (Onuchic et al., 1992) of electron-transfer paths between the heme and blue copper sites in various configurations of the cyt(II)/pc(II) complex confirmed the

experimental findings by showing that the configuration that optimizes the surface interactions does not optimize the heme-copper electronic coupling (Ullmann & Kostić, 1995). Motions of the cytochrome *c* molecule, whose basic patch explores the area within or near the broad acidic patch in plastocyanin, enhance this electronic coupling. In this way configurational fluctuation improves the intrinsic electron-transfer reactivity. Analysis of enthalpy of activation ($\Delta H^{\neq}$) for the reaction in eq 3 in terms of solvation effects (Ivković-Jensen & Kostić, 1996) answered some but not nearly all the questions concerning the dynamic process of gating. All the previous studies of the reactions in eqs 3-6 have been done with wild-type plastocyanin. Now we report a systematic comparison of the wild-type form and nine mutants of plastocyanin in the reaction in eq 3 at different ionic strengths and viscosities. Analysis of kinetic results reveals a likely trajectory for the cytochrome *c* motion on the plastocyanin surface.

Materials and Methods

Chemicals. Distilled water was demineralized to a resistivity greater than 17 MΩ•cm. Chromatography resins and gels were purhcased from Sigma Chemical Co. and Pharmaica. Nitrogen, HF, and ultrapure argon were purchased from Air Products Co. All other chemicals were purchased from Fisher Chemical Co.

Buffers. All buffers were made fresh from the solid salts $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7\ H_2O$ and had ionic strength (μ) of 2.5 or 10 mM and pH of 7.00 ± 0.05 at 293 K. Ionic strength was raised from 10 to 100 mM by addition of NaCl. In all of these preparations we took into consideration dependence of the pK_a of $H_2PO_4^-$ on ionic strength.

A computer program for calculating buffer compositions is given in the Supporting Information.

Temperature and Viscosity. Temperature was kept at 20.0 ± 0.2 °C with a 30-L circulating bath Forma Scientific CH/P 2067. Viscosity was adjusted by adding glycerol to the buffered solution, up to the concentration of 80% w/w.

Zinc Cytochrome *c*. Horse-heart cytochrome *c* was purchased from Sigma Chemical Co. The iron-free (so-called free-base) form was made, purified, and reconstituted with zinc(II) by a modification (Ye et al., 1996) of the original procedure (Vanderkooi & Ericińska, 1975; Vanderkooi et al., 1976). The product, zinc cytochrome *c*, was handled at 4 °C, in the dark. Two of the criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$. The absorptivity is $\varepsilon_{423} = 2.43 \times 10^5\ M^{-1}cm^{-1}$ (Vanderkooi et al., 1976).

Plastocyanin. Recombinant wild-type protein and nine mutants were prepared by the previously published method for overexpression in *E. coli* (Nordling et al., 1990; Sigfridson et al., 1995, 1996), with the expression vector pUG101t_r (Nordling et al., 1991). The polymerase chain reaction and its modifications were reported before (Landt et al., 1990; Nordling et al., 1991). The protein was chromatographically purified first with a DE32 column and then with a 26/10 Q Sephasose high-performance FPLC column by Pharmacia. The blue fraction was concentrated by dialysis against dry polyethyleneglycol (PEG 2000) and passed through a gel-filtration column Sephacryl S-100 HR. The amount of holo-plastocyanin was determined spectrophotometrically, under oxidizing conditions, on the basis of the absorptivity $\varepsilon_{597} = 4900\ M^{-1}cm^{-1}$ (Katoh et al., 1962).

Only the fractions of the highest purity were used in this study. Despite the most careful handling, the mutants Leu12Glu, Leu12Asn, and Tyr83His irreversibly lost a fraction of the copper(II) ions. Solutions in a 100 mM Tris buffer were made 100 and 150 μM in these mutants, 100 and 200 μM in $CuSO_4$, and 150 mM in NaCl. Incubation overnight and repeated chromatography, as described above, temporarily lowered the absorbance quotient A_{280}/A_{597} from 1.8-2.0 to 1.5-1.6; the quotient increased later. Therefore, the presence of some of apoplastocyanin in these three mutants had to be tolerated. The absorbance ratios of the other six mutants were 1.4 or less.

Flash Kinetic Spectrophotometry. So-called laser flash photolysis at the resolution of one point per μs was done with a standard apparatus (Zhou & Kostić, 1991a,b, 1992b, 1993a,b). The triplet state ^{3}Zncyt was created by 0.4-μs pulses of light from a Phase-R (now Luminex) DL1100 laser containing the dye Rhodamine 590. The concentration of zinc cytochrome *c* was always 10 μM. Appearance and disappearance of ^{3}Zncyt and Zncyt$^+$ were monitored at 460 and 675 nm, respectively.

Concentration of the triplet state depended on the intensity of the laser pulse and was ca. 1.0 μM, much lower than the cupriplastocyanin concentration, which was adjusted to 2.5, 5.0, 7.5, 10, 15, 20, 25, 30, 40, and 50 μM. Kinetic conditions for the pseudo-first order were thus achieved. The protein solutions, prepared with deaerated buffers, were thoroughly deaerated further in the stream of wet argon, without frothing, for 10 min after each addition of plastocyanin. At each set of conditions (cupriplastocyanin concentration, ionic strength, and viscosity) multiple traces were recorded: six at the ionic strength of 100 mM and ten at

the ionic strengths of 10 and 2.5 mM. The change of absorbance with time was analyzed with the software SigmaPlot v1.02, from Jandel Scientific, Inc.

Kinetic Effects of Viscosity. Because we are interested in the effects of solution viscosity on the unimolecular component of quenching, the reaction in eq 3, we did these experiments in the sodium phophate buffer at the low ionic strength of 2.5 mM and with the high cupriplastocyanin concentration of 50 μM. The relative viscosity ($\eta/\eta o$) of the buffered solution was adjusted with glycerol (CRC Handbook). These experiments with the wild-type form and mutants of plastocyanin were done like the previous experiments with the wild-type form only (Zhou & Kostić, 1993b). Given $\eta_o = 1.002$ cp for water at 25 °C and the fact that the buffered solutions were dilute, the relative viscosity is practically equal to the absolute viscosity (η).

Fittings of Data. Least-squares averaging, with SigmaPlot v1.02, of the results from separate fittings of kinetic traces obtained by successive flashes gave better results that fittings of averaged traces. The former method lessens the undue influence of so-called outliers on the average result. The correlation coefficient of the rate constant was greater than 0.990. The error margins for all results include two standard deviations and correspond to the confidence limit greater than 95%; they are rounded to one significant figure, for clarity.

Molecular Modeling and Graphics. Structures of cytochrome *c* (Takano & Dickerson, 1981) and plastocyanin (Guss & Freeman, 1983) were taken from the crystallographic studies. The three configurations of the diprotein complex were those designated maximum-overlap (max ov); maximum-overlap, rotated (max ov rot); and

northern equatorial (n/eq) in the original study (Roberts et al., 1991) and afterwards (Ullmann & Kostić, 1995). For the sake of consistency, we retain these designations and abbreviations. The program package QUANTA 4.0 was used on a SiliconGraphics workstation IRIS 4D. Protein structures were drawn with the program MolScript v1.3 (Kraulis, 1991).

Results

Natural Decay of the Triplet State, ^{3}Zncyt. The rate constant for this monoexponential process is 120 ± 10, 100 ± 10, and 80 ± 10 s^{-1} at the ionic strengths of 2.5, 10, and 100 mM, respectively. See Supporting Information, Figure S1. When the buffer at the ionic strength of 2.5 mM is made 80% w/w in glycerol, the rate constant decreases to 75 ± 10 s^{-1}. When the rate constant ceased to decrease during the passing of argon, the sample solution was considered deaerated.

Quenching of ^{3}Zncyt by Cupriplastocyanin. For a typical experiment showing this quenching, see Supporting Information, Figure S2. The mechanism is shown in Scheme 1. The subscripts in the symbols for the intracomplex rate constants k_F (for the unimolecular reaction) and k_f (for the bimolecular reaction) are reminders that both of these are so-called forward reactions, which are defined above. The two subscripts are not identical because the persistent complex, which exists at low ionic strength, and the transient complex, which is involved at higher ionic strength, are not necessarily identical. We retain these symbols from our previous publications, for the sake of consistency.

At the ionic strength of 100 mM the overall quenching is monoexponential, i.e., the reaction is purely bimolecular (Zhou & Kostić, 1991a). At the intermediate, but already low, ionic strength of 10 mM the quenching by the wild-type form and by seven of the mutants is

biexponential. Quenching by Asp42Asn and by the double mutant Glu59Lys/Glu60Gln remains monoexponential. The only component of quenching by these two mutants, and the slower component in the case of the wild-type form and seven other mutants, corresponds to the bimolecular reaction in Scheme 1. The faster component, which is evident with the latter eight but not with the former two quenchers, corresponds to the unimolecular reaction in Scheme 1. The relative amplitude of the bimolecular component decreases from 100 to less than 20%, while that of the unimolecular component increases from 0 to ca. 70%, as the cupriplastocyanin concentration is raised from 2.5 to 40 μM. These approximate values are averages for all the mutants.

We succeeded in observing directly the unimolecular component of the quenching (k_F) by all the mutants when we lowered the ionic strength to 2.5 mM. The overall quenching is practically biexponential for the wild-type form and the following six mutants: Phe35Tyr, Gln88Glu, Asp42Asn, Glu43Asn, Tyr83Phe, and the double mutant Glu59Lys/Glu60Gln. Representative kinetic data for the bimolecular component of quenching are shown in Figure 1 and in Supporting Information, Figure S2. The quenching is triexponential for the following three mutants: Leu2Glu, Leu12Asn, and Tyr83His. Representative kinetic data are shown in Supporting Information, Figure S3. The third phase was accepted or rejected on the basis of the standard deviation and the statistical null hypothesis with a confidence level of 95%. The relative amplitudes varied with cupriplastocyanin concentration as they did at the ionic strength of 10 mM, except that the unimolecular component dominated the overall quenching when the quencher concentration was 40 μM.

The Slowest Component of Quenching and the Magnitude of Transient Absorbance. The slowest component, the third phase, was prominent for the mutants Leu12Glu, Leu12Asn, and Tyr83His, only at the ionic strength of 2.5 mM. When this component was observed, its rate constant was more than 3000 times higher than that for the natural decay and ca. ten times lower than that for the bimolecular component. As Figure S4 in the Supporting Information shows, this component resembles the bimolecular component of quenching. Its amplitude (contribution to the total transient absorbance) is negligible at the lowest concentrations of cupriplastocyanin but becomes a major (ca. 40%) or even dominant (ca. 60%, in the case of Leu12Glu) fraction of the total amplitude at the higher concentrations of cupriplastocyanin.

Kinetic experiments at the ionic strength of 2.5 mM gave identical results when performed in cuvettes made of quartz and of polystyrene. Evidently, the third phase of quenching is not due to adsoption of proteins to the quartz surface.

We estimated the concentration of apoplastocyanin in the samples of the mutants. We set $\varepsilon_{597} = 4{,}900\ M^{-1}cm^{-1}$ for all the mutants (Katoh et al., 1962; Sigfridsson et al., 1995, 1996) and calculated the expected absorbance at 280 nm for those mutants that differ from the wild-type protein in aromatic residues (Gill & Von Hippel, 1989). The calculated absorbance quotients A_{597}/A_{280} for each mutant agreed nicely with the lowest value recorded during the purification; see Supporting Information, Table S1. The amount of apoplastocyanin correlates well with the amplitude of the third phase.

In most experiments the magnitude of the signal (the transient absorbance of ^{3}Zncyt at 460 nm) was in the range 0.080-0.14. Given the noise level of ca. 0.005 absorbance units,

these signals allowed for good precision and relaible fittings. The three aformentioned mutants that contained large fractions of apoproteins presented a problem, however. At the ionic strength of 2.5 mM, upon each addition of these mutants the transient absorbance became smaller. It decreased to ca. 0.020, the lowest value measurable with acceptable accuracy, before the mutant concentration increased to 40 μM. The problem became easier when the ionic strength was raised 10 mM and dissappeared at the ionic strength of 100 mM. Fortunately, at all ionic strengths the absorbance remained constant upon repeated flashing; as many as 30 traces were recorded. This finding is correct, because the back reactions in eqs 4 and 6 regenerate zinc cytochrome *c*. Since we have fewer than nine k_{obs} values for each of these three mutants at the ionic strengths of 2.5 and 10 mM, fittings concerning the bimolecular component could not be done well. Therefore three k_f values are missing in Table 1. This was not a serious limitation because the intracomplex rate constants for all the forms of plastocyanin were determined directly, as k_F for the unimolecular component of quenching. The k_f values for the remaining seven forms nicely agree with the corresponding k_F values.

Kinetic Effects of Viscosity. As the representative findings in Figure 2 show, the intramolecular rate constant k_F smoothly decreases and levels off as the solution viscosity increases.

Discussion

Plastocyanin Mutants. The structure of poplar plastocyanin, determined by crystallography (Guss & Freeman, 1983), closely resembles the structure of the bean protein in solution, determined by nmr spectroscopy (Moore et al., 1991). The acidic patch of

carboxylate groups consists of two clusters, on either side of Tyr83. The lower cluster, residues 42-45, is larger than the upper cluster, residues 59-61. The hydrophobic patch, made up mostly of nonpolar residues, surrounds His87, a ligand to the copper atom. These structual features are shown schematically in Figure 3.

Before this study, plastocyanin mutants were used to investigate reactions with the two physiological partners of this protein. Mutations in the hydrophobic patch (Gly10, Leu12, and Ala90) hinder the electron transfer to photosystem I (Haehnel et al., 1994; Sigfridsson et al., 1996), whereas the mutation Asp42Asn apparently hinders the association but not the subsequent electron-transfer step (Sigfridsson et al., 1996). It is accepted that cuproplastocyanin uses its acidic patch for recognition of, and its hydrophobic patch for transfering an electron to, photosystem I (Haehnel et al., 1994). Conversion into amides of certain carboxylate anions in the acidic patch hinders electron transfer from cytochrome *c* and cytochrome *f*, as in eq 2, but conversion of others has no significant effect (Modi et al., 1992b; Lee et al., 1995; Sigfridsson et al., 1996). The mutants Tyr83Phe and Tyr83Leu resemble the wild-type cupriplastocyanin in their reactions with ferrocytochrome *f* and ferrocytochrome *c*, respectively (Modi et al., 1992a; He et al., 1991). The reaction with ferrocytochrome *c* is only partially analyzed, and this study is a contribution to its full understanding. Even though the two proteins are not physiological partners, the mechanism of their reaction is interesting because it shows the essence of gating.

We work with the nine mutants listed in Table 1. They have been characterized by UV-vis spectrophotometry, EPR spectroscopy, and isoelectric focusing; their redox potentials have been determined; and they have been used in previous kinetic studies with proteins

other than cytochrome *c* (Kyritsis et al., 1993; Sigfridsson et al., 1996). Nonpolar, neutral Leu12 was changed into two polar residues — the neutral Asn and the anionic Glu. The nonpolar, conserved Phe35 was changed into the somewhat polar Tyr. The mutation Gln88Glu introduced a negative charge between the hydrophobic and acidic patches. The anionic residues Asp42 and Glu43 in the acidic patch were neutralized by conversion into the amides. In the double mutation the pair of anions Glu59 and Glu60 were converted into a cation and a neutral residue, Lys and Gln. The prominent residue Tyr83, which separates the two acidic clusters, was replaced with two aromatic residues — the nonpolar Phe and the polar His. All of the aforementioned estimates of charge at pH 7.0 are based on assumptions that the side chains under consideration have normal pK_a values.

Mechanism of Quenching and the Rate Constants. Previous studies in this laboratory (Zhou & Kostić, 1991a, 1993b) with the natural plastocyanin gave much evidence for redox quenching of ^{3}Zncyt, that is, for Scheme 1. Systematic experiments at ten ionic strengths spanning the interval 2.5 mM to 3.00 M showed that at $\mu \leq 10$ mM the reaction can be made to occur mostly by a unimolecular mechanism, within the persistent complex, whereas at $\mu > 40$ mM the reaction occurs solely by a bimolecular mechanism, within the transient complex. Equality of the corresponding rate constants, $k_F = (2.5 \pm 0.4) \times 10^5$ s^{-1} and $k_f = (2.8 \pm 0.6) \times 10^5$ s^{-1}, was an early evidence that, in the case of wild-type plastocyanin, the two complexes are the same or that they rearrange into another complex common to both reaction pathways in Scheme 1. Equality of the corresponding activation parameters $\Delta H^{\neq}$ (13 ± 2 and 13 ± 1 kJ/mol) and $\Delta S^{\neq}$ (-97 ± 4 and -96 ± 3 J/Kmol) is firm evidence that the two complexes are the same, i.e., that the wild-type plastocyanin associates

with zinc cytochrome *c* similarly at different ionic strengths (Ivković-Jensen & Kostić, 1996). In both complexes, however, electron transfer is gated by a rearrangement, which was quantitatively studied by analyzing the dependence of k_F on solution viscosity (Zhou & Kostić, 1992a, 1993b). The aforementioned intracomplex rate constant actually corresponds to the rate-limiting rearrangement process; the electron-transfer step is faster than that and is not directly observed.

In this study, working with the recombinant protein, we reproduced the previous results for the wild-type plastocyanin. Reassured by this reproducibility, we compared the reactivity of the nine mutants. The ionic strengths of 100, 10, and 2.5 mM brought out the relevant mechanistic features in Scheme 1. The rate constant k_F was observed directly. The other rate constants were obtained from fittings to eq 7, which is derived from the so-called improved steady-state approximation (Espenson, 1995) and which accounts for both the equilibrium step (k_{on} and k_{off}) and the electon-transfer step (k_f). The quencher concentration, designated [pc(II)], is less than the total concentration, designated $[pc(II)]_o$, because of association with zinc cytochrome *c*; see eq 8.

$$k_{obs} = \frac{k_{on}\, k_f\, [pc(II)]}{k_{off} + k_f + k_{on}[pc(II)]} \tag{7}$$

$$[pc(II)] = [pc(II)]_o - \frac{1}{2}\left\{[Zncyt]_o + [pc(II)]_o + \frac{k_{off}}{k_{on}} - \sqrt{\left([Zncyt]_o + [pc(II)]_o + \frac{k_{off}}{k_{on}}\right)^2 - 4[Zncyt]_o[pc(II)]_o}\right\} \tag{8}$$

At μ = 100 mM quenching of ^{3}Zncyt by all the forms of plastocyanin is monoexponential. The plots of k_{obs} vs. $[pc(II)]_o$ are linear up to the quencher concentration

of 40 μM. Evidently, only the bimolecular mechanism in Scheme 1 operates. At μ = 10 mM the quenching reactions are biexponential for all the forms of plastocyanin except the mutants Asp42Asn and Glu59Lys/Glu60Gln, which associate to zinc cytochrome *c* relatively weakly and therefore do not show the unimolecular mechanism in Scheme 1. At μ = 2.5 mM the quenching is biexponential for all the forms of plastocyanin; both the bimolecular and the unimolecular mechanisms in Scheme 1 operate. Most important, the rate constants for the same reaction determined at different ionic strengths are equal, within the error margins.

We explained above the third phase in the quenching reaction, found with only three mutants and only at the ionic strength of 2.5 mM. Since apoplastocyanin is redox-inactive and incapable of directly quenching ^{3}Zncyt (Zhou & Kostić, 1991a), its effect must be indirect. It competes with the quencher, cupriplastocyanin, for association with zinc cytochrome *c*. The reactive species, ^{3}Zncyt, must dissociate from apoplastocyanin and reassociate with cupriplastocyanin. We took into account the concentration of apoplastocyanin in fittings of the experimental data to eqs 7 and 8. Since this protein is colorless, association constants for it and zinc cytochrome *c* could not be determined. We had to assume that these constants are the same as for the holo-forms of the mutants in question.

Kinetic Effects of Viscosity. One of the solvent effects is to modulate protein motion. We know of only several prior studies of protein reactions in which viscosity was varied (Gavish & Werber, 1979; Beece et al., 1980; Khoshtariya et al., 1991; Ansari et al., 1992; Nocek et al., 1991). Studies from this laboratory (Zhou & Kostić, 1992a, 1993b; Qin & Kostić, 1994) showed that buffered mixtures of water and several viscous liquids, glycerol

among them, do not perturb the spectroscopic and photophysical properties of zinc cytochrome *c* and plastocyanin. These studies also showed that the smooth dependences of the kind shown in Figure 2 and Table 2 are caused by changes in viscosity, not in other properties of the solution.

Analysis of the Viscosity Effects. Acccording to Kramers's theory (Kramers, 1940), the rate of crossing a diffusive barrier in a unimolecular reaction is inversely proportional to viscous friction. A configurational change of a diprotein complex depends on the friction of the proteins with each other and with the solvent. In the empirical eq 9 the two frictions are considered additive. The constant *c* has units of frequency, σ is protein friction and has units of viscosity, η is solvent viscosity, and *E* is the barrier separating the configurations of the diprotein complex. Combination of eq 9 with eq 10 from the theory of transition states yields eq 11 for the modified Kramers's theory. The term $(1 + \eta)$ *RT* corresponds to the constant *c* in eq 9. Because the empirical eq 12 proved useful in a previous study (Qin & Kostić, 1994), we use it again.

The parameter δ defines the dependence of the rearrangement rate on the solution viscosity. It is related, but not equal, to the protein friction (σ) in eq 9. Fitting of the rate constant (k_F) and the viscosity (η) to either eq 11 or eq 12 gave the free energy of activation for the rearrangement ($\Delta G^{\ddagger}$) and the friction parameter σ or δ.

$$k_F = \frac{c}{\sigma + \eta} \exp\left(\frac{-E}{RT}\right) \tag{9}$$

$$k = \frac{k_B T}{h} \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right) \tag{10}$$

$$k_F = \frac{k_B T}{h} \propto \frac{1 + \eta}{\alpha + \eta} \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right) \tag{11}$$

$$k_F = \frac{k_B T}{h}\ \eta^{-\delta} \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right) \qquad (12)$$

The two fittings gave the results in Table 2, which are consistent with each other and with those in Table 1. The wild-type plastocyanin and all but one of the single mutants behave alike. The higher value of $\Delta G^{\neq}$ for the double mutant can be attributed to electrostatic repulsion between the basic patch in cytochrome *c* and the pair of residues in the upper cluster whose combined charge was reversed from -2 in the wild-type protein to +1 in the double mutant. The relatively small variation of the friction parameters σ and δ among the various forms of plastocyanin indicates that solution viscosity similarly affects configurational dynamics of diprotein complexes containing these various forms. In other words, zinc cytochrome *c* follows more or less the same trajectory on the surfaces of all the plastocyanin mutants, but the obstacles along the way vary as mutations alter the electrostatic potential. This finding justifies the following analysis of the rearrangement pathways in terms of the rate constants.

Possible Pathways of Rearrangement. A systematic search for the best match of the electrostatic fields of cytochrome *c* and plastocyanin and for the strongest electrostatic attraction yielded five families of stabilized configurations, which have similar electrostatic energies (Roberts et al., 1991). Of these five only three provide relatively good paths for electon tunneling from the heme to the copper site (Ullmann & Kostić, 1995). For the sake of consistency, we retain the original (Roberts et al., 1991) designations and symbols for these configurations. The so-called maximum overlap (max ov) configuration allows for optimal docking, whereas the so-called maximum-overlap, rotated (max ov rot) and northern

equatorial (n/eq) configurations provide more efficient electron-transfer paths. With different parametrizations in the theoretical analyses, either of the latter two configurations emerged as the best; the max ov configuration never did (Ullmann & Kostić, 1995). As Figure 4 shows, rearrangements of the first configuration into the second and the third amounts to rotation of the cytochrome *c* molecule by ca 180° or its gliding on the plastocyanin surface toward, or beyond, the upper edge of the acidic patch. We describe these fluctuations as configurational, rather that conformational, because both protein molecules are treated as rigid bodies.

In the initial configuration (max ov) the exposed heme edge and the cationic lysine residues around it abut the two acidic clusters. During the rotation by ca. 180°, which yields the configuration max ov rot, this basic patch in cytochrome *c* remains in contact with the acidic patch as it moves over both the upper and the lower clusters in this patch. During the simultaneous translation and counterclockwise rotation by ca. 90°, which yields the configuration n/eq, the basic patch crosses over the upper cluster but not over the lower one as it approaches the residue no. 88.

Kinetics of the Rearrangement. As explained above, the rate constants in Table 1 pertain to the configurational fluctuation that is gating the faster electron-transfer reaction. Equation 13 shows the conversion of the initial (i) to the rearranged (r) configuration and subsequent electron transfer. The wild-type and mutant forms of plastocyanin can be compared on the basis of the k_F values, for the persistent complex in Scheme 1.

$$^3\text{Zncyt/pc(II)}_i \underset{}{\overset{k_F}{\rightleftharpoons}} {}^3\text{Zncyt/pc(II)}_r \xrightarrow{\text{ET}} \text{Zncyt}^+\text{/pc(I)} \qquad (13)$$

The k_f values, for the transient complex, nicely agree with them. Because the error margins (rounded to one digit) include two standard deviations on either side of the fitted value of k_{obs}, differences exceeding the margins are significant. The small effects of replacing Leu12 with other residues are most likely due to conformational perturbations of the active site, which are evident in changes of the redox potential and the EPR and UV-vis spectra of these mutants (Sigfridsson et al., 1996). Indeed, the replaced atoms of Leu12 in the wild-type protein approach the copper atom to 4 Å.

Only two mutants, Gln88Glu and the double mutant Glu59Lys/Glu60Gln, truly differ from the wild-type protein in the kinetics of rearrangement. In the analysis of these differences, we assume that mutation alters the energetics of rearrangement, but not the initial docking configuration and the rearrangement trajectory. This assumption is justified by the results in Figure 2 and Table 2. These results, which were discussed above, show that solution viscosity identically affects the complexes ^{3}Zncyt/pc(II) containing the wild-type form and all the mutants of plastocyanin.

The results in Table 1 clearly show that mutations in the lower cluster do not affect the rearrangement, whereas those in the upper cluster and at the position no. 88 do. Although the electrostatic changes in the lower cluster are smaller than those in the upper one, the kinetic results are precise enough and the pattern consistent enough to warrant our conclusion. Moreover, the direction of change agrees with the electrostatic considerations. Decreasing negative charge in the upper cluster weakens the attraction for the basic patch of cytochrome *c*; increasing positive charge in the upper cluster repels the basic patch; and introducing a negative charge at position no. 88 attracts the basic patch. Moreover, the

residue Gln88 is involved in the most efficient electron-tunneling path in the configuration n/eq (Ullmann & Kostić, 1995). Both the effects and the noneffects of mutations consistently point at the northern equatorial (n/eq) configuration, or one similar to it, as the reactive one.

Conclusion and Prospects

A recent analysis of electron-tunneling paths between the heme and the blue copper site indicated two likely pathways for the rearrangement of the diprotein complex cyt/pc from the configuration optimal for the docking *interaction* to the configuration optimal for the electron-transfer *reaction* (Ullmann & Kostić, 1995). This theoretical study guided us in the present experimental study. The effects of ionic strength and of viscosity on the protein rearrangement involving the wild-type form and nine mutants of cupriplastocyanin showed which of the two pathways is likely for this rate-limiting rearrangement of the complex ^{3}Zncyt/pc(II). On the basis of this study, we will design new plastocyanin mutants and explore in greater detail the dynamics of configurational fluctuations that gate the interprotein electron-transfer reaction.

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Scheme 1.

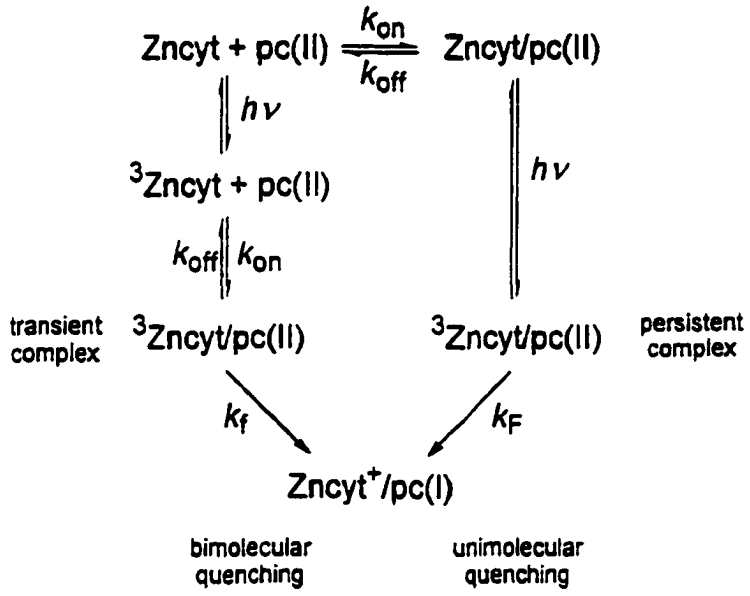

Table 1. Properties of recombinant plastocyanin

pc(II) mutant	A_{280}/A_{597} ratio			% Znpc	maximum amplitude of the third phase (%)
	calculated	lowest obtained in purification	final		
wild type	1.1	1.08	1.34	15	19
Leu12Glu	1.1	1.15	3.22	58	64
Leu12Asn	1.1	1.13	1.90	32	41
Phe35Tyr	1.5	1.41	1.83	16	23
Gln88Glu	1.1	1.08	1.38	18	22
Glu59Lys/ Glu60Gln	1.1	1.15	1.39	14	17
Tyr83Phe	0.8	0.78	1.06	21	26
Tyr83His	0.8	0.77	2.16	53	64
Asp42Asn	1.1	1.08	1.38	14	22
Glu43Gln	1.1	1.08	1.38	17	22

Table 2. Protein association and rearrangement of the diprotein complex ^{3}Zncyt/pe(II) from the docking configuration into the electron-transfer configuration[a]

surface location	pc(II) mutant	local charge[b] wild type	local charge[b] mutant	$10^{-9}k_{on}$ (M^{-1} s^{-1})	$10^{-3}k_{off}$ (s^{-1})	$10^{-5}K_a$ (M^{-1})	$10^{-5}k_f$ (s^{-1})	$10^{-5}k_r$ (s^{-1})
	wild type			100	3.3	300 ± 200	2.0 ± 0.4	2.1 ± 0.1
hydrophobic patch	Leu12Glu	0	−1					1.6 ± 0.2
	Leu12Asn	0	0					2.4 ± 0.5
	Phe35Tyr	0	0	41	4.2	100 ± 50	2.1 ± 0.3	2.0 ± 0.2
between the patches	Gln88Glu	0	−1	200	8.0	300 ± 200	3.1 ± 0.6	3.1 ± 0.3
upper acidic cluster	Glu59Lys/Glu60Gln	−2	+1	2.6	14	2 ± 1	0.18 ± 0.04	0.16 ± 0.02
between the clusters	Tyr83Phe	0	0	340	3.8	900 ± 500	2.1 ± 0.3	2.0 ± 0.2
	Tyr83His	0	0/+1					1.6 ± 0.4
lower acidic cluster	Asp42Asn	−1	0	110	28	40 ± 20	2.3 ± 0.3	2.4 ± 0.2
	Glu43Asn	−1	0	51	9.1	60 ± 30	2.0 ± 0.5	2.2 ± 0.3

[a] This rearrangement gates the electron-transfer reactions in eqs 3 and 5. The conditions were as follows: sodium phosphate buffer at pH 7.0 and an ionic strength of 2.5 mM at 293 K. [b] Assuming normal pK_a values.

Table 3. Fittings to two equations of the dependence on the solution viscosity of the rate constant k_F for the rearrangement of the diprotein complex ^{3}Zncyt/pc(II) from the docking configuration into the electron-transfer configuration[a]

	eq 11		eq 12	
mutant	$\Delta G^{\ddagger}$ (kJ/mol)	σ	$\Delta G^{\ddagger}$ (kJ/mol)	δ
wild type	43 ± 1	0.7 ± 0.2	43 ± 1	0.8 ± 0.1
Leu12Asn	43 ± 1	1.2 ± 0.2	43 ± 1	0.7 ± 0.1
Phe35Tyr	43 ± 1	0.9 ± 0.2	43 ± 1	0.7 ± 0.1
Gln88Glu	43 ± 1	1.5 ± 0.2	43 ± 1	0.6 ± 0.1
Glu59Lys/ Glu60Gln	49 ± 1	0.7 ± 0.2	49 ± 1	0.9 ± 0.1
Tyr83Phe	43 ± 1	0.9 ± 0.2	43 ± 1	0.7 ± 0.1
Asp42Asn	43 ± 1	0.7 ± 0.2	43 ± 1	0.8 ± 0.1
Glu43Asn	43 ± 1	1.3 ± 0.2	43 ± 1	0.7 ± 0.1

[a] This rearrangement gates the electron-transfer reactions in eqs 3 and 5. For the conditions, see Table 2.

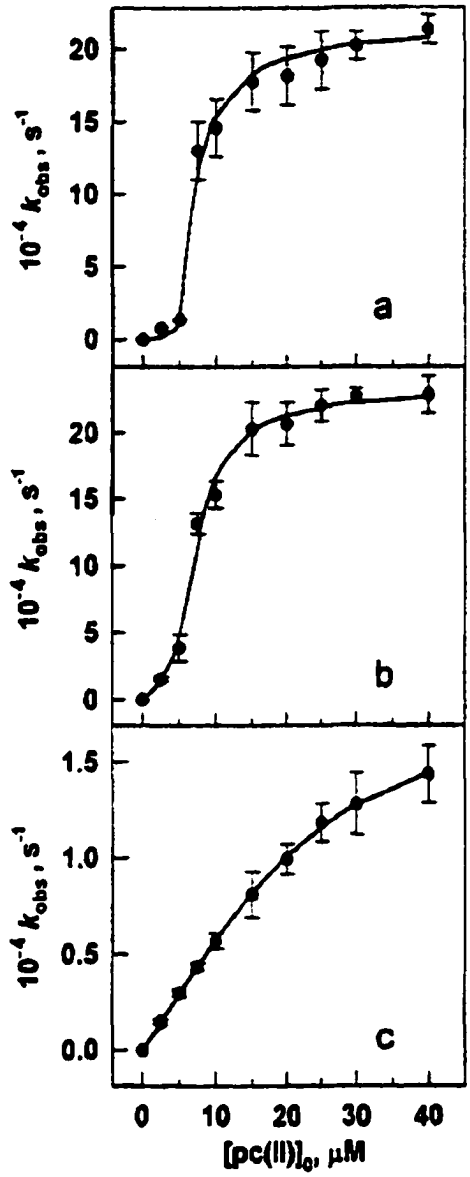

Figure 1. Dependence of the bimolecular rate constant k_{obs} for the reaction in eq 5 on the concentration of cupriplastocyanin. The conditions were as follows: sodium phosphate buffer at pH 7.0 and ionic strength of 2.5 mM at 293 K. (a) Wild-type protein, (b) single mutant Asp42Asn, and (c) double mutant Glu59Lys/Glu60Gln. The lines are fittins to eqs 7 and 8. The error bars include two standard deviations.

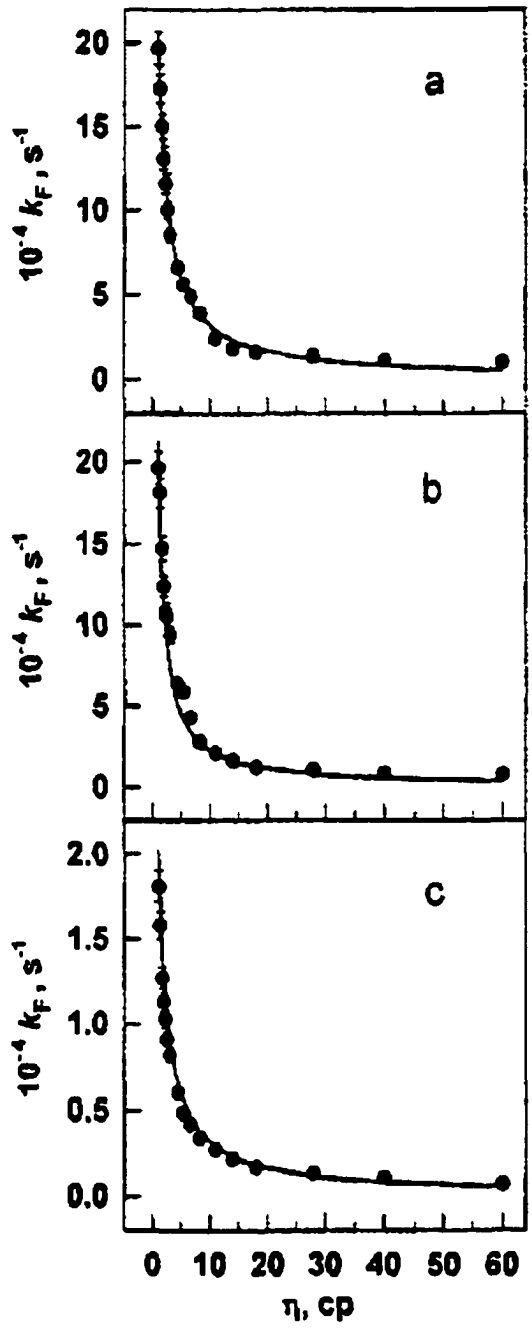

Figure 2. Dependence on solution viscosity of the unimolecular rate constant k_F for the rearrangement of the diprotein complex ^{3}Zncyt/pc(II) from the docking configuration into the electron-transfer configuration. Viscosity (η) of a sodium phosphate buffer having pH 7.00 ± 0.05 and ionic strength of 2.5 mM was adjusted with glycerol. (a) Wild-type cupriplastocyanin; (b) single mutant Asp42Asn; double mutant Glu59Lys/Glu60Gln. The lines are fittings to eq 12. The error bars include two standard deviations.

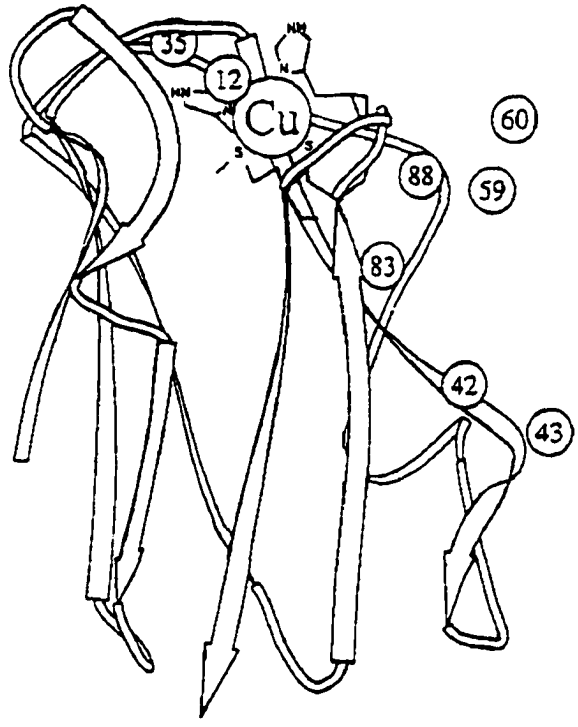

Figure 3. The structure of wild-type plastocyanin showing the copper site and locations of the mutated residues. All the circled numerals mark the α-carbon atoms, except for no. 60, which marks the γ-carbon atom in order to avoid overlap.

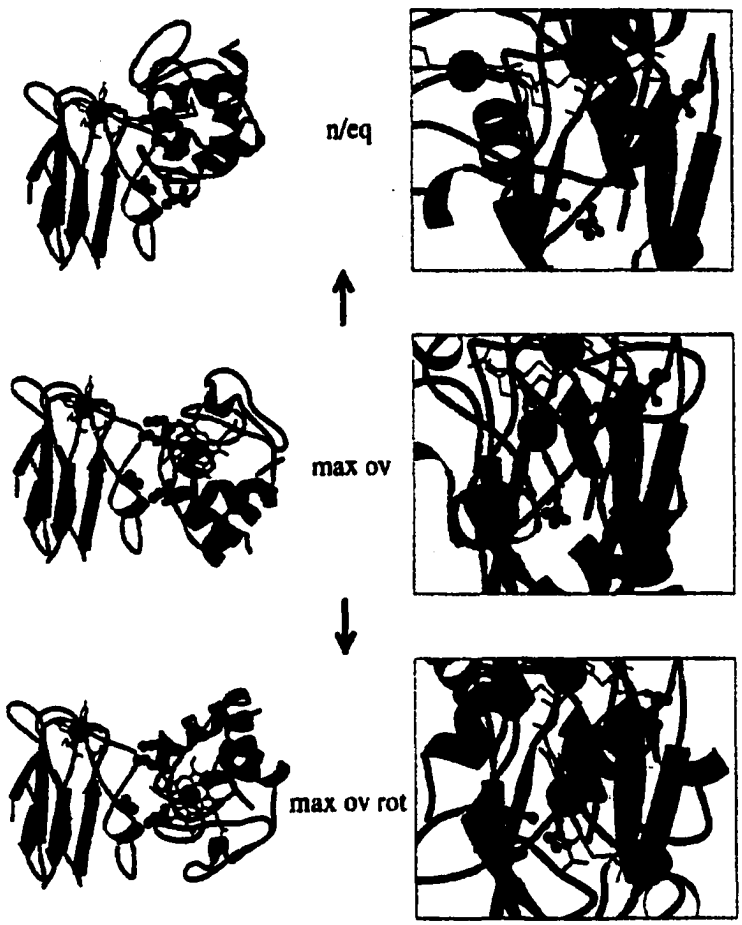

Figure 4. Two trajectories, shown with arrows, for rearrangement of the diprotein complex. Plastocyanin (blue) is stationary, while cytochrome *c* (red) moves. The atomic coordinates are taken from Roberts et al., 1991. The initial configuration, designated max ov, provides optimal docking. The rearranged configurations, designated n/eq and max ov rot, provides more efficient electron-tunneling paths from the heme to the copper site. The two metal atoms are highlighted; all the ligands to copper and the porphyrin ring are shown as wireframe models. The basic (positively charged) patch around the exposed heme edge moves across the acidic (negatively charged) patch in plastocyanin. In the configuration n/eq the basic patch reaches the upper edge of the acidic patch and the area between the acidic and hydrophobic patches, marked by the residues nos. 59, 60, and 88. In the configuration max ov rot the cytochrome *c* molecule is rotated by ca. 180°, mostly over the lower part of the acidic patch, marked by the residues nos. 42 and 43. Shown on the left side are the intact protein molecules. Shown on the right side is a magnified, local view of the plastocyanin surface through the molecule of cytochrome *c*. The acidic residues Asp42, Glu43, Glu59, and Glu60 in the wild-type plastocyanin, the mutations of which proved particularly informative, are highlighted as ball-and-stick models; their carboxylate oxygen atoms are shown in red.

CHAPTER 3. REDOX REACTIVITY AND REORGANIZATION ENERGY OF ZINC CYTOCHROME *C* CATION RADICAL

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Abstract

Little is known about transient intermediates in photoinduced electron-transfer reactions of metalloproteins. Oxidative quenching of the triplet state of zinc cytochrome *c*, ^{3}Zncyt, is done at 20 °C, pH 7.00 and ionic strength of 1.00 M, conditions that suppress the thermal back-reaction and prolong the lifetime of the cation radical, Zncyt$^+$. This species is reduced by $[Fe(CN)_6]^{4-}$, $[W(CN)_8]^{4-}$, $[Os(CN)_6]^{4-}$, $[Mo(CN)_8]^{4-}$, and $[Ru(CN)_6]^{4-}$ complexes of similar structures and the same charge. The rate constants and thermodynamic driving forces for these five similar electron-transfer reactions were fitted to Marcus theory. The reorganization energy of Zncyt$^+$ is λ=0.38(5) eV, lower than that of native cytochrome *c*, because redox orbital of the porphyrin cation radical is delocalized and possibly because Met80 is not an axial ligand to the zinc(II) ion in the reconstituted cytochrome *c*. The rate constant for electron self-exchange between Zncyt$^+$ and Zncyt, k_{11}=1.0(5)·10^7 M^{-1}s^{-1}, is large owing to the extended electron delocalization and relatively low reorganization energy. These results may be relevant to zinc(II) derivatives of other heme proteins, which are often used in studies of photoinduced electron-transfer reactions.

Introduction

Electron-transfer reactions of metalloproteins play essential role in many biological processes. Replacement of iron ions in the active site of myoglobin, hemoglobin, cytochrome *c* peroxidase, cytochrome *c*, and other heme proteins has been used extensively to probe the structure, function, and reactivity of these proteins. Cytochrome *c*,[1,2] designated cyt, is particularly well suited for studies of electron-transfer reactions since it is very well characterized and since its structures both in crystal and in solution are precisely known. Although electron-transfer reactions involving cytochrome *c* [2-4] and its derivatives [5-7] have been much studied, many questions remain unanswered.

Replacement of iron(II) with zinc(II) does not perturb the overall structure of the protein.[8] Zinc cytochrome *c*, designated Zncyt, offers many advantages over the native species. It is easily excited by the laser pulse and converted to the triplet state, ^{3}Zncyt. This long-lived state has been used as a strong reducing agent in several studies.[5,6,9-11] Recently it was used also as an oxidizing agent, in reactions with hexacyanoferrate(II) anion and conjugate bases of ethylenediaminetetraacetic acid (EDTA).[12] The advantages of ^{3}Zncyt are that external redox reagents are not needed to initiate the reaction between Zncyt and its partner. In these photoinduced reactions short-lived intermediates are formed: zinc cytochrome *c* cation radical ($Zncyt^+$) in oxidative quenching of ^{3}Zncyt and zinc cytochrome *c* anion radical ($Zncyt^-$) in reductive quenching of the triplet state. In subsequent thermal reactions the ion radical intermediates are converted back to Zncyt, which then becomes available for the next cycle. The thermal reaction is usually faster than the quenching

reaction since radical intermediates are usually more reactive than the triplet excited state. Thus the difficulty in detecting the radical intermediates.

$$\text{Zncyt}^{+} + \left[\text{M(CN)}_{n}\right]^{4-} \xrightarrow{k_r} \text{Zncyt} + \left[\text{M(CN)}_{n}\right]^{3-} \quad (1)$$

The cation Zncyt^{+} has been partially characterized by UV-vis spectrophotometry and differential-pulse polarography.[5] To our knowledge, however, its reactivity has not been studied. Here we examine the kinetics of reaction in eq 1, between Zncyt^{+} and transition-metal cyano complexes. We analyze the rate constant k_r for Zncyt^{+} reduction in terms of Marcus theory of electron-transfer reactions, determine reorganization energy and electron self-exchange rate constant, and discuss the implications of our findings for electron-transfer reactions of cytochrome *c*.

Experimental Section

Chemicals. Distilled water was demineralized to a resistivity greater than 17 MΩcm. Chromatography resins and gels were purchased from Sigma Chemical Co. and Amersham Pharmacia Biotech, Inc. Nitrogen, hydrogen fluoride, and ultrapure argon were purchased from Air Products, Co. Potassium tetrahydroborate was synthesized by a standard procedure.[13] Potassium hexacyanoruthenate(II) was purchased from Alfa Æsar. All other chemicals were purchased from Fisher Chemical Co.

Synthesis and Purification of Cyano Complexes. The following complexes salts were synthesized by standard methods: $K_4[Mo(CN)_8]\cdot 2H_2O$;[14] $K_4[Os(CN)_6]\cdot 3H_2O$;[15] $K_4[W(CN)_8]\cdot 2H_2O$;[16] and $K_3[W(CN)_8]\cdot 2H_2O$.[17] The salts were dissolved in several mL of water, methanol was added until precipitation started.[18] The solution was cooled to 4 °C and

left for one day. Translucent and opalescent crystals were filtered and rinsed twice with ethanol. The purification was done in the dark, to avoid complex decomposition.[19]

Buffers. All buffers were made fresh. Phosphate buffer at pH 7.00 had ionic strength of 1.00 M: 10 mM from the phosphate salts and 990 mM from NaCl. The pK_a value of the $H_2PO_4^-$ ion depends on ionic strength, and we took this dependence into account in adjusting the pH. Acetate buffer at pH 4.00 had ionic strength of 40 mM.

Proteins. Horse-heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co. The iron-free form was made, purified, and reconstituted with zinc(II) ions by a modification[20] of the original procedure.[21] The product, zinc cytochrome *c*, was handled at 4 °C in the dark. The criteria of its purity were the absorbance ratios, $A_{423}/A_{549}>15.4$ and $A_{549}/A_{585}<2.0$, and the rate constant of natural decay of the triplet state, less than 120 s^{-1}.[22] Concentration of Zncyt was determined spectrophotometrically, based on its molar absorptivity $\varepsilon_{423}=2.43\cdot10^5$ $M^{-1}cm^{-1}$.[22]

Recombinant spinach plastocyanin was prepared by the previously published method for overexpression in *Escherichia coli*.[23] The protein was purified first with a DE32 column and then with a 26/10 Q Sepharose high-performance FPLC column from Amersham Pharmacia Biotech, Inc. Plastocyanin was oxidized with excess of $K_3[Fe(CN)_6]$ and reduced with a small excess of ascorbic acid. The protein was concentrated and buffer was exchanged with Centricon (Amicon, Millipore Corporation) and Macrosep (Filtron Technology Co.) concentrators. The criterion for purity was $A_{278}/A_{597}<1.2$. The concentration of the protein was determined spectrophotometrically, on the basis of its molar absorptivity $\varepsilon_{597}=4700$ $M^{-1}cm^{-1}$. The reduction potential of plastocyanin was taken to be $E_{½}=0.384$ V.[23]

Solutions of both proteins in a phosphate buffer at pH 7.00 and ionic strength of 2.5 mM were stored at 77 K.

Physical Measurements. Absorption spectra were recorded with a Perkin-Elmer Lambda 18 spectrophotometer. The ^{1}H and ^{13}C NMR spectra of solutions in D_2O were recorded with a Bruker DRX-400 spectrometer. Ethanol was used as an internal standard, and 10,000 $\{^{1}H\}^{13}C$ scans were recorded 5 s apart.

Laser flash spectroscopy on microsecond time scale was done with a standard apparatus.[9,24] The triplet state ^{3}Zncyt was created by 0.4-μs pulses of light from a Phase-R (now Luminex) DL1100 laser containing the dye rhodamine 590. The samples were deaerated for 30 min by gentle flushing with ultrapure argon. The cell jacket was connected to a 30-L circulating bath Forma 2067, which maintained the temperature at 20.0±0.2 °C. The concentration of zinc cytochrome *c* was always 10 μM. The triplet state, ^{3}Zncyt, was monitored at 460 nm, where the difference in absorbance between the triplet and the ground state is greatest.[22] The formation and disappearance of the zinc cytochrome *c* cation radical, $Zncyt^{+}$, were monitored at 675 nm, where the difference in absorbance between the cation radical and the ground state is largest.[5,8] Kinetic traces at 460 nm were averaged over five flashes, while kinetic traces at 675 nm were averaged over ten flashes. The concentration of the triplet state depended on the intensity of the laser pulse and was ca. 1 μM, much lower than the cupriplastocyanin concentration. Thus kinetic conditions for pseudo-first order reactions were achieved.

Cyclic voltammograms of metal complexes were recorded with a RDE4 bipotentiostat and a disk electrode having a surface area of 0.165 cm^2 from Pine Instrument,

Co. A three-compartment electrochemical cell separated the working, reference, and counter electrodes. A saturated calomel electrode from Fisher Scientific Co. provided the reference potential. The counter electrode was a coiled platinum wire with the surface area of ca. 5 cm^2. Supporting electrolyte was a phosphate buffer at pH 7.00 and ionic strength of 1.00 M. Data were collected with a personal computer equipped with an AT-MIO-16XE-50 board from National Instruments and with LabView software. The scan rate was 50 mVs^{-1} for $Ru(CN)_6^{4-}$ because of the high background current at potentials greater than 1.2 V, and 100 mVs^{-1} for the other complexes.

Kinetic Calculations. Kinetic traces from separate flashes were fitted, and results statistically analyzed, with SigmaPlot v3.06 software, using the least-square averaging method. The correlation coefficients R^2 for the linear plots that provided the rate constants were greater than 0.990. The error margins for all results include two standard deviations and correspond to the confidence limit greater than 95%. The work terms, w_{ij}, and correction factors, W_{12} and f_{12}, were calculated with Microsoft Excel 97. Rate constant for diffusion and for activation-controlled electron transfer were calculated with modifications of the program kindly provided by Professor Claudia Turro.[25]

Results

Synthesis and Characterization of Cyano Complexes. Absorptivities at 190 nm of $K_4[Mo(CN)_8]\cdot 2H_2O$, $K_4[Os(CN)_6]\cdot 3H_2O$, and $K_3[W(CN)_8]\cdot 2H_2O$ were greater than the published value[26,27] because KCN, used in syntheses, was in excess and cocrystallized with the complex salts. For example, the salt $K_4[Os(CN)_6]\cdot 3H_2O$ showed two ^{13}C NMR

resonances, at 142.3 and 165.1 ppm. The former is due to the cyano ligand; the latter, to the free CN^- ion.[28] Recrystallization of crude salts from water failed because both they and KCN are very soluble in water.[29] Fractional crystallization from aqueous methanol succeeded. Absorption spectra of pure salts agreed with the published ones; ^{13}C NMR spectra of pure salts contained only one resonance, due to the cyano ligands.

Because of a discrepancy in one report[30] about the reduction potentials of $Os(CN)_6^{4-/3-}$ and $Fe(CN)_6^{4-/3-}$, we determined the $E_{½}$ values of all the complex anions. Phosphate buffer at pH 7.00 and ionic strength of 1.00 M was used to record background current. Cyclic voltammograms of each complex had two well-resolved peaks, corresponding to oxidation and reduction by one electron. The results in Table 1 completely agree with the other reports.[31-34]

Reduction Potential of Zncyt$^+$. The reduction potential of the Zncyt$^+$/Zncyt couple at ionic strength of ca. 0.200 M is 0.80±0.05 V.[5] Our kinetic experiments, however, were done at the ionic strength of 1.00 M. Reduction potential of horse-heart cytochrome *c* changes only slightly, from 0.260 to 0.240 V, when ionic strength is changed from 0.200 M to 1.00 M.[2] We calculated, by Debye-Hückel theory, the change in activity coefficients as ionic strength is raised from 0.200 to 1.00 M. The corresponding change in the reduction potential of Zncyt$^+$ was 0.03 V, less than the error in the differential-pulse polarography (0.05 V).[5] Therefore we set the reduction potential of Zncyt$^+$ at 0.80 V in our calculations.

Formation of Zncyt$^+$. Laser pulse excites the porphyrin chromophore in zinc cytochrome *c*, and we observe the triplet state (eq 2). This excited state decays to the ground state monoexponentially (eq 3), with the rate constant of 80±5 s^{-1}.

$$\text{Zncyt} \xrightarrow{h\upsilon} {}^3\text{Zncyt} \tag{2}$$

$${}^3\text{Zncyt} \xrightarrow{\textit{natural decay}} \text{Zncyt} \tag{3}$$

$${}^3\text{Zncyt} + \text{pc(II)} \xrightarrow{k_f} \text{Zncyt}^+ + \text{pc(I)} \tag{4}$$

When cupriplastocyanin, pc(II), is present at ionic strength of 1.00 M, the triplet state disappears monoexponentially, as shown in Figure 1a. This quenching is the so-called forward electron-transfer reaction[9] in eq 4, the rate of which is linearly proportional to the concentration of pc(II). Change in absorbance at 675 nm (Figure 1b, upper trace) indicates the formation of the cation radical, Zncyt$^+$.[5,9] Traces at 675 nm were fitted to eq 5, in which a_1 and a_2 are the amplitudes, and k_1 and k_2 are the first-order rate constants, for appearance and disappearance of Zncyt$^+$. If this were the only species absorbing at 675 nm, the amplitudes a_1 and a_2 would be the same.[35] But the triplet state also absorbs at this wavelength and disappears at the same rate, k_1, at which the cation radical appears. Therefore, there are only two, rather than three, exponential terms.[36]

$$\Delta A = -a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \tag{5}$$

The change in absorbance was ca. five times smaller at 675 nm than at 460 nm. To improve accuracy in fittings of the smaller signals to eq 5, we determined k_1 precisely from the strong signals for the disappearance of ^{3}Zncyt at 460 nm, fixed this k_1 value, and allowed a_1, a_2, and k_2 to vary. As more pc(II) was added, the maximum transient concentration of Zncyt$^+$ increased as well. In the thermal (so-called back) reaction (eq 6), direction of electron transfer is reverse of that in the forward reaction (eq 4), and the system returns to the state prior to the laser pulse, namely Zncyt and pc(II).

$$Zncyt^+ + pc(I) \xrightarrow{k_b} Zncyt + pc(II) \quad (6)$$

Initial concentration of pc(II) was always 30 μM because then we were able to observe $Zncyt^+$ clearly. Under the aforementioned conditions the observed first-order rate constants were k_1=700±20 s^{-1} and k_2=20±1 s^{-1}.

Reduction of $Zncyt^+$ by Cyano Complexes and Side Reactions. When $[Os(CN)_6]^{4-}$, $[Mo(CN)_8]^{4-}$, or $[Ru(CN)_6]^{4-}$ complex is added to the reaction in eq 4, the rate constant k_2 (for disappearance of $Zncyt^+$) increases, as the lower trace in Figure 1b shows. This first-order rate constant is linearly proportional to the concentration of the cyano complex, as Figure 2 shows, and the intercept of fitted lines with *y*-axis is the first-order rate constant for the 'background' reaction of $Zncyt^+$ and pc(I). The rate constants k_2 are given in Table S1 in the Supporting Information. Since the cyano complexes were present in excess over $Zncyt^+$, the pseudo-first order conditions were satisfied. Plots of k_2 versus the concentration of the cyano complexes gave the second-order rate constants k_r for the reaction of interest (eq 1), which are given in Table 1.

With $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ complexes the first-order kinetic plots showed slight curvature. Moreover, the rate of ^3Zncyt quenching in the presence pc(II) increased with addition of these two complexes. To check whether the triplet was directly quenched by the cyano complexes, we separately treated ^3Zncyt with relatively high concentrations (up to 40 μM) of all five 4- complexes in the absence of pc(II). None of the $[M(CN)_n]^{4-}$ complexes quenched ^3Zncyt. We concluded that the observed quenching in the complete reaction mixtures containing $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ is due to the $[Fe(CN)_6]^{3-}$ and $[W(CN)_8]^{3-}$ complexes, products of oxidation of the corresponding 4- complexes by pc(II). To test this

hypothesis, we treated a 30 μM solution of pc(II) with various concentrations of $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ ions and easily detected bleaching of the blue color (reduction of the protein) by UV-vis spectrophotometry. This side reaction is shown in eq 7, in which M is Fe or W and n is 6 or 8. The equilibrium constant, K_e, is 21±5 for $[Fe(CN)_6]^{4-/3-}$ and 1200±200 for $[W(CN)_8]^{4-/3-}$. Those results agree with the K_e values determined from the reduction potentials, which are 17 and 1070.

$$pc(II) + [M(CN)_n]^{4-} \overset{K_e}{\rightleftharpoons} pc(I) + [M(CN)_n]^{3-} \qquad (7)$$

The 3- cyano complexes formed in one side reaction (eq 7) undergo another side reaction, shown in eq 8. In two series of control experiments, we separately determined the rate constants k_f' listed in Table 1. Because the reaction k_f' competes with the reaction k_f, and in both of them electron transfer occurs in the forward direction, the corresponding rate constants have similar symbols.

$$^3Zncyt + \left[M(CN)_n\right]^{3-} \xrightarrow{k_f'} Zncyt^+ + \left[M(CN)_n\right]^{4-} \qquad (8)$$

We can quantitatively determine the contributions of the reactions k_f (eq 4) and k_f' (eq 8) to the observed rate of ^{3}Zncyt quenching in the presence of both pc(II) and the $[M(CN)_6]^{3-}$ complex of Fe(III) or W(V). For example, the rate constant of ^{3}Zncyt quenching by 30 μM pc(II) is k_1=600±20 s^{-1}. Upon addition of 10 μM $[W(CN)_8]^{4-}$ the rate constant becomes k_1=1200±100 s^{-1}, an increase of ca. 600 s^{-1}. The concentration of $[W(CN)_8]^{3-}$, calculated from the given concentrations of the reactants and the known K_e in eq 7, is 0.47 μM. Therefore, the contribution to k_1 from the side reaction in eq 8 is $(1.15\cdot10^9\ M^{-1}s^{-1})\times(4.7\cdot10^{-7}\ M)=540\ s^{-1}\approx600\ s^{-1}$. The additional quenching of ^{3}Zncyt by the 3-

complex is accounted for. Because the 4- complexes of Os(II), Mo(IV), and Ru(II) have too high reduction potentials to be oxidized by pc(II), they do not undergo the side reaction in eq 7, the corresponding 3- complexes are not formed, and the side reaction in eq 8 does not occur. Although the side reactions in eqs 7 and 8 with the iron and tungsten complexes are unavoidable, we studied their kinetics in detail and separated these reactions from the reaction of interest, k_r.

Concentrations of the 4- complexes of Fe(II) and W(IV) in the reaction mixture could not be determined simply from the amounts added to the mixture, because of the side reaction in eq 7; we calculated these concentrations on the basis of K_c. In the cases of Os(II), Mo(IV), and Ru(II), the concentrations of the 4- complexes could be determined simply. Plots of first-order rate constants k_2 vs concentration of the $[M(CN)_n]^{4-}$ complexes were linear. The slopes of these plots yielded the second-order rate constants k_r for the reduction of the cation radical $Zncyt^+$ by $[M(CN)_n]^{4-}$ complexes (eq 1).

Reoganization Energy of $Zncyt^+$ and its Reaction with Cyano Complexes. Driving force, ΔG_r^0, for the reaction in eq 1 was calculated from the reduction potentials of $Zncyt^+$ and the $[M(CN)_n]^{4-}$ complexes corrected for the work required to bring the reactants and the products together ($\Delta G_r^0 = \Delta G^0 - w_{12} + w_{21}$). The rate constants k_r and ΔG_r^0, listed in Table 1, were fitted to eqs 9-13.[25,37] The result is shown in Figure 3.

$$k_r = \frac{k_{act} \cdot k_{diff}}{k_{act} + k_{diff}} \tag{9}$$

$$k_{act} = \frac{4\pi N_0}{1000} \int_r^{\infty} dr \cdot r^2 \cdot k_{et}(r) \cdot e^{-\frac{U(r)}{k_b T}} \tag{10}$$

$$k_{et}(r) = \frac{4\pi^2 H_{DA}^2}{h N_0 \sqrt{4\pi\lambda RT}} \exp\left(-\frac{\left(\Delta G_r^0 + \lambda\right)^2}{4\lambda RT} \right) \tag{11}$$

$$H_{DA} = H_{DA}^0 \cdot \exp\left[-\frac{\beta_0 (d - d_0)}{2} \right] \tag{12}$$

$$k_{diff} = \frac{4\pi N_0 D}{1000 \int_r^{\infty} dr \cdot r^2 \cdot e^{-\frac{U(r)}{k_b T}}} \tag{13}$$

$$U(r) = \frac{z_1 z_2 e^2}{D_s r\left(1 + \beta r \sqrt{\mu}\right)} \tag{14}$$

$$\beta = \sqrt{\frac{8\pi N_0 e^2}{D_s k_B T}} \tag{15}$$

For bimolecular electron-transfer reaction, the expression for observed rate constant k_r has the form of a consecutive reaction mechanism consisting of activated (k_{act}) and diffusional (k_{diff}) rate constants, as in eq 9.[38,39] The latter, calculated from eqs 13-15, is $1.8 \cdot 10^{10}$ $M^{-1}s^{-1}$; this value is marked with a horizontal line in Figure 3. The activated electron transfer occurs over a range of distances and the rate constant k_{act} is obtained by integrating the rate constant for the electron transfer $k_{et}(r)$ over the equilibrium distribution of reactant separation distances ($r^2 \cdot \exp(-U(r)/k_bT)$. The electronic coupling between the electron donor and the electron acceptor (so-called tunneling matrix element), H_{DA}, in cm^{-1} was calculated from eq 12. H_{DA}^0 is the value of H_{AD} when $d=d_0$ taken to be 200 cm^{-1} for cytochrome *c*[40,41] and d_0 is the edge-to-edge distance between cytochrome *c* and cyano complexes, defined as

3 Å to account for electronic clouds of the outermost atoms of each reactant;[37] The distance d at which the electron is transferred from cyano complex to cytochrome c is related to the sum of the reactants' radii $d = r - \sigma_i - \sigma_j$ where σ_i and σ_j are radii of cytochrome c and cyano complex. Fittings of this kind are done to different forms of the same equations, in which quantities have various units. There are many ways to do this task correctly so we briefly explain our fittings to avoid confusion. In eqs 10-13 h=6.625·10^{-34} Js; N_0=6.023·10^{23} mol^{-1}; λ is the reorganization energy in Jmol^{-1}; R=8.314 JK^{-1}mol^{-1}; T is in K; and ΔG^0_r is in Jmol^{-1}. Intermolecular potential U(r) between two highly charged spheres (adequate representations of cytochrome c and the cyano complex)[37] is dominated by electrostatic interactions. We treat this potential according to Debye-Hückel theory, eq 14. In eqs 13 and 14 D=8.08·10^{-10} m^{2}s^{-1} is the sum of diffusion coefficients for cytochrome c and $[M(CN)_n]^{4-}$; D_s=8.89·10^{-9} Fm^{-1} is the static dielectric constant of the solvent; k_b=1.38·10^{-23} JK^{-1} is Boltzman's constant; and β=1.05·10^8 M$^{-½}$m^{-1} is a factor at 293 K, defined in eq 15.

In the fittings of ΔG^0_r and k_r to eqs 9-12 there were only two variable parameters, all the other quantities were known. The best fit, shown in Figure 3, yielded d=3.8 Å and λ=0.35±0.05 eV.

Calculation of Self-Exchange Rate Constant for Zncyt$^+$ and Zncyt. The self exchange redox reaction in eq 16 cannot be studied directly, but the reaction in eq 1 can. We used the Marcus cross-relation (eq 18) to estimate the rate constant k_{11}. The rate constant k_r (in general case it is k_{12}) is proportional to the self-exchange rate constants k_{11} (eq 16) and k_{22} (eq 17). The equilibrium constant K_r (in general case it is K_{12}) for the reaction of interest (eq 1), the factor f_{12}, and the work W_{12} required to bring two reactants together are unitless

numbers calculated from eqs 19-22; see Table 1. The other quantities are as follows: z is number of electrons exchanged (one in our case), $\Delta E_{1/2}$ is in volts, F=96485 Fm^{-1}, R=8.314 $JK^{-1}mol^{-1}$, T=293 K, and the collision frequency $Z=1\cdot10^{11}$ $M^{-1}s^{-1}$.[37,42]

$$\text{Zncyt} + \text{Zncyt}^{+} \xrightarrow{k_{11}} \text{Zncyt}^{+} + \text{Zncyt} \tag{16}$$

$$\left[M(CN)_n\right]^{4-} + \left[M(CN)_n\right]^{3-} \xrightarrow{k_{22}} \left[M(CN)_n\right]^{3-} + \left[M(CN)_n\right]^{4-} \tag{17}$$

$$k_{11} = \frac{k_r^2}{k_{22}\cdot K_r\cdot f_{12}\cdot W_{12}^2} \tag{18}$$

$$K_r = \exp\left(-\frac{z\,F\,\Delta E_{1/2}}{RT}\right) \tag{19}$$

$$f_{12} = \exp\left[\frac{\left(\ln K_r + \frac{w_{12}-w_{21}}{RT}\right)^2}{4\cdot\left(\ln\frac{k_{11}\cdot k_{22}}{Z^2} + \frac{w_{11}+w_{22}}{RT}\right)}\right] \tag{20}$$

$$W_{12} = \exp\left(-\frac{w_{12}+w_{21}-w_{11}-w_{22}}{2RT}\right) \tag{21}$$

$$w_{ij} = \frac{z_i\,z_j\,e^2\left(\frac{\exp(\beta\sigma_i\sqrt{\mu})}{1+\beta\sigma_i\sqrt{\mu}} + \frac{\exp(\beta\sigma_j\sqrt{\mu})}{1+\beta\sigma_j\sqrt{\mu}}\right)}{2D_s\,r\cdot\exp(\beta r\sqrt{\mu})} \tag{22}$$

When the reactants bear opposite charges, the Marcus cross-relation may be inapplicable.[37] The problems can be minimized by raising ionic strength, as we did. Furthermore, we used Debye-Hückel theory and corrected with so-called work terms, w_{ij}, the driving forces for the reactions in eqs 1, 16, and 17.

In eq 22, σ_i and σ_j are the sums of radii (in m) of reacting ions and predominant counter ions: Zncyt and $Zncyt^+$ ($17.5 \cdot 10^{-10}$ m) with Cl^- ($1.81 \cdot 10^{-10}$ m) and $[Fe(CN)_6]^{4-}$ ($4.50 \cdot 10^{-10}$ m), $[Ru(CN)_6]^{4-}$ ($4.70 \cdot 10^{-10}$ m), and $[W(CN)_8]^{4-}$, $[Os(CN)_6]^{4-}$, and $[Mo(CN)_8]^{4-}$ ($4.80 \cdot 10^{-10}$ m each) with Na^+ ($1.10 \cdot 10^{-10}$ m).[34] Also in eq 22 μ is ionic strength in $molm^{-3}$; charges z_i and z_j are +7 for $Zncyt^+$, +6 for Zncyt, -4 for $[M(CN)_n]^{4-}$, and -3 for $[M(CN)_n]^{3-}$; elemental charge $e = 1.6 \cdot 10^{-19}$ C; dielectric constant of the medium is $D_s = 4 \varepsilon \varepsilon_0 = 8.89 \cdot 10^{-9}$ Fm^{-1}; and radius of transient complex, r, is the sum of the $Zncyt^+$ and $[M(CN)_n]^{4-}$ radii in meters.

$$k_{11} = \exp(2 \times intercept) \tag{23}$$

Since the self-exchange rate constant k_{11} is needed for the calculation of f_{12}, we used the experimental result[43] for cytochrome *c* , $k_{11} = 5 \cdot 10^4$ M^{-1} s^{-1}, as an approximate starting value in our calculations. The self-exchange rate constant of interest, k_{11}, can be determined from Figure 4 and eq 23. The intercept with the vertical axis was calculated iteratively, with the k_{11} value from the previous cycle as input for the next. The iterations converged when the self-consistent result was obtained: $k_{11} = (1.0 \pm 0.5) \cdot 10^7$ $M^{-1}s^{-1}$.

Discussion

The Chemical Reactions. All the reactions occurring in this intricate system are shown in Scheme 1. Excitation (*hv*) of the porphyrin chromophore in Zncyt indirectly produces the long-lived triplet state, ^{3}Zncyt. In the absence of a quencher, this state decays to the ground state. The blue copper protein cupriplastocyanin, pc(II), quenches ^{3}Zncyt in a reaction that is much faster than natural decay; the rate constant of this forward

electron-transfer reaction is designated k_f. The copper site becomes reduced into pc(I), and the porphyrin becomes oxidized into Zncyt$^+$. This cation radical is short-lived because it undergoes the back electron-transfer reaction, designated k_b. In this thermal reaction, an electron moves from the copper site to the porphyrin; the Zncyt$^+$ is reduced and converted back to Zncyt. The cycle is completed.

The transient species Zncyt$^+$ is the main object of our study. We separately add five cyano complexes $[M(CN)_n]^{4-}$ to reduce Zncyt$^+$, in competition with pc(I). The fraction of Zncyt$^+$ reduced by $[M(CN)_n]^{4-}$ depends on the concentration of the cyano complex and the relative magnitudes of k_r and k_b.[36] Reduction of Zncyt$^+$ by $[M(CN)_n]^{4-}$ produces Zncyt and $[M(CN)_n]^{3-}$. This 3- complex oxidizes pc(I) to regenerate the 4- complex and pc(II). Again, the cycle is completed.

Kinetics becomes further complicated if a fraction of the $[M(CN)_n]^{4-}$ complex is oxidized to $[M(CN)_n]^{3-}$ by pc(II). This was the case with $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$, the only two complexes thermodynamically capable of this reaction. Because this equilibrium (shown in eq 7) is established prior to the laser pulse, it is omitted from Scheme 1. The important consequence of this side reaction is that, in addition to Zncyt, pc(II), and $[M(CN)_n]^{4-}$, which are added to the solution, the reaction mixture contains also some $[M(CN)_n]^{3-}$. This is a new species, which undergoes a new reaction, k_f'. The equilibrium process in eq 7 also causes a small conversion of pc(II) to pc(I). We quantified this conversion in all experiments and adjusted the concentration of both forms of plastocyanin in kinetic calculations concerning the reactions k_f and k_b. The measured rate constant k_b used in these calculations is $(3.6\pm0.2)\cdot10^6\ M^{-1}s^{-1}$.

Requirements of the Reductants for Zncyt$^+$. To serve as a reducing agent in reaction of interest (eq 1 and k_r in Scheme 1), a compound should meet several requirements. Because Zncyt$^+$ bears a charge +7 at pH 7.00, its reaction partner should bear a negative, preferably high, charge, for favorable association. The compound should undergo only one-electron oxidation, to match the oxidizing capacity of Zncyt$^+$. The compound should be stable in both the reduced and the oxidized form, that is, as both the reactant and the product in eq 1. Because we trigger the thermal redox reaction photochemically, the reductant and its oxidized form must not absorb in the wavelength ranges in which Zncyt is excited (by the laser pulse) and in which ^{3}Zncyt and Zncyt$^+$ are monitored (by the spectrophotometric beam). The five transition-metal cyano complexes in Table 1 meet all of these requirements. Moreover, they have similar, highly symmetrical shapes and the same charge. Because of this uniformity, we did not have to be concerned with the effects of binding affinity (i.e., the association constant) on the bimolecular rate constant k_r.[44] These five complexes constitute an ideal series of reductants with which to test the reactivity of Zncyt$^+$ as an oxidant.

To reduce Zncyt$^+$, an agent also must be a member of a redox couple with reduction potential of ca. 0.80 V or lower. To avoid reducing pc(II), whose reduction potential is 0.384 V, the reduction potential of the suitable couple must be ca. 0.40 V or higher. The values near this limit posed a problem; both $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ ions partially reduce pc(II). We overcame this problem by careful studies of the reactions in eqs 7 and 8. Knowing the exact concentration of $[Fe(CN)_6]^{3-}$ and $[W(CN)_8]^{3-}$, we were able to account for contributions to the quenching from both rate constants, k_f' and k_r. Because the complexes $[Mo(CN)_8]^{4-}$ and $[Ru(CN)_6]^{4-}$ belong to redox couples with reduction potentials slightly

greater than 0.80 V, their reactions k_r occur "uphill" in the thermodynamic sense. We easily solved this problem by employing an excess of each cyano complex over $Zncyt^+$. Because, however, the 4- cyano complex has to compete with pc(I) for reduction of $Zncyt^+$, the reaction k_r has to be faster than the reaction k_b. Fortunately, this was the case with the complexes $[Mo(CN)_8]^{4-}$ and $[Ru(CN)_6]^{4-}$. Because weaker reducing reagents would not necessarily meet this kinetic requirement, we could not go beyond the reduction potential of 0.907 V.

Considering all of the various requirements of the reducing agents and constraints imposed by the "clean" photochemical method that we used, we were fortunate to find a series of five similar reducing agents for a meaningful plot in Figure 3. But before we studied the reaction k_r (eq 1 and Scheme 1) we had to examine some side reactions.

Side Reactions. When the reaction mixture contained $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$, quenching of ^3Zncyt by pc(II), which we initially considered the only quencher, was faster than we expected on the basis of the rate constant k_b. To study the reaction of interest, k_r, we had to use relatively high concentrations of these 4- complexes, a condition that favored the side reaction in eq 7. The products of this side reaction, complexes $[Fe(CN)_6]^{3-}$ and $[W(CN)_8]^{3-}$, turned out also to quench ^3Zncyt, as in eq 8. We prepared these two 3- complexes, unexpected ingredients of the reaction mixtures and determined the equilibrium constants K_e (eq 7) and the rate constants k_f' (eq 8). Both quenching reactions k_f (eq 4) and k_f' (eq 8) are shown in Scheme 1. Because k_f' is six times greater in the case of $[Fe(CN)_6]^{3-}$ and hundred times greater in case of $[W(CN)_8]^{3-}$ then k_f, even the low concentration of these 3- complexes in the reaction mixture markedly enhanced the observed quenching of ^3Zncyt.

Knowing both k_f and k_f' values, we quantitatively accounted for this enhanced rate. Then we returned to our main purpose — study of the reaction k_r and of the properties of Zncyt$^+$ that govern its redox reactions.

Formation and Lifetime of Zncyt$^+$. This cation radical is formed by oxidative quenching of the triplet state, ^{3}Zncyt, in reactions k_f and k_f'. To prolong the lifetime of Zncyt$^+$ so we can study its reactivity (eq 1), we had to disfavor the back reaction, k_b.

At first, we tried to keep the reaction mixture at pH 4.00 and ionic strength of 40 mM, at which plastocyanin is easily reduced (so that reaction k_f would occur) but not reoxidized (so that reaction k_b would be hampered).[45] These experiments failed because of partial denaturation of pc(I) in the acidic solution.

The reactants Zncyt$^+$ and pc(I) bear charges of +7 and –9 at pH 7.00. To disfavor the undesirable back-reaction, k_b in Scheme 1, we added NaCl to the buffer at pH 7.00. By keeping the ionic strength at 1.00 M, we were able to prolong the lifetime of Zncyt$^+$ and make it accessible to the cyano complexes $[M(CN)_n]^{4-}$.

Reduction of Zncyt$^+$ by the Cyano Complexes $[M(CN)_n]^{4-}$. The reaction in eq 1 is bimolecular at the ionic strength of 1.00 M. The linear plots of the pseudo-first order rate constants k_2 versus the concentration of the $[M(CN)_n]^{4-}$ complexes gave the bimolecular rate constants k_r. They and the ΔG_r^0 values, both in Table 1, are plotted in Figure 3.

The leveling off, predicted by Marcus theory,[37] could be due to two causes. First, the reaction could have became diffusion-limited ($k_{act} >> k_{diff}$ in eq 9) and therefore independent of the driving force (ΔG_r^0). In this case, the diffusion limit would have to be approximately equal to, or a little greater than, $7 \cdot 10^7$ $M^{-1}s^{-1}$, the value at which the plot in Figure 3 levels off.

Second, the reaction could be a case of true electron transfer ($k_{act}<<k_{diff}$ in eq 9) that became nearly activationless ($\Delta G_r^0 \approx \lambda$ in eq 11) with the $[Fe(CN)_6]^{4-}$ as the reductant. In this case the diffusion limit would be grater than $1 \cdot 10^8$ $M^{-1}s^{-1}$.

To distinguish between these two cases we calculated the rate constant for diffusion since experimental determination is proven to be very difficult.[37,46] To calculate the rate constant for diffusion k_{diff} we applied the Smoluchowski equation[47] to $Zncyt^+$ and $[Fe(CN)_6]^{4-}$. The product of their charges is (+7)×(-4)=-28, the rate constant for diffusion, $k_{diff}=1.8 \cdot 10^{10}$ $M^{-1}s^{-1}$. This result generally agrees with the experimental value of $4.2 \cdot 10^9$ $M^{-1}s^{-1}$, obtained for a somewhat similar pair of reactants, pc(II) with a net charge -8 and $[Ru(bpy)_3]^{2+}$, in a reaction with a high driving force (ΔG=1.2 eV) which is believed to be diffusion controlled.[37] The calculated rate constant for diffusion from eqs 13-16 for pc(II) and $[Ru(bpy)_3]^{2+}$ is $6 \cdot 10^9$ $M^{-1}s^{-1}$. Electrostatic effects are slight at ionic strength of 1.00 M, but a greater (negative) product of charges still corresponds to a three times faster diffusion which is in excellent agreement with our calculated value. We may tentatively conclude that diffusion limit lies well above the plateau in Figure 3, i.e., that the reaction k_r has not became diffusion-controlled in the case of $[Fe(CN)_6]^{4-}$.

Because Smoluchowski equation tends to overestimate k_{diff} (while still realistically predicting its relative values for related systems),[48] we further tested the nature of the reaction in eq 1. We know the experimental rate constant, $k_f^{'}=7.7 \cdot 10^8$ $M^{-1}s^{-1}$, for the reaction in eq 8, which involves essentially the same reactants except with different charges, whose product is (+6)×(-3)=-18. If the reaction $k_f^{'}$ were diffusion-controlled, than we could estimate the rate constant of interest, k_r. The product of charges -28 would correspond to

k_r=1.2·10^9 M^{-1}s^{-1}, a value greater than k_f'. The experimental result, k_r=7·10^7 M^{-1}s^{-1} at the plateau, is much lower than the estimate and the known k_f'. Clearly, it is not onset of diffusion control that causes the plateau in Figure 3. We conclude that the reaction in eq 1 is a case of true electron transfer, which is approaching or it has reached the activationless regime. Fitting to Marcus equation is justified.

Fitting to Marcus Equation and Reorganization Energy of Zncyt$^+$. Because the five cyano complexes in eq 1 have similar structures and the same charge, the fitting in Figure 3 is meaningful. One of its results is the edge-to-edge distance between the heme and cyano complex is d=3.9±0.2 Å. This result is in excellent agreement with the previously determined reaction distances for cytochrome *c* and anionic complexes,[49] but the negatively charged cyano complexes seem to react at the closer distance than the positively charged Ru^{2+} complexes.[25] We conclude that the electron is transferred at the exposed-heme edge and that the significant localization of the unpaired spin on the *meso*-carbon atoms[50,51] may render singly-occupied a_{2u} orbital accessible to the $[M(CN)_n]^{4-}$ complex. Because of inherent errors associated with estimate of reaction distance[49] we sustain from further discussion.

The other, and more interesting, result of our finding is the total reorganization energy, λ=0.35±0.05 eV. As eq 24 shows, this quantity is approximately equal to the average reorganization energy of the two reactants.[52] Each of these energies is made up of inner-sphere and outer-sphere terms. For the cyano complexes (eq 25) these separate terms have been estimated at 0.031 and 0.29 eV, respectively;[28] evidently, the 4- and 3- forms of the complexes have very similar structures (bond lengths), but are solvated to different extents.

$$\lambda \approx \frac{\lambda_{\text{Zncyt}^+} + \lambda_{[M(CN)_n]^{4-}}}{2} \tag{24}$$

$$\lambda_{[M(CN)_n]^{4-}} = \lambda_{in} + \lambda_{out} \tag{25}$$

Given $\lambda_{[M(CN)_n]^{4-}}$ =0.32 eV, from eq 24, we estimate λ_{Zncyt^+}=0.38 eV. This result can be compared with the reorganization energy of native cytochrome *c* (in ferric and ferrous oxidation states). Estimates of this quantity vary widely, between 0.5[37] and 1.2 eV.[53] Experimental study of the self-exchange redox reaction gave the result of 0.72 eV.[43] Recent theoretical studies yielded the values of 0.69,[54] 0.87,[55] and 0.68 eV.[56] The last four results are fairly consistent, and we take the reorganization energy of native cytochrome *c* to be their average, or 0.74 eV.

Oxidation and reduction of the iron protein occur predominantly at this transition metal, on which are largely localized the redox orbitals of the heme.[5] Particularly affected is the Fe—S bond involving the axial ligand Met80, which changes by 0.08 Å.[57] Because the protein surface is not significantly perturbed by the replacement of the metal ion,[8] solvation, the chief outer-sphere factor, may be considered unchanged. Oxidation and reduction of the zinc protein occur at the porphyrin ring. Changes in the occupancy of the delocalized redox orbital are expected to have only slight effect on the porphyrin geometry. The zinc(II) ion seems to be five-coordinate, detached from Met80.[20] Without this bond, Met80 probably is not affected by the oxidation state of the heme; yet another source of inner-sphere reorganization energy is absent in zinc cytochrome *c*.

Conformational and other structural differences between the iron(II) and iron(III) forms of cytochrome *c* are still being debated, [58-63] and their contributions to the

reorganization energy are difficult to estimate.[64] Without the firm knowledge about the native protein, we can only suppose that the decrease in total reorganization energy from 0.74 to 0.38 eV is due mainly to a decrease in the internal structural rearrangement from native to zinc-substituted cytochrome *c*.

Electron Self-Exchange between Zncyt and Zncyt$^+$. The rate constant k_{11} (eq 16) is 200 times greater than the corresponding rate constant for native cytochrome *c*, at the ionic strength of 1.00 M. We attribute this greater intrinsic reactivity to the greater accessibility of the redox orbital and lower reorganization energy, properties discussed in the preceding subsection.

Conclusion

The iron and zinc forms of the same protein, cytochrome *c*, differ markedly in their properties as redox agents. For meaningful fittings to Marcus theory, kinetic results obtained with heme proteins and their reconstituted forms should not be used in the same plot.[5,65] Ground-state and excited-state redox reactions should not be treated together, as they sometimes are in studies of this kind.

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Supporting Information Available. A table of first-order rate constants k_2 for the reaction in eq 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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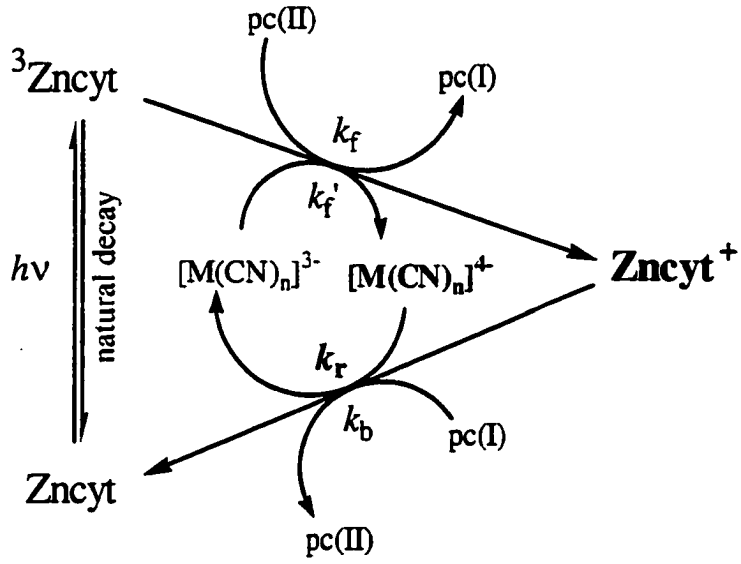
Table 1. Properties of the cyano complexes and other quantities dependent on these properties: reduction potential ($E_{½}$), driving force for the reaction in eq 1 (ΔG^0_r), rate constant for the self-exchange reaction in eq 17 (k_{22}), so-called work terms in the Marcus theory (w_{12}, w_{21}, w_{22}, and W_{12}), the factor f_{12}, rate constant for the reaction in eq 8 (k_f), and rate constant for the reaction in eq 1 (k_r)

complex	$[Fe(CN)_6]^{3-/4-}$	$[W(CN)_8]^{3-/4-}$	$[Os(CN)_6]^{3-/4-}$	$[Mo(CN)_8]^{3-/4-}$	$[Ru(CN)_6]^{3-/4-}$
$E_{½}$ / V, vs NHE	0.454	0.560	0.661	0.810	0.907
ΔG^0_r / eV	0.346	0.240	0.139	-0.010	-0.107
k_{22} / 10^4 $M^{-1}s^{-1}$ [a]	1.12	9.84	8.90	16.4	5.50
w_{12} / J mol^{-1}	-629	-563	-563	-563	-584
w_{21} / J mol^{-1}	-404	-362	-362	-362	-375
w_{22} / J mol^{-1}	2625	2153	2153	2153	2299
W_{12}	2.126	1.887	1.887	1.887	1.958
f_{12}	0.147	0.274	0.719	0.997	0.814
k_f' / 10^7 M^{-1} s^{-1}	77(1)	115(5)	-	-	-
k_r / 10^7 M^{-1} s^{-1}	7(1)	9(1)	1.42(5)	0.35(5)	0.0194(2)

[a] from ref 28


pc(II)
^{3}Zncyt
pc(I)
k_f
k_f'
$h\nu$
natural decay
$[M(CN)_n]^{3-}$
$[M(CN)_n]^{4-}$
Zncyt$^+$
k_r
k_b
pc(I)
Zncyt
pc(II)

Scheme 1

Figure Captions

Figure 1. Transient absorbance changes in a solution containing 10 μM zinc cytochrome *c* and 30 μM cupriplastocyanin in a phosphate buffer at pH 7.00, ionic strength adjusted to 1.00 M with NaCl and 293 K. (a) The triplet state, ^{3}Zncyt. (b) The cation radical, Zncyt$^+$, in the absence of cyano complexes (upper trace) and in the presence of 4.0 μM $[Os(CN)_8]^{4-}$ complex.

Figure 2. Rate constant k_2 for the disappearance of the cation radical Zncyt$^+$ as a function of the concentration of the $[M(CN)_n]^{4-}$ complexes in a phosphate buffer at pH 7.00, ionic strength adjusted to 1.00 M with NaCl and 293 K.

Figure 3. The curve is so-called Marcus plot for the reaction in eq 1, the best fit to eqs 9-12. The plotted quantities are in Table 1. The horizontal line is at k_{diff}, the result of the calculation according to eqs 13-15.

Figure 4. Determination of the rate constant k_{11}, for the self-exchange reaction between Zncyt$^+$ and Zncyt (eq 16) based on the kinetics of the reaction in eq 1 involving five $[M(CN)_n]^{4-}$ complexes. The straight line is the best fit of these kinetic results to eqs 18-23.

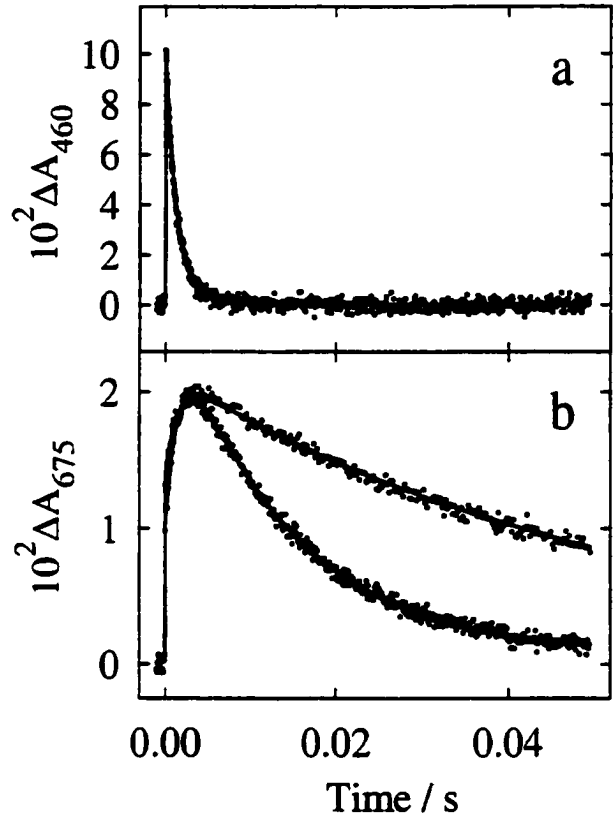

a
b
$10^2\Delta A_{460}$
$10^2\Delta A_{675}$
10
8
6
4
2
0
2
1
0
0.00
0.02
0.04
Time / s

Figure 1

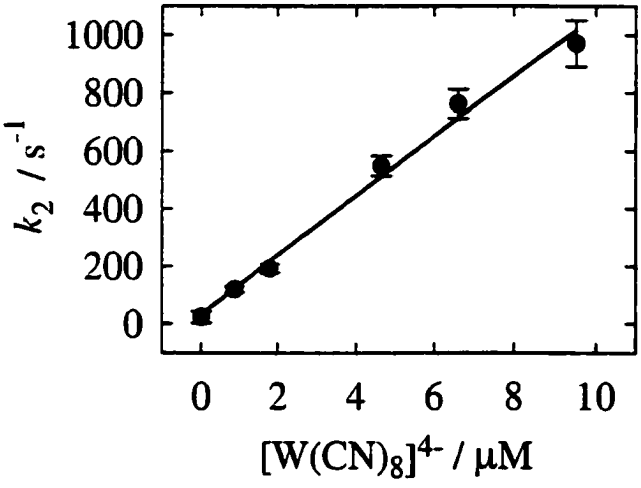


Figure 2

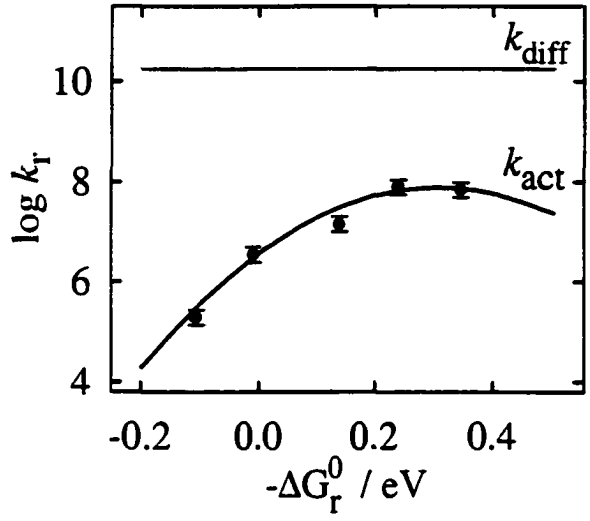


Figure 3

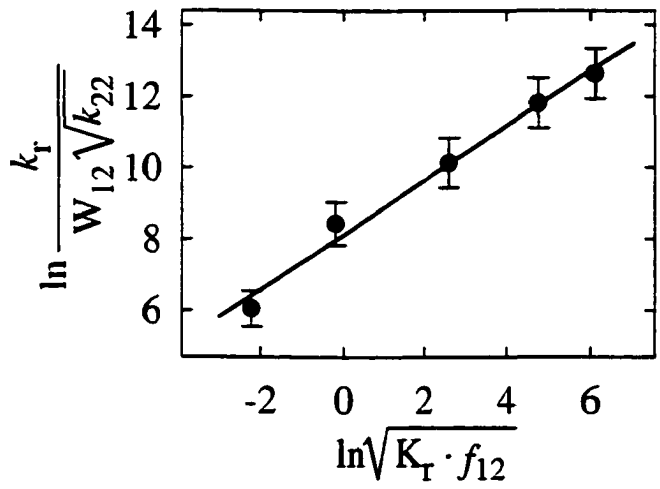


Figure 4

CHAPTER 4. EFFECTS OF PH ON PROTEIN ASSOCIATION. THE CASE OF CYTOCHROME C AND PLASTOCYANIN. EXTENSION OF THE PROTON-LINKAGE MODEL AND EXPERIMENTAL VERIFICATION OF THE EXTENDED MODEL

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All kinetic experiments and fittings were done by primary author. Interpretation of results is the equal contribution from M. M. C. and G. M. U.

Introduction

Association of proteins with other biomolecules and with each other is an important step in various biological processes. Specific recognition between hormones and receptors, enzymes and inhibitors, antibodies and antigens, and electron carriers and redox enzymes depends on their selective association. Protonation state, and therefore charge, of titratable groups plays a decisive role in association.[1] When this process involves transfer of H+ ions, it will depend on pH.[2,3]

Studies of the effects of pH on protein-protein association are scarce. Among the few systems examined are cytochrome *c* and cytochrome b_5,[4,5] proteinases and their inhibitors,[6] and cytochrome *c* and cytochrome *c* peroxidase.[7] The obvious method of studying these systems – determining and interpreting pH dependence of association constant – has not been systematically applied.

To develop this method, we chose a well-characterized system of two prototypical electron carriers, negatively-charged blue copper protein plastocyanin (pc) and positively-charged heme protein cytochrome *c* (cyt). Each of them contains many charged amino-acid residues suited for electrostatic interactions. The negative charge in high-plant plastocyanins is localized mostly in the so-called acidic patch, which consists of the lower cluster— residues Asp42, Glu43, and Glu44 — and the upper cluster— residues Glu59, Glu60, and Asp61. Some of the acidic residues seem to have unusually high pK_a values,[8] but consequences of this decreased acidity have not been investigated. The positive charge in mitochondrial cytochromes *c* is found in the basic patch surrounding the heme edge exposed at the protein surface. These patches in the two proteins have been implicated in their reactions with charged metal complexes,[8-12] other proteins,[13-17] and each other.[18-21] The goal of this study is not to find out more about the protein pair in eq 1 and Figure 1, but to develop a method for studying noncovalent protein association exemplified by this pair.

$$\text{cyt} + \text{pc} \overset{K}{\rightleftharpoons} \text{cyt / pc} \qquad (1)$$

To determine the association constant, K, we take advantage of the redox activity of the proteins and measure the bimolecular rate constant for their electron-transfer reaction at different pH values. Kinetic studies with native proteins would require use of external redox agents, which would interact with the proteins and perturb their association. We avoid these complications by studying the photoinduced reaction in eq 2. Replacement of iron(II) by zinc(II) in the heme does not markedly affect the structure of cytochrome *c* and its association with other proteins.[23-25] The oxidation of the triplet (excited) state of the zinc

derivative, ^{3}Zncyt, by copper(II)plastocyanin, pc(II), has extensively been studied in our laboratory.[19,21,26-30] Kinetics is a "clean" method by which to dependence of K on pH.

$$^3\text{Zncyt} + \text{pc(II)} \underset{}{\overset{K}{\rightleftharpoons}} {}^3\text{Zncyt} / \text{pc(II)} \rightarrow \text{Zncyt}^+ / \text{pc(I)} \qquad (2)$$

The so-called linkage model relates the chemical potential of external ligand and the free energy of its association with a receptor.[31] In the proton-linkage model, th ligand is H^+ ion. The number of H^+ ions released or taken up during a reaction (q) is related to the pH dependence of free energy of this reaction. If association constant K at one pH value and the number q are known, then K at another pH value can be calculated with eq 2. This model has been applied to protein-protein association,[4,7,32] protein unfolding,[33] and redox reactions.[34,35]The model should apply also to treat the release of different species, such as dioxygen, iron or calcium for instance. Its main shortcoming is that it treats the reacting system as a reservoir of H^+ ions and does not give information about individual reactants and the titratable groups involved in the transfer of H^+ ions.

$$\log K(pH_2) = \log K(pH_1) + \int_{pH_1}^{pH_2} q \, d\text{pH} \qquad (3)$$

In this study, we extend the familiar proton-linkage model so it can give information about individual proteins and even about amino-acid residues involved in protein association. In particular, we extract from the experimental results the composite pK_a values, both in separate and in combined proteins, of groups of residues that change their protonation state upon protein association. We allow for interactions among the residues as theoretically calculate individual pK_a values of all titratable residues in proteins. We study the effects of

several plastocyanin mutations on the pH dependence of association and explain these effects in terms of the extended model. Finally, we compare the calculated effects of the mutations on individual pK_a values and their observed effects on protein association and identify some of the residues in plastocyanin are deprotonated as this protein associates with cytochrome *c*.

Materials and Methods

Chemicals. Distilled water was demineralized to resistivity greater 16 MΩ cm. Chromatography gels were purchased from Sigma Chemical Company; triethanolammonium chloride, from Aldrich Chemical Company; hydrogen fluoride, nitrogen, and ultrapure argon, from Air Products Co. All other chemicals were purchased from Fisher Chemical Company.

Buffers. The buffers kept the ionic strength of 2.50 mM over the entire pH range, from 5.4 to 9.0.[36] This full range was covered with a buffer made by dissolving 2.50 mmol (0.4641 g) of triethanolammonium chloride and 2.50 mmol (142.8 μL) of glacial acetic acid in 1.000 L of water. The pH interval from 5.4 to 7.2 was covered also with a 2.50 mM solution of sodium cacodylate, and the pH interval from 6.6 to 9.0 was covered also with a 2.50 mM solution of triethanolammonium chloride. The basic component of the first two buffers and the acidic component of the third were created in situ by adding 0.100 M solutions of NaOH or HCl, respectively. These solutions of base and acid were used also to adjust the pH, which was measured with a Fisher Accumet 805 MP pH meter equipped with an Aldrich combination microelectrode. The values of association constant (K) in the overlapping region, from pH 6.6 to pH 7.2, were the same regardless of the buffer chosen.

Proteins. Horse-heart cytochrome *c* was purchased from Sigma Chemical Co. The iron-free (so-called free-base) form was made, purified, and reconstituted with zinc(II) by a

modification[25] of the original procedure.[37,38] The product, zinc cytochrome *c*, was handled at 4 °C, in the dark. The criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$. The absorptivity at 423nm is $2.43 \cdot 10^5$ $M^{-1}cm^{-1}$.[37] Spinach plastocyanin and its three single mutants were prepared by overexpression in *E. coli*[39] with the vector pUG101t_r[13] and purified first with a DE32 column and then with a 26/10 Q Sepharose high-performance FPLC column from Pharmacia. The blue fraction was concentrated by dialysis against dry polyethyleneglycol (PEG 20000) and passed through a gel-filtration column Sephacryl S-100 HR. The amount of copper(II)-plastocyanin was determined spectrophotometrically in the presence of $K_3[Fe(CN)_6]$, on the basis of the absorptivity at 597 nm, which is 4700 M^{-1} cm^{-1}.[39] The UV-vis spectra were recorded with a Perkin-Elmer Lambda 18 spectrophotometer.

Flash Kinetic Spectrophotometry. The so-called laser flash photolysis on a microsecond time scale was done with a standard apparatus.[40] Argon for deaeration was first passed through water and then gently through the buffered solution of the proteins for 30 minutes. The sample cuvette was kept at 293.0(2) K with the 30-L circulating bath Forma 2067. Concentration of Zncyt was kept at 10.0 μM, while concentration of pc(II) was varied from 2.0 to 30.0 μM. Initial volume of the protein solution was 1.000 mL. Upon each addition of a small portion (1.00 to 4.00 μL) of a 0.100 M solution of NaOH or HCl, the pH was measured with the freshly-calibrated pH meter, and the solution was deaerated for 10 more minutes. Calibration of the pH meter was checked after the last adjustment of pH. Formation and decay of the triplet sate, ^{3}Zncyt, was monitored by change in the absorbance

at 460 nm, ΔA_{460}. For each concentration of pc(II) at a given pH value, four or more laser pulses were delivered, and kinetic traces were recorded.

Regression Analysis of the Rate Constant k_{obs} and Association Constant K. The kinetic traces were fitted to eq 4 by a least-squares nonlinear regression method, with the program SigmaPlot v4.01, obtained from SPSS Inc. We confirmed[19,20,26,27,41] that

$$\Delta A_{460} = a_1 \exp(-k_F t) + a_2 \exp(-k_{obs} t) \tag{4}$$

the first-order rate constant k_F does not depend on concentration of pc(II) and corresponds to unimolecular quenching of ^{3}Zncyt by pc(II), while the pseudo first-order rate constant k_{obs} depends on the concentration of pc(II) and corresponds to bimolecular quenching of ^{3}Zncyt by pc(II). Fitting of k_{obs} to eqs 5 and 6 gave the rate constants for association, k_{on}, and dissociation, k_{off}, of the Zncyt/pc(II) complex, as in our previous studies.[19,28,42] The typical fit is shown in Figure S1 in the Supporting Information. In this study, for the first time, we analyze the association constant K, calculated from eq 7. The error margins for all reported values correspond to two standard deviations and the confidence limit greater than 95%.

$$k_{obs} = \frac{k_{on}\, k_F\, [\mathrm{pc(II)}]}{k_{off} + k_F + k_{on}\, [\mathrm{pc(II)}]} \tag{5}$$

$$[\mathrm{pc(II)}] = [\mathrm{pc(II)}]_0 - \frac{1}{2}\left\{[\mathrm{Zncyt}]_0 + [\mathrm{pc(II)}]_0 + \frac{k_{off}}{k_{on}} - \sqrt{\left([\mathrm{Zncyt}]_0 + [\mathrm{pc(II)}]_0 + \frac{k_{off}}{k_{on}}\right)^2 - 4[\mathrm{Zncyt}]_0[\mathrm{pc(II)}]_0}\right\} \tag{6}$$

$$\mathrm{K} = \frac{k_{on}}{k_{off}} \tag{7}$$

Calculations of pK_a Values of Individual Residues. The crystal structures of horse cytochrome *c* (1HRC)[43] and spinach plastocyanin (1AG6)[44] were taken from the Protein

Data Bank. Since the latter structure is known only for the Gly8Asp mutant, we changed Asp8 back to Gly with the program CHARMM.[45] We then created the mutants Asp42Asn, Glu59Gln, and Glu60Gln by the same method. In these slight mutations of the carboxylic acid to the corresponding amide the coordinates of nonhydrogen atoms in the side chains were kept fixed. The hydrogen atoms were added by the HBUILD method,[46] and their energy was minimized in 3000 steps of steepest descent in the CHARMM program.

The pK_a values of amino-acid residues were calculated by a standard method,[3,47] starting from pK_a values of model compounds, N-formyl methylamide derivatives of amino acids.[48] The partial charges were taken from AMBER94[49] force field for atoms in side chains and from a quantum-mechanical calculation[50] for the blue-copper site. If all the residues in the protein were electroneutral, a titratable side chain would have the so-called intrinsic pK_a value. The intrinsic pK_a values and the interaction energies between titratable groups were obtained by the MEAD program.[51] solving the linearized Poisson-Boltzmann equation on a grid in two focusing steps.[52,53] The dielectric constants of the protein interior and of water were set to 4.0 and 80.0, respectively.[54] The ionic strength was set to 2.50 mM. The solvent-accessible and the ion-exclusion layers were probed with a sphere having the radius of 1.4 Å and 2.0 Å, respectively. The grid was centered on the geometric center of the model compound or the protein in the first step and on the titratable group in the second. The first grid had a spacing of 1.0 Å; the second, of 0.25 Å. The two grids had the same length in all three dimensions: 61 Å for the calculations of the model compounds and 81 Å for the calculations of the proteins. Calculated ΔG° for transferring the titratable group from aqueous solution to the protein in which all other residues are electroneutral was added to the pK_a

value of the appropriate model compound. One of us (G.M.U.) wrote a program that uses the output of the program MEAD and determines the protonation probabilities of all titratable residues in a protein on the basis of free energies of protonation calculated by a hybrid of a statistical-mechanical method and the Tanford-Roxby method.[55] The program is available at http://www.scripps.edu/~ullmann/program.html.

The pK_a value of an isolated titratable side chain is the pH value at which the protonation probability, $\langle x \rangle$, is 0.50. Because an isolated side chain obeys the Henderson-Hasselbalch equation, $pK_a = pK_{1/2}$. But a protein exists in multiple protonation states, and the $pK_{1/2}$ value of a side chain no longer reflects the probability of its protonation.[3] Consequently, the pK_a value depends on pH, as Figure S2 in Supporting Information shows. Fortunately, this dependence is very small over the pH range of our experiments.[56] For the sake of accuracy, $\langle x \rangle$ were calculated over the entire range $5.4 \leq pH \leq 9.0$ in steps of 0.05 pH units, and at each step the pK_a result was obtained with eq 8. An average of all the pK_a results corresponding to $\langle x \rangle > 0.0010$ for a given titratable side chain is the pK_a value of this side chain.

$$pK_a = pH + \log \frac{\langle x \rangle}{1 - \langle x \rangle} \tag{8}$$

Extended Proton-Linkage Model. We extend the original linkage model[31] to extract from it not only the number of H^+ ions released or taken up upon association, but also the pK_a values of the groups of residues involved in association. When m_k amino acid residues each lose a H^+ ion in the same pH interval, we designate them *isoacidic residues.* The width of the pH interval and other criteria for residues to be considered isoacidic will be

analyzed in the Discussion section. The system (protein pair) has M groups of isoacidic residues, which are indexed with k. Each group acts collectively and has one composite pK_a value in the separate ($pK^s_{a_k}$) and another composite pK_a value in the combined ($pK^c_{a_k}$) proteins.

Each group of isoacidic residues can lose m_k hydrogen ions. When a total of $\sum_{k=1} m_k$ ions are bound, all residues in all isoacidic groups are protonated, and only one protonation state is possible. We take it as the reference protonation state of the protein pair, for which the association constant is K_o. The other protonation states are defined with respect to this one.

When i of the M isoacidic groups are deprotonated, the number of possible protonation states is p_i, given by eq 9. The protonation states with the same number of deprotonated groups are indexed with j, which runs from 1 to p_i. Each protonation state of the protein pair is designated by two indexes, i and j. Going from the reference (fully-protonated)

$$p_i = \binom{M}{M-i} = \frac{M!}{(M-i)!i!} \tag{9}$$

$$a_i^j = \sum_{k=1}^{M} x_i^{jk} m_k \tag{10}$$

$$L_i^{js} = \prod_{k=1}^{M} x_{ik}^j \left(K^s_{a_k}\right)^{m_k} = 10^{-\sum_{k=1}^{M} x_{ik}^j m_k pK^s_{a_k}} \tag{11}$$

$$L_i^{jc} = \prod_{k=1}^{M} x_{ik}^j \left(K^c_{a_k}\right)^{m_k} = 10^{-\sum_{k=1}^{M} x_{ik}^j m_k pK^c_{a_k}} \tag{12}$$

state to the *i,j* protonation state, the protein pair loses a_i^j hydrogen ions, the number given by eq 10. The component x_{ik}^j of a protonation-state vector shows whether the *k*-th group of *M* isoacidic groups is protonated (0) or not (1). The constant L_i^j, defined for separate (superscript s) proteins in eq 11 and for combined (superscript c) proteins in eq 12, is the equilibrium constant between the reference state and the *i,j* state (for a detailed explanation, see Supporting Information). These equilibrium constants can be related to the $pK_{a_k}^s$ and $pK_{a_k}^c$ values, as the right-hand sides of eqs 11 and 12 show.

$$K = K_0 \frac{\sum_{i=0}^{M} \sum_{j=1}^{p_i} \left[H^+\right]^{-a_i^j} L_i^{jc}}{\sum_{i=0}^{M} \sum_{j=1}^{p_i} \left[H^+\right]^{-a_i^j} L_i^{jc}} \qquad (13)$$

Protein association (within the system) can be accompanied by release and uptake of H^+ ions (by the system). Therefore the association constant K depends on the concentration of H^+ ions. We experimentally determine this dependence and fit it to eqs 9-13. We estimated the initial values of the fitting parameters in eqs 11 and 12 from the experimentally-determined dependence of K on pH. According to eq 3, the slope of the plot of log K versus pH is q, defined in eq 14. Positive and negative values of q correspond to release and uptake, respectively, of H^+ ions upon protein association. We round to integers the maxima and minima of q and use their absolute values as initial values of m_k in the fittings to eqs 9-13. In the program SigmaPlot, m_k values had to be treated as constants and changed manually. Fittings of the experimental

$$q = \frac{d \log K}{d\,pH} \tag{14}$$

results to eqs 9-13 yielded K_o, three $pK^s_{a_k}$ values for three groups of isoacidic residues in separate (free) proteins, and three $pK^c_{a_k}$ values for these groups in combined (associated) proteins. Fidelity of the fit to the experimental results was judged by the correlation coefficients (R^2). We considered the fitting successful if the fitted line passed through all 19 experimentally-determined points (within the error bars); all such fittings gave $R^2 \geq 0.990$. The fitted values of m_k, $pK^s_{a_k}$, $pK^c_{a_k}$ are reasonable, clearly related to the known properties of the proteins under study. That reasonableness, not merely the high statistic quality of the fits, reassured us about the soundness of these results.

A detailed derivation of the extended proton-linkage model and an example of its application to our experimental results are given in the Supporting Information.

Results

Oxidative Quenching of ^{3}Zncyt by pc(II). In the absence of copper(II)plastocyanin, the excited state ^{3}Zncyt decays with the rate constant of 80±10 s^{-1}. In the presence of copper(II)plastocyanin, this excited state is oxidatively quenched. As concentration of copper(II)plastocyanin increases the rate constant increases as well, as shown in Figure S1 in the Supporting Information. These findings confirm the previous detailed studies in this laboratory and were analyzed as before.[20,26,41] In this study, we are interested in the bimolecular reaction between the two proteins, whose rate constant is k_{obs} and whose amplitude is a_2.

When the concentration of copper(II)plastocyanin is kept constant and pH is raised from 5.4 to 9.0, the rate constant k_{obs} increases, reaches a maximum, and then decreases, as seen in Figure S3 in Supporting Information. Simultaneously, the amplitude a_2 decreases, reaches a minimum, and increases, as seen in Figure S4 in the Supporting Information.

Dependence of the Association Constant K on pH. Kinetics of electron transfer is not a goal of this study but a reliable means of investigating the association of plastocyanin and cytochrome *c*. We experimentally determined K at nineteen pH values in the interval 5.4 to 9.0 and did so for four protein pairs, those that cytochrome *c* forms with the wild-type plastocyanin and its three mutants, Figures 2a—d. When pH is raised from 5.4 to 9.0, association constant K increases, reaches a maximum, and then decreases. The wild-type plastocyanin and all three mutants show the same qualitative trend, but quantitative differences among them are important.

The Fitted m_k Values. All four plots in Figure 3 were successfully fitted in the same way. Reasonable initial values of m_k were available from experimental data – absolute values of the q extrema, as explained above. For example, the initial m_k values for fitting of results in Figure 3a were m_1=2, m_2=1, and m_3=3. We kept changing the values until an excellent fit was achieved, with correlation coefficient $R^2 \geq 0.990$. But we did not stop there. To make sure that the fitting was not accidental, we changed the m_k values trying to spoil the fit. Some of these attempts are shown in Figure S5 in Supporting Information. If a set of m_k values was unsatisfactory, no adjustment of the six pK_a values could produce a good fit. Of the approximately 300 attempted fittings, only eight were satisfactory. These eight sets of fitted parameters are the eight columns in Table 1, two columns each for wild-type plastocyanin and its three mutants; each of them interacts with cytochrome *c*.

The Fitted Composite pK_a Values. Criterion for Isoacidic Residues. The estimated error in the pK_a values is ±0.10 pH units. Changes of this magnitude in the pK_a values were more than sufficient to divert the fitted line from the error bars of some of the experimentally-determined points, as seen in Figures S6-S8 in Supporting Information. Given the precision of the experimental results, the individual pK_a values of the residues belonging to the same isoacidic group differ by ±0.30 pH units or less. We determined this interval in many attempts to spoil the fittings by imposing spreads of various magnitude on these individual pK_a values. When the spread was within ±0.30 pH units, the fitted lines passed through the error bars of all experimentally-determined points for each of the four protein pairs.

Because association of cyt with all four forms of pc obeys the same equations, because excellent fits were nearly unique, and because the fitted parameters were quite reasonable, we accepted the success in fitting as verification of the extended proton-linkage model.

Calculated Individual pK_a values. We used an advanced electrostatic model to calculate the pK_a values of all titratable residues in all four forms of plastocyanin and cytochrome *c*; see Tables S1 and S2 in Supporting Information. The values for the residues in the acidic patch of plastocyanin, which will be discussed, are highlighted in Table 2.

Discussion

Protein Association. Protein association involves hydrophobic interactions, which do not depend on pH, and electrostatic (ionic and polar) interactions, which do.[57] By changing

the pH value, one can adjust the protonation state of titratable side chains and study the dependence of the association constant, K, on this state.

Association constant can be determined by NMR spectroscopy or calorimetry, but there are many pitfalls in these experiments. Exchange of H^+ ions, nonspecific perturbation of NMR resonances, interactions of the proteins with the buffer as well as with each other, and the deviations from ideal behavior when the protein concentrations are relatively high—all of these factors complicate the experiments.[57] To complicate matters further, these interactions and deviations may also depend on pH. Even if the technical problems are solved, the final results may be difficult to interpret. If the protein association is a step in a chemical reaction, especially if multiple protein complexes are possible, it may not be clear whether the constant K corresponds to the reactive complex.

This fundamental ambiguity is avoided if the constant K is determined by studying the kinetics of the reaction in which the association of interest is a step. Kinetic studies require relatively low concentrations of proteins and can give precise values of the association constant.

Because deprotonation of the carboxyl group and protonation of the amino group are endoergic processes, these electroneutral groups are unfavorable as precursors of salt-bridges. The salt-bridges are preferentially formed from the preexisting carboxylate anions and ammonium cations.

The Protein Pair. To study in detail the effect of pH on association between proteins, we chose a pair of stable and well-characterized metalloproteins having complementary electrostatic properties. The prototypical blue copper protein plastocyanin, designated pc, has an anionic (acidic) patch consisting of two clusters: Asp42, Glu43, and Glu44 make up the

so-called upper cluster, while Glu59, Glu60, and Asp61 make up the so-called lower cluster.[11,58-60] The prototypical heme protein cytochrome *c*, designated cyt, has a cationic (basic) patch consisting mostly of Lys residues.[61-63] Chemical modification of cyt,[64] saturation kinetics in the electron-transfer reaction,[19,26,41] NMR spectroscopy,[65] site-directed mutations in pc,[28,30] and computational simulation of docking[66,67] all agree that the acidic patch in pc abuts the basic patch of cyt upon association, and it is not a purpose of this study to verify this fact. Marked dependence of association constant on ionic strength indicates large contribution of polar interactions to this association.[41,68] Because these two proteins undergo electron-transfer reactions, their association constant, K, is precisely determined by kinetic experiments.

Dependence of Association Constant, K, on pH. We will always consider the effects of the increasing pH value. As Figures 2a—d show, affinity of cytochrome *c* for each of the four forms of plastocyanin follows the same general trend— growth from 5.4 to ca. 6.5, then decline to 9.0. We will first explain this general increase and decrease in K, and will later analyze the inflections in the plots.

The initial increase can be due to decreasing repulsion or increasing attraction between the two proteins as pH is raised. The first explanation can be true if cationic side chains existed on the interacting surfaces of both proteins at pH 5.4, so that their deprotonation abolishes the repulsion. Since, however, the acidic patch in plastocyanin lacks cationic side chains, we reject the hypothesis of decreasing repulsion. Deprotonation of carboxyl groups in the acidic patch in plastocyanin favors salt-bridges with the basic patch in cytochrome *c*, attraction increases, and the K reaches its maximum around pH 6.5.

The decrease in K as the pH is raised further can, similarly, be due to increasing repulsion or decreasing attraction between the proteins. The first hypothesis requires that interacting surfaces in both proteins contain acidic side chains, whose deprotonation would cause repulsion between the resulting carboxylate anions. Because, however, the basic patch in cytochrome *c* lacks acidic side chains, we reject this hypothesis of increasing repulsion. Deprotonation of cationic residues in this basic patch disfavors salt-bridges with the acidic patch in plastocyanin, and the K decreases. To summarize, as pH is raised, first the acidic residues in plastocyanin and then the basic residues in cytochrome *c* loose H^+ ions to the solvent.

To test this reasonable explanation and to explain quantitatively the dependence of K on pH, we needed a suitable theoretical model for this dependence. Only one such model seems to be commonly used now in chemistry and biochemistry.

The Proton Linkage Model. This established model[31] has been used to study protein-protein association[4,7,32] and redox potentials of proteins.[34,35] In principle, the model applies to dependence of binding affinity on any ligand. When the variable is pH, the ligand is the H^+ ion. The model (eq 3) correlates the effect of pH on association to the number of H^+ ions (q) transferred between the system and the solvent. The transfer can be release or uptake by the system; the system is a pair of proteins, cytochrome *c* and plastocyanin considered together; and the solvent is the buffer. As Figure 1 shows, the system can exist in two states: the proteins are either separate or combined. When the system changes state, H^+ ions may be transferred between it and the buffer. If a titratable side chain is affected by the change of state of the system, this side chain will have different individual pK_a values in the two states. Consequently, the change of state may cause (de)protonation of

this side chain. A swap of H^+ ions within the system (i.e., between the proteins) cannot be detected. We are interested in those cases in which protein association triggers uptake or release of H^+ ions by the protein pair (viewed as a whole). If this H^+ transfer occurs in the experimental pH range, the transfer can be detected in the plot of K vs. pH.

The proton-linkage model treats the system as a reservoir of H^+ ions and yields only the number of these ions involved in a pH-dependent process. Because the model does not recognize the specific sources of these ions, it can not give detailed information about the process. If the pK_a values of these sources could be determined, the model would become a useful tool for the study of chemical and biochemical reaction mechanisms. We extended the model and estimated the composite pK_a values of groups of amino-acid residues involved in the protein association.

Extended Proton-Linkage Model. In principle, our analysis holds equally for increasing for increasing or decreasing pH, association or dissociation of the protein pair, and uptake or release of H+ ions by the pair. By convention, however, we consider the changes in the direction of increasing pH, where protein association may trigger release of ions to the buffer.

We introduce the concept of *isoacidic residues*, those that lose a H^+ ion each in the same pH interval. This concept and definition apply also to residues such as lysine that are normally termed as basic. Because the protonated form of lysine is acidic and looses a H^+ ion as pH is raised. Individual pK_a values of isoacidic residues are clustered within ±0.30 pH units, as explained above. The width of this interval depends on the specific system under study and precision of the experimental measurements and results. A combination of these values is a *composite pK_a value* for the *isoacidic group* of residues.

Because the system has two states (separate and combined proteins), each isoacidic group has two pK_a values designated pK_a^s and pK_a^c, respectively. If an isoacidic group is affected by change in state (i.e. protein association), pK_a^s and pK_a^c will differ. If the transfer of H^+ ions between an isoacidic group and the buffer occurs in the pH range covered in the experiments, this isoacidic group can be detected.

Scheme 1 shows the effects of protein association and of pH on a carboxyl side chain, representing a group of isoacidic aspartic or glutamic residues. This conceptual Scheme applies regardless of the state of the basic residues; in this case they happen to be protonated, as cations. Association and dissociation of the proteins are represented by horizontal arrows; release or uptake of H^+ ions, by vertical arrows; the two processes combined, by the downward diagonal arrow. When $pH<pK_a^c$ the neutral isoacidic group predominates in both separate and combined proteins. Upon association (the upper horizontal), the system does not release a H^+ ion (to the buffer). A salt-bridge cannot form, but a hydrogen bond (dotted line in lightface) may form. At $pK_a^c<pH<pK_a^s$, the side chain is predominantly neutral when the proteins are separate but predominantly protonated when they are combined. Association (the diagonal) triggers release of a H^+ ion, which can be detected. A salt-bridge (dotted line in boldface) can form. At $pH>pK_a^s$, the carboxylate anion predominates in both separate and combined proteins. Upon association (the lower horizontal) the system does not release a H^+ ion, and a salt-bridge can form.

Scheme 2 shows the effects of association and of pH change on an ammonium side chain, representing a group of isoacidic lysine residues. The acidic residues in plastocyanin happen to be deprotonated. As before, association and H^+ loss are represented by rightward

and downward arrows, respectively; combination of association and H^+ uptake is represented by diagonal arrow. As before, the (de)protonation state, and therefore the charge, of the isoacidic group depends on the relation among the pH, pK_a^s, and pK_a^c values. The main consequence of these relations is the possibility for forming salt-bridges or merely hydrogen bonds.

When the oppositely-charged interaction surfaces in separate proteins are hydrophilic, as in the system understudy, upon association acidic groups tent to lose H^+ ions, while basic groups tend to gain H^+ ions. For this reason, the diagonal arrows in Schemes 1 and 2 are placed as they are. The arrows along the other diagonal are omitted for the sake of clarity, although the extended model is equally applicable to the processes shown and not shown.

Extended proton-linkage model allows quantitative treatment of the release or uptake of the H^+ ions by the system triggered by the change of the system state. Given the large number of titratable side chains in proteins, their individual pK_a values are intractable. An attempt to consider individual side chains would require too many parameters, with which fittings of experimental results would be meaningless. Therefore we recognize the existence of isoacidic residues, which act as a group. Because composite pK_a values of these groups are relatively few, fittings with them are possible and meaningful.

Application of the Extended Proton-Linkage Model to Wild-Type Plastocyanin and Cytochrome *c*. The Number of Isoacidic Residues. The results are shown in Figure 3a. The general trend was explained above: as pH is raised, an increase in K due to the acidic residues in plastocyanin, followed by a decrease due to the so-called basic (actually acidic when protonated) residues in cytochrome *c*. Now we will analyze subtler, but more revealing, inflection points in the curve. There are three of them, corresponding to three groups of

isoacidic residues. The first one is in plastocyanin, the next two in cytochrome c. Each isoacidic group contains m_k residues and has a pK_a^s value in the separate and pK_a^c in the combined proteins. As explained in Materials and Methods, the fitting yields ten parameters: three sets of m_k, pK_a^s, pK_a^c values and one K_o value.

Fitting of Figure 3a to eqs 9-13 is very sensitive to the choice of m_k values and sensitive to the choice of the other parameters. If the m_k triplet was wrong, variations in other parameters could never result in a good fit.

We often tested fits by deliberately spoiling them— changing m_1, m_2, or m_3 a little to see if the fit will markedly worsen. We rejected seemingly good fits that failed to be spoiled in this way. A few of these tests are shown in Figure S5 in Supporting Information. In the end, out of nearly 100 attempts at fitting with different m_k triplets, only two met all the criteria and are given in the first two columns in Table 1.

The m_k values show that association involves a group of three isoacidic residues in wild-type plastocyanin and also one group of two or three and another group of three isoacidic residues in cytochrome *c*. The apparent mismatch between the salt-bridge partners, three in plastocyanin versus five or six in cytochrome c is not worrisome. The other residues in plastocyanin that form salt-bridges have typical, relatively low, pK_a values and are undetectable in the pH range covered.

A direct titration of cytochrome *c* showed that this protein loses ca. five H^+ ions in this pH range.[69] Indeed, our fitting gave the sum m_2+m_3 of five or six. Evidently, the extended proton-linkage model gives realistic numbers of isoacidic residues.

Application of the Extended Proton-Linkage Model to Wild-Type Plastocyanin and Cytochrome *c*. The pK_a Values. A kinetic study of electron-transfer reactions of plastocyanin suggested that some residues in this protein have pK_a values around 6.[8] Indeed, our fittings gave $pK^s_{a_1}$ of 6.4 and 6.3 for the first isoacidic group in separated proteins.

To test the sensitivity of the fits to composite pK_a values of isoacidic groups, we systematically changed these values, one at the time, in the first two columns in Table 1. Some of these attempts are shown in Figures S6-S8 in the Supporting Information. Deviations from the best values by as little as ±0.10 clearly spoil the fits. We conclude that the error margin for the composite pK_a values in this study is ±0.10 pH units. It was achieved with 19 values of K covering a range of 3.6 pH units, shown in Figure 3a. The precision of fitting depends on the soundness of the theoretical model and on the number and quality of the experimental results.

The two successful fittings in Table 1 are quite consistent; only the $pK^s_{a_2}$ values in the two columns differ by more than 0.10 pH units. The following interpretation is consistent with both fittings. That $pK^s_{a_1} > pK^c_{a_1}$, whereas $pK^s_{a_2} < pK^c_{a_2}$ and $pK^s_{a_3} < pK^c_{a_3}$ means that upon association carboxylate anions in plastocyanin and ammonium (lysine) cations in cytochrome *c* are favored. This findings is symptomatic of salt-bridges in a polar environment.

The possibility of extracting composite pK_a values of groups of residues and analyzing their shifts upon protein association is the chief benefit of our extension of the standard proton-linkage model.

Application of Extended Proton-Linkage Model to Plastocyanin Mutants and Cytochrome *c*. In each of the three mutants one carboxyl group in the acidic patch is changed to the corresponding carboxamide, Asp to Asn or Glu to Gln. This noninvasive[13,17] change alters electrostatic but not steric properties of the side chain[29] and can show how association with cytochrome *c* will change when a residue that can form salt-bridge is noninvasively replaced by the one that cannot. We will pay attention to the changes in m_1, $pK^s_{a_1}$, and $pK^c_{a_1}$ parameters upon each mutation in plastocyanin.

Out of some 200 attempts at fitting, each of the plots in Figures 2b—d was accurately reproduced (correlation coefficients $R^2 \geq 0.990$) with only two sets of parameters. The consistency of the two fits can be assessed by comparing columns in each pair in Table 1. For Asp42Asn, m_3 differs by one, and the pK_a values agree. For Glu59Gln, m_2 differs by one, and only $pK^s_{a_3}$ values differ slightly. For Glu60Gln, m_3 differs by one, and two pK_a values differ by more than 0.3 pH units. Although these differences are relatively small, we interpret the parameters cautiously.

Plastocyanin Residues Involved in Association with Cytochrome *c*. In our experiments we could not go bellow pH 5.4 and observe the pK_a values of ordinary acidic residues because these conditions would damage plastocyanin.[70] The residues belonging to the isoacidic set m_1 have unusually high pK_a values, a symptom of their mutual interactions within plastocyanin. Deprotonation of one carboxyl group makes the deprotonation of another more difficult, and their pK_a values increase. We do not use the pK_a values of individual residues in Table 2 to interpret the inflection points in Figures 2a—d because individual residues are subsumed in isoacidic groups, with their composite pK_a values. The

calculated values in Table 2 are, however, useful for detection and qualitative assessment of effects of mutations in plastocyanin. Because the theoretical calculations allowed interactions among the residues, the individual pK_a values can show indirect effects of mutation of a given residue on other residues in plastocyanin. In the following discussions we use results from both Tables 1 and 2.

Effects of the Asp42Asn Mutation. Residue 42 belongs to the lower cluster in the acidic patch. Because its pK_a in all the plastocyanin forms is 4.3 or 4.4, well outside the pH range covered, it is unlikely to contribute to an isoacidic group whose composite pK_a^s is 6.1. We conclude that Asp42 cannot directly contribute to the dependence in Figure 3a. Indeed, the m_1 value remains unchanged upon mutation of Asp42, evidence that this residue is not deprotonated upon association with cytochrome *c*. But this residue can contribute to the pH dependence of K indirectly, through its interactions with other residues. As Table 2 shows, upon mutation Asp42Asn individual pK_a values of the neighboring residues Glu43 and Asp44 are lowered by 0.4 and 0.8 units, respectively. This change probably contributes to the slight lowering, by 0.2 or 0.3 units (see Table 1), of the composite $pK_{a_1}^s$ value upon mutation.

Effects of the Glu59Gln Mutation. Residue Glu59 belongs to the upper acidic cluster. With the individual pK_a value of 5.5, it likely contributes to the isoacidic group whose composite $pK_{a_1}^s$ is ca. 5.6 or 5.7. As Table 1 shows, m_1 is 2 for this mutant (and 3 for the wild-type plastocyanin.) Evidently, when ionization of Glu59 is prevented (by mutation), the number of ionisable residues in plastocyanin is lowered by one. It is tempting to conclude, that Glu59 is one of the three isoacidic residues in wild-type plastocyanin. But mutation of

Glu59 could conceivably act indirectly, by lowering the pK_a value of a proximate residue so that this other residue stays deprotonated over the entire pH range studied and can no longer be seen in Figure 5. As Table 2 shows, mutation Glu59Gln lowers the individual calculated pK_a values of Glu60 and Asp61 relatively little, by 0.6 and 0.3 units, respectively. The large indirect effect is unlikely. We return to the straightforward conclusion that Glu59 itself is a member of the isoacidic group in plastocyanin.

To test this conclusion, we mutated the adjacent residue, Glu60, in an attempt to affect Glu59 indirectly. If our reasoning is correct, ionization of Glu59 in plastocyanin upon association with cytochrome *c* could be thwarted by lowering the individual pK_a value of Glu59.

Effects of the Glu60Gln Mutation. Calculated pK_a of Glu60 is 4.3, probably too low to make this residue a member of the first isoacidic group, which is ionized in the experimental pH range. Although our experiments can not detect ionization of Glu60, if it occurs upon association, our experiments in combination with theoretical calculations of pK_a values can reveal effects of Glu60 on other acidic residues in plastocyanin. As Table 2 shows, mutation to Gln lowers the individual pK_a of Glu59 by 0.6 and of Asp61 by 1.2 units. The values of 4.9 and 5.2 are probably low enough to preclude detectable ionization of Glu59 and Asp61 (as members of the isoacidic group) in the experimental pH range, which starts at pH 5.4.

Indeed, the m_1 value of 1 or 2 (in the last two columns in Table 1) is smaller than the m_1 value of 3 (in the first two columns). Upon mutation Glu60Gln, one or two acidic residues in plastocyanin, likely residues other than Glu60, cease contributing to the release of H^+ ions

upon association with cytochrome *c* in the pH range covered. According to our evidence, one of these residues likely is Asp61; the other, if there are two of them, probably is Glu59.

Implications for the Photosynthetic Function of Plastocyanin. This protein carries electrons from cytochrome *f* to photosystem I in the tylakoid of the chloroplasts, which has pH<5.0.[71] Our experimental results (Table 1) and theoretical calculations (Table 2) consistently show that some of the acidic side chains in plastocyanin retain the H^+ ions, and remain electroneutral, even at pH>5.0, under physiological conditions. To assume that acidic side chains are deprotonated and therefore anionic, as is routinely done when considering electrostatic properties of proteins, would cause mistakes in the study of interaction of plastocyanin with its physiological partners.

Conclusions

We extended the familiar proton-linkage model so that it can reveal some molecular details about pH-dependent phenomena in chemical and biochemical systems. The extended model, in combination with theoretical calculations of pK_a values of individual titratable side chains, can be used to determine composite pK_a values of those side chains that act concertedly or approximately so in exchanging H^+ ions with the solvent upon some change of the system.

This, the first application of the extended proton-linkage model, concerns the pH dependence of the association between two proteins. Intricate dependence of the association constant on pH and effects on this dependence of several site-directed mutations were consistently explained and fitted with nearly-unique sets of reasonable parameters in each

case. Interpretation of these parameters revealed general and specific features of the protein-protein interaction.

The extended proton-linkage model is a general one. Besides protein association, it is applicable to various pH-dependent reactions and interactions, such as binding of substrates and cofactors to enzymes, of proteins to nucleic acids and membranes, of anitgens to antibodies, and of metal ions to biomolecules. Encouraged by the outcome of this study, we will explore some of these new applications of the extended proton-linkage model.

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Table 1. Parameters with which the pH Dependence of Association Constant K in Figures 2a—d were Fitted to Eqs 9-13. Association of Cytochrome *c* with Each of the Four Forms of Plastocyanin was Successfully Fitted with Two Sets of Parameters: m_k is the Number of Isoacidic Residues in a Group; pK_a^s and pK_a^c are Composite pK_a Values for Isoacidic Residues in, Respectively, Separate and Combined (Associated) Proteins; and K_o is the Association Constant for Fully-Protonated Proteins. Subscript 1 Designates Plastocyanin; Subscripts 2 and 3, Cytochrome *c*

	pc form							
parameter[e]	wild type[a]		Asp42Asn[b]		Glu59Gln[c]		Glu60Gln[d]	
m_1	3	3	3	3	2	2	1	2
m_2	2	3	2	2	1	2	1	1
m_3	3	3	2	3	1	1	1	1
$pK_{a_1}^s$	6.4	6.3	6.1	6.1	5.7	5.6	6.3	6.0
$pK_{a_2}^s$	6.3	6.6	6.0	6.0	6.4	6.6	6.3	6.8
$pK_{a_3}^s$	7.9	7.8	8.1	8.1	8.6	8.3	8.2	8.1
$pK_{a_1}^c$	5.8	5.9	5.9	5.9	5.2	5.1	5.0	5.7
$pK_{a_2}^c$	7.0	6.9	6.4	6.4	6.6	6.7	7.0	7.0
$pK_{a_3}^c$	8.3	8.3	8.2	8.1	8.9	8.7	8.6	8.4
10^{-4} K_o	8(1)	7(1)	4.7(6)	4.7(6)	1.3(6)	0.9(4)	0.9(3)	2.6(3)

[a] Fittings shown in Figure 3a.

[b] Fittings shown in Figure 3b.

[c] Fittings shown in Figure 3c.

[d] Fittings shown in Figure 3d.

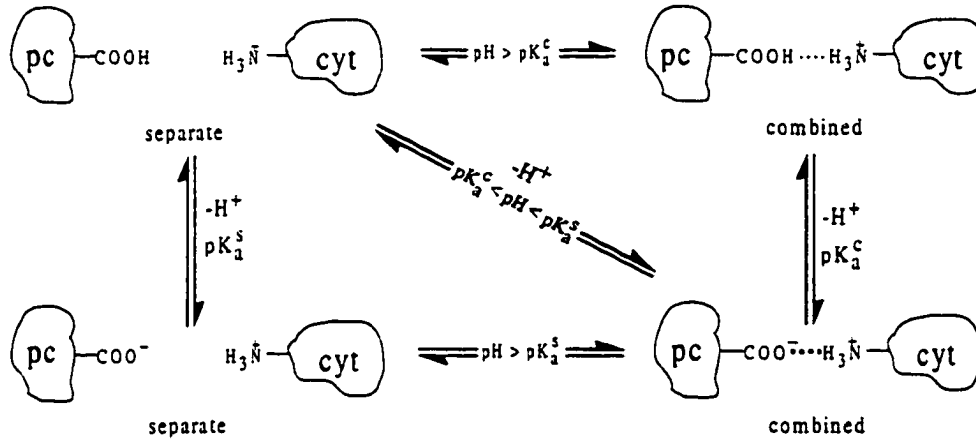
[e] Margins of error for the pK_a values is ±0.10.

Table 2. Calculated pK_a values of Amino-Acid Residues in the Acidic Patch of Plastocyanin, at Ionic Strength of 2.50 mM and 293 K.

	pc form			
residue	wild type	Asp42Asn	Glu59Gln	Glu60Gln
Asp42	4.4	[a]	4.3	4.3
Glu43	6.2	5.8	6.0	6.1
Asp44	6.2	5.4	6.1	6.1
Glu59	5.5	5.4	[a]	4.9
Glu60	4.3	4.3	3.7	[a]
Asp61	6.3	6.3	6.0	5.2

[a] Residue absent in the mutant

Scheme 1. The effect of pH (vertical arrows) and of association (horizontal arrows) on acidic residues in plastocyanin that is separate from and combined with cytochrome *c*. One carboxyl side chain represents a group of several isoacidic residues. Protein association in a certain pH interval triggers release of H^+ ions to the buffer. Boldface dots represent a salt-bridge; lightface dots, a hydrogen bond.



Scheme 2. The effect of pH (vertical arrows) and of association (horizontal arrows) on basic residues in cytochrome *c* that is separate from and combined with plastocyanin. One ammonium side chain represents a group of several isoacidic residues. Protein association in a certain pH interval triggers uptake of H^+ ions to the buffer. Boldface dots represent a salt-bridge; lightface dots, a hydrogen bond.

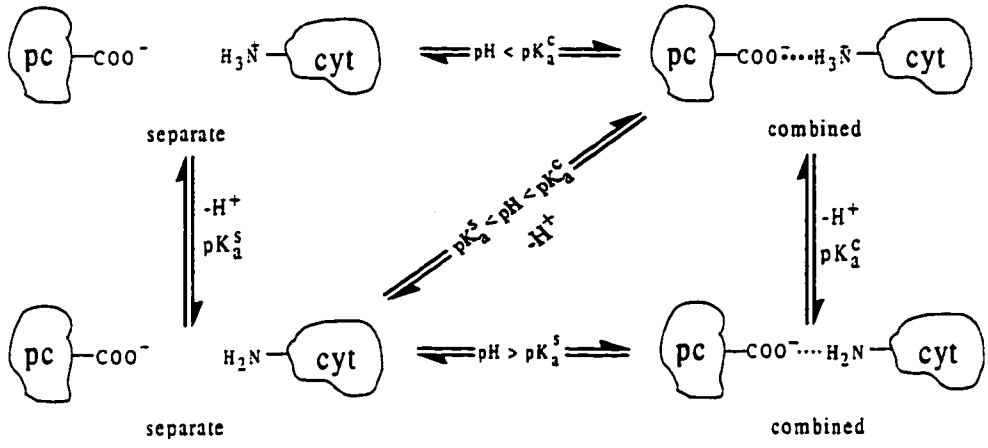
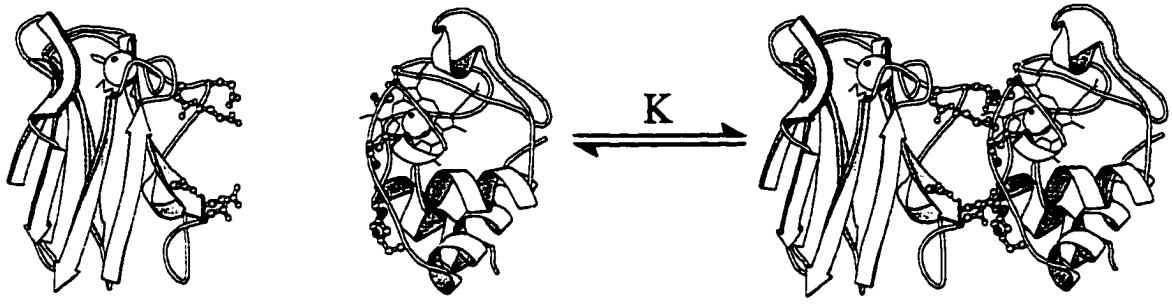


Figure Captions

Figure 1. Reversible, noncovalent association between the blue copper protein plastocyanin (pc) and the heme protein cytochrome c (cyt). The two metal atoms are shown as spheres, while the porphyrin ring and the four ligands to the copper atom are shown as wire-frame models. The acidic residues Asp42, Glu43, Asp44, Glu59, Glu60, and Asp61 in plastocyanin, and the basic residues Lys13, Lys25, Lys27, and Lys86 in cytochrome *c* are highlighted as ball-and-stick models. The protein structures were drawn with the program MolScript v2.1.[22]

Figure 2. Dependence on pH of the number of H^+ ions (q) released to the buffer by the protein pair shown in Figure 1 upon their association at ionic strength of 2.50 mM and 293 K, calculated with eq 14. The error bars include two standard deviations. The line is calculated with eq 14 on the basis of the fitted line from Figure 3a.

Figure 3. Dependence on pH of association constant K for association of cytochrome *c* with (a) wild-type plastocyanin and the following three mutants: (b) Asp42Asn, (c) Glu59Gln, and (d) Glu60Gln at ionic strength of 2.50 mM and 293 K. Error bars for the experimental results include two standard deviations. The two nearly overlapping lines in each case are fittings to eqs 9-13. The two sets of fitted parameters are given paired columns in Table 1.



pc cyt pc / cyt

separate combined

Figure 1

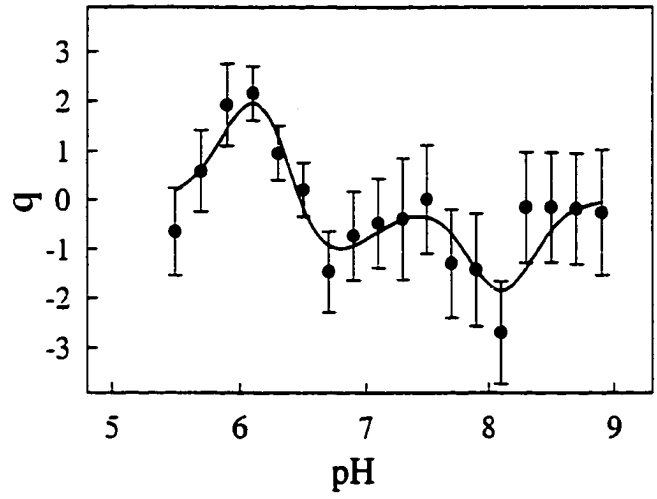


Figure 2

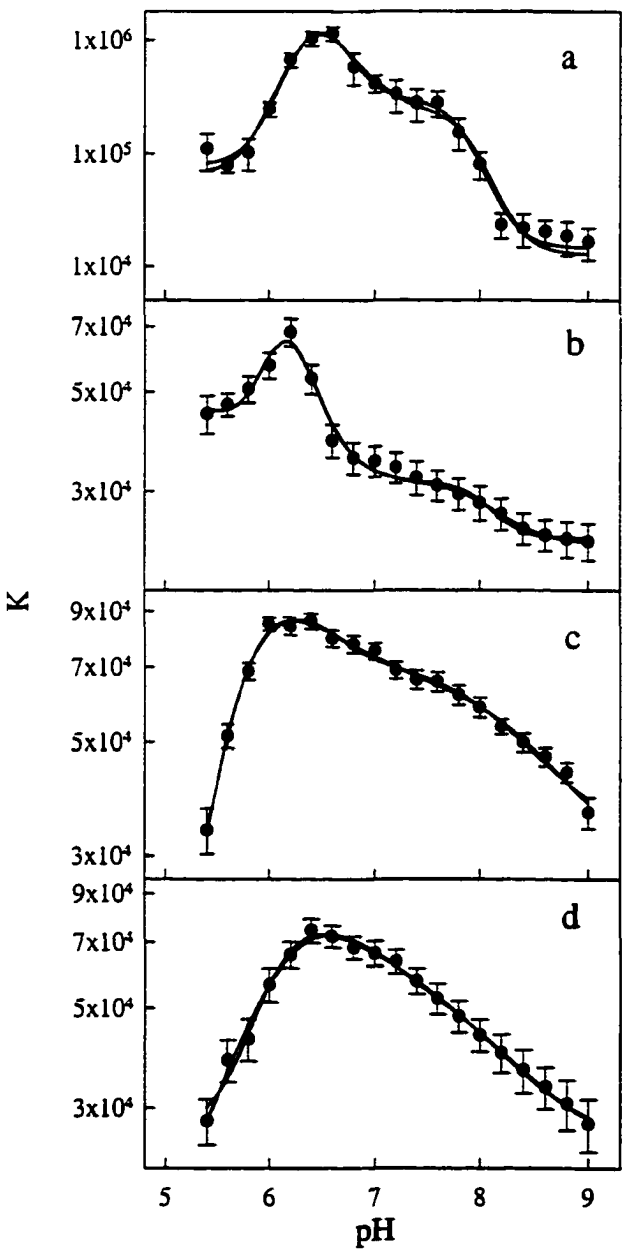

1x10⁶
1x10⁵
1x10⁴
a
7x10⁴
5x10⁴
3x10⁴
b
9x10⁴
7x10⁴
5x10⁴
3x10⁴
c
9x10⁴
7x10⁴
5x10⁴
3x10⁴
d
K
5
6
7
8
9
pH

Figure 3

CHAPTER 5. EFFECTS OF PH ON KINETICS OF THE STRUCTURAL REARRANGEMENT THAT GATES THE ELECTRON-TRANSFER REACTION BETWEEN ZINC CYTOCHROME *C* AND PLASTOCYANIN. ANALYSIS OF PROTONATION STATES IN DIPROTEIN COMPLEX

Milan M. Crnogorac and Nenad M. Kostić

Abstract

Electron transfer from zinc cytochrome *c* to copper(II)plastocyanin in the electrostatically-stabilized complex Zncyt/pc(II) is gated by the structural rearrangement of this complex [Crnogorac, M. M., Shen, C., Young, S., Hansson, O., Kostić, N. M. (1996) *Biochemistry 35*, 16465-74]. Now we report the effects of pH on this rearrangement in four complexes Zncyt/pc(II), which zinc cytochrome *c* makes with the wild-type form and the single mutants Asp 42 Asn, Glu 59 Gln, and Glu 60 Gln of plastocyanin. The rate constant for the rearrangement, k_F, differs for the four forms of plastocyanin but is independent of pH from 5.4 to 9.0 in all four cases. That k_F is affected by the single mutations but not by pH changes suggests that the residues Asp 42, Glu 59, and Glu 60 in the wild-type plastocyanin remain deprotonated (i.e., as anions) within the Zncyt/pc(II) complex throughout the pH range examined. Analysis of the protein rearrangement suggests that loss of salt bridges in the initial configuration of the complex is compensated by formation of new salt bridges in the rearranged configuration.

Introduction

Metalloproteins act as electron carriers in many biological processes, such as photosynthesis and respiration. Despite much recent research,[1-7] recognition and electron transfer between metalloproteins is only partially understood. A pair of metalloproteins can form multiple complexes in solution, and configuration that is optimal for recognition need not be optimal for electron transfer.[8-17] The proteins may rearrange from the binding configuration to the reactive configuration. If this rearrangement is the rate-limiting step, the electron-transfer reaction is said to be gated.[7]

The heme protein cytochrome *c*[18-20] and the blue-copper protein plastocyanin,[21-26] designated cyt and pc, are well suited for kinetic and mechanistic studies since their three-dimensional structures in both crystal and solution are known. At pH 7.0 copper(II)plastocyanin, pc(II), has a charge of –8 while iron(II)cytochrome *c* has a charge of +6. The negative charge in plastocyanins from high plants is found mostly in the acidic patch, which consists of the lower cluster— residues Asp 42, Glu 43, and Glu 44 — and the upper cluster— residues Glu 59, Glu 60, and Asp 61. This patch has been implicated in reactions of pc with small inorganic complexes and with other proteins.[21,27-32] Some of the acidic residues seem to have unusually high pK_a values.[33]

The replacement of iron(II) with zinc(II) does not perturb the conformation of cyt and its association with other proteins.[34-37] The zinc(II) derivative, designated Zncyt, is excited by the laser pulse to its triplet state, ^{3}Zncyt, a strong electron-donor (E° = -0.88 V vs. NHE) that reduces pc(II). The reaction in eq 1 is gated by a structural rearrangement of the

electrostatically-stabilized complex ^{3}Zncyt/pc(II). The first-order rate constant k_F corresponds to this rearrangement, not electron transfer.[37-39]

$$^3\text{Zncyt}/\text{pc(II)} \xrightarrow{k_F} \text{Zncyt}^+/\text{pc(I)} \qquad (1)$$

The effects and non-effects of ionic strength, viscosity, thermodynamic driving force, temperature, and site-directed mutation revealed much, but not all, about the mechanism of this rearrangement.[27,38-42] Here we study the effects of neutralizing certain residues in the acidic patch. Besides the wild-type form, we chose the following three mutants of spinach pc: Asp 42 Asn, Glu 59 Gln, and Glu 60 Gln. The kinetics results reveal the protonation state of these residues in the dynamic diprotein complex. This information may not be obtainable by other experimental methods.

Materials and Methods

Chemicals. Distilled water was demineralized to resistivity greater 16 MΩ cm. Chromatography gels were purchased from Sigma Chemical Company. Triethanolammonium chloride was purchased from Aldrich Chemical Company. Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co. All other chemicals were purchased from Fisher Chemical Company.

Buffers. The buffers kept the ionic strength of 2.50 mM over the entire pH range, from 5.4 to 9.0.[43] The full range was covered with a buffer made by dissolving 2.50 mmol (0.4641 g) of triethanolammonium chloride and 2.50 mmol (142.8 μL) of glacial acetic acid in 1.000 L of demineralized water. We refer to this solution as the complete buffer. The pH interval from 5.4 to 7.2 was covered also with a 2.50 mM solution of sodium cacodylate, termed the acidic buffer. The pH interval from 6.6 to 9.0 was covered also with a 2.50 mM

solution of triethanolammonium chloride, termed the basic buffer. The basic component of the first two buffers and the acidic component of the third were created by adding 0.100 M solutions of NaOH or HCl, respectively. These two solutions were used also to adjust the pH, which was measured with a Fisher Accumet 805 MP pH meter equipped with an Aldrich combination microelectrode. Conductivity was measured with the instrument HI 8733, by HANNA Instruments, Inc. The k_F values in the overlapping region, from 6.6 to 7.2, were the same regardless of the buffer chosen.

Proteins. Cytochrome *c* from horse heart was purchased from Sigma Chemical Co. The iron-free (so-called free-base) form was made, purified, and reconstituted with zinc(II) by a modification[44] of the original procedure.[34,45] The product, zinc cytochrome *c*, was handled at 4 °C, in the dark. The criteria of purity were the absorbance ratios $A_{423}/A_{549} >$ 15.4 and $A_{549}/A_{585} < 2.0$. The absorptivity is $\varepsilon_{423}=2.43\cdot10^5$ $M^{-1}cm^{-1}$.[45] Wild-type plastocyanin from spinach and three single mutants were prepared by overexpression in *E. coli*,[46] with the vector pUG101t_r[47] and purified first with a DE32 column and then with a 26/10 Q Sepharose high-performance FPLC column from Pharmacia. The blue fraction was concentrated by dialysis against dry polyethyleneglycol (PEG 2000) and passed through a gel-filtration column Sephacryl S-100 HR. The amount of holo-plastocyanin was determined spectrophotometrically, in the presence of $K_3[Fe(CN)_6]$, on the basis of the absorptivity $\varepsilon_{597}=4700$ M^{-1} cm^{-1}.[46] The UV-vis spectra were recorded with a Perkin-Elmer Lambda 18 spectrophotometer.

Flash Kinetic Spectrophotometry. The so-called laser flash photolysis on a microsecond time scale was done with a standard apparatus.[48] Argon for deaeration was first

passed through water and then through the buffered solution of the proteins for 30 minutes. The cell jacket was connected to the 30-L circulating bath Forma 2067, which maintained the temperature at 293.0(2) K. Concentration of Zncyt was kept at 10.0 μM, while concentration of pc(II) was varied from 2.0 to 30.0 μM. Initial volume of the solution was 1.000 mL. Upon each addition of a small portion, 1.00 to 4.00 μL, of a 0.100 M solution of NaOH or HCl, the pH was measured with the freshly-calibrated pH meter, and the solution was additionally deaerated for 10 more minutes. Formation and decay of the triplet sate, ^{3}Zncyt, was monitored by change in absorbance at 460 nm, ΔA_{460}. At each concentration of pc(II) and each pH value, four or more laser pulses were delivered and kinetic traces recorded. Calibration of the pH meter was checked after the last adjustment of pH.

Regression Analysis of the Rate Constant k_F and Modeling of the Rearrangement. The kinetic traces were fitted to eq 2 by a least-squares nonlinear regression method, with the program SigmaPlot v4.01,

$$\Delta A_{460} = a_1 \exp(-k_F t) + a_2 \exp(-k_{obs} t) \qquad (2)$$

from SPSS Inc. The pseudo-first order rate constant k_{obs} depends on the concentration of pc(II) and corresponds to bimolecular quenching of ^{3}Zncyt by pc(II). We focus on the first-order rate constant, k_F, which corresponds to unimolecular quenching in the complex ^{3}Zncyt/pc(II), and on its amplitude a_1. The error margins for all reported values correspond to two standard deviations and the confidence limit greater than 95%.

The rearrangement involves the migration of Zncyt on the pc surface from the binding configuration, in which the basic patch in Zncyt abuts the acidic patch in pc, to the reactive configuration, in which Zncyt sits near the upper edge of the acidic patch or between

the acidic and hydrophobic patch in pc; see Figure 1.[38,39,41,42] The respective models for the two configurations are so-called max-ov and n/eq complexes found in a thorough computational search.[49] The rearrangement of the Zncyt/pc complex was modeled and viewed with the program RasMol v2.4.

Results

Redox Quenching of ^{3}Zncyt by Wild-type pc(II) and its Mutants. The rate constant for the natural decay of the triplet ^{3}Zncyt remains 110 s^{-1} over the entire pH range, from 5.4 to 9.0.[37,50,51] In the presence of the quencher, any of the four forms of pc(II), the triplet decay is biphasic throughout the pH range. Because we are interested in the properties of the Zncyt/pc(II) complex, we study the unimolecular phase, that corresponding to the first term in eq 2. As the pc(II) concentration increases, the amplitude a_1 increases, but the rate constant k_F remains the same. At a constant pc(II) concentration, a_1 depends on pH (see Figure 2), but k_F does not (see Figure 3). As Figure 2 and Figure S1 in the Supporting Information show, the maximum in a_1 occurs approximately at pH 6.8, 6.2, 6.4, and 6.3 for the wild-type, Asp 42 Asn, Glu 59 Gln, and Glu 60 Gln forms of pc(II), respectively. For sufficiently accurate determination of k_F, the concentration of Zncyt/pc(II) complex should be greater than ca. 1.0 μM. Because the mutants Glu 59 Gln and Glu 60 Gln have diminished affinity for Zncyt, even 30.0 μM concentration of them could not produce a sufficient concentration of Zncyt/pc(II) at low and high ends of the pH range. For this reason, the plots in Figures 3c and 3d are a little shorter than the others. All four plots in Figure 3 span the pH ranges over which k_F could be determined well. The main result, shown in Table 1 and Figure 3, is that the rearrangement of the complex Zncyt/pc(II) is affected by mutation in pc but not by pH changes.

Discussion

Properties of the Buffers. The pH interval from 5.4 to 9.0 is as wide as was safely possible. Staying in it, we avoided protonation of His 87 in pc[52] and nonspecific damage to the proteins. Because association of Zncyt and pc depends on ionic strength, we took pains to keep it constant while varying pH.[43] If the acidic and the basic component of a buffer bear different charges, when pH changes so will change the concentrations of these two ions and the ionic strength. We avoided this problem by making each buffer from an ion (cacodylate anion or triethanolammonium cation) and its uncharged conjugate partner (cacodylic acid or triethanolamine). When HCl is added to the former buffer, the anion is replaced by the Cl^- anion; when NaOH is added to the latter buffer, the cation is replaced by the Na^+ cation. In each case ionic strength is kept constant.

To have sufficient buffering capacity of the same buffer over the entire pH range, we used a mixture of two acid-base pairs. To keep the ionic strength constant, we chose the two acidic members of these pairs with widely different pK_a values: acetic acid (4.64) and triethanolammonium ion (7.78). To compensate for the variation of buffer capacity with pH, we varied the portions of added NaOH solution from 1.00 μL (at the acidic end) to 2.00 - 4.00 μL (around the neutral point), down to 2.00 and 1.00 μL (at the basic end) of this complete buffer. The total volume of added NaOH was 30.0 μL, or less than 3 % of the sample. Titrations shown in Figure S2 in the Supporting Information gave identical pH values and conductivity with the complete buffer alone and with a 10.0 μM solution of cytochrome *c* in the complete buffer. Evidently, the protein does not alter the buffer properties. As Figure S3 in the Supporting Information shows, the calculated ionic strength remains 2.50 mM throughout the pH range. Because a_1 and k_F are independent of the buffer

(at a given pH value) we rule out specific interactions between the proteins and the buffer. Clearly, the changes in Figures 2 and 3 and in Figure S1 in the Supporting Information are due solely to changes in pH.

Independence of the Protein Rearrangement of pH. There are relatively few studies of the effects of pH on redox reactions and accompanying processes involving metalloproteins.[53-55] The rate constant for a true electron-transfer reaction within ascorbate oxidase is independent of pH.[56] We do not, however, know any previous study of a gated electron-transfer reaction at various pH values. This study of protein rearrangement in an electrostatically-stabilized complex may be the first of its kind.

The surprising finding in Figure 3, that the rate of rearrangement is independent of pH over a fairly wide range, may have three explanations, depending on the (de)protonation state of those side chains that are affected by protein association. First, this state changes but is irrelevant for the rate of rearrangement. Second, this state changes but multiple changes cancel one another so that the plot in Figure 3a remains horizontal. Third, this state does not change.

To distinguish among these three explanations, we compared the behavior of three complexes, those that Zncyt makes with the wild-type pc and with the mutants Asp 42 Asn and Glu 59 Gln; see Figures 3a, 3b, and 3c. Carboxylic acid and carboxamide differ in electrostatic but not steric properties. Any effect of the mutations, if observed, would be due to electrostatic differences between the acid and its amide.

As Table 1 shows, the k_F value for the Zncyt/pc(II) complex containing Asp 42 Asn is higher, and that for the complex containing Glu 59 Gln lower, than the k_F value for the complex containing wild-type pc. Evidently, neutralization of charge by amidation of Asp 42

and of Glu 59 in pc mutants does affect the rearrangement of the diprotein complex. Similarly, neutralization of charge by protonation of Asp 42 and Glu 59 in the wild-type pc is expected to raise and lower, respectively, the k_F value for Zncyt/pc(II). The first explanation is refuted.

Simultaneous deprotonation of Asp 42 and Glu 59 in the wild-type pc would both assist and hinder the rearrangement and, conceivably, produce little or no net change of k_F with pH. In this case, the single mutation Asp 42 Asn would abolish the former, and Glu 59 Gln the latter, effect and make the k_F sensitive to the deprotonation of the other acidic residue. Horizontal plots in Figures 3b and 3c, however, rule out this scenario. The second explanation above is refuted, too. Evidence favors the third possible explanation.

To test the conclusion that the acidic residues in pc within the complex remain deprotonated throughout the pH range, we examined also the complex Zncyt/pc(II) containing the mutant Glu 60 Gln; see Figure 3d. As Table 1 shows, the protein rearrangement is markedly impeded by this amidation. Neutralization by protonation likewise would impede the rearrangement. Absence of this effect, the horizontal plot in Figure 3a, shows that protonation does not occur in the pH interval covered. Evidently, Glu 60 remains deprotonated. Our study of the reaction in eq 1 at various pH values gave detailed information about the interface of the associated proteins that could not be obtained in other ways.

A Closer Look at Rearrangement. There is much evidence for the general orientation of cytochrome *c* and high-plant plastocyanin in the electrostatically-stabilized binary complex: the basic patch around the exposed-heme edge in the former abuts the broad acidic patch in the latter.[57-62] A systematic computational search for the best match of these

proteins' electrostatic fields gave further information about the association.[49] Recent studies in our laboratory confirmed these previous findings about the configuration of the initial or binding complex and added new information about the reactive complex.[39,42] During the rearrangement, the basic patch in cytochrome *c* moves from the acidic patch to an area near the upper edge of the acidic patch and between the acidic and hydrophobic patches, in the vicinity of Gln 88, in plastocyanin.

Our new finding, that some key residues in both acidic clusters in plastocyanin remain deprotonated during the rearrangement, prompted us to take a closer look at the protein interface in the two configurations. If the binding and reactive configurations of the Zncyt/pc(II) complex approximately correspond to the so-called max-ov and n/eq configurations found in the aforementioned computational study,[49] this preference for the anionic form can be attributed to salt bridges. Each configuration has four of them. The bridge between Lys 86 and Asp 44 survives the rearrangement. The residue Lys 13 switches from Glu 43 to Glu 59. While Lys 25 breaks, Lys 8 makes a salt bridge with Glu 60. While Lys 27 breaks a salt bridge with Glu 59, Lys 87 makes a similar one with Glu 43. All in all, three salt bridges seem to be lost and three gained in the rearrangement. This compensation may be a reason for the relatively low activation enthalpy for the rearrangement[41,63] and for the independence of its rate constant (k_F) on pH.

Conclusion

If relevant mutants are available, studying possible effects of pH on a dynamic protein complex allow inferences about the (de)protonation states of particular side chains. Although NMR spectroscopy may in some cases reveal these states, this information would

not necessarily pertain to the dynamic process. Analyzing these (de)protonation states on the basis of kinetic results ensures that the conclusion will be relevant to the reactivity of the protein complex.

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Supporting information available. A plot showing the relative amplitude of the unimolecular phase (rearrangement of the Zncyt/pc(II) complexes) containing mutants of pc; a plot showing the conductivity and pH of the complete buffer as a function of the volume of NaOH added; and a plot showing the concentrations of ions (i.e., contributions to the ionic strength) in this buffer.

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Table 1. The Maximum Relative Amplitude of the Unimolecular Phase (Structural Rearrangement) in the Reaction in eq 1 and the Rate Constant k_F when the Concentrations of Zncyt and pc(II) are 10.0 μM each, Temperature is 293.0 K, and Ionic Strength is 2.50 mM. The Error Margins Correspond to Two Standard Deviations and the Confidence Limit Greater than 95%

plastocyanin form	max of $\frac{a_1}{a_1 + a_2}$	$10^{-5}\, k_F / s^{-1}$
wild-type	0.68(3)	2.0(1)
Asp 42 Asn	0.23(4)	2.3(1)
Glu 59 Gln	0.21(3)	1.6(1)
Glu 60 Gln	0.23(4)	1.1(1)

Figure Captions

Figure 1. Rearrangement of the diprotein complex Zncyt/pc(II) from the binding to the reactive configuration, corresponding to the so-called max-ov and n/eq structures found in a computational study.[49] The two metals are highlighted, while the porphyrin ring and the ligands to copper are shown as wire-frame models. Plastocyanin (on the left) is stationary, while cytochrome *c* (on the right) moves. In the binding configuration Lys 13, Lys 86, Lys 25, and Lys 27 residues in cytochrome *c* form salt bridges respectively with Glu 43, Asp 44, Glu 59, and Glu 60 residues in plastocyanin. In the reactive configuration Lys 87, Lys 86, Lys 13, and Lys 8 residues in cytochrome *c* form salt bridges respectively with Glu 43, Asp 44, Glu 59, and Glu 60 residues in plastocyanin. All these residues are highlighted as ball-and-stick models. Protein structures were drawn with the program MolScript v2.1[64]

Figure 2. Dependence on pH at the constant ionic strength of 2.50 mM of the relative amplitude of the unimolecular phase (structural rearrangement) in the reaction in eq 1. Concentrations of Zncyt and pc(II) were 10.0 μM each.

Figure 3. Independence of pH of the rate constant k_F for the structural rearrangement of the diprotein complex ^{3}Zncyt/pc(II) containing four forms of copper(II)plastocyanin: (a) wild-type, (b) Asp 42 Asn, (c) Glu 59 Gln, and (d) Glu 60 Gln. Each line is the fit to the slope of zero and had $R^2 \geq 0.97$.

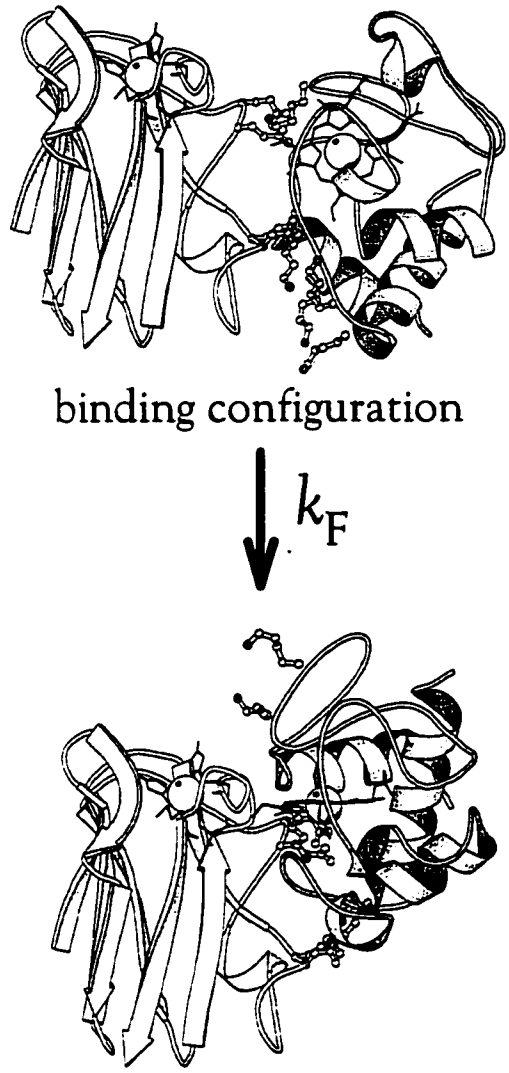

Figure 1

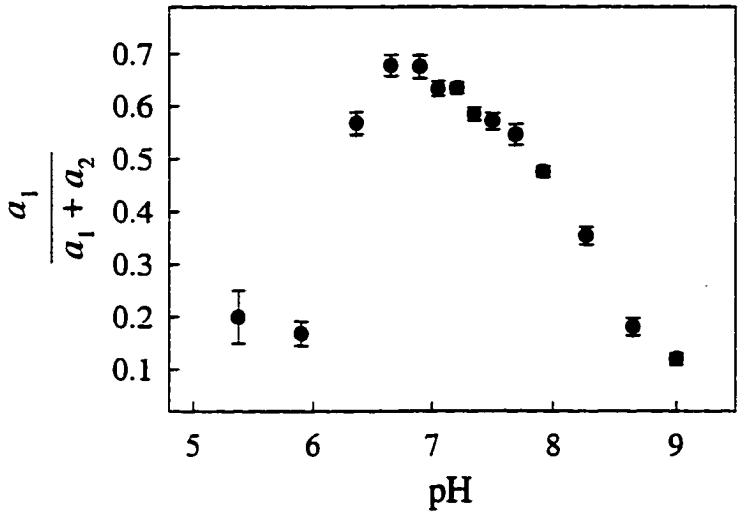

Figure 2

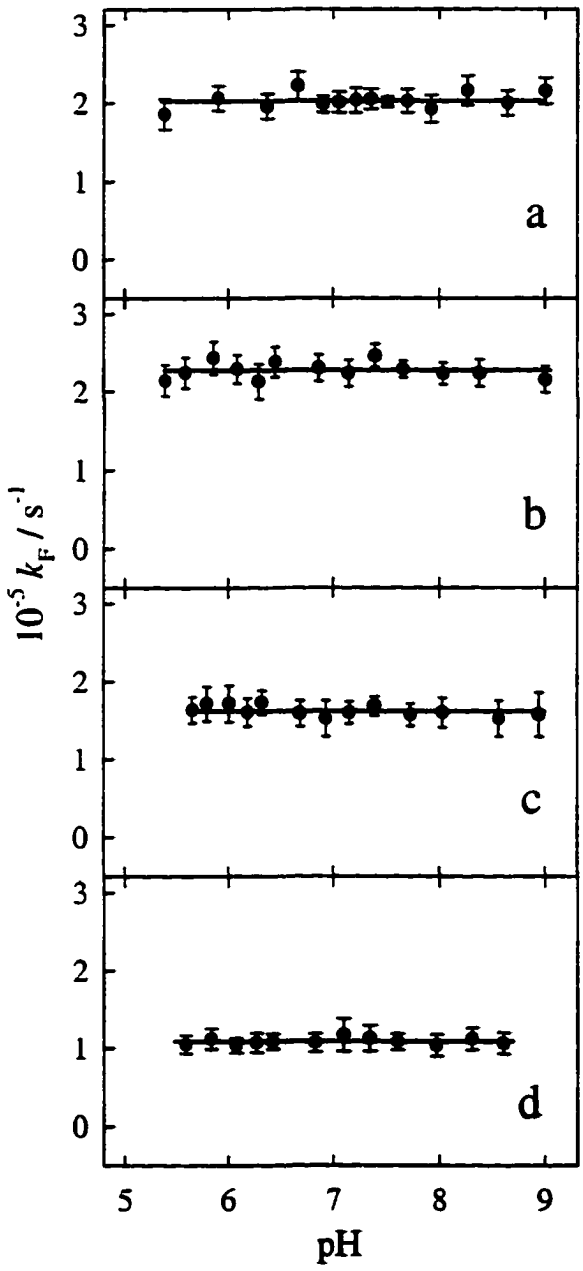

Figure 3

CHAPTER 6. CONCLUSIONS

We investigated the effects of site-directed mutations and viscosity on rate-limiting rearrangement within the complex of cytochrome *c* and plastocyanin and identified the reactive configuration of the complex. In the reactive configuration of the complex cytochrome *c* heme edge is between plastocyanin acidic and hydrophobic patch.

Reactive intermediate in the reaction of cytochrome *c* and plastocyanin, the zinc cytochrome *c* cation radical, exhibits different reactivity from iron form of the protein. The reorganization energy and electron self-exchange rate constant of the cation radical show that methionine, ligand in iron form of the protein, is uncoordinated in the zinc form of the protein.

We extended the proton-linkage model so it can give information about individual proteins and even about amino-acid residues involved in protein association. We extract from the experimental results the composite pK_a values, both in separate and in combined proteins, of groups of residues that change their protonation state upon protein association. The extended model was applied to intricate effects of pH on association of cytochrome *c* with wild-type and several mutant forms of plastocyanin. We extract from the experimental results the composite pK_a values, both in separate and in combined proteins, of groups of residues that change their protonation state upon protein association. Interpretation of these parameters revealed general and specific features of the protein-protein interaction.

The effects of pH on protein rearrangement were studied. The rearrangement is independent of pH and amino acid side chains in the acidic patch of plastocyanin are deprotonated in the complex of cytochrome *c* and plastocyanin. Study of effects of pH on a

dynamic protein complex allow inferences about the protonation states of particular side chains.

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