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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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 strengths, and within the transient diprotein complex, which is involved at higher ionic strengths, 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 42 and 43), replacement of Tyr83 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 59 and 60) impedes the rearrangement. Creation of an anion at position 88, between the upper acidic cluster and the hydrophobic patch, facilitates the rearrangement. 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. Mutations that affect protein association (i.e., change the binding constant) do not necessarily affect the reaction between the associated proteins (i.e., the rate constant) and vice versa. All of the kinetic and thermodynamic effects and noneffects of mutations consistently indicate that in the protein rearrangement the basic patch of zinc cytochrome *c* moves from a position between the two acidic clusters to a position at or near the upper acidic cluster.

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 forward slant represents protein association, i.e., the diprotein complex. The Roman numerals are the oxidation states of iron and

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[1] Abbreviations: cyt, cytochrome *c*; cyt(III), ferricytochrome *c*; cyt(II), ferrocytochrome *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*; Znpc, zinc plastocyanin.

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copper. In zinc cytochrome *c*, the oxidation state of zinc is always II, and an electron is given and accepted by the porphyrin ring.

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 strengths. Much evidence shows that in the cyt/pc complexes 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 cyt(III)/pc(II) complex, 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., 1997; 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−c, 1993a,b; Qin & Kostić, 1994, 1996; Kostić, 1996; Ivković-Jensen & Kostić, 1996). The reactions in eqs 3 and 5, in

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

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

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

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

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

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

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 a 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 a driving force of ca. 1.2 eV; in them, the protein rearrangement is the 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 the enthalpy of activation ($\Delta H^{\ddagger}$) for the reaction in eq 3 in terms of solvation effects (Ivković-Jensen & Kostić, 1996) answered some but not nearly all of 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 spinach 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 Pharmacia. 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 $Na_2HPO_4 \cdot 7H_2O$ and had ionic strengths (μ) of 2.5 or 10 mM and a pH of 7.00 ± 0.05 at 293 K. The ionic strength was raised from 10 to 100 mM by addition of solid NaCl. In all of these preparation, we took into consideration dependence of the pK_a of $H_2PO_4^-$ on ionic strength.

Temperature and Viscosity. The temperature was kept at 20.0 ± 0.2 °C with a Forma Scientific CH/P 2067 30 L circulating bath. The 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., 1997) of the original procedure (Vanderkooi & Erecińska, 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 that the absorbance ratio A_{423}/A_{549} was >15.4 and A_{549}/A_{585} was <2.0. The absorptivity $\epsilon_{423} = 2.43 \times 10^5$ M^{-1} cm^{-1} (Vanderkooi et al., 1976).

Plastocyanin. Recombinant wild-type protein from spinach and nine of its mutants were prepared by the previously

Table 1: Properties of Recombinant Plastocyanin

pc(II) mutant	A_{280}/A_{597} ratio: calculated	A_{280}/A_{597} ratio: lowest obtained in purification	A_{280}/A_{597} ratio: final	% Znpc	maximum amplitude of the third phase (%)
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

published method for overexpression in *Escherichia 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 Sepharose high-performance FPLC column from Pharmacia. The blue fraction was concentrated by dialysis against dry polyethylene glycol (PEG 20000) and passed through a Sephacryl S-100 HR gel filtration column. The amount of holoplastocyanin was determined spectrophotometrically, under oxidizing conditions, on the basis of an absorptivity $\epsilon_{597} = 4900\ M^{-1}\ cm^{-1}$ (Katoh et al., 1962).

Only the fractions of the highest purity were used in this study. Despite the most careful handling, the mutants contained some apo and zinc forms, which could not be oxidized with $K_3[Fe(CN)_6]$. Solutions in a 100 mM Tris buffer were made 100 and 150 μM in the 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. Because traces of zinc(II) ions are present in reagent-grade chemicals, some zinc form of the mutants had to be tolerated. The properties of the mutants are given in Table 1.

Flash Kinetic Spectrophotometry. So-called laser flash photolysis at the resolution of one point per microsecond 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.

The 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 (η/η_o) of the buffered solution was adjusted with glycerol (Weast, 1986). 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 that $\eta_o = 1.002$ 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 than 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 afterward (Ullmann & Kostić, 1995). For the sake of consistency, we retain these designations and abbreviations. The rearrangement was analyzed by the program package QUANTA 4.0, used on a SiliconGraphics IRIS 4D workstation. Protein structures were drawn with the program MolScript v1.3 (Kraulis, 1991).

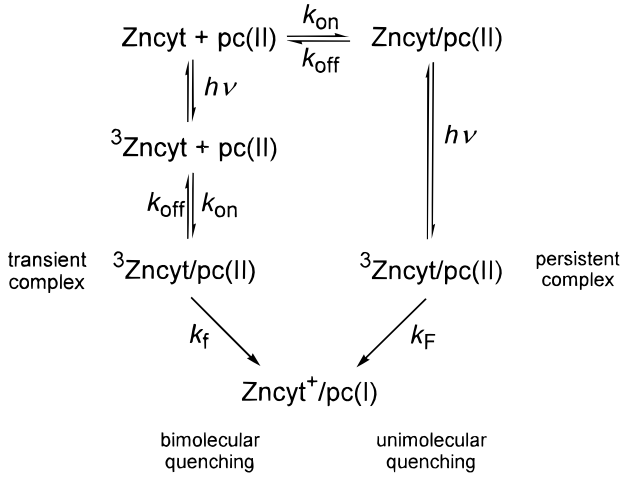
RESULTS

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. 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 Figure S1 of the Supporting Information. 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 strengths, and the transient complex, which is involved at higher ionic strengths, 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

Scheme 1



low, ionic strength of 10 mM, the quenching by Asp42Asn and by the double mutant Glu59Lys/Glu60Gln remains monoexponential. Quenching by the wild-type form and by seven of the mutants is biexponential. The only component of quenching by the former two mutants, and the slower component in the case of the wild-type form and the 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. As Table 1 of the Supporting Information shows, 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. Relative amplitudes are not accurate enough for calculation of the association constants, K_a.

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 Figure S2 of the Supporting Information. The quenching is clearly triexponential for the following three mutants: Leu12Glu, Leu12Asn, and Tyr83His. Representative kinetic data are shown in Figure S3 of the Supporting Information. 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.

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. 10 times lower than that for the bimolecular component. As Figure S4 of the Supporting Information shows, this

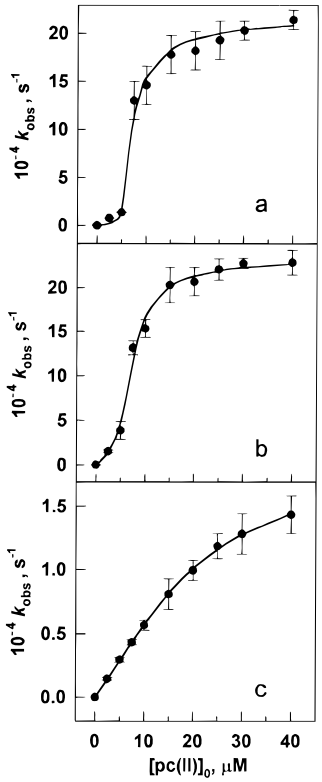


FIGURE 1: Dependence of the bimolecular rate constant k_{obs} for the reaction in eq 5 on the concentration of cupriplastocyanin. The conditions were as follows: sodium phosphate buffer at pH 7.0 and an ionic strength of 2.5 mM at 293 K. (a) Wild-type protein, (b) single mutant Asp42Asn, and (c) double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

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 zinc plastocyanin in the samples of the mutants. We set ϵ_{597} equal to 4900 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_{280}/A_{597} for each mutant agreed with the lowest value recorded during the purification. The amount of zinc plastocyanin correlates well with the amplitude of the third phase. See Table 1.

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 unit, these signals allowed for good precision and reliable fittings. The three aforementioned mutants that contained large fractions of zinc proteins 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

Table 2: Protein Association and Rearrangement of the Diprotein Complex ^{3}Zncyt/pc(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})	$10^{-5}k_F$ (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.

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 2. 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.

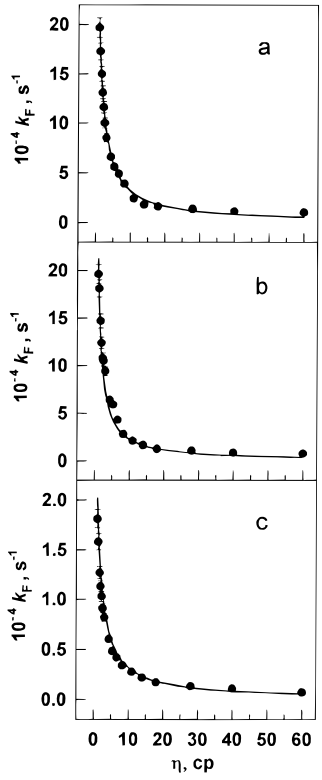


FIGURE 2: Dependence on solution viscosity of the unimolecular rate constant k_F for the rearrangement of the diprotein complex ^{3}Zncyt/pc(II) from the docking configuration into the electron-transfer configuration. The viscosity (η) of a sodium phosphate buffer at pH 7.0 and an ionic strength of 2.5 mM at 293 K was adjusted with glycerol: (a) wild-type cupriplastocyanin, (b) single mutant Asp42Asn, and (c) double mutant Glu59Lys/Glu60Gln. The lines are fittings to eq 12. The error bars include two standard deviations.

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 transferring 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 were compared with the wild-type cupriplastocyanin in their electron-transfer 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 2. 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

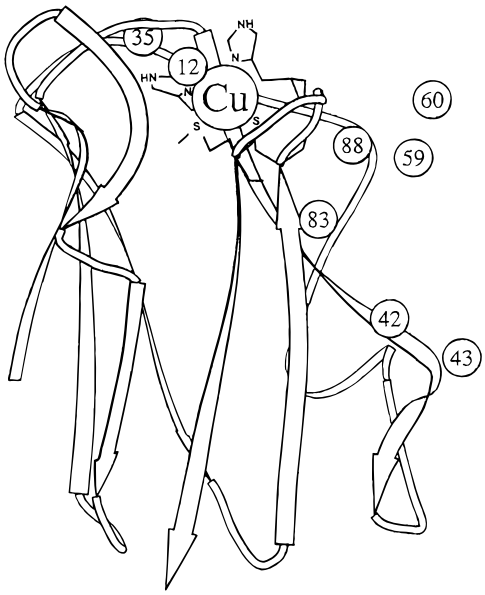


FIGURE 3: Structure of wild-type plastocyanin showing the copper atom, its four ligands, and the locations of the mutated residues. All the circled numerals mark the α-carbon atoms, except for 60, which marks the γ-carbon atom in order to avoid overlap.

kinetic studies with proteins other than cytochrome *c* (Kyritsis et al., 1993; Sigfridsson et al., 1996; S. Young, unpublished results). 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, anions Glu59 and Glu60 were converted into a cation and a neutral residue, Lys and Gln, respectively. 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 from French bean gave much evidence for redox quenching of ^{3}Zncyt, that is for Scheme 1. Systematic experiments at 10 ionic strengths spanning the interval from 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. The 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 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. That equality of the corresponding activation parameters $\Delta H^{\ddagger}$ (13 ± 2 and 13 ± 1 kJ/mol) and $\Delta S^{\ddagger}$ (-97 ± 4 and -96 ± 3 J/K mol) 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 rearrangement step (k_f). The quencher concentration, designated [pc(II)], is less than the total concentration, designated $[pc(II)]_o$, because of the association with zinc cytochrome *c*; see eq 8.

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

$$[pc(II)] = [pc(II)]_o - {}^1/_2\left[[Zncyt]_o + [pc(II)]_o + \frac{k_{off}}{k_{on}} - \sqrt{\left([Zncyt]_o + [pc(II)]_o + \frac{k_{off}}{k_{on}}\right)^2 - 4[Zncyt]_o[pc(II)]_o}\right] \tag{8}$$

At $\mu = 100$ 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 $\mu = 10$ 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 $\mu = 2.5$ mM, the quenching is triexponential for all the forms of plastocyanin; both the bimolecular and the unimolecular mechanisms in Scheme 1 operate.

We explained above the third phase in the quenching reaction, found only at the ionic strength of 2.5 mM. Since zinc plastocyanin 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 zinc plastocyanin and reassociate with cupriplastocyanin. We took into account the concentration of zinc plastocyanin 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 copper(II) form of the mutants in question.

The intracomplex rate constant k_f in Scheme 1 is independent of ionic strength (in the range of ionic strengths in which this reaction is observed). This independence, which was known for wild-type plastocyanin and which persists at different temperatures (Zhou & Kostić, 1993b; Ivković-Jensen & Kostić , 1996), is now demonstrated also for the mutants of this protein, at 293 K. The values of k_F in Table

2 remain the same, within the error margins, at ionic strengths of 2.5 and 10 mM and so do the values of k_f at ionic strengths of 2.5, 10, and 100 mM. Whenever both the unimolecular and the bimolecular reaction are observed at a given ionic strength, the rate constants k_F and k_f are equal.

The ionic strength affects the degree of protein association, i.e., the concentration of the diprotein complex. The ionic strength, however, does not seem to affect the electron-transfer properties of this complex, even though it is clearly a dynamic system. In studies of various protein pairs, the dependence of the observed rate constant on ionic strength, sometimes at only two values of it, has been taken as evidence for rearrangement of the protein complex (Kostić, 1991). This reasoning may be correct in particular cases, but intuitive equating of stability and rigidity is ambiguous.

Because of the presence of zinc plastocyanin and the consequent occurrence of the third component in the quenching reaction, the rate constants k_{on} and k_{off} in Table 2 are relatively imprecise, having error margins of up to 60%. Hence, the wide error margins of the association constant, K_a exists. Fortunately, the K_a values differ considerably, as will be discussed below.

Kinetic Effects of Viscosity. One of the effects of the solvent is modulation of 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 3 are caused by changes in viscosity, and not by changes 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 $(1 +$

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

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

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

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

$\eta)k_BT/h$ 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 12 gave the free energy of activation for the rearrangement ($\Delta G^{\ddagger}$) and the friction parameter σ or δ.

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.

The two fittings gave the results in Table 3, which are consistent with each other and with those in Table 2. The wild-type plastocyanin and the mutants behave in a like manner. The higher value of $\Delta G^{\ddagger}$ 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. Although the rate constant k_F is slightly higher for Gln88Glu than for other mutants (Table 2), the parameter $\Delta G^{\ddagger}$ is approximately the same, within the error margins of the fitting, for all the single-residue mutants (Table 3). The large kinetic effect of the double mutation is clearly evident in the $\Delta G^{\ddagger}$ value. 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 electron 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. See Figure 4. 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

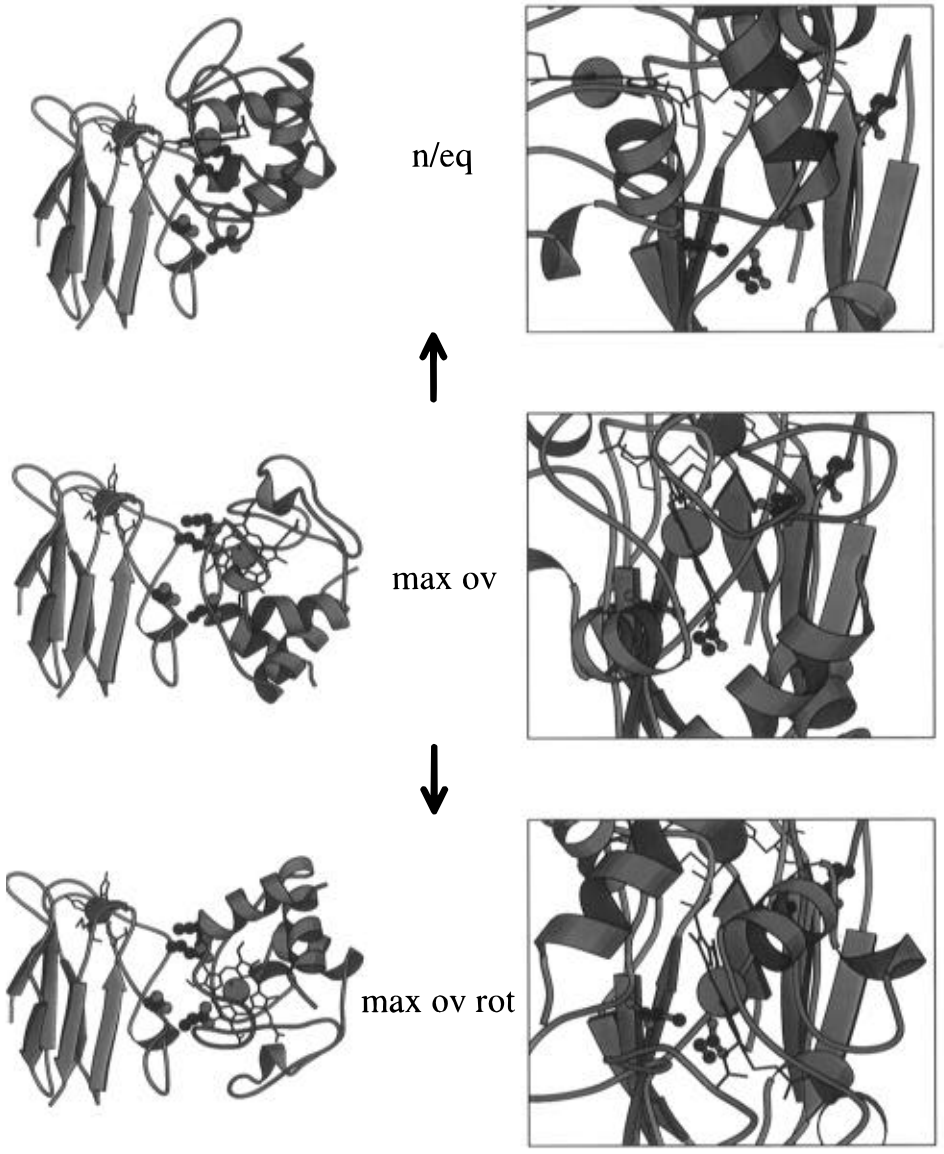


FIGURE 4: Two trajectories, shown with arrows, for rearrangement of the diprotein complex. Plastocyanin (blue) is stationary, while cytochrome *c* (red) moves. The atomic coordinates are taken from Roberts et al. (1991). The initial configuration, designated max ov, provides optimal docking. The rearranged configurations, designated n/eq and max ov rot, provide more efficient electron-tunneling paths from the heme to the copper site. The two metal atoms are highlighted; the porphyrin ring and all the ligands to copper are shown as wire-frame models. The basic (positively charged) patch around the exposed heme edge moves across the acidic (negatively charged) patch in plastocyanin. In the n/eq configuration, the basic patch reaches the upper edge of the acidic patch and the area between the acidic and hydrophobic patches, marked by residues 59, 60, and 88. In the max ov rot configuration, the cytochrome *c* molecule is rotated by ca. 180°, remaining over the acidic patch, so that the heme edge points toward Tyr83. 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.

shows, rearrangements of the first configuration into the second and the third amount to rotation of the cytochrome *c* molecule by ca 180° and its gliding on the plastocyanin surface toward, or beyond, the upper edge of the acidic patch. We describe these fluctuations as configurational, rather than conformational, because both protein molecules are treated as rigid bodies.

In the initial configuration (max ov), the exposed heme edge and cationic lysine residues 7, 8, 13, 27, and 72 around it abut the two acidic clusters. During the rotation by ca. 180°, which yields the max ov rot configuration, this basic patch in cytochrome *c* remains in contact with the acidic patch as it moves over both the upper and the lower cluster in this patch. Both of these clusters, therefore, present potential barriers to this rearrangement. During the simultaneous translation and counterclockwise rotation by ca. 90°, which yields the n/eq configuration, the basic patch crosses over the upper cluster but not over the lower one as it approaches residue 88. Only the upper cluster, therefore, presents a potential barrier to this rearrangement. See Figure 4.

Kinetics of the Rearrangement. As explained above, rate constants k_f and k_F in Table 2 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.

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

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. 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_F, 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, those atoms of Leu12 in the wild-type protein that are replaced in the mutant are located only 4 Å from the copper atom.

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 3. These results, which were discussed above, show that solution viscosity identically affects the ^{3}Zncyt/pc(II) complexes containing the wild-type form and all the mutants of plastocyanin.

The rate constants k_f and k_F in Table 2 clearly show that mutations in the lower cluster do not affect the rearrangement, whereas those in the upper cluster and at position 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 88 attracts the basic patch of cytochrome *c*. Moreover, residue Gln88 is involved in the most efficient electron-tunneling path in the n/eq configuration (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.

The reversal of local charges brought about by the double mutation has a great effect on the protein association, as the K_a values in Table 2 show. Neutralization of a single charge of Asp42 or Glu43, in the lower cluster, has a lesser but significant effect. Indeed, the mutants having the lowest K_a values, namely 2×10^5 and 4×10^6 M^{-1}, did not show the unimolecular component of the quenching at the ionic strength of 10 mM. In these two cases, the concentration of the ^{3}Zncyt/pc(II) complex was too low for the reaction k_F to be observed. (Fortunately, all the mutants, including these two, showed the unimolecular component at the ionic strength of 2.5 mM.) Single mutations in positions 42 and 43, however, do not detectably alter the kinetics of rearrangement, as the rate constants k_f and k_F show. Introduction of a negative charge between the acidic and the hydrophobic patch, in the mutant Gln88Glu, does not affect the protein association but does assist their rearrangement in the associated state. Evidently, residue 88 is not involved in the initial docking of the proteins but is involved in the configurational fluctuation.

In studies of dynamic metalloprotein complexes, relationships are sometimes sought, and claimed, between association constants and rate constants. This reasoning may be correct in a given case, but intuitive equating of stability and reactivity is ambiguous. The position of an equilibrium between free proteins and their complex is a matter of thermodynamics, whereas the rate at which this equilibrium is established and the rate at which the complex may rearrange or react are matters of kinetics.

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 cyt/pc diprotein complex 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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SUPPORTING INFORMATION AVAILABLE

A table, showing relative amplitudes of the three components of quenching, and four figures, showing natural decay and quenching of ^{3}Zncyt, dependence of k_{obs} on pc(II) concentration, triexponential quenching of ^{3}Zncyt, and dependence of the rate constant of the third phase of quenching on pc(II) concentration (5 pages). Ordering information is given on any current masthead page.

REFERENCES

Angiolillo, P. J., & Vanderkooi, J. M. (1995) *Biophys. J. 68,* 2505.
Anni, H., Vanderkooi, J. M., & Mayne, L. (1995) *Biochemistry 34,* 5744.
Ansari, A., Jones, C. M., Henry, E. R., Hofrichter, J., & Eaton, W. A. (1992) *Science 256,* 1796.
Bagby, S., Driscoll, P. C., Goodall, K. G., Redfield, C., & Hill, H. A. O. (1990) *Eur. J. Biochem. 188,* 413.
Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., Reinisch, L., Reynolds, A. H., Sorensen, L. B., & Yue, K. T. (1980) *Biochemistry 19,* 5147.
Brunschwig, B. S., & Sutin, N. (1989) *J. Am. Chem. Soc. 111,* 7454.
Burch, A. M., Rigby, S. E. J., Funk, W. D., MacGilliwray, R. T. A., Mauk, M. R., Mauk, A. G., & Moore, G. R. (1990) *Science 247,* 831.
Chen, L., Poliks, R., Hamada, K., Chen, Z., Mathews, F. S., Davidson, V. L., Satow, Y., Huizinga, E.,Vellieux, F. M. D., & Hol, W. G. J. (1992) *Biochemistry 31,* 4959.
Chen, L., Durley, R. C. E., Mathews, F. S., & Davidson, V. L. (1994) *Science 264,* 86.
Espenson, J. H. (1995) *Chemical Kinetics and Reaction Mechanisms,* 2nd ed., McGraw Hill, New York.

Feitelson, J., & McLendon, G. (1991) *Biochemistry 30*, 5051.
Gavish, B., & Werber, M. M. (1979) *Biochemistry 18*, 1269.
Gill, S. C., & Von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319.
Gross, E. L. (1993) *Photosynth. Res. 37*, 103.
Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521.
Haehnel, W., Jansen, T., Gause, K., Klösgen, R. B., Stahl, B., Michl, D., Huvermann, B., Karas, M., & Herrmann, R. G. (1994) *EMBO J. 13*, 1028.
Harris, T. K., & Davidson, V. L. (1993) *Biochemistry 32*, 14145.
He, S., Modi, S., Bendall, D. S., & Gray, J. C. (1991) *EMBO J. 10*, 4011.
Hoffman, B. M., & Ratner, M. A. (1987) *J. Am. Chem. Soc. 109*, 6237.
Hoffman, B. M., & Ratner, M. A. (1988) *J. Am. Chem. Soc. 110*, 8267.
Hoffman, B. M., Ratner, M. A., & Wallin, S. A. (1990) *Adv. Chem. Ser. 226*, 125.
Hoffman, B. M., Natan, M. J., Nocek, J. M., & Wallin, S. A. (1991) *Struct. Bonding (Berlin) 75*, 86.
Ivković-Jensen, M., & Kostić, N. M. (1996) *Biochemistry 35*, 15095.
Katoh, S., Shiratori, I., & Takamiya, A. (1962) *J. Biochem. (Tokyo) 51*, 32.
Khoshtariya, D. E., Hammerstad-Pedersen, J. M., & Ulstrup, J. (1991) *Biochim. Biophys. Acta 1076*, 359.
King, J. C., Binstead, R. A., & Wright, P. E. (1985) *Biochim. Biophys. Acta* 106, 262.
Kostić, N. M. (1991) *Met. Ions Biol. Syst. 27*, 129.
Kostić, N. M. (1996) in *Metal-Containing Polymeric Materials* (Pitman, C. U., Jr., et al., Eds.) Plenum Publishing, New York.
Kramers, H. A. (1940) *Physica (Utrecht) 7*, 284.
Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946.
Kyritsis, P., Dennison, C., McFarlane, W., Nordling, M., Vänngård, Young, S., & Sykes, A. G. (1993) *J. Chem. Soc., Dalton Trans.*, 2289.
Landt, O., Grunert, H.-P., & Hahn, U. (1990) *Gene 96*, 125.
Lee, B. H., Hibino, T., Takabe, T., Weisbeek, P. J., & Takabe, T. (1995) *J. Biochem. (Tokyo) 117*, 1209.
Mauk, A. G. (1991) *Struct. Bonding (Berlin) 75*, 131.
Mauk, M. R., Ferrer, J. C., & Mauk, A. G. (1994) *Biochemistry 33*, 12609.
McLendon, G. (1991a) *Struct. Bonding (Berlin) 75*, 160.
McLendon, G. (1991b) *Met. Ions Biol. Syst. 27*, 183.
McLendon, G., & Hake, R. (1992) *Chem. Rev. 92*, 481.
McLendon, G., Zhang, Q., Wallin, S. A., Miller, R. M., Billestone, W., Spears, K. G., & Hoffman, B. M. (1993) *J. Am. Chem. Soc. 115*, 3665.
Meyer, T. E., Zhao, C. G., Cusanovich, M. A., & Tollin, G. (1993) *Biochemistry 32*, 4552.
Modi, S., He, S., Gray, J. C., & Bendall, D. S. (1992a) *Biochim. Biophys. Acta 1101*, 64.
Modi, S., Nordling, M., Lundberg, L. G., Hansson, Ö., & Bendall, D. S. (1992b) *Biochim. Biophys. Acta 1102*, 85.
Moore, G. R., & Pettigrew, G. W. (1990) *Cytochrome c: Evolutionary, Structural, and Physiochemical Aspects*, Springer-Verlag, Berlin.
Moore, J. M., Lepre, C. A., Gippert, G. P., Chazin, W. J., Case, D. A., & Wright, P. E. (1991) *J. Mol. Biol. 221*, 533.
Nocek, J. M., Stemp, E. D. A., Finnegan, M. G., Koshy, T. I., Johnson, M. K., Margoliash, E., Mauk, A. G., Smith, M., & Hoffman, B. M. (1991) *J. Am. Chem. Soc. 113*, 6822.
Nordling, M., Olausson, T., & Lundberg, L. G. (1990) *FEBS Lett. 276*, 98.
Nordling, M., Sigfridsson, K., Young, S., Lundberg, L G., & Hansson, Ö. (1991) *FEBS Lett. 29*, 327.
Northrup, S. H., Boles, J. O., & Reynolds, J. C. L. (1988) *Science 241*, 67.
Onuchic, J. N., Beratan, D. N., Winkler, J. R., & Gray, H. B. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 349.
Peerey, L. M., & Kostić, N. M. (1989) *Biochemistry 28*, 1861.
Peerey, L. M., Brothers, H. M., II, Hazzard, J. T., Tollin, G., & Kostić, N. M. (1991) *Biochemistry 30*, 9297.
Pelletier, H., & Kraut, J. (1992) *Science 258*, 1748.
Pettigrew, G. W., & Moore, G. R. (1987) *Cytochrome c: Biological Aspects*, Springer-Verlag, Berlin.
Qin, L., & Kostić, N. M. (1992) *Biochemistry 31*, 5145.
Qin, L., & Kostić, N. M. (1993) *Biochemistry 32*, 6273.
Qin, L., & Kostić, N. M. (1994) *Biochemistry 33*, 12592.
Qin, L., & Kostić, N. M. (1996) *Biochemistry 35*, 3379.
Redinbo, M. R., Yeates, T. O., & Merchant, S. (1994) *J. Bioenerg. Biomembr. 26*, 49.
Roberts, W. A., Freeman, H. C., Getzoff, E. D., Olson, A. J., & Tainer, J. A. (1991) *J. Biol. Chem. 266*, 13431.
Rodgers, K. K., Pochapsky, T. C., & Sligar, S. G. (1988) *Science 240*, 1657.
Scott, R. A., & Mauk, A. G., Eds. (1996) *Cytochrome c: A Multidisciplinary Approach*, University Science Books, Sausalito, CA.
Sigfridsson, K., Hansson, Ö., Karlsson, B. G., Baltzer, L., Nordling, M., & Lundberg, L. G. (1995) *Biochim. Biophys. Acta 1228*, 28.
Sigfridsson, K., Young, S., & Hansson, Ö. (1996) *Biochemistry 35*, 1249.
Sullivan, E. P., Jr., Hazzard, J. T., Tollin, G., & Enemark, J. H. (1992) *J. Am. Chem. Soc. 114*, 9662.
Sykes, A. G. (1991a) *Adv. Inorg. Chem. 36*, 377.
Sykes, A. G. (1991b) *Struct. Bonding (Berlin) 75*, 177.
Takano, T., & Dickerson, R. E. (1981) *J. Mol. Biol. 153*, 79.
Therien, M. J., Chang, J., Raphael, A. L., Bowler, B. E., & Gray, H. B. (1991) *Struct. Bonding (Berlin) 75*, 110.
Ullmann, G. M., & Kostić, N. M. (1995) *J. Am. Chem. Soc. 117*, 4766.
Vanderkooi, J. M., & Erecińska, M. (1975) *Eur. J. Biochem. 60*, 199.
Vanderkooi, J. M., Adar, F., & Erecińska, M. (1976) *Eur. J. Biochem. 64*, 381.
Walker, M. C., & Tollin, G. (1992) *Biochemistry 31*, 2798.
Wallin, S. A., Stemp, E. D. A., Everest, A. M., Nocek, J. M., Netzel, T. L., & Hoffman, B. M. (1991) *J. Am. Chem. Soc. 113*, 1842.
Weast, R. C., Ed. (1986) *CRC Handbook of Chemistry and Physics*, 66th ed., p D-232, CRC Press, Boca Raton, FL.
Wendoloski, J. J., Matthew, J. B., Webber, P. C., & Salemme, F. R. (1987) *Science 238*, 794.
Willie, A., Steyton, P. S., Sligar, S. G., Durham, B., & Millett, F. (1992) *Biochemistry 31*, 7237.
Winkler, J. R., & Gray, H. B. (1992) *Chem. Rev. 92*, 369.
Ye, S., Shen, C., Cotton, T. M., & Kostić, N. M. (1997) *J. Inorg. Biochem.* (in press).
Zhou, J. S., & Kostić, N. M. (1991a) *J. Am. Chem. Soc. 113*, 6067.
Zhou, J. S., & Kostić, N. M. (1991b) *J. Am. Chem. Soc. 113*, 7040.
Zhou, J. S., & Kostić, N. M. (1992a) *J. Am. Chem. Soc. 114*, 3562.
Zhou, J. S., & Kostić, N. M. (1992b) *Biochemistry 31*, 7543.
Zhou, J. S., & Kostić, N. M. (1992c) *Spectrum 5* (no. 2), 1.
Zhou, J. S., & Kostić, N. M. (1993a) *Biochemistry 32*, 4539.
Zhou, J. S., & Kostić, N. M. (1993b) *J. Am. Chem. Soc. 115*, 10796.
Zhou, J. S., & Hoffman, B. M. (1994) *Science 265*, 1693.
Zhou, J. S., Brothers, H. M., II, Neddersen, J. P., Peerey, L. M., Cotton, T. M., & Kostić, N. M. (1992) *Bioconjugate Chem. 3*, 382.
Zhou, J. S., Nocek, J. M., DeVan, M. L., & Hoffman, B. M. (1995) *Science 269*, 204.

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