Effects of Single and Double Mutations in Plastocyanin on the Rate Constant and Activation Parameters for the Rearrangement Gating the Electron-Transfer Reaction between the Triplet State of Zinc Cytochrome *c* and Cupriplastocyanin†

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ABSTRACT: The unimolecular rate constant for the photoinduced electron-transfer reaction ^{3}Zncyt/pc(II) → Zncyt$^+$/pc(I) within the electrostatic complex of zinc cytochrome *c* and spinach cupriplastocyanin is k_F. We report the effects on k_F of the following factors, all at pH 7.0: 12 single mutations on the plastocyanin surface (Leu12Asn, Leu12Glu, Leu12Lys, Asp42Asn, Asp42Lys, Glu43Asn, Glu59Gln, Glu59Lys, Glu60Gln, Glu60Lys, Gln88Glu, and Gln88Lys), the double mutation Glu59Lys/Glu60Gln, temperature (in the range 273.3−302.9 K), and solution viscosity (in the range 1.00−116.0 cP) at 283.2 and 293.2 K. We also report the effects of the plastocyanin mutations on the association constant (K_a) and the corresponding free energy of association (ΔG_a) with zinc cytochrome *c* at 298.2 K. Dependence of k_F on temperature yielded the activation parameters $\Delta H^‡$, $\Delta S^‡$, and $\Delta G^‡$. Dependence of k_F on solution viscosity yielded the protein friction and confirmed the $\Delta G^‡$ values determined from the temperature dependence. The aforementioned intracomplex reaction is not a simple electron-transfer reaction because donor−acceptor electronic coupling (H_{AB}) and reorganizational energy (λ), obtained by fitting of the temperature dependence of k_F to the Marcus equation, deviate from the expectations based on precedents and because k_F greatly depends on viscosity. This last dependence and the fact that certain mutations affect K_a but not k_F are two lines of evidence against the mechanism in which the electron-transfer step is coupled with the faster, but thermodynamically unfavorable, rearrangement step. The electron-transfer reaction is gated by the slower, and thus rate determining, structural rearrangement of the diprotein complex; the rate constant k_F corresponds to this rearrangement. Isokinetic correlation of $\Delta H^‡$ and $\Delta S^‡$ parameters and Coulombic energies of the various configurations of the Zncyt/pc(II) complex consistently show that the rearrangement is a facile configurational fluctuation of the associated proteins, qualitatively the same process regardless of the mutations in plastocyanin. Correlation of k_F with the orientation of the cupriplastocyanin dipole moment indicates that the reactive configuration of the diprotein complex involves the area near the residue 59, between the upper acidic cluster and the hydrophobic patch. Kinetic effects and noneffects of plastocyanin mutations show that the rearrangement from the initial (docking) configuration, which involves both acidic clusters, to the reactive configuration does not involve the lower acidic cluster and the hydrophobic patch but involves the upper acidic cluster and the area near the residue 88.

Electron-transfer reactions are essential for life. Among biomolecules that constitute electron-transport chains, there are numerous metalloproteins. The mechanism of interactions between them and general correlation of their structure and function can be studied by site-directed mutagenesis. In the case of electron carriers, there are two main questions that these studies can answer: how these proteins associate, and how electron transfer between them occurs.

Some of the properties that govern the rate of electron transfer are driving force, donor−acceptor electronic coupling, and reorganizational energy (*1*). If the rate of electron transfer is great, the rate-determining factor may be some nonredox process. In this case, the reaction may be gated by or coupled with this process (*2*−*10*). Two metalloproteins in solution form a dynamic binary complex (*5*, *11*−*21*). The orientation optimal for binding may not be optimal for electron transfer (*17*, *22*−*26*).

Plastocyanin (pc)[1] and cytochrome *c* (cyt) are fully characterized in both oxidized and reduced states (*27*−*31*). Replacement of iron in cytochrome *c* by zinc(II) does not perturb the conformation of the protein (*32*−*34*) and its association with other metalloproteins (*30*, *35*−*38*). Spectroscopic and chemical investigations show that both cyto-

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chrome *c* and zinc cytochrome *c* bind to plastocyanin via their basic patch surrounding the exposed heme edge. Plastocyanin binds to each of them with its broad acidic patch (*39*−*43*). There are no crystal structures of plastocyanin mutants, but comparison of spectroscopic and electrochemical properties (UV−vis, CD, EPR, and NMR spectra and reduction potentials) of the mutants used here with those properties of the wild-type protein shows that the overall conformation and the copper site are not detectably perturbed by mutation of surface residues in the acidic and hydrophobic patches (*44*−*49*).

Mutants of plastocyanin were used in experiments with photosystem I (*47*, *50*), with cytochrome *f* (*45*, *46*, *51*−*53*), and with cytochrome *c* (*45*, *46*, *54*). The results indicate that plastocyanin can use for electron-transfer both the negative patch around Tyr 83 and hydrophobic patch near the copper atom.

Studies of the quenching of the triplet states ^{3}Zncyt and ^{3}Sncyt by French-bean cupriplastocyanin (*43*, *55*−*63*) showed that, at low ionic strength, at which the diprotein complex persists, each intracomplex (unimolecular) reaction in eqs 1 and 2 can be observed as the faster of the two kinetic phases.

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

$$^3\text{Sncyt/pc(II)} \xrightarrow{k_F} \text{Sncyt}^+\text{/pc(I)} \quad (2)$$

Independence of the rate constant k_F of the driving force for electron transfer (1.2 and 0.80 eV in the eqs 1 and 2, respectively) and smooth decrease of k_F as solution viscosity increases show that k_F actually represents a structural rearrangement of the diprotein complex. The viscous solvents impede the rearrangement process that occurs in the electrostatic but not in the covalent diprotein complex (*57*, *61*). Similar results were obtained also for the reaction between the triplet state of zinc cytochrome *c* and ferricytochrome b_5 (*64*). In the protein pairs zinc cytochrome *c*/plastocyanin, tin cytochrome *c*/plastocyanin, and zinc cytochrome *c*/ferricytochrome b_5, the rate-limiting process seems to be a configurational fluctuation. This process was investigated by changing experimental conditions (*54*, *65*, *66*) and by theoretical analysis of electron-transfer paths between the heme and blue copper sites in various configurations of the cyt(II)/pc(II) complex (*25*). The configuration that optimizes the surface interactions does not optimize the heme-copper electronic coupling. Motions of the cytochrome *c* molecule, whose basic patch explores the area within or near the acidic patch in plastocyanin, enhance the electronic coupling.

To understand the unimolecular reaction in eq 1, we first examined the effects of temperature and ionic strength at constant viscosity (*65*), determined the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the rearrangement k_F, and interpreted these parameters in terms of molecular surfaces (*67*) of the two proteins. Next, we greatly widened the viscosity range by varying temperature as well as the glycerol concentration (*66*) and showed that the activation parameters depend on viscosity, but not on the way it is adjusted. We proposed models for the rearrangement.

A previous study of the reaction in eq 1 with mutants of plastocyanin (*54*) concluded that the rearrangement involves the upper cluster of the acidic patch. The double mutation Glu59Lys/Glu60Gln, in which the local charge was changed by 3 units, lowered the rate constant k_F. In this work, we separate the two single mutations that were combined in this double mutation and investigate the effects of these two and 11 other single mutations on the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the protein rearrangement.

MATERIALS AND METHODS

Chemicals. Distilled water was demineralized to a resistivity greater than 17 MΩ cm. Chromatography gels (CM Sephadex C-50, Sephadex G-25 and G-75, and Sephadex DEAE A-25) were purchased from Sigma Chemical Co. Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co. All other chemicals were purchased from Fischer Chemical Co.

Buffers. Sodium dihydrogen phosphate and sodium monohydrogen phosphate were used to make buffers with ionic strength of 2.5 mM and pH 7.0.

Viscosity. The absolute viscosity (η) of water and of aqueous solutions of glycerol at different temperatures were taken from tables (*68*−*70*). The relative viscosity (η/η_o) of buffers with and without glycerol was measured with a thermostated glass viscometer; the absolute error was ±0.05 cP. Given $\eta_o = 1.002$ cP, the absolute viscosity was calculated. Both calculations and experiments showed the contribution of salts to viscosity to be negligible. The contribution of proteins, at micromolar concentrations, was neglected.

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 (*34*) of the original procedure (*35*, *36*). 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 third was the rate constant for natural decay of the triplet state, $k_d < 110$ s^{-1}; it was checked before each series of kinetic experiments.

Plastocyanin. Plastocyanin was isolated from spinach by a standard procedure (*71*) and purified repeatedly by gel-filtration chromatography on Sephadex G-25 and G-75 columns and by an ion-exchange chromatography on a Sephadex DEAE A-25 column; the criterion of purity was the absorbance quotient $A_{278}/A_{597} < 1.20$. Recombinant wild-type protein from spinach and 13 mutants of it were prepared by the published method (*72*) and purified chromatographically (*54*). All proteins were desalted, transferred into a 2.5 mM phosphate buffer at pH 7.0, and stored in liquid nitrogen. Before each series of kinetic experiments, plastocyanin was treated with a small excess of dissolved $K_3[Fe(CN)_6]$, which was then removed with Centricon ultrafiltration cells. Concentrations of the proteins were determined from their UV−vis spectra, on the basis of the known absorptivities:

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

$\epsilon_{423} = 2.43 \times 10^5\ M^{-1}\ cm^{-1}$ for zinc cytochrome *c* (*36*) and $\epsilon_{597} = 4700\ M^{-1}\ cm^{-1}$ for all the mutants of cupriplastocyanin (*72*).

Flash Kinetic Spectrophotometry. So-called laser flash photolysis on the microsecond scale was done with a standard apparatus (*43*, *55*, *56*, *60*, *61*). Argon was passed first through water and then through the buffered solution to be deaerated. The required volume of the buffer was deaerated in a 10 mm cuvette for 30 min. Next, the cell jacket was connected to a 30-L circulating bath Forma 2067, which maintained the temperature within ±0.2 °C. The actual temperature in the cell was calibrated with an Omega HH82 digital thermometer and was known with a precision of ±0.1 °C. The temperature range was 273.3−302.9 K. After the temperature of the buffer was adjusted, other solutions were added. The concentration of zinc cytochrome *c* was always 10.0 μM. After each exposure to air, the solution in the cuvette was gently deaerated for 10−30 min, longer for more viscous solutions. Determinations of k_d in control experiments proved this deaeration to be thorough enough. Formation and decay (natural or by quenching) of the triplet state, ^{3}Zncyt, were monitored at 460 nm. Its concentration depended on the intensity of the laser pulse and was ca. 1.0 μM, much lower than the cupriplastocyanin concentration. Kinetic conditions for the pseudo-first order were thus satisfied. At each set of conditions (temperature and glycerol concentration), 5−10 laser pulses were delivered. Error bars in the figures enclose all the corresponding experimental values.

Kinetics. All the rate constants were obtained from the change in the absorbance at 460 nm with time. The absorbance decrease corresponds to the disappearance of the ^{3}Zncyt. The traces were analyzed with kinetic software from OLIS, Inc. and with the fitting program SigmaPlot 1.02, from Jandel Scientific Co. The standard errors of fitting, which are given in the tables, are computed by dividing the standard deviation by the square root of the number of measurements.

Energies and Geometrical Properties of the Diprotein Complex in Three Configurations. We examined three configurations of the electrostatic complex between ferrocytochrome *c* from tuna (*73*) and cupriplastocyanin from poplar (*74*). These configurations were designated maximum-overlap (max ov); maximum-overlap, rotated (max ov rot); and northern equatorial (n/eq) in the original study (*41*) and afterward (*54*, *65*, *66*). For the sake of consistency, we retain these designations and abbreviations. The structure of horse-heart ferrocytochrome *c* was obtained from the Protein Data Bank (1hrc) (*75*). Hydrogen atoms were added with CHARMM (*76*). The structure of spinach cupriplastocyanin was obtained from the structure of poplar cupriplastocyanin (1plc) (*77*) by "mutating" the nonidentical residues. The conformation of each new residue was matched to that of the original residue. The structure was then minimized with constrained atoms that had not changed upon "mutation" and unconstrained new atoms that had no counterparts in the structure of poplar plastocyanin. Dipole moments were calculated according to the center of mass (*78*), assuming usual p*K* values at pH 7 and CHARMM partial charges for all atoms. The coordinates of the configurations designated max ov, max ov rot, and n/eq were taken from Roberts et al. (*41*). The structures of horse-heart ferrocytochrome *c* and of spinach cupriplastocyanin were superimposed on the proteins in these three configurations using Kabsch algorithm (*79*). In each configuration, the energies of monopole−monopole (eq 3), monopole−dipole (eqs 4 and 5), and dipole−dipole (eq 6) interactions and the total Coulombic energy (eq 7) were calculated by standard formulas below (*80*).

$$E_{qq} = \frac{1}{4\pi\epsilon\epsilon_0}\frac{q_1 q_2}{r} \tag{3}$$

$$E_{\mu q} = \frac{1}{4\pi\epsilon\epsilon_0}\frac{\mu_1 q_2 \cos\theta_1}{r^2} \tag{4}$$

$$E_{q\mu} = \frac{1}{4\pi\epsilon\epsilon_0}\frac{q_1 \mu_2 \cos\theta_2}{r^2} \tag{5}$$

$$E_{\mu\mu} = \frac{1}{4\pi\epsilon\epsilon_0}\frac{\mu_1\mu_2}{r^3}(2\cos\theta_1\cos\theta_2 + \sin\theta_1\sin\theta_2\cos\phi) \tag{6}$$

$$E = E_{qq} + E_{\mu q} + E_{q\mu} + E_{\mu\mu} \tag{7}$$

The symbols in eqs 3−7 have their usual meanings: ϵ is permittivity of water, ϵ_0 is permittivity of vacuum, q_1 and q_2 are the net charges at pH 7 of pc(II) and cyt(II), μ_1 is the dipole moment of pc(II), μ_2 is the dipole moments of cyt-(II), θ_1 is the angle in pc(II) between the positive end of the dipole moment and the vector that connects the centers of mass the two proteins, θ_2 is the angle in cyt(II) between the positive end of the dipole moment and the vector that connects the centers of mass of the two proteins, ϕ is the torsion angle defined by the dipole-moment vectors and the centers of mass of the two proteins, and r is the distance between their centers of mass. Angles and torsion angles were obtained with the program HyperChem. All energies were calculated with the program SigmaPlot 1.02, from Jandel Scientific Co.

RESULTS

Quenching of ^{3}Zncyt by Cupriplastocyanin. The quenching of ^{3}Zncyt in the presence of high concentrations of cupriplastocyanin (wild-type and mutants alike) is biphasic. The faster phase (amplitude a_1 and rate constant k_F) is the unimolecular reaction within the diprotein complex (eq 1), and the slower phase (amplitude a_2 and rate constant k_{obs}) is the bimolecular reaction between the unassociated protein molecules (*55*, *65*, *66*). The unimolecular rate constant k_F and the composite rate constant k_{obs} were obtained from eq 8. In this work, we are interested only in the unimolecular reaction, shown in eq 1.

The Unimolecular Reaction with Mutants. Table 1 shows the mutants of plastocyanin that were used in the experiments. The positions of the mutated residues are shown in Figure 1. Effects of temperature on the unimolecular reaction in eq 1 were studied at ionic strength of 2.5 mM and at pH 7.0. Results are shown in Figure 2 and in Supporting Information, Table S1. The data were fitted to the Eyring

Table 1: Properties of Cupriplastocyanin: Total Charge (q_1), Difference in q_1 between the Mutant and the Wild-Type Protein (Δq), Charge of the Acidic Patch (q_p), Dipole Moment (μ_1), Orientation of the Dipole Moment (α), Reduction Potential ($E°$), Calculated Driving Force for the Unimolecular Reaction in eq 1 ($\Delta G°$), Binding Constant with Zinc Cytochrome *c* (K_a), and Rate Constant of the Unimolecular Reaction in eq 1(k_F)

pc(II) species	abbreviation	q_1[a]	Δq[a]	q_p[a]	μ_1 (D)	α[b] (deg)	$E°$ (mV)	$\Delta G°$ (kJ/mol)	$K_a \times 10^{-5}$ [c] (M^{-1})	$k_F \times 10^{-5}$ [d] (s^{-1})
wild-type	WT	−8	0	−7	324	84	381[e]	−122	9 ± 2	2.3
Leu12Asn	L12N	−8	0	−7	300	85	362[f]	−120	4 ± 2	1.9
Leu12Glu	L12E	−9	−1	−7	337	73	354[f]	−119	6 ± 3	2.4
Leu12Lys	L12K	−7	1	−7	310	102	411[f]	−125	3 ± 2	1.1
Asp42Asn	D42N	−7	1	−6	276	83	387[e]	−122	1.3 ± 0.7	2.3
Asp42Lys	D42K	−6	2	−5	221	77	391[g]	−123	0.4 ± 0.1	2.4
Glu43Asn	E43N	−7	1	−6			384[e]	−122	1.0 ± 0.5	2.0
Glu59Gln	E59Q	−7	1	−6	253	89			2.7 ± 0.7	2.3
Glu59Lys	E59K	−6	2	−5	184	103			0.6 ± 0.1	0.26
Glu59Lys/Glu60Gln	E59K/E60Q	−5	3	−4	148	108	406[e]	−124	0.2 ± 0.1	0.20
Glu60Gln	E60Q	−7	1	−6	264	89			1.5 ± 0.3	1.2
Glu60Lys	E60K	−6	2	−5	180	102			0.8 ± 0.1	0.66
Gln88Glu	Q88E	−9	−1	−7	369	80	375[e]	−121	9 ± 5	2.8
Gln88Lys	Q88K	−7	1	−7	255	89			1.0 ± 0.5	1.7

[a] Assuming usual p*K* values at pH 7. [b] Angle between the negative end of the dipole moment and vector that connects the center of mass of cupriplastocyanin with the copper atom. [c] Estimated from the fractional contribution of the faster phase at 298.2 K. [d] At 298.2 K. Error is ± 10%. [e] From ref *53*. [f] From ref *47*. [g] From ref 49.

equation (eq 9) in a nonlinear form. The symbols in eqs 8−14 have their usual meanings:

$$\Delta A = a_1 \exp(-k_F t) + a_2 \exp(-k_{obs} t) + b \quad (8)$$

$$k_F = \frac{k_B T}{h} \exp\frac{\Delta S^{\ddagger}}{R} \exp\frac{-\Delta H^{\ddagger}}{RT} \quad (9)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (10)$$

$$k_F = \frac{4\pi^2 H_{AB}{}^2}{h\sqrt{4\pi\lambda\, RT}} \exp\left[\frac{-(\Delta G° + \lambda)^2}{4\lambda\, RT}\right] \quad (11)$$

$$k_F = k_0 \exp[-\beta(r - r_0)] \exp\left[\frac{-(\Delta G° + \lambda)^2}{4\lambda\, RT}\right] \quad (12)$$

$$k_F = \frac{k_B T}{h} \frac{1+\sigma}{\eta+\sigma} \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right) \quad (13)$$

$$k_F = \frac{k_B T}{h} \frac{1+\sigma}{\eta+\sigma} \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \exp\left(\frac{-\Delta H^{\ddagger}}{RT}\right) \quad (14)$$

k_B is the Boltzmann constant, h is the Planck constant, R is the gas constant, $\Delta G°$ is the driving force, H_{AB} is the electronic coupling between the heme and the copper site, λ is the reorganizational energy, k_0 is the nuclear frequency (10^{13} s^{-1}), r_0 is the contact distance (3.0 Å), β is the attenuation of electronic coupling (1.1 and 1.4 $Å^{-1}$), and r is the donor−acceptor distance. The results of fitting to eq 9 are the activation parameters for the reaction in eq 1 with different mutants of plastocyanin; these parameters are listed in Table 2. Free energy of activation can be calculated from these two parameters by eq 10. The $\Delta G^{\ddagger}$ values in Table 3 are given at 283.2 and 293.2 K, the two temperatures at which the viscosity experiments were done. Reorganizational energies, donor−acceptor electronic couplings, and the electron-transfer distances were obtained from the Marcus equations 11 and 12; these parameters are shown in Supporting Information, Table S2. Viscosity effects on the unimolecular reaction in eq 1 were studied at ionic strength of 2.5 mM and at pH 7.0; viscosity was changed at 283.2 and 293.2 K. The results are shown in Figure 3. The results of fitting to eqs 13 and 14 are shown in Table 3. The fractional amplitudes of the unimolecular reaction, at one or more concentrations of plastocyanin, were used to estimate association constants given in Table 1. Association constants were then converted to the free energies of association at 298.2 K, which are shown in Supporting Information, Table S3 and Figure S1.

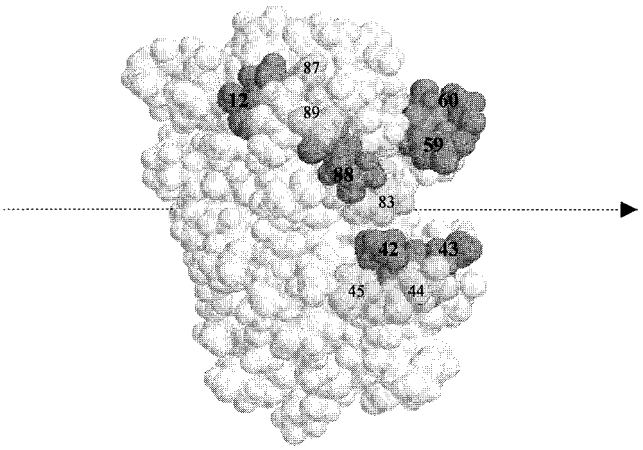

FIGURE 1: Structure of wild-type spinach plastocyanin showing positions of mutated residues (Leu12, Asp42, Glu43, Glu59, Glu60, and Gln88), the dipole moment vector, and other residues mentioned in the text (Asp44, Glu45, His87, Tyr83, and Gly89). This figure was generated with the program RasMol.

DISCUSSION

Choice of Experimental Conditions Depends on Protein Association. Zinc cytochrome *c* and cupriplastocyanin associate at low ionic strength because the complementary patches on the protein surfaces bear opposite charges. Only at low ionic strength and at high concentration of cupriplastocyanin can both unimolecular and bimolecular reactions be observed, as two phases. At high ionic strength and at low concentration of plastocyanin, only the bimolecular reaction can be observed. Since we are interested only in the unimolecular reaction, we chose the ionic strength of 2.5 mM. We used different concentrations of plastocyanin

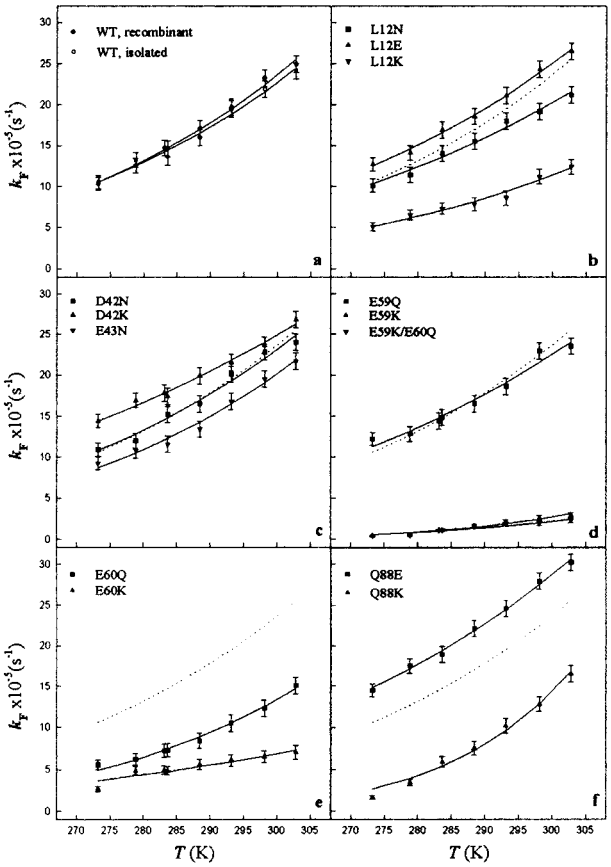

FIGURE 2: Dependence on temperature of the unimolecular rate constant (k_F) for the intracomplex reaction in eq 1, in sodium phosphate buffer at pH 7.0 and ionic strength 2.5 mM involving the following variants of spinach cupriplastocyanin: (a) wild type, both recombinant and isolated; (b) mutants in the hydrophobic patch Leu12Asn, Leu12Glu, and Leu12Lys; (c) mutants in the lower acidic cluster Asp42Asn, Asp42Lys, and Glu43Asn; (d) mutants in the upper acidic cluster Glu59Gln, Glu59Lys, and the double mutant, Glu59Lys/Glu60Gln; (e) mutants in the upper acidic cluster Glu60Gln and Glu60Lys; and (f) mutants between the upper acidic cluster and the hydrophobic patch Gln88Glu and Gln88Lys. The lines are fittings to eq 9. For easy comparison, the fitting for the recombinant wild-type spinach cupriplastocyaninin in frame a is shown, as a dotted line, also in other frames.

Table 2: Effects of Mutations in Plastocyanin on the Activation Parameters for the Unimolecular Reaction in Eq 1, Obtained by Fitting of the Rate Constant k_F to Eyring Equation (Eq 9)

pc(II) species	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol K)
recombinant wild-type	18.1 ± 0.7	−82 ± 3
isolated wild-type	17 ± 2	−85 ± 4
Leu12Asn	15 ± 1	−94 ± 4
Leu12Glu	15 ± 1	−91 ± 2
Leu12Lys	18 ± 2	−88 ± 6
Asp42Asn	17 ± 2	−86 ± 5
Asp42Lys	12 ± 1	−103 ± 3
Glu43Asn	19 ± 2	−80 ± 5
Glu59Gln	15 ± 2	−92 ± 5
Glu59Lys	37 ± 5	−38 ± 2
Glu59Lys/Glu60Gln	31 ± 6	−60 ± 2
Glu60Gln	23 ± 2	−70 ± 4
Glu60Lys	14 ± 3	−107 ± 9
Gln88Glu	15 ± 1	−92 ± 2
Gln88Lys	40 ± 3	−13 ± 10

(30−60 μM), depending on properties of the plastocyanin variant. By mutating the residues on the surface of plastocyanin that are involved in the association, we change the association constant with zinc cytochrome *c*. If the association constant is low (as it is, for example, for the double mutant E59K/E60Q), a high concentration of plastocyanin mutant is required to detect a faster phase (unimolecular reaction) even at very low ionic strength. (In this example, concentration of the double mutant E59K/E60Q higher than 50 μM is required even at ionic strength of 2.5 mM).

Choice of Mutations Is Guided by Previous Experiments. Mutations on the plastocyanin surface explore the areas highlighted in Figure 1 (and numbered as in the wild-type spinach protein), which were implicated in docking or electron transfer in previous studies of plastocyanin (*44*−*47*, *53*, *54*, *81*, *82*). The negatively charged or acidic patch surrounding Tyr83 is made up of the lower (residues 42−45) and upper (residues 59−61) clusters. The following three single mutations resulted in neutralization or reversal of charges in the lower cluster: Asp42Asn, Asp42Lys, and Glu43Asn. The double mutation Glu59Lys/Glu60Gln in the upper cluster is "dissected" into the constituent single mutations, Glu59Lys and Glu60Gln. The charges of these crucial residues are reversed, one at the time, in the mutations Glu59Gln and Glu60Lys. The electroneutral or hydrophobic patch surrounding the partially exposed residue His87, which is a ligand to the copper ion, is probed by mutations of Leu12 into electroneutral (Asp), anionic (Glu), and cationic (Lys) residues. In the region between the hydrophobic patch and the upper cluster of the acidic patch, charges are introduced by replacing the electroneutral Gln88 with Glu and Lys. The mutants having Gln in positions 59 or 60 and those having Lys in positions 42, 59, 60, or 88 are used in kinetic experiments for the first time in this study.

Effects of Plastocyanin Charge on k_F and K_a. Assuming usual p*K* values, zinc cytochrome *c* has an overall charge of +6, wild-type spinach cupriplastocyanin has an overall charge of −8, and its mutants have overall charges between −5 and −9, all at pH 7.0 (Table 1). The mutations in plastocyanin simultaneously change the overall charge (q_1), the dipole moment (μ_1), and the charge of the acidic patch (q_p). The rate constant k_F decreases as the charge becomes less negative; see Table 1 and Supporting Information, Table S1 and Figure S1a. (The correlation, however, is poor: $0.41 < R_c^2 < 0.56$; see also Supporting Information, Table S4.) Clear evidence that variation in k_F cannot be attributed simply to charge is the difference in k_F for the mutants Glu59Lys and Asp42Lys, which have the same charge, and the identity of k_F for Leu12Glu and Asp42Lys, which have different charges.

Binding constants K_a for association of cupriplastocyanin with zinc cytochrome *c* at 298.2 K are shown in Table 1. Free energies of association, ΔG_a, were calculated from K_a and are given in Supporting Information, Table S3 and Figure S1c. As the charge of plastocyanin becomes less negative, the association constant decreases, and, consequently, the free energy of association becomes less negative. The correlations of ΔG_a with the overall charge (q_1) and with the charge of the acidic patch (q_p) are of similar degrees; the respective values of R_c^2 are 0.87 and 0.76. The association is affected by mutations in either acidic cluster.

Dipole Moments of Plastocyanin Mutants Have Different Magnitudes and Orientations. Figure 1 shows the dipole moment of wild-type spinach cupriplastocyanin; the arrow marks the negative end of the dipole. The general orientation

Table 3: Effect of Temperature and Plastocyanin Mutation on the Free Energy of Activation and on the Protein Friction for the Unimolecular Reaction in Eq 1 at the Ionic Strength of 2.5 mM

pc(II) species	T (K)	eq 10, $\Delta G^{\ddagger}$ (kJ/mol)	eq 13		eq 14, σ (cP)
			σ (cP)	$\Delta G^{\ddagger}$ (kJ/mol)	
wild-type	293.2	42 ± 1	0.8 ± 0.2	42.0 ± 0.1	1.1 ± 0.3
	283.2	41 ± 1	0.9 ± 0.2	40.9 ± 0.1	2.1 ± 0.6
Leu12Asn	293.2	43 ± 1	1.2 ± 0.2[a]	43 ± 1[a]	
Leu12Lys	293.2	44 ± 2	1.4 ± 0.2	44.0 ± 0.1	0.7 ± 0.3
Asp42Asn	293.2	42 ± 2	0.7 ± 0.2[a]	43 ± 1[a]	
Asp42Lys	293.2	42 ± 1	1.0 ± 0.2	41.8 ± 0.1	1.3 ± 0.3
	283.2	41 ± 1	1.7 ± 0.4	40.5 ± 0.1	3.0 ± 0.8
Glu43Asn	293.2	42 ± 2	1.3 ± 0.2[a]	43 ± 1[a]	
Glu59Gln	283.2	41 ± 2	0.9 ± 0.2	40.9 ± 0.1	1.3 ± 0.2
Glu59Lys	283.2	48 ± 5	1.9 ± 0.2	47.1 ± 0.2	5 ± 2
Glu59Lys/Glu60Gln	293.2	49 ± 6	0.7 ± 0.2[a]	49 ± 1[a]	
	283.2	48 ± 6	1.7 ± 0.3	47.2 ± 0.1	5 ± 3
Glu60Gln	283.2	43 ± 2	2.1 ± 0.4	42.6 ± 0.1	3.0 ± 0.6
Glu60Lys	283.2	44 ± 3	2.2 ± 0.5	43.6 ± 0.1	6 ± 3
Gln88Glu	293.2	42 ± 1	1.5 ± 0.2[a]	43 ± 1[a]	
Gln88Lys	293.2	44 ± 3	0.6 ± 0.5	43.5 ± 0.2	1.1 ± 0.6

[a] From ref *54*.

of the dipole moment in mutants remains the same, the vector protruding between the two clusters in the acidic patch. The magnitude, however, varies from 148 to 369 D; see Table 1. To describe the change in the orientation of the dipole moment, we consider angles defined by an atom in plastocyanin, the center of mass of this protein, and the negative end of the dipole moment. One such angle is α, involving the copper atom. Its values in Table 1 show how much the dipole moment in various mutants points away from the copper atom. Other angles are listed in Supporting Information, Table S5; α-carbon atoms are used as reference points because their positions are most accurately known.

The magnitudes and orientations of the dipole moments were correlated to the rate constants of bimolecular electron-transfer reactions involving proteins (*43*, *60*, *62*, *64*, *83*−*87*). A crucial assumption in these analyses is that the orientation of the two reacting molecules is governed by their dipolar interactions. This assumption is justified when the dipole moments are sufficiently large and the ionic strength is sufficiently low. Indeed, plastocyanin and cytochrome *c* interact in such a way that their dipole moments align approximately head-to-tail (*39*−*43*).

Our study differs from the previous ones in that we analyze the *unimolecular reaction k_F between the proteins associated in a dynamic complex*. The question is how the dipole moments affect the rearrangement that is involved in the electron-transfer reaction in eq 1.

Effects of Temperature and of Plastocyanin Mutation on the Rate Constant k_F. Figure 2, which shows simultaneously both of these effects, clearly reveals something that can be surmised from the k_F values at a single temperature (Table 1), namely that the rate constant k_F is affected by the mutations in the upper acidic cluster. The large effect of the double mutation Glu59Lys/Glu60Gln is mostly due to the single mutation Glu59Lys.

The rate constant k_F tends to increase as the magnitude of the dipole moment increases (see Supporting Information, Figure S1b and Table S4), but the correlation is relatively poor. As Figures S2 and S3 in the Supporting Information show, the rate constant k_F increases as the dipole moment shifts toward the residues 12, 59, 60, 83, and 87−89 and toward the copper atom and as it shifts away from the lower acidic patch, residues 42−45. The quality of the correlation varies with the choice of the reference point (the angle in Table S5) and temperature; the range $0.24 < R_c^2 < 0.88$ encompasses all the values in Supporting Information, Table S6. Under certain assumptions, these correlations can be used to estimate the area on the plastocyanin surface to which zinc cytochrome *c* migrates in the rearrangement process k_F that precedes the electron-transfer step. If the process k_F is the same in all the mutants and if the rearranged configuration of the complex ^{3}Zncyt/pc(II) is governed by dipolar interactions, then the trajectory of the ^{3}Zncyt migration is away from the lower acidic cluster and toward the hydrophobic patch. The "destination" apparently lies between the residues 59 and 87. Indeed, the calculation of electron-tunneling paths showed that the lower acidic cluster is unfavorable for electron transfer, whereas the upper acidic cluster and the area between it and the hydrophobic patch are favorable.

A simple model outlined in Supporting Information, Appendix S1, leads to a similar conclusion. Under the assumptions that zinc cytochrome *c* initially docks at the site at which the negative end of the dipole moment emerges from the plastocyanin surface and that the angular velocity of zinc cytochrome *c* is the same in the complexes with all the plastocyanin mutants, the reactive configuration of these complexes involves the area near residue 59 on the plastocyanin surface.

Effects of Viscosity and of Plastocyanin Mutation on the Rate Constant k_F. Figure 3 shows these two effects at two temperatures. Effects of solution viscosity on reactions of proteins have been studied in different systems (*5*, *88*−*91*). As previous studies in our laboratory show (*61*, *65*, *66*), it is the viscosity, and not other properties of the added viscosigens, that is responsible for the dependence of the rate constant k_F on the solvent composition. When the solvent molecules are much smaller than the solute (protein) molecules, eqs 13 and 14 apply, as we have shown previously (*66*). The contribution of the protein friction to the total friction is designated σ (*91*). Since η is absolute viscosity, σ also has the units of viscosity. Either of these equations is valid in the following three regimes: at low solution viscosity ($\eta < \sigma$), protein friction dominates; at intermediate

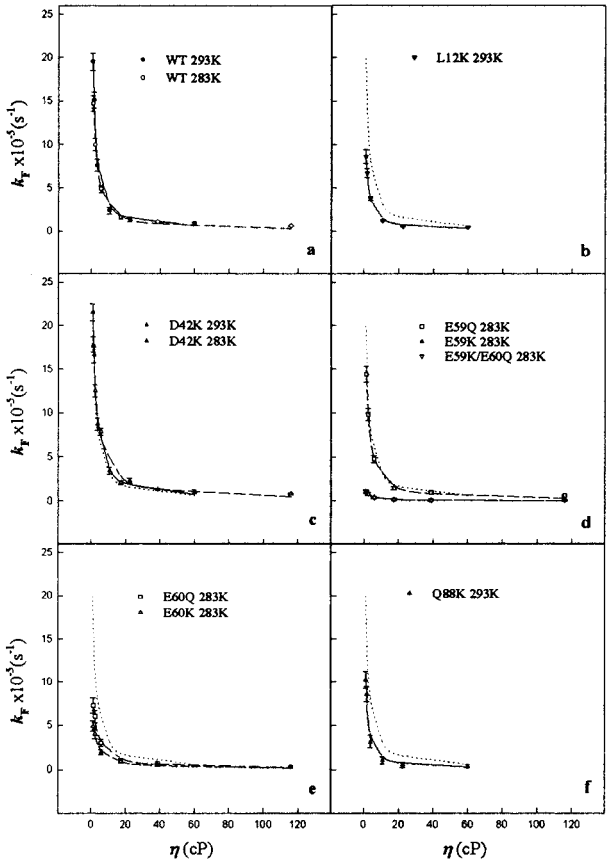

FIGURE 3: Dependence on viscosity of the unimolecular rate constant (k_F) for the intracomplex reaction in eq 1, in sodium phosphate buffer at pH 7.0 and ionic strength 2.5 mM, and temperatures shown, involving the following variants of spinach cupriplastocyanin: (a) recombinant wild type at 293.2 and 283.2 K; (b) mutant in the hydrophobic patch Leu12Lys, at 293.2 K; (c) mutant in the lower acidic cluster Asp42Lys, at 293.2 and 283.2 K; (d) mutants in the upper acidic cluster Glu59Gln, Glu59Lys, and the double mutant Glu59Lys/Glu60Gln, at 283.2 K; (e) mutants in the upper acidic cluster Glu60Gln and Glu60Lys, at 283.2 K; and (f) mutant between the upper acidic cluster and the hydrophobic patch Gln88Lys, at 293 K. The black symbols are for 293.2 K, and the white symbols are for 283.2 K. The lines are fittings to eq 13. For easy comparison, the fitting for the recombinant wild-type spinach cupriplastocyaninin at 293.2 K is shown, as a dotted line, also in other frames.

solution viscosity ($\eta \approx \sigma$), both kinds of friction are important; and at high solution viscosity ($\eta > \sigma$), solvent friction dominates. In this last case, the rate constant becomes inversely proportional to the solvent viscosity. This regime is called the overdamped Kramers limit (*92*). Viscosity in our experiments spans all three regimes. The results of fittings to eq 13, with two variables, are shown in Table 3; $\Delta G^{\ddagger}$ is assumed to be independent of viscosity. The activation parameter $\Delta G^{\ddagger}$ for the reaction in eq 1 varies from 40 to 49 kJ/mol, and its values obtained from eqs 10 and 13 are equal. When the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, obtained from the dependence of k_F on temperature, are used in eq 14, then dependence of k_F on viscosity can be fitted with a single parameter, σ. Values of σ obtained by the one-variable fittings to eq 14 are approximately the same as those obtained by the two-variable fittings to eq 13.

Most of the mutants resemble the wild-type plastocyanin in the value of σ for the complex ^{3}Zncyt/pc(II). Mutations in the upper acidic cluster, however, tend to raise this friction;

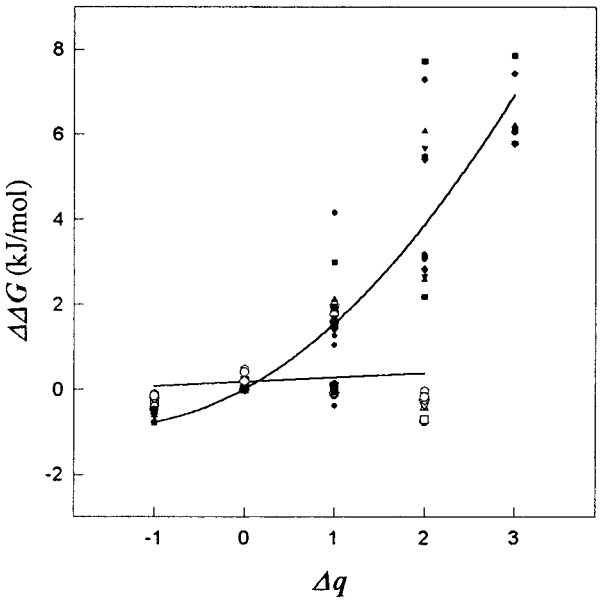

FIGURE 4: Interaction energy $\Delta\Delta G$ calculated by eq 15, for the reaction in eq 1, involving wild-type spinach cupriplastocyanin and the mutants in positions 12, 42, and 43 (white symbols) and in positions 59, 60, and 88 (black symbols) at the following seven temperatures: 273.3, 278.9, 283.7, 288.5, 293.2, 298.2, and 302.9 K. Differently shaped symbols represent data at different temperatures. The quantity Δq, given in Table 1, is the difference in total charge between the mutant and the wild-type plastocyanin. Many data points unavoidably overlap. The straight line is a first-order regression fit for the wild-type protein and the mutants in positions 12, 42, and 43. The curve is a second-order regression fit for the wild-type protein and the mutants in positions 59, 60, and 88.

see the values for Glu59Lys, Glu59Lys/Glu60Gln, Glu60Lys, and Glu60Gln in Table 3. The lysine residue introduced at those positions may enhance the protein friction by its electrostatic, steric, or both properties. Effects of both temperature and viscosity on k_F show that positions 59 and 60 have different properties and behave differently with respect to the interaction with cytochrome *c*. In all cases where viscosity dependencies of k_F are known at two temperatures for a particular mutant, protein friction is lower at the higher temperature. This generalization is consistent with our previous work (*66*) and with the notion that the diprotein complex is more dynamic at higher temperature.

Consideration of "Interaction Energies" $\Delta\Delta G$. After having considered the effect of plastocyanin charge on the rate constant k_F, we now revisit this issue by analyzing so-called interaction energy (*53*, *82*), a difference in the ΔG values for the reaction in eq 1 involving plastocyanin mutants (k_F^{mut}) and the wild-type protein (k_F^{wt}). The interaction energy is defined in eq 15. Figure 4 shows that mutations

$$\Delta\Delta G = RT \ln \frac{k_F^{wt}}{k_F^{mut}} \qquad (15)$$

in positions 12, 42, and 43 do not noticeably affect the energetics of the reaction, while mutations in positions 59, 60, and 88 do. The hydrophobic patch and the lower acidic cluster are probably not involved in the rearrangement, whereas the upper cluster and residue 88 are. When the charge in the latter region becomes more positive, k_F decreases, presumably because the rearrangement involves

movement of the positively charged patch of cytochrome *c* over or toward this region on the plastocyanin surface.

Interpretations of the Activation Parameters. As Figure 2 shows, the unimolecular rate constant k_F increases as the temperature increases. Fittings of this dependence to Eyring equation (eq 9) yielded the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Fitting of subsets of data points from smaller temperature intervals gave somewhat different activation parameters; thus, it is important that experiments span as wide a range of temperature as possible. From enthalpy and entropy of activation, the free energy of activation, $\Delta G^{\ddagger}$, at any temperature may be calculated with eq 10. Its values at 283.2 and 293.2 K are given in Table 3, together with the values of $\Delta G^{\ddagger}$ obtained from the viscosity dependence of k_F. The values $\Delta H^{\ddagger} = 13$ kJ/mol and $\Delta S^{\ddagger} = -97$ J/mol K for the reaction in eq 1 between zinc cytochrome *c* and wild-type French-bean plastocyanin were successfully explained in our earlier study (*65*). The enthalpy change was quantitatively reproduced by calculations concerning the change in the character of the exposed surfaces in the rate-determining rearrangement (configurational fluctuation) of the diprotein complex ^{3}Zncyt/pc(II). The negative entropy change was attributed to the closer approach of the two protein molecules as the complex tightens for the fast electron-transfer reaction to occur. A decrease in the rate constant for the rearrangement with the increasing viscosity was attributed to the change in $\Delta H^{\ddagger}$ (*65*), and it was shown that $\Delta S^{\ddagger}$ decreases (becomes more negative) as the viscosity increases (*66*). There is a compensation between enthalpy of activation and entropy of activation upon the change in viscosity.

Table 2 shows a marked effect of mutations in plastocyanin upon the activation parameters. Mutants that have lysine in position 59 or 88 have a particularly low rate constant k_F (Figure 2) because their $\Delta H^{\ddagger}$ values are more positive, even though their $\Delta S^{\ddagger}$ values are less negative, than those values for the other mutants. The positively charged and bulky lysine residue in the pathway of rearrangement will impede the motion of the positively charged zinc cytochrome *c* (the effect on $\Delta H^{\ddagger}$) and hinder its close approach to plastocyanin (the effect on $\Delta S^{\ddagger}$). The case of Asp42Lys, where $\Delta H^{\ddagger}$ is slightly lowered, shows that the residue 42 is not involved in the pathway of rearrangement; conversion of a negative to a positive charge seems to facilitate the rearrangement. The mutations in the position 12 do not significantly affect the activation parameters. All of these findings are consistent with the notion that the upper acidic cluster and part of the area between this cluster and the hydrophobic patch are favorable for electron transfer. Mutations such as Gln88Glu and Asp42Lys facilitate the movement of Zncyt from the initial docking site, at the center of the acidic patch, toward the hydrophobic patch and thus lower the enthalpy of activation. As Table 2 shows, the same mutations in positions 59 and 60 have different effects on the rearrangement of the diprotein complex. Whereas the residue 59 lies at the interface between cytochrome *c* and plastocyanin, the residue 60 lies on the edge of this interface in all three configurations of the diprotein complex that are examined here. Since $\Delta H^{\ddagger}$ does not increase in the mutant Glu60Lys, we conclude that residue 60 does not lie in the pathway of the rearrangement. In the max ov configuration, Glu59 interacts with lysines 13, 25, and 27 in cytochrome *c*, and Glu60 interacts with lysines 25 and 27. In the n/eq configuration, Glu59 interacts with lysines 5, 7, 8, 13, 86, and 87 and Arg59, and Glu60 interacts with lysines 5, 7, 8, and 13 (*41*). Clearly, the anionic residues 59 and 60 in plastocyanin differ in their interactions with the cationic residues in cytochrome *c*. The main conclusion from the effects of mutation on the activation parameters is that the residues 59 and 88 lie in the pathway of rearrangement, while the residue 60 does not.

Isokinetic Correlations. The linear dependence of the activation parameters expressed in eq 16 is called isokinetic correlation (*39*). This dependence has seldom been sought

$$\Delta H^{\ddagger} = \beta \Delta S^{\ddagger} + C \qquad (16)$$

in studies of electron-transfer reactions between metalloproteins (*39*, *93*−*97*), but it can reveal important features of the reaction mechanism. Fourteen pairs of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values, for the reaction in eq 1 involving the wild-type and all 13 mutants of spinach plastocyanin, fall on the straight line, within the margins of error. The high correlation ($R_c^2 = 0.95$) of this dependence, shown in Figure 5a, is evidence that the reaction in eq 1 has the same mechanism for all the mutants of spinach plastocyanin. Moreover, the activation parameters for this reaction of the wild-type French-bean plastocyanin at eight values of the solution viscosity (*65*) define eight additional points that also fall on the same line; see Figure 5b. These findings justify our analyses in the preceding subsections, in which the constancy of this mechanism between the spinach and French-bean proteins and among mutants of the spinach protein was tacitly assumed. Now we find a compensation between enthalpy and entropy of activation both upon mutation and upon changing the viscosity of the solution. Fitting to eq 16 of the combined data from this and our previous study, shown in Figure 5b, gives a large correlation coefficient ($R_c^2 = 0.94$) and the compensation temperature $\beta = 342 \pm 20$ K.

Activation parameters for the unimolecular electron transfer at pH 5.5 and 7.5 from spinach cuproplastocyanin to the oxidized photosystem I within the complex of these two (associated) proteins (*97*) define the points that still fall on the same extended, linear plot in Figure 5b, which now covers wide intervals of $\Delta H^{\ddagger}$ (ca. 40 kJ/mol) and $\Delta S^{\ddagger}$ (ca. 140 J/mol K). The coefficient R_c^2 and β remain unchanged, 0.94 and 321 ± 20 K.

The isokinetic correlation, which holds well for the aforementioned unimolecular reactions, does not apply to the redox reaction between ferrocytochrome *c* and cuprilastocyanin. Activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were reported in studies in which this reaction was induced by mixing (*39*) and by intramolecular reduction of ferricytochrome *c* by a ruthenium(II) complex attached to the surface of this protein (*98*). The former reaction, between the native proteins, is certainly bimolecular. The latter reaction involves chemically modified cytochrome *c* and native plastocyanin; the deviation from the isokinetic correlation established for the unimolecular reactions raises the question of the molecularity of this reaction.

The discussion in this subsection shows that kinetic effects of temperature are worth studying because the activation parameters so determined reveal important aspects of the mechanism of the redox reactions between metalloproteins.

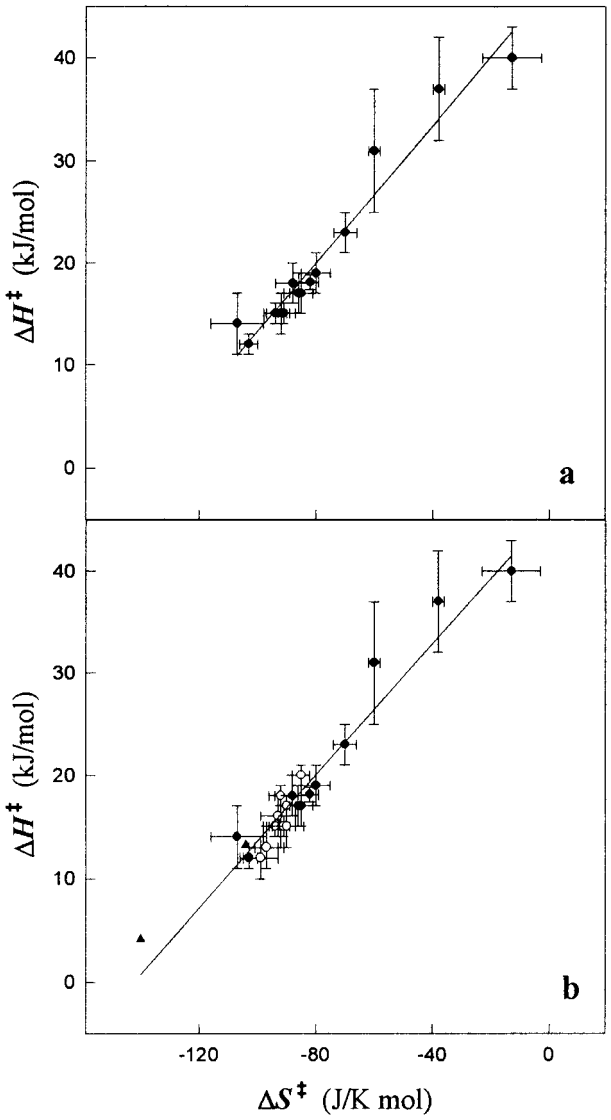

FIGURE 5: Isokinetic relationships between the activation enthalpy and activation entropy for the unimolecular oxidoreduction reactions within metalloprotein complexes. (a) The reaction in eq 1 involving wild-type spinach cupriplastocyanin and its mutants, in sodium phosphate buffer at pH 7.0 and ionic strength 2.5 mM. (b) The data from part a, shown as black circles, plus the following two sets of data: the reaction in eq 1 involving wild-type French-bean plastocyanin at eight values of solution viscosity, in sodium phosphate buffer at pH 7.0 and ionic strength 2.5 mM, shown as white circles (from ref *65*), and the reaction between wild-type spinach cuproplastocyanin and the oxidized form of photosystem I, shown as black triangles (from ref *97*). The lines are fittings to eq 16.

Fittings to Marcus Theory. The thermodynamic driving force for electron-transfer reactions of metalloproteins is usually equated to the difference between the reduction potentials of the acceptor and the donor. The correction for the work needed to bring the reactants together is often neglected even in bimolecular reactions. In this study, since the reaction in eq 1 is unimolecular, this last approximation is justified. We calculate the thermodynamic driving force for the oxidoreduction reaction in eq 1 as the negative value of the sum of the potentials for oxidation of ^{3}Zncyt to Zncyt$^+$ (0.88 V) and for the reduction of pc(II) to pc(I). Reduction potentials of some of the mutants are known (*47*, *49*, *53*) and listed in Table 1. For other mutants, the wild-type value (0.381 V) is used. The perturbation of potentials upon association between cytochrome *c* and plastocyanin is found to be minimal (*22*). The temperature dependence of the driving force when the work terms are negligible amounts to the temperature dependence of the potentials for the two half-reactions. The change of the driving force with temperature is calculated from the thermodynamic parameters ($\Delta H°$ and $\Delta S°$) for the reduction potentials of cytochrome *c* and plastocyanin (*99*); this change is very small in the temperature range studied, no more than ± 2 kJ/mol. Because redox potentials of these (and other) metalloproteins depend only slightly on temperature, we can assume that so does the potential of ^{3}Zncyt. Because reduction potentials of the cupriplastocyanin mutants are very similar and because they very likely resemble the wild-type protein in that their reduction potentials depend only slightly on temperature (*99*), the calculated driving force, $\Delta G°$ in Table 1, is practically constant over the temperature interval covered. In principle, the driving force for the reaction in eq 1 may depend on the difference of the binding constants for the two complexes (the reactant and the product). We studied, both experimentally and theoretically, the association of ^{3}Zncyt with the cupriplastocyanin mutants. The experimentally determined K_a and ΔG_a values are given in Tables 1 and S3. The calculated Coulombic energies for the Zncyt/pc(II) complexes containing each of the mutants are given in Table 4. These energies are correlated with, but are much more negative than, the ΔG_a values in Table S3 because the calculations did not allow for the effects of ionic strength. We estimated theoretically the difference in binding affinities for the ^{3}Zncyt/pc(II) and Zncyt$^+$/pc(I) complexes by calculating the Coulombic interactions, *E* in eq 7, for both of these complexes, containing each of the mutants. The difference ΔE varies from 14 to 17 kJ/mol for different mutants. As approximate as these calculations are, they nevertheless suggest that the difference in binding affinities between the reactant and the product in eq 1 is relatively small and almost independent of the plastocyanin mutant involved. Because calculated driving forces $\Delta G°$ are large, ca. −120 kJ/mol, their small variations among the mutants would be unlikely to cause the marked changes in k_F discussed below even if this rate constant were that of a true electron-transfer reaction.

Virtual independence of the driving force of temperature makes possible the fittings of the temperature dependence of k_F to eqs 11 and 12 with the same value for the driving

Table 4. Total Coulombic Energies Calculated with Eq 7 for Three Configurations[a] of Diprotein Complexes

	E (kJ/mol)		
pc(II)	max ov	n/eq	max ov rot
wild-type	−78.4	−73.0	−76.3
Leu12Asn	−76.4	−70.3	−74.2
Leu12Glu	−85.5	−83.6	−83.7
Leu12Lys	−69.1	−59.0	−66.6
Asp42Asn	−67.8	−62.5	−65.8
Asp42Lys	−56.1	−51.9	−54.4
Glu59Gln	−66.1	−61.7	−64.4
Glu59Lys	−53.3	−49.5	−51.7
Glu59Lys/Glu60Gln	−42.9	−40.6	−42.0
Glu60Gln	−66.9	−63.1	−65.3
Glu60Lys	−52.8	−49.5	−51.5
Gln88Glu	−88.5	−84.3	−86.4
Gln88Lys	−65.9	−59.3	−63.8

[a] Described in refs *41*, *25*, *65*, and *66*.

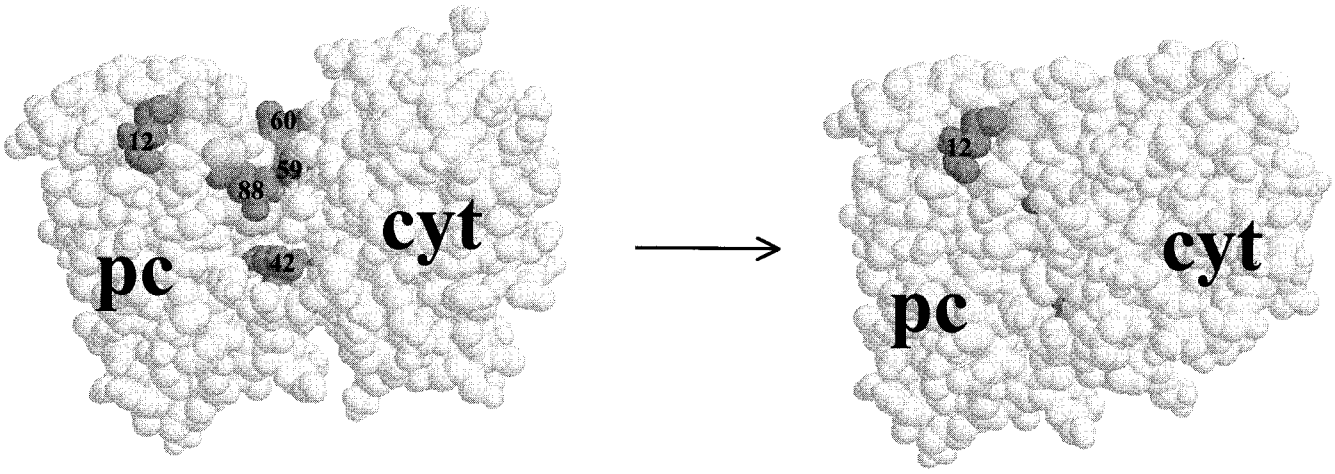

FIGURE 6: The model for the rearrangement of the diprotein complex ^{3}Zncyt/pc(II) from the max ov to the n/eq configuration, which limits the rate of the gated electron-transfer reaction in eq 1. This figure was generated with the program RasMol.

force at all temperatures (*1*). The results are given in Supporting Information, Table S2, and discussed in the remainder of this subsection.

The reorganizational energy (λ) and donor−acceptor coupling (H_{AB}) for wild-type spinach plastocyanin are 277 ± 4 kJ/mol and 6×10^{-23} J, and for French-bean plastocyanin they are 250 kJ/mol and 3×10^{-23} J (*66*). The agreement proves once again that the protein variants from different green plants react similarly and are comparable. The values of λ obtained from fittings to eq 11 of the kinetic results with mutants at the ionic strength of 2.5 mM fall in the range 245−380 kJ/mol, or 2.5−3.9 eV. The reorganizational energies (λ) for electron-transfer reactions within single metalloproteins labeled with ruthenium complexes are 1.2, 1.3, and 0.9 eV, respectively, for cytochrome *c*, myoglobin, and azurin (*100*). The reorganizational energies for reactions involving pairs of metalloproteins are 0.8, 0.9, 1.4, 1.9, and 2.3 eV, respectively, for the following pairs: cytochrome *c* and cytochrome b_5 (*101*), hemoglobin and cytochrome b_5 (*102*), cytochrome *c* and cytochrome *c* peroxidase (*17*), methanol dehydrogenase and cytochrome c_{551i} (*103*), and methylamine dehydrogenase and amicyanin (*104*). The reorganizational energy is 2.1 eV for hemoglobins partially reconstituted with zinc(II) ions (*105*, *106*).

The values of H_{AB} obtained from fittings to eq 11 of the kinetic results with mutants at the ionic strength of 2.5 mM fall in the range 2×10^{-23}−400×10^{-23} J or 1−200 cm^{-1}. This value is 11.6 cm^{-1} for methylamine dehydrogenase and amicyanin (*104*). Although the electronic coupling between the heme and the copper site may be affected by surface mutations, any correlation of the H_{AB} values with properties of the mutants would be unwarranted because the relevant interactions inside the protein molecules are unknowable at present.

The values of λ and *r* obtained from fittings to eq 12, using $\beta = 1.4$ Å^{-1}, of the kinetic results with mutants at the ionic strength of 2.5 mM, are 238−374 kJ/mol and 3.7−11.9 Å. The values of *r* obtained from fittings to eq 12, with the values of λ from the fittings to eq 11, fall in the interval 3.4−11.2 Å. The values of *r* obtained from fittings to eq 12 using $\beta = 1.1$ Å^{-1} fall in the interval 4−14 Å. Evidently, the choice between two reasonable values of β (*100*) little affects the results of fitting. The exceptionally high λ values, some high H_{AB} values, and some *r* values lower than those expected from the structure of the complex, taken together, indicate that the reaction in eq 1 is not a true electron-transfer reaction.

Free Energies of Association. The association constant of the diprotein complex (K_a) depends on ionic strength but not on temperature (*65*) and glycerol concentration (*66*). The K_a values at the ionic strength of 2.5 mM are listed in Table 1. Free energies of association (ΔG_a) calculated from these association constants are shown in Supporting Information, Table S3 and Figure S1c,d. There are clear correlations of ΔG_a with overall charge and with the magnitude of the dipole moment; in both cases $R_c^2 = 0.86$. Although Coulombic interactions are not the only ones responsible for protein association (*26*, *107*), these interactions correlate well with the relative stabilities of the complexes that zinc cytochrome *c* forms with different variants of plastocyanin.

Coulombic Energies of Three Configurations of the Complex Zncyt/pc(II). The configurational fluctuation involved in the reaction in eq 1 amounts to the rearrangement from the configuration max ov to the configuration resembling n/eq or max ov rot (*25*, *54*, *65*). These designations are adopted from the study by Roberts et al. (*41*). Now we test whether this model, proposed for the complex Zncyt/pc(II) containing wild-type plastocyanin, applies also to the complexes containing mutants. Electrostatic interactions between the two proteins can be expressed in terms of their dipole moments (*83*−*87*). Total Coulombic energies and contributions to them from different interactions in the configurations max ov, n/eq, and max ov rot are calculated with eqs 3−7 and listed in Table 4. The angles θ_1, θ_2, and ϕ are listed in Supporting Information, Table S7. The total Coulombic energies (*E*), calculated by this simple method, reproduce the main results of the intricate computations by Roberts et al. (*41*). The following two generalizations hold for all the mutants studied: the most stable configuration of the complex with ferrocytochrome *c* is max ov, and the three configurations of the complex have similar Coulombic energies. Our simple analysis gives useful qualitative results, although it neglects several non-Coulombic contributions to the stability of protein complexes, interactions that proved important in a quantitative investigation of association between ferrocytochrome *f* and plastocyanin (*26*). As

Figures S4 and S5 in the Supporting Information show, rotation around the axis that connects the centers of mass of the protein (change in ϕ) affects the total Coulombic energy very slightly, by 0−4 kJ/mol; variations in the angles θ_1 and θ_2 alter the total energy more. Still, in the extreme, the protein complex containing the double mutant Glu59Lys/Glu60Gln in different configurations lies no more then 5 kJ/mol above the minimum in energy.

These simple electrostatic calculations give two main results. First, the rearrangement of the complex Zncyt/pc(II) involved in the redox reaction in eq 1 was aptly described as configurational fluctuations (*54*, *65*); indeed, the diffusion of the two proteins on each other's surfaces are accompanied by very small changes in energy. Second, the model of the rearrangement that was proposed in the study with wild-type plastocyanin applies also to the numerous mutants examined here. This second conclusion justifies the preceding discussion in this article.

Interplay Between the Rearrangement and Electron Transfer. We accept classification of oxidoreduction reactions between metalloproteins proposed by Brunschwig and Sutin (*2*) and refined by Davidson (*10*). There are three types of reaction. One is true electron transfer. When this step is combined with a fast but unfavorable interconversion, an equilibrium process, the overall reaction is termed "coupled". When the electron-transfer step is combined with a slower, and thus rate-limiting, structural rearrangement, the overall reaction is termed "gated". Although various structural processes and the electron-transfer step can variously affect one another, a more detailed classification would not be justified by the present understanding of metalloprotein oxidoreduction reactions.

Various evidence indicates that the process in eq 1 is not a simple electron-transfer reaction. The rate constant k_F varies greatly with solution viscosity. The viscosity effects show that the process in eq 1 includes a structural rearrangement of the diprotein complex. In the case of so-called coupled electron transfer (*10*) the observed rate constant would be a product of an equilibrium constant and an electron-transfer rate constant. Because both of these microscopic constants are expected to be independent of viscosity (*89*, *108*), evidence in Figure 3 argues against "coupling". Table 1 shows that every mutation to lysine in plastocyanin lowers its association constant with zinc cytochrome *c*, but Figure 2 shows that not every mutation to lysine lowers the rate of rearrangement of the resulting diprotein complex. The observed rate constant does not include the microscopic equilibrium constant that is expected to be affected by mutations; this is further evidence against "coupling". In the current classification, mentioned above, the only remaining possibility is that the reaction is gated. Fittings to the Marcus equations gave the parameters λ, H_{AB}, and r that, as a set, deviate from the expectations based on the precedents for true electron-transfer reactions.

We conclude that the reaction in eq 1, irrespective of the plastocyanin mutant used, is gated by a rearrangement of the diprotein complex, a step slower than the electron-transfer step. A model of this rearrangement in Figure 6 is based on the effects and noneffects of the numerous mutations in plastocyanin on the rate constant k_F. This rearrangement amounts to configurational fluctuation of the two associated protein molecules.

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SUPPORTING INFORMATION AVAILABLE

Seven tables, showing k_F at different temperatures, Marcus parameters, free energy of association, correlation coefficients for the plots of k_F and ΔG_a vs charges and vs dipole moments, angles within plastocyanin mutants, correlation coefficients for plots of k_F vs angles, and angles within the diprotein complexes; five figures, one showing regressions for the plots of k_F and ΔG_a vs charges and vs dipole moments, two showing regressions for the plots of k_F vs angles, and two showing changes in E upon rotations θ_1, θ_2, and ϕ; an appendix, outlining calculations with a geometrical model of rearrangement (14 pages). Ordering information is given on any current masthead page.

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