Protein Docking and Gated Electron-Transfer Reactions between Zinc Cytochrome *c* and the New Plastocyanin from the Fern *Dryopteris crassirhizoma*. Direct Kinetic Evidence for Multiple Binary Complexes

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Abstract: A new plastocyanin from the fern *Dryopteris crassirhizoma* markedly differs from other plastocyanins in having a very large acidic surface, which extends into the area that is hydrophobic in other plastocyanins. The exceptionally large dipole moment of 439 D has a completely different orientation and protrudes through the "northwest" region of the surface, which is now acidic. Consequently, the new plastocyanin differs from its congeners in the photoinduced reaction with zinc cytochrome *c*: ^{3}Zncyt + pc(II) → Zncyt$^+$ + pc(I). At ionic strength ≤20 mM and solution viscosity ≤1.8 cp, at least three exponentials are needed to describe the oxidative quenching of ^{3}Zncyt. Besides a bimolecular phase, there are two distinct unimolecular phases corresponding to electron transfer within two different persistent complexes ^{3}Zncyt/pc(II). So-called normal and reverse titrations yield consistent values of the unimolecular rate constants: k_1 is $(3.3 \pm 0.7) \times 10^5$ s^{-1} and $(3.2 \pm 0.4) \times 10^5$ s^{-1}, and k_2 is $(7.6 \pm 0.8) \times 10^3$ s^{-1} and $(8.2 \pm 1.2) \times 10^3$ s^{-1}. The respective $\Delta H^{\ddagger}$ values also differ (16 ± 2 and 27 ± 7 kJ/mol), but $\Delta S^{\ddagger}$ values are the same (-88 ± 7 and -78 ± 23 J/K mol). Viscosity effects and also unrealistic reorganizational energies obtained in fittings of temperature effects to Marcus theory reveal that both unimolecular electron-transfer reactions (k_1 and k_2) are gated by structural rearrangement of the respective binary complexes. Additional evidence for multiple persistent binary complexes is dependence on ionic strength of the apparent rate constant k_{app} for electron transfer in the transient binary complex ^{3}Zncyt/pc(II). Analysis of this dependence indicates that rearrangement of the protein complexes involves relatively large migration of zinc cytochrome *c*, which is facilitated at higher ionic strength. When zinc cytochrome *c* is present in excess, a transient, but not persistent, ternary complex Zncyt/pc/Zncyt is formed; both reverse titration and analysis of the effects of protein association on the ^{1}H NMR chemical shifts support this conclusion. Existence of a ternary complex is consistent with the existence of multiple binary complexes. Monte Carlo simulations show possible docking configurations of the binary Zncyt/pc complexes. These theoretical calculations, in conjunction with our kinetic data, suggest that the faster (k_1) and slower (k_2) intracomplex reactions seem to occur when ^{3}Zncyt docks, respectively, in the "northeast" and "northwest" surface regions of fern plastocyanin (in the conventional orientation). The new type of docking, on the "northwest" side of the plastocyanin surface, is favored by new acidic residues in this region.

Introduction

Electron transfer between metalloproteins is a key step in many biological processes, and it is important to understand the molecular mechanisms of these reactions. The heme protein cytochrome *c*[1−3] and the blue copper protein plastocyanin,[4−6] although not physiological partners, make an excellent model system for studies of electron transfer. At low ionic strengths, the two proteins associate because of the complementary charges on their surfaces. Their association has been examined by various methods.[7−13] Several model configurations for the complex have been created from the crystal structures of separate proteins,[14] and electron-tunneling paths between iron(II) and

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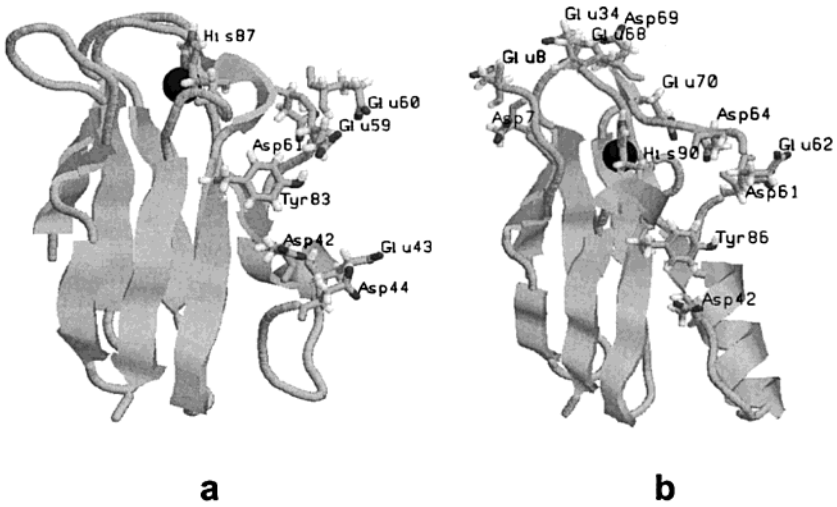

Figure 1. Schematic representations of the structures of cupriplastocyanin from (a) poplar, a high plant, and (b) *D. crassirhizoma*, a fern.[31,88] The residues Tyr83 and Tyr86 on the "east" side, His87 and His90 on the "north" side, and the negatively charged residues forming the acidic patch are highlighted. In high-plant plastocyanins (a), the "north" side is hydrophobic, and the "east" side is acidic. In the fern plastocyanin (b), the "north" surface is incorporated into the much-extended acidic patch.

copper(II) sites in these configurations have been analyzed in our laboratory by the *Pathways* method.[15] The dynamic properties of this diprotein system allow study of the interplay between the structural rearrangement and electron transfer,[11−13,16−18] a phenomenon of intense current interest.[19−22]

Plastocyanin has two surface regions for recognition of redox partners: a hydrophobic patch adjacent to the copper site, which contains the ligand His87, and an acidic patch remote from the copper site, which consists of two clusters of acidic residues surrounding Tyr83. Despite their different distances from the copper site, these two patches are approximately equally coupled to this redox site.[15,23−25] Cytochrome *c*, which is positively charged, binds to the large acidic patch of plastocyanin, and electron transfer occurs in this general configuration.[7,8,14,26−30] Previous research in our laboratory showed that mobility within the diprotein complex is necessary for efficient electron transfer[16,27] and that the complex with optimal electrostatic interactions is not optimal for electron transfer; motions of the proteins enhance the electronic coupling between their redox sites.[15] In the photoinduced reaction, the electron-transfer step has a high driving force and is gated by a rearrangement of the diprotein complex, a step slower than the electron-transfer step.[16,17] Experiments with mutants of high-plant plastocyanins revealed that in the rearrangement of the complex 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 on the plastocyanin surface.[11,13,18]

Crystal structure of a new plastocyanin from the fern *Dryopteris crassirhizoma* showed great differences in surface properties from the high-plant plastocyanins: the acidic patch is extended toward the copper-bound His residue, and several acidic residues disappear from the remote patch (Figure 1).[31] This unusual charge distribution allows us to study the dynamic aspects of protein−protein recognition and electron transfer. Extension of the acidic patch in fern plastocyanin creates a larger binding surface, which might incorporate multiple binding sites. Moreover, appearance of a new cluster of acidic residues away from Tyr86 may favor new electron-transfer pathways through the protein. The new plastocyanin has the same active site as other plastocyanins but very different electrostatic properties. To test the effects of this structural change on reactivity, we study binding and electron transfer of fern plastocyanin with cytochrome *c*.

Very recent studies with metal complexes as redox probes have concluded that the electron-transfer properties of fern and high-plant plastocyanins are quite similar.[31] In this study, using another protein to probe this unique protein, we come to a different conclusion. Because cytochrome *c* and plastocyanin are not physiological partners, our study is not biological in the narrow sense. We use cytochrome *c* to explore the surface of the new protein and to learn about electron transfer and dynamics of the protein interface.

To eliminate complications arising from thermal reactions and to directly detect the dynamic process of interest, we make the redox step photoinduced. Replacement of heme iron with zinc(II) does not significantly perturb the structure of cytochrome *c* and its interactions with other proteins.[32−37] The

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excited triplet state of the zinc porphyrin, ^{3}Zncyt, is produced by a laser flash. The triplet is a strong reducing agent and is quenched oxidatively by cupriplastocyanin, pc(II) (see eq 1). The resulting cation radical, Zncyt$^+$, returns to the ground state, Zncyt, in the thermal (so-called back) electron-transfer reaction with cuproplastocyanin, pc(I) (see eq 2). Our group has extensively studied both of these reactions using wild-type and various mutants of high-plant plastocyanins.[11−13,17,18,25,26,38−41] Here we report a detailed kinetic study of photoinduced electron transfer between Zn-substituted cytochrome *c* and the unique plastocyanin from fern.

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

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

A pair of redox proteins, even physiological partners, can form multiple complexes in solution.[22,42−47] In principle, these complexes may differ in the electron-transfer reactivity, but in very few cases has this difference been detected experimentally. Hoffman et al. elegantly showed that cytochrome *c* can bind to cytochrome *c* peroxidase at two different sites,[22,48−50] but the electron-transfer rate constants for these two sites could not be determined directly and had to be extracted from the fittings. In another recent report from the same laboratory, two distinct intracomplex processes caused by different protein configurations have been identified in the thermal back-reaction after the photoinduced oxidation of zinc myoglobin by cytochrome b_5.[47] Our study may be the first in which different electron-transfer reactions are detected within the electrostatic complex of the same protein pair and thoroughly characterized.

Materials and Methods

Chemicals. Distilled water was demineralized to a resistivity greater than 17 MΩ·cm. Chromatography resins and gels were purchased from Sigma Chemical Co. Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co; all other chemicals were from Fisher Chemical Co.

Buffers. All buffers were prepared fresh from $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ and had pH of 7.00 ± 0.05. The ionic strengths (μ) higher than 10 mM were set with NaCl.

Temperature. Temperature in the range from 0.1 to 35 °C was kept with a 30-L circulating bath Forma Scientific CH/P 2067. Most experiments (ionic strength dependence, viscosity dependence, and reverse titration) were done at 25.0 ± 0.2 °C.

Viscosity. The kinetic effects of viscosity were studied at the ionic strength of 2.5 mM and with a cupriplastocyanin concentration of 30 μM for detailed study of one of the two unimolecular phases of ^{3}Zncyt quenching. Glycerol was added incrementally to the buffered solution, up to the concentration of 85% w/v. The absolute viscosity (η) of the solutions was interpolated from tables.[51]

Proteins. Horse-heart cytochrome *c* was obtained from Sigma Chemical Co. Iron was removed, and the free-base protein was purified and reconstituted with zinc(II), by a modification of the original procedure.[34,35] The criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$ and also the rate constant for natural decay of the triplet state, $k_d < 110\ s^{-1}$. Plastocyanin from leaves of the fern *D. crassirhizoma* Nakai was isolated by the published method.[31] The criterion of purity was the absorbance ratio $A_{278}/A_{590} < 1.4$. Concentrations were determined on the basis of known absorptivities: $\epsilon_{423} = 243\ mM^{-1}\ cm^{-1}$ for zinc cytochrome *c* and $\epsilon_{590} = 4700\ M^{-1}\ cm^{-1}$ for cupriplastocyanin.[31,35] Apoplastocyanin and cobalt(II) plastocyanin were prepared and purified by standard methods.[52] All proteins were stored in liquid nitrogen. Before each series of kinetic experiments, protein stock solutions were exchanged into working buffer using Centricon-10 ultrafiltration cells obtained from Amicon Co.

Flash Kinetic Spectrophotometry. Laser flash photolysis on the microsecond time scale was performed with a standard apparatus.[39] The triplet state ^{3}Zncyt was created by 0.4-μs pulses from a Phase-R (now Luminex) DL1100 laser containing the dye rhodamine 590. For deaeration, argon was passed first through water and then through the buffer. After the temperature of the buffer was adjusted, protein solutions were added. For the so-called normal titration experiments, the concentration of zinc cytochrome *c* was kept at 10.0 μM, and the concentration of cupriplastocyanin was varied between 2.0 and 40 μM. For the so-called reverse titrations, the concentration of cupriplastocyanin was kept at 20 μM, and the concentration of zinc cytochrome *c* was varied between 2.0 and 80 μM. After each addition of proteins to the cell, the solution was gently deaerated for 15−40 min, longer for the more viscous solutions. Decay of the triplet state, ^{3}Zncyt, was monitored at 460 nm, where the transient absorbance reaches the maximum. The concentration of ^{3}Zncyt depended on the intensity of the laser pulse but was always much lower than the concentrations of cupriplastocyanin, apoplastocyanin, or cobalt(II) plastocyanin. Formation and disappearance of the cation radical, Zncyt$^+$, were monitored at 675 nm, where the difference between the absorbances of the cation and the triplet is greatest. To enhance signals, at least 30 shots were collected and averaged. Experiments on a nanosecond scale were done with the second harmonic (532 nm) of a Q-switched Nd:YAG laser that produced 6-ns pulses.[53]

NMR Spectroscopy. Proton NMR experiments at 25 °C were performed with a Bruker DRX500 spectrometer and analyzed with Bruker Xwinnmr 2.5 software.

Aliquots of a solution that contained 4.3 mM ferrocytochrome *c* and 2.0 mM sodium ascorbate in a sodium phosphate buffer at pH 7.0 and ionic strength of 2.5 mM were added to the NMR tube containing a solution in the same buffer that contained 1.0 mM cuproplastocyanin and 10% D_2O. One-dimensional ^{1}H NMR spectra were recorded for plastocyanin alone and after each addition of cytochrome *c*. The acquisition parameters were: FID size 16 000, 256 transients, and spectral width of 20 ppm. The solvent signal was suppressed by a

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presaturation pulse during the relaxation delay. External reference was the methyl signal of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in the aforementioned buffer. Resonances well resolved throughout the titration were analyzed. Their chemical shifts were determined manually, minding the effects of overlap on the peak shape.

Several of the cuproplastocyanin resonances significantly shifted in the titration were assigned with 2D nuclear Overhauser effect spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) spectra of this protein. In both NOESY and TOCSY experiments, each of 400 acquired FIDs was an average of 80 scans and consisted of 1024 complex points. Mixing times were 125 ms (NOESY) and 60 ms (TOCSY). Solvent resonance was suppressed with the WATERGATE method. In the TOCSY experiment, isotropic mixing was done with the TOWNY sequence.

Treatment of Electrostatic Interactions. The dependence of the bimolecular rate constant on ionic strength was analyzed in terms of net charges (Z) and dipole moments (vectors **P** with magnitudes P) of the protein molecules,[54] as in eq 3. In this equation, k and k_{inf} are, respectively, the

$$\ln k = \ln k_{inf} - [Z_1Z_2 + (Z\mathbf{P})(1 + \kappa R) + (\mathbf{PP})(1 + \kappa R)^2]\frac{e^2}{4\pi\epsilon_0\epsilon k_B TR} f(\kappa) \quad (3)$$

bimolecular rate constants at a given and at infinite ionic strengths; Z_1 and Z_2 are net charges; R_1 and R_2 are the radii of each protein, $R = R_1 + R_2$; the other symbols have the usual meanings.[54] The function of ionic strength is defined in eq 4. The monopole−dipole (eq 5) and dipole−dipole (eq 6) interactions depend on the location of the reactive

$$f(\kappa) = \frac{1 - \exp(-2\kappa R_2)}{2\kappa R_2(1 + \kappa R_1)} \quad (4)$$

$$Z\mathbf{P} = \frac{Z_1P_2\cos\theta^2 + Z_2P_1\cos\theta_1}{eR} \quad (5)$$

$$\mathbf{PP} = \frac{P_1P_2\cos\theta_1\cos\theta_2}{(eR)^2} \quad (6)$$

sites on the protein surfaces with respect to the dipole vectors. The angles θ_1 and θ_2 are defined by the positive end of the dipole vector and the vector from the center of mass to the reactive surface in each protein. For convenience, we use the supplementary angles, θ_1' and θ_2': $\theta_i' = 180° - \theta_i$. The radii of cytochrome *c* and plastocyanin are 18.5 and 15.5 Å.[55] In eqs 3−6, the subscript 1 (Z_1, P_1, R_1, and θ_1) designates zinc cytochrome *c*, the larger protein; the subscript 2 (Z_2, P_2, R_2, and θ_2) designates plastocyanin. In the fittings, Z_1, P_1, R_1, θ_1, Z_2, P_2, and R_2 were constants. The dipole moment of zinc cytochrome *c* is 281 D; its orientation was reported earlier.[16] Because cytochrome *c* undergoes electron transfer via its exposed heme edge, which is located at about 30° with respect to the positive end of the dipole vector,[10] the parameter θ_1 was set at 30°.

The dipole moment of fern cupriplastocyanin was calculated from its atomic coordinates (entry 1KDI in the Protein Data Bank).[31] Hydrogen atoms were added with CHARMM.[56] Partial charges for most atoms were taken from the CHARMM force field,[57] and the usual pK_a values were assumed. Atomic partial charges for the copper site were kindly supplied by Professor E. I. Solomon.[58] Calculations of the dipole moment included partial charges of all atoms.

Fittings of Kinetic Data. The rate constants were obtained from changes of the absorbance at 460 and 675 nm with time. The change at 460 nm corresponds to the disappearance of the triplet ^{3}Zncyt and is a sum of several exponentials (eq 7). The change at 675 nm is caused by the triplet ^{3}Zncyt and by the cation radical Zncyt$^+$ and is described by eqs 8−11.[18] The contribution of the ^{3}Zncyt to the absorbance change at 675 nm is given in eq 9, in which a_t is the instantaneous absorbance after the laser flash. The contribution of the Zncyt$^+$ is analyzed with the simplifying eq 11. Because at low ionic strength the signal at 675 nm from the Zncyt$^+$ is too weak for reliable analysis, fittings to more complex equations are unwarranted. Kinetic data were analyzed with the software SigmaPlot v.4.0, from SPSS Inc. All error margins include two standard deviations and correspond to the confidence limit greater than 95%.

$$\Delta A_{460} = \sum_i a_i \exp(-k_i t) + b \quad (7)$$

$$\Delta A_{675} = \Delta A_{triplet} + \Delta A_{cation} \quad (8)$$

$$\Delta A_{triplet} = a_t[\sum_i f_i \exp(-k_i t)] \quad (9)$$

$$f_i = \frac{a_i}{a_1 + a_2 + a_3} \quad (10)$$

$$\Delta A_{cation} = a_c[\exp(-k_{fall}t) - \exp(-k_{rise}t)] \quad (11)$$

Monte Carlo Calculations. The docking was simulated with ferrocytochrome *c* and cupriplastocyanin because their net charges are relevant to the experimental studies. In the search for the encounter complex(es) the protein molecules were rigid bodies. The center of mass of plastocyanin was placed in the center of two concentric cubes having edges of 100 and 200 Å and of two concentric spheres having radii of 60 and 80 Å. The dielectric constant ϵ was set at 80.0. The Coulombic potential of plastocyanin was mapped on two cubic grids, with spacings of 0.5 Å in the inner cube and of 1.0 Å in the outer. The energy of cytochrome *c* in this field was evaluated by multiplying the atomic charges taken from CHARMM22 with the field values obtained by linear interpolations at the atomic positions. The configurations of the diprotein complex were simulated by an annealing procedure described earlier.[58] The temperature was gradually lowered from 300 to 0 K.

Results

Natural Decay of ^{3}Zncyt. In the absence of a quencher, the decay of this triplet excited state to the ground state is monoexponential. The rate constant, k_d, depends on temperature and viscosity but not on protein concentration.[12] At 25 °C and viscosity of 0.89 cp, the intrinsic rate constant k_d is 90 ± 10 s^{-1}.

Oxidative Quenching of ^{3}Zncyt by Fern Cupriplastocyanin at High and Intermediate Ionic Strength. In the presence of fern cupriplastocyanin, at ionic strengths from 3000 to 30 mM, the decay of the triplet state ^{3}Zncyt became faster but remained monoexponential. The rate constants for disappearance of ^{3}Zncyt and appearance of Zncyt$^+$ are equal within the error bounds, as Figure 2 shows. The pseudo-first-order rate constants are directly proportional to the cupriplastocyanin concentration and depend on ionic strength. The second-order rate constants, obtained from the slopes of the linear plots, are listed in Table 1.

Oxidative Quenching of ^{3}Zncyt by Fern Cupriplastocyanin at Low Ionic Strength. In the presence of fern cupriplastocyanin, as ionic strength is lowered further, the kinetics becomes

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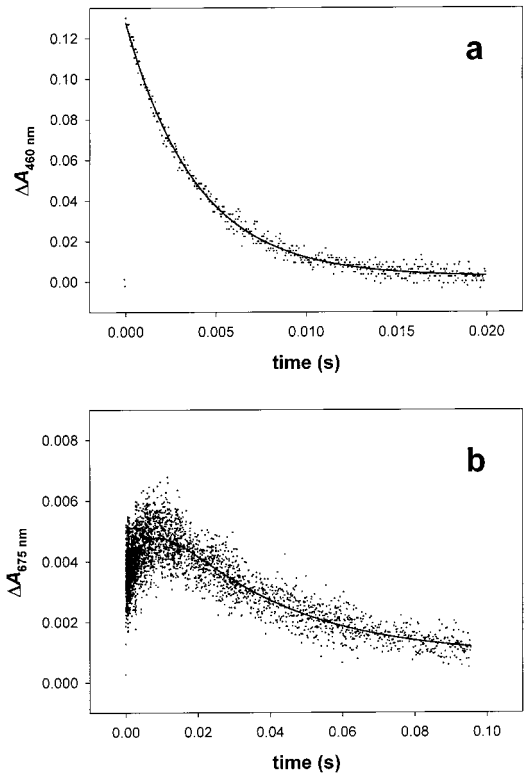

Figure 2. Transient absorbance changes in a solution initially containing 10 μM zinc cytochrome *c* and 15 μM fern cupriplastocyanin in a sodium phosphate buffer at pH 7.0 and ionic strength of 1000 mM at 25 °C. (a) Disappearance of the triplet ^{3}Zncyt, monitored at 460 nm. The line is a single-exponential fit. (b) Appearance and disappearance of the cation radical Zncyt$^+$, monitored at 675 nm. The line is a fit to eqs 8−11.

Table 1. Bimolecular Rate Constants ($k_{bim} \times 10^{-6}$, M^{-1} s^{-1}) for the Reaction of ^{3}Zncyt with Cupriplastocyanin from Different Plants at pH 7.0, 25 °C, and Different Ionic Strengths (μ)

	pc source	
μ, mM	fern	bean[a]
30	770 ± 20	n.d.
40	320 ± 20	700
50	340 ± 10	310
60	150 ± 5	230
80	140 ± 20	150
100	100 ± 5	130
200	18 ± 2	40
500	13 ± 1	14
1000	9.5 ± 0.4	9.4
3000	5.8 ± 0.4	6.7

[a] From ref 16.

more intricate. The ^{3}Zncyt decay is best described with a triexponential function (eq 12), as Figure 3 shows. Many attempts to analyze the absorbance change with time in terms of biexponential functions failed. (See Figure S1 in the Supporting Information.)

$$\Delta A = a_1 \exp(-k_1 t) + a_2 \exp(-k_2 t) + a_3 \exp(-k_{obs} t) + b \quad (12)$$

Rate constants for two of the three phases, $k_1 = (3.3 \pm 0.7) \times 10^5$ s^{-1} and $k_2 = (7.6 \pm 0.8) \times 10^3$ s^{-1}, are independent of the cupriplastocyanin concentration and ionic strength; evidently, they correspond to intracomplex reactions. The relative amplitudes of these phases increase as the quencher concentration increases. (See Figures S2 and S3 in the Supporting Information.) The third rate constant, k_{obs}, depends on both the cupriplastocyanin concentration and ionic strength. (See Figure 4.) The relative amplitude of this phase decreases as the cupriplastocyanin concentration increases and as ionic strength decreases. (See Figures S2 and S3 in the Supporting Information.) At high quencher concentrations, the rate constant k_{obs} shows saturation owing to protein association.

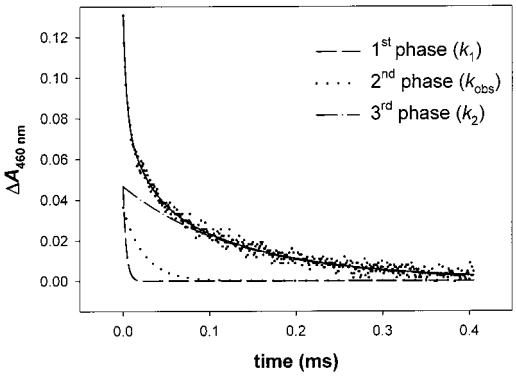

Figure 3. Triexponential quenching of the triplet ^{3}Zncyt by 30 μM fern cupriplastocyanin in a sodium phosphate buffer at pH 7.0 and ionic strength of 2.5 mM at 25 °C. The solid line is a fit to eq 12. The three phases are designated by the corresponding rate constants: k_1, k_2, and k_{obs}.

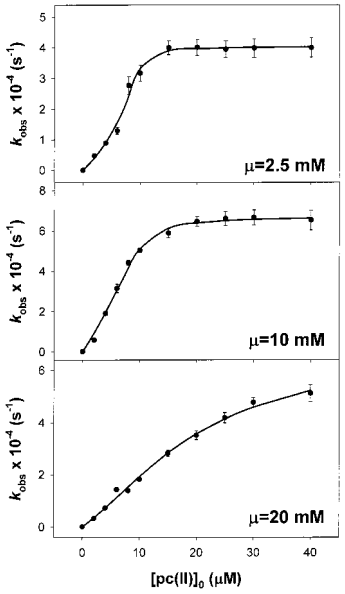

Figure 4. Results of so-called normal titrations: dependence of the rate constant k_{obs} on the concentration of fern cupriplastocyanin at low ionic strength (μ). The solvents were sodium phosphate buffers of indicated ionic strengths at pH 7.0 and 25 °C. The lines are fits to eq 15. Error bars smaller than the dots cannot be seen.

The cation radical Zncyt$^+$, formed as the triplet ^{3}Zncyt is oxidized by cupriplastocyanin, is observed also at low ionic strength. (See Figure 5.) At ionic strength of 2.5 mM, the absorbance grows at the rate of $(7.9 \pm 0.9) \times 10^5$ s^{-1} and declines at the rate of $(1.5 \pm 0.1) \times 10^5$ s^{-1}. The decrease of the absorbance matches the average rate constant, k_{av}, for the decay of the triplet ^{3}Zncyt (eq 13), obtained from the absorbance change at 460 nm. The increase of the absorbance at 675 nm is caused by the back-reaction, and the decrease is caused by the forward reaction.[38]

$$k_{av} = f_1 k_1 + f_2 k_2 + f_3 k_{obs} \quad (13)$$

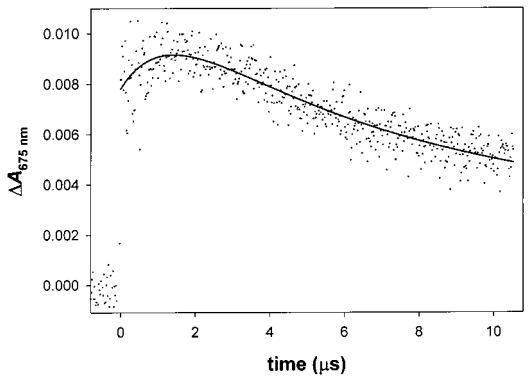

Figure 5. Appearance and disappearance of the cation radical Zncyt$^+$, monitored at 675 nm, in a sodium phosphate buffer at pH 7.0 and ionic strength of 2.5 mM at 25 °C. Initial concentrations of the proteins were 10 μM zinc cytochrome *c* and 40 μM fern cupriplastocyanin. The line is a fit to eqs 8−11.

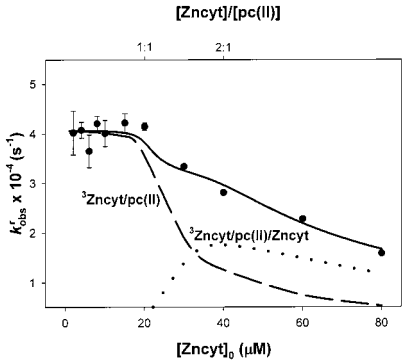

Figure 6. Results of so-called reverse titrations: dependence of the rate constant k^r_{obs} on the concentration of zinc cytochrome *c* in a solution containing 20 μM fern cupriplastocyanin. The solvent is a sodium phosphate buffer at pH 7.0 and ionic strength of 2.5 mM at 25 °C. The dashed line is the theoretical prediction for a 1:1 model of binding with the parameters obtained from the so-called normal titration experiments. The solid line is a fit to a 2:1 model of binding. The dotted line is the difference between the experimental data and the simulated points for a 1:1 model of binding. Error bars smaller than the dots cannot be seen.

In a so-called reverse (superscript r) titration, at the ionic strength of 2.5 mM and 25 °C, zinc cytochrome *c* was added successively to a solution containing a fixed concentration of fern cupriplastocyanin. Again, decay of the triplet ^{3}Zncyt is triexponential. The intracomplex rate constants are $k^r_1 = (3.2 \pm 0.4) \times 10^5$ s^{-1} and $k^r_2 = (8.2 \pm 1.2) \times 10^3$ s^{-1}. The third component, k^r_{obs}, depends on zinc cytochrome *c* concentration and shows saturation. (See Figure 6.)

Nonredox Quenching of ^{3}Zncyt. The rates of the ^{3}Zncyt decay in the presence of copper-free (apo-) and cobalt(II) forms of fern plastocyanin were measured at the ionic strength of 2.5 mM. Apoplastocyanin associates with zinc cytochrome *c*, but does not quench the triplet by either energy transfer or electron transfer. Upon association, however, radiationless decay of ^{3}Zncyt is enhanced by 190 ± 50 s^{-1}. Reduction of cobalt(II) plastocyanin is improbable, but energy transfer is possible. The observed enhancement of the ^{3}Zncyt decay by 250 ± 20 s^{-1} is a combined effect of a radiationless process and energy transfer. The rate constant for dipole−dipole energy transfer is proportional to the overlap between the emission spectrum of the donor, here ^{3}Zncyt, and the absorption spectrum of the quencher.[59] On the basis of the difference between the absorption spectra of the cobalt(II) and copper(II) forms of fern plastocyanin, the estimated rate constant for energy transfer to the latter is ca. 600 s^{-1}.[38]

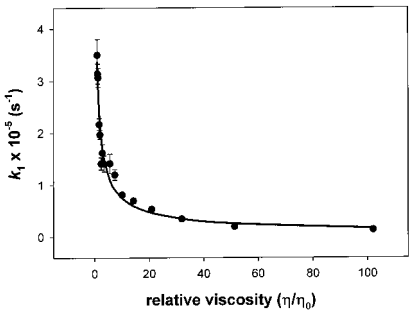

Figure 7. Dependence on solution viscosity of the unimolecular rate constant k_1. The viscosity of a sodium phosphate buffer at pH 7.0 and ionic strength of 2.5 mM at 25 °C was adjusted with glycerol. The solid line is a fit to eq 21. Error bars smaller than the dots cannot be seen.

Table 2. Friction Parameter (δ) and Activation Parameters for the Unimolecular Reaction of ^{3}Zncyt with Cupriplastocyanin from Different Plants at pH 7.0 and Ionic Strength of 2.5 mM, Obtained by Fittings to Two Equations

		eq 21			eq 14	
pc source		T (K)	δ	$\Delta G^‡$ (kJ/mol)	$\Delta H^‡$ (kJ/mol)	$\Delta S^‡$ (J/K mol)
fern[a]	k_1	298	0.66 ± 0.05	41.7 ± 0.2	16 ± 2	−88 ± 7
	k_2				27 ± 7	−78 ± 23
bean		298	0.45 ± 0.07[b]	41.8 ± 0.2[b]	13 ± 2[c]	−97 ± 4[c]
spinach		293	0.8 ± 0.1[d]	43 ± 1[d]	17 ± 2[e]	−85 ± 4[e]

[a] Two unimolecular reactions, k_1 and k_2, are observed with this protein, only one with each of the other proteins. [b] From ref 17. [c] From ref 12. [d] From ref 11. [e] From ref 13.

Kinetic Effects of Viscosity. The reaction in eq 1 was studied at ionic strength of 2.5 mM and 25 °C. The decay of ^{3}Zncyt was triphasic in the range from 0.89 to 1.8 cp, and biphasic at higher viscosities. The amplitude of the fastest phase was unaffected by viscosity. (See Figure S4 in the Supporting Information.) Apparently, the two slower phases converge at higher viscosities. We therefore analyze only the fastest component. The unimolecular rate constant k_1 (for an intracomplex process) smoothly decreases and levels off as the solution viscosity increases. (See Figure 7.)

Effects of Temperature on Unimolecular Rate Constants k_1 and k_2. The dependencies on temperature of the rate constants k_1 and k_2 at ionic strength 2.5 mM are shown in Figure S5 in the Supporting Information. Fitting to Eyring equation (eq 14) gave the activation parameters $\Delta H^‡$ and $\Delta S^‡$, which are listed in Table 2. The amplitudes of the kinetic phases change only slightly with temperature. (See Figure S6 in the Supporting Information.)

$$k = \frac{k_B T}{h} \exp\frac{\Delta S^‡}{R} \exp\frac{-\Delta H^‡}{RT} \qquad (14)$$

Association of Fern Cuproplastocyanin and Ferrocytochrome *c*. To avoid complications in the NMR spectra, titrations were done with the reduced forms of both cytochrome *c* and plastocyanin. Numerous resonances, corresponding to protons in both proteins, were affected by the presence of the other protein, but changes in their chemical shifts were 0.06 ppm or less, varying in magnitude and direction. Two resonances are

(59) Förster, T. *Modern Quantum Chemistry*; Sinanoğlu, O., Ed.; Academic Press: New York, 1965; Part III, p 93.

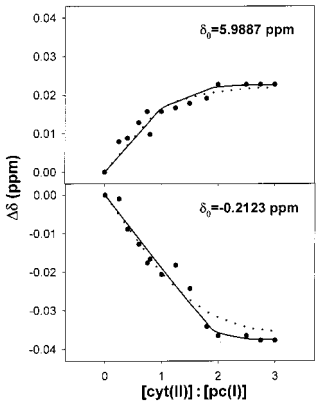

Figure 8. Titration of fern cuproplastocyanin with ferrocytochrome *c*, followed by ^{1}H NMR spectroscopy. Changes in chemical shifts ($\Delta\delta$) for two of the plastocyanin peaks, whose chemical shifts in free cuproplastocyanin are δ_0, are plotted against the mole ratio. The dotted lines are fittings to a 1:1 model of binding. The solid lines are fittings to a 2:1 model of binding.

chosen for Figure 8. Some resonances in cuproplastocyanin broadened upon addition of ferrocytochrome *c*. These observations are consistent with protein association. Absence of resonance splitting suggests that the exchange between the complex and the free proteins is fast on the NMR time scale.

Dipole Moment of Fern Cupriplastocyanin. The calculated magnitude is 439 D. The positive and the negative ends of the vector penetrate the protein surface near the backbone oxygen atom of Thr82 and near the nitrogen atom of Glu68, respectively.

Monte Carlo Simulations of the Docking Configurations. Extensive calculations resulted in 10 000 successful events, docking configurations corresponding to local minima of energy. More than 50% of these configurations fell below the average of the highest and the lowest Coulombic energies. (See Figure 9.) Each dot represents the center of mass of a cytochrome *c* molecule; the heme always points toward fern plastocyanin. Simulations with cytochrome *c* and spinach plastocyanin showed that in most of the electrostatically stabilized diprotein complexes cytochrome *c* is docked at the remote (acidic) patch, in the vicinity of the residue Tyr83.

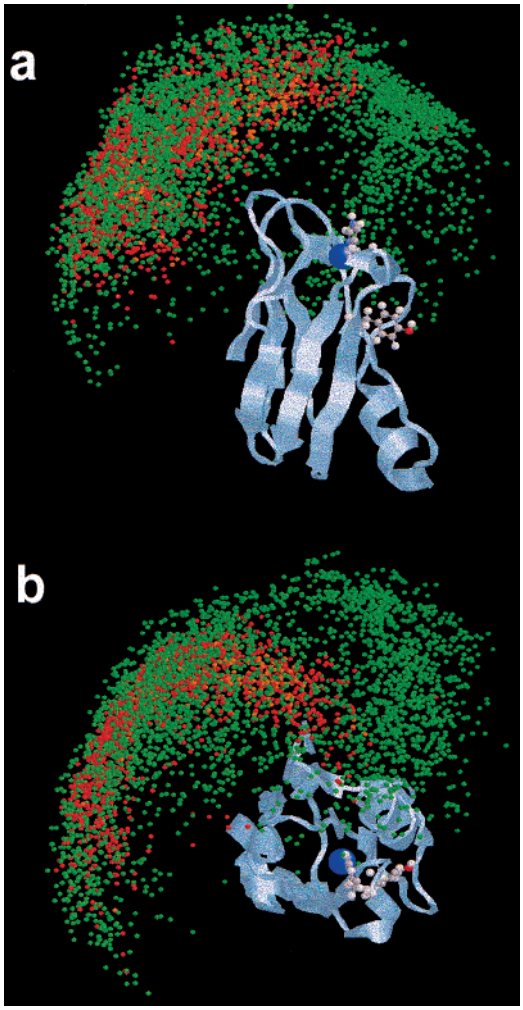

Figure 9. Representations of the 5000 most stable (of the 10 000 found) configurations of the binary complex simulated by the Monte Carlo method. Fern plastocyanin is shown explicitly, with residues Tyr86 and His90 highlighted. Each dot represents the center of mass of cytochrome *c*. The density of dots reflects probability of docking. The particularly stable orientations of cytochrome *c* are represented with red dots. (a) View from the conventional direction and (b) view from the top. The pictures were prepared with the program RasMol, version 2.6.

Discussion

Protein Docking and Electron Transfer. Rate and specificity of electron-transfer reactions depend on association of the reactants and on electron tunneling between the redox sites.[60] In some reactions the rate-limiting step is simply the electron transfer. A pair of proteins, however, can associate in multiple configurations, and an orientation that is optimal for binding might not be optimal for the electron transfer. In such cases, the electron-transfer reaction involves a rearrangement of the complex from the initial, docking configuration to a different, reactive configuration.[16,25,42,45] If this interconversion is fast but thermodynamically unfavorable, the overall electron-transfer reaction is called "coupled"; if the rearrangement is slower than the electron-transfer step, the overall reaction is called "gated".[21]

Both protein docking and dynamic rearrangement affect the electron-transfer reaction, and it is important to understand these interactions. Here we combine kinetic and NMR spectroscopic experiments and theoretical simulations to explore the binding surface and electron-transfer reactivity of fern plastocyanin, a blue copper protein that contains a very large acidic patch on its surface.

Mechanism of ^{3}Zncyt Quenching by Fern Cupriplastocyanin. Previous studies in this laboratory showed that high-plant cupriplastocyanin quenches the triplet state of zinc cytochrome *c* by electron transfer, as in eq 1.[11,38,61] The reaction is biphasic at low ionic strength ($\mu \leq 20$ mM) and monophasic at intermediate and high ionic strength ($\mu \geq 30$ mM). The faster phase is the unimolecular reaction within the persistent diprotein complex, Zncyt/pc(II), which exists before the laser flash. The slower phase at low ionic strength, and the only one at intermediate and high ionic strength, is the bimolecular reaction between unassociated proteins, within the transient (collisional) complex. The persistent and transient complexes have similar rate constants, $(2.5 \pm 0.4) \times 10^5$ s^{-1} and $(2.8 \pm 0.6) \times 10^5$ s^{-1},[38] and activation parameters $\Delta H^{\ddagger}$ (13 ± 2 and 13 ± 1 kJ/mol) and $\Delta S^{\ddagger}$ (-97 ± 4 and -96 ± 3 J/K mol).[12] Evidently, the proteins associate similarly in the persistent and the transient complex and rearrange to the same redox-active configuration.

(60) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265−322.

(61) Zhou, J. S.; Kostić, N. M. *Biochemistry* **1993**, *32*, 4539−4546.

In this study, we did similar kinetic experiments with fern cupriplastocyanin. At $\mu \geq 30$ mM, the results are as before. The reaction with ^{3}Zncyt is bimolecular; the two proteins form only a transient complex. At lower ionic strength, however, the fern protein differs markedly from its high-plant congeners. At least three kinetic phases are necessary to describe the ^{3}Zncyt decay in the presence of fern cupriplastocyanin. Two of the three kinetic phases are unimolecular processes, corresponding to intracomplex reactions. We conclude that at least two different configurations of the persistent complex Zncyt/pc(II) exist in solution before the laser flash. Because both proteins are chromatographically pure and the relative amplitudes of the different phases change with solution conditions, the multiphasic kinetics is not caused by heterogenity of the individual proteins. Multiphasic kinetics is caused by multiple complexes, which differ in reactivity.

Various evidence shows that all three phases of the ^{3}Zncyt decay in the presence of fern cupriplastocyanin are caused by the electron-transfer reaction, as shown in eq 1. Control experiments with apo- and cobalt(II) forms of this plastocyanin show that nonredox modes of quenching are negligible. Moreover, the cation radical Zncyt$^+$ was detected in all three phases of the reaction.

The complete mechanism of the photoinduced reaction is given in Scheme 1. At ionic strength lower than 30 mM, the rate constants k_1 and k_2 are observed directly. The apparent electron-transfer rate constant, k_{app}, and the overall apparent association constant, K_a, are obtained from eq 15,[62] which contains the concentration of cupriplastocyanin that acts as a quencher in the bimolecular reaction. Because this concentration is less than the total concentration (subsript 0), eq 16 is needed. This kinetic treatment does not incorporate microscopic association constants and rate constants for the individual diprotein complexes. Possibly more than two complexes exist in solution and are not distinguished experimentally. Moreover, these forms may interconvert (see below). Addition of more parameters in fittings would not give firm results. We use the simplest kinetic model conservatively to estimate the overall association constants K_a for the diprotein complexes and the "average" electron-transfer rate constant k_{app} within these complexes. These apparent constants are given in Table 3.

$$k_{obs} = \frac{k_{on}k_{app}K_a[pc(II)]}{k_{on} + k_{app}K_a + k_{on}K_a[pc(II)]} \quad (15)$$

$$[pc(II)] = [pc(II)]_0 - \frac{1}{2}\{[Zncyt]_0 + [pc(II)]_0 + K_a^{-1} - \sqrt{([Zncyt]_0 + [pc(II)]_0 + K_a^{-1})^2 - 4[Zncyt]_0[pc(II)]_0}\} \quad (16)$$

At ionic strengths of 30 mM and higher, the quenching of ^{3}Zncyt by fern cupriplastocyanin is a monoexponential process and the dependence of the observed rate constant, k_{obs}, on the cupriplastocyanin concentration does not show saturation. Now only the bimolecular rate constant, k_{bim}, is determined (see eq 17).[63] In this case, the association constant K_a affects the value of k_{bim}, but K_a cannot be determined from kinetics.

$$k_{obs} = K_a k_{app}[pc(II)] = k_{bim}[pc(II)] \quad (17)$$

Scheme 1

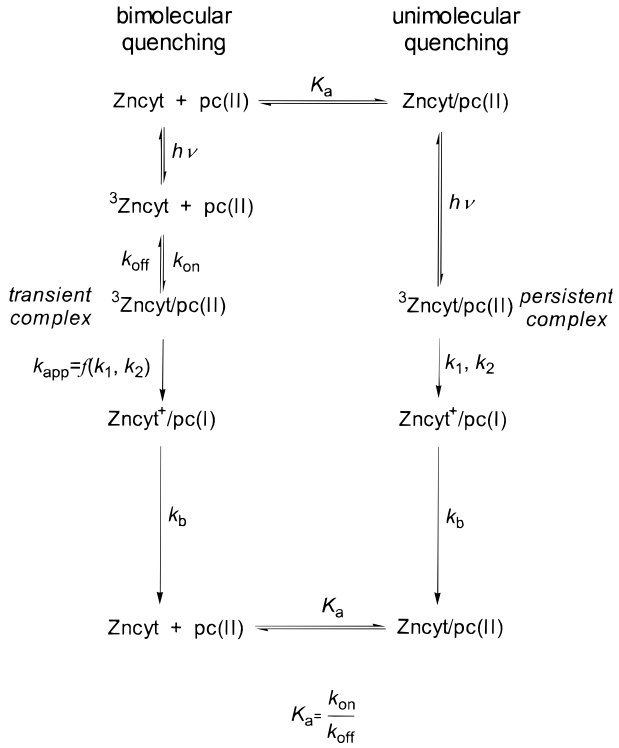

Table 3. Apparent Electron-Transfer Rate Constants,[a] k_{app}, and Apparent Association Constants,[b] K_a, for the Reaction of ^{3}Zncyt with Fern Cupriplastocyanin at pH 7.0 and 25 °C

μ, mM	$k_{app} \times 10^{-4}$, s^{-1}	K_a, M^{-1}
1.25	3.2 ± 0.2	n.d.[c]
2.5	4.1 ± 0.1	$(6.8 \pm 2.7) \times 10^6$
5.0	~5.2	n.d.[c]
10	6.8 ± 0.2	$(1.8 \pm 0.6) \times 10^6$
20	7.7 ± 0.8	$(1.9 \pm 1.0) \times 10^5$

[a] The k_{app} values are obtained from fits to eq 15 and are well determined by the limiting asymptote in Figure 4. [b] $K_a = k_{on}/k_{off}$. [c] Not determined because the data points were few.

Electrostatic Interactions and Protein−Protein Orientations in the Bimolecular Reactions of ^{3}Zncyt with High-Plant and Fern Cupriplastocyanins. Assuming normal pK_a values, zinc cytochrome *c* and fern cupriplastocyanin at pH 7.0 have overall charges of +6 and −6, respectively. The bimolecular rate constant k_{bim} in Table 1 decreases with increasing ionic strength, as expected for the oppositely charged proteins. The bimolecular rate constants are very similar to those reported for the analogous reaction of high-plant cupriplastocyanin, especially at higher ionic strength. (See Table 1.)

Zinc cytochrome *c* and plastocyanin have not only large net charges, but also large dipole moments. Dipolar interactions can strongly influence the dependence on ionic strength of rate constants and association constants for the reactions of macromolecules.[25,26,45,61,64−68] The semiquantitative van Leeuwen theory (eq 3) provides information about the protein−protein orientation in the transient complex.[54] Previous studies

(62) Espenson, J. H., Ed. *Chemical Kinetics and Reaction Mechanisms*, 2nd ed.; McGraw-Hill: New York, 1995.

(63) Equation 17 is obtained from eq 15 under the following condition: $k_{off} > k_{on}[pc(II)] + k_{app}$.

(64) Brothers, H. M., II; Zhou, J. S.; Kostić, N. M. *J. Inorg. Organomet. Polym.* **1993**, *3*, 59−76.

(65) Cheng, J.; Zhou, J. S.; Kostić, N. M. *Inorg. Chem.* **1994**, *33*, 1600−1606.

(66) Davidson, V. L.; Jones, L. H. *Biochemistry* **1995**, *34*, 1238.

(67) Dixon, D. W.; Hong, X.; Woehler, S. E. *Biophys. J.* **1989**, *56*, 339−351.

(68) Sokerina, E. V.; Ullmann, G. M.; van Pouderoyen, G.; Canters, G. W.; Kostić, N. M. *J. Inorg. Biol. Chem.* **1999**, *4*, 111−121.

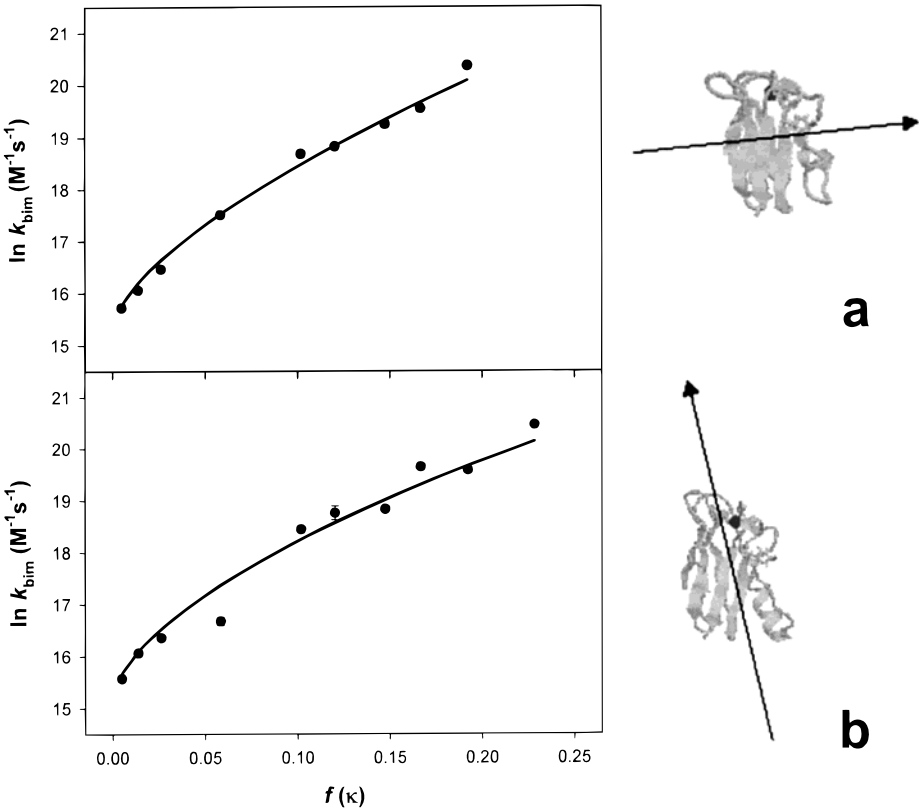

Figure 10. Fits to eq 3 of the bimolecular rate constants in Table 1 for the reaction of ^{3}Zncyt with cupriplastocyanins from (a) bean and (b) fern, both in a sodium phosphate buffer at pH 7.0 and 25 °C. Notice the great elongation and reorientation of the dipole moment in the fern protein, a consequence of the radical change in the distribution of charges (Figure 1). The function $f(\kappa)$ is defined in eq 4. Error bars smaller than the dots cannot be seen.

in our laboratory, based on this electrostatic theory, showed that in the bimolecular reaction french-bean (later in the text, simply bean) plastocyanin uses its remote (acidic) patch for interaction with zinc cytochrome *c*.[61]

Table 1 and Figure 10 allow comparison of bean and fern plastocyanins in the same reaction (shown in eq 1). In fittings to eq 3 two parameters were allowed to vary: the bimolecular rate constant k_{inf} at infinite ionic strength and the angle θ_2' that specifies the position of the reaction site on the plastocyanin surface with respect to this protein's dipole vector. The best fits gave $k_{inf} = (6.4 \pm 1.2) \times 10^5\ M^{-1}\ s^{-1}$ and $\theta_2' = 17 \pm 30°$. Although the θ_2' value defines a band of possible sites equatorial to the dipole vector, rather than a unique site on the plastocyanin surface, this value allows a distinction among relatively distant areas on the surface. The fitted value of θ_2' is consistent with the location of a new acidic patch near His90 and inconsistent with the position of Tyr86. These two residues correspond to His87 and Tyr83 in high-plant plastocyanins.

For comparison, similar fittings for the reaction of bean plastocyanin gave $k_{inf} = (1.3 \pm 0.3) \times 10^6\ M^{-1}\ s^{-1}$ and $\theta_2' = 36 \pm 15°$.[61] The plastocyanins from bean and fern differ in overall charge (−8 vs −6), magnitude of the dipole moment (362 vs 439 D), and, especially important, orientation of the dipole moment (see Figure 10). In both plastocyanins the predicted reaction site sits close to the negative end of the dipole vector. Indeed, in the most stable configuration of the diprotein complex the dipole moments seemed to be oriented head-to-tail.[13] The dipole moment is reoriented from the bean to fern plastocyanin, and therefore the predicted reaction site on the surface changes completely. The binding surface of fern plastocyanin differs from that of other plastocyanins. With the creation of the new cluster of acidic (anionic) residues in the "northwest" region, the dipole vector reorients and protrudes out of this cluster.

Application of van Leeuwen theory to bean plastocyanin correctly implicated the acidic patch in the reaction with zinc cytochrome *c*,[61] but did not reveal the full complexity of this reaction. Other studies suggested that this reaction is gated by dynamic rearrangement of the complex.[16] When the rate of the rearrangement is independent of ionic strength, the reactive configuration cannot be deduced from the analysis of electrostatic interactions. Moreover, van Leeuwen theory is inapplicable to proteins with multiple binding sites. For these reasons, we consider the results of van Leeuwen analyses only as hints to the unique properties of fern plastocyanin. Firmer evidence comes from studies to be discussed next.

Experimental Evidence for Multiple Binding Sites on the Surface of Fern Plastocyanin. Analysis of the kinetic traces for ^{3}Zncyt decay in the presence of fern cupriplastocyanin revealed that there are not one but two (or possibly more) forms of the Zncyt/pc(II) complex. The prime evidence for them, discussed above, is direct observation of two different unimolecular (intracomplex) electron-transfer reactions. Additional experimental evidence leads to the same conclusion.

I. Effects of Ionic Strength. The apparent electron-transfer rate constant k_{app} within the transient Zncyt/pc(II) complex depends on ionic strength, as Table 3 shows. The rate constant for true electron transfer within a unique complex is not expected to depend on ionic strength.[21] If, however, multiple complexes exist, a change in their relative populations with ionic strength will cause a change in the rate constant k_{app}.[49] Others reported similar effects of ionic strength on electron-transfer rates within protein complexes[45,69,70] and attributed them to two causes: coupling between conformational change and electron transfer

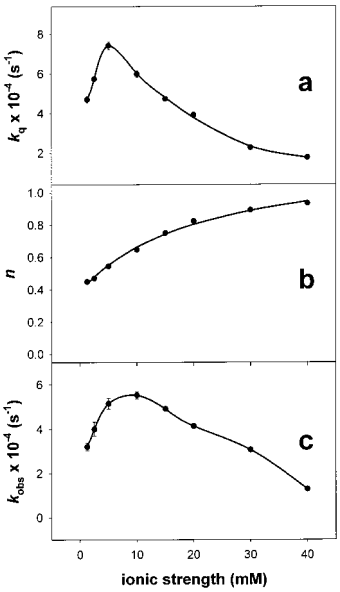

Figure 11. Results of fitting to a stretched-exponential function. Dependence on ionic strength of the parameters (a) k_q and (b) n, both from eq 18, and (c) the pseudo-first-order rate constant k_{obs} from eq 12, for the reaction between ^{3}Zncyt and fern cupriplastocyanin in a sodium phosphate buffer at pH 7.0 and 25 °C. Initial concentrations of the proteins were 10 μM zinc cytochrome *c* and 40 μM fern cupriplastocyanin. The lines simply connect the points. Error bars smaller than the dots cannot be seen.

after the complex formation,[45] or change in the relative binding affinities of two different binding sites.[49] Finding that k_{app} depends on ionic strength supports our conclusion about two (or more) binding sites on the surface of fern plastocyanin. Interestingly, the apparent electron-transfer rate constant k_{app} increases as ionic strength increases, suggesting higher reactivity of fern plastocyanin at higher ionic strength.

Decay of the triplet ^{3}Zncyt can be described not only with multiple exponentials (eq 7), but equally well with a stretched-

$$\Delta A = A_0 \exp\{(-k_d t) - (k_q t)^n\} + b \qquad (18)$$

exponential function.[71] In eq 18, the quenching rate constant k_q is the most probable rate constant within the family of reactive species; k_d is the intrinsic rate constant (in our case, natural decay of ^{3}Zncyt); and n is the distribution parameter. In the limit $n = 1$, all reactive species show the same quenching rate constant, and eq 18 reduces to a monoexponential function. As Figure 11 shows, at low ionic strength $n < 1$; as ionic strength increases, n increases and approaches the limit $n = 1$. We conclude that multiple protein complexes (which can be transient or persistent) exist at low ionic strength. As ionic strength increases, these complexes rapidly interconvert, or all of them convert to the same configuration. Both the rate constant k_q, from fittting to a stretched exponential (Figure 11a), and the rate constant k_{obs}, determined from triexponential fittings (Figure 11c), first increase and then decrease as ionic strength increases further. The drop is easily explained; it is expected for the reaction of two oppositely charged proteins and is caused by a decrease in the association constant, K_a.

The initial increase in the rate constants k_q and k_{obs} is more subtle. Clearly, as ionic strength increases (in this interval), the contribution of the more reactive complex increases. But as ionic strength increases, the relative amplitudes of both unimolecular reactions (k_1 and k_2) decrease; moreover, this decrease is steeper for the faster (k_1) than for the slower (k_2) reaction. (See Figure S3 in the Supporting Information.) This experimental result argues against preferential formation of the more reactive complex from the free proteins as ionic strength is raised; the initial increase in Figures 11a and 11c cannot be caused by the change in the association constant. We take this initial increase as evidence for rearrangement of the diprotein complex that becomes easier as ionic strength increases (but remains low). As ionic strength increases in the range from 0 to 10 mM, interconversions between diprotein complexes become faster. The less reactive complex(es) can rearrange to the more reactive one(s). The most reactive complex can be the faster of the two that we detect (the one corresponding to the rate constant k_1), or some complex that we do not detect.

Dependencies similar to those in Figure 11 have been found in several other systems and taken as evidence that a "tight" or "less reactive" protein complex formed at low ionic strength rearranges into a "flexible" or "more reactive" complex at higher ionic strength.[29,43,70,72−76] The finding that ionic strength affects the rate of rearrangement of the complex of zinc cytochrome *c* and fern plastocyanin is surprising, because ionic strength does not affect the rate of the similar rearrangement involving bean plastocyanin.[38] For this high-plant plastocyanin, the rearrangement involves movement of the basic patch of zinc cytochrome *c* in the vicinity of the initial binding site within the acidic patch; various evidence argues against large migration of zinc cytochrome *c*.[11,13] We suggest that a larger migration occurs in the new system involving fern plastocyanin, namely from a less reactive to a more reactive site on the plastocyanin surface. Surface diffusion would be promoted if the two binding sites shared one or more anionic residues as a "bridge". Migration could also be accomplished by breakup and re-formation of the diprotein complex.

II. Detection of the Transient Ternary Complex. Whether fern plastocyanin has more than one binding site competent for electron transfer could be tested by finding whether electron transfer is possible between the binary complex Zncyt/pc(II) and free zinc cytochrome *c*. If two binding sites on the cupriplastocyanin surface do not overlap and can simultaneously hold two molecules of zinc cytochrome *c*, a ternary complex Zncyt/pc(II)/Zncyt can be formed. If spatial constraints or electrostatic repulsion preclude simultaneous and persistent binding of two molecules of zinc cytochrome *c*, the ternary complex may perhaps form transiently.

The reverse titration procedure, introduced by Zhou and Hoffman, allows study of binding and photoinduced electron-transfer reactions of multisite proteins.[49] We use the superscript r for the rate constants determined in these experiments.

In the reverse titration at low ionic strength the quenching of the triplet ^{3}Zncyt by fern cupriplastocyanin was again

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triphasic. Both intracomplex rate constants agree with those determined by the normal titration. As Figure 6 shows, the rate constant for the third component, k^{r}_{obs}, stays at its maximum value as long as [Zncyt]/[pc(II)] < 1 and then monotonically decreases with further additions of zinc cytochrome *c*. In both normal and reverse titrations, the rate constants k_{obs} and k^{r}_{obs} change their behavior toward saturation as the ratio [Zncyt]/[pc(II)] approaches 1. Saturation occurs when essentially all zinc cytochrome *c* molecules are bound. The results are consistent with tight 1:1 binding. There is no indication of a persistent ternary complex. The titration curve, however, qualitatively differs from that expected if only binary Zncyt/pc(II) complex were involved in the quenching of the triplet ^{3}Zncyt. Results of the reverse titration in Figure 6 cannot be fitted with the parameters obtained from the normal titration. We conclude that a transient ternary complex Zncyt/pc(II)/Zncyt contributes to the quenching rate. The data are fitted successfully to the model involving the binary complex and the second molecule of zinc cytochrome *c* that are in rapid exchange, i.e., dissociation of the transient ternary complex is much faster than the intracomplex quenching.[77,78] This fitting gives the following estimates for the ternary complex (superscript t): the association constant $K^{t}_{a} \sim 4 \times 10^5$ M^{-1} and the apparent electron-transfer rate constant $k^{t}_{app} = (4.8 \pm 1.5) \times 10^4$ s^{-1}. The second molecule of zinc cytochrome *c* binds more weakly because the first one has already neutralized the negative charge on the cupriplastocyanin surface. The apparent rate constant k^{t}_{app} is equal, within the error bounds, to the corresponding constant for the binary complex, k_{app}. The second molecule of zinc cytochrome *c* can bind transiently at more than one plastocyanin site in persistent binary complexes that seem to coexist. Protein association is nonspecific because the acidic patch in fern plastocyanin is very broad. In conclusion, the results of reverse titration confirm that zinc cytochrome *c* binds to fern plastocyanin at more than one surface site capable of the electron-transfer reaction.

III. NMR Spectroscopic Studies of the Protein Association. To learn more about the stoichiometry of the protein complex and possible location of the two binding sites, we used ^{1}H NMR spectroscopy. Although the interactions between the binary complex and an additional molecule of cytochrome *c* are only transient, they can be detected in ^{1}H NMR titration experiments.[79]

The titration curves for 12 selected resonances were first fitted to a 1:1 model of binding.[9] In eq 19, $\Delta\delta$ is the change in chemical shift, $\Delta\delta_\infty$ is this change when the ratio [cyt(II)]/[pc(I)] tends to infinity, and K_a is the association constant for a 1:1 complex. Global fittings of all 12 data sets yielded one association constant K_a. As Figure 8 shows, fitting to the 1:1 model is adequate ($R^2 = 0.939$), but the parameter $K_a = (3.9 \pm 1.1) \times 10^3$ M^{-1} is unrealistic, far lower than new kinetic results for fern plastocyanin and the earlier kinetic results for high-plant plastocyanin, both at low ionic strength.[8,9,27,29,80] We rejected the 1:1 model.

$$\Delta\delta = \frac{1}{2}\Delta\delta_\infty\{[\text{cyt(II)}] + [\text{pc(I)}] + K_a^{-1} - \sqrt{([\text{cyt(II)}] + [\text{pc(I)}] + K_a^{-1})^2 - 4[\text{cyt(II)}]\,[\text{pc(I)}]}\} \quad (19)$$

Next, we tried a 2:1 model, 2 cyt to 1 pc.[81] There are new symbols in eq 20: x_b and x_t are the molar fractions of plastocyanin bound in the binary and the ternary complex, respectively; f_b and f_t are the respective coefficients describing the sensitivity of the plastocyanin chemical shifts. To minimize the number of the fitted parameters, we used in the model overall association constants and not the constants for particular sites. The association constants for the binary and the ternary

$$\Delta\delta = (x_b f_b + x_t f_t)\Delta\delta_\infty \quad (20)$$

complex, K^{b}_{a} and K^{t}_{a}, are routinely related to the molar fractions of these complexes, x_b and x_t. For some of the resonances, two association constants could not be distinguished in fitting to the 2:1 model. Because a global fitting was unwarranted, curves for individual resonances were fitted separately. The fitting having the highest value of R^2 and yielding two association constants gave $K^{b}_{a} = (3.0 \pm 0.2) \times 10^6$ M^{-1} and $K^{t}_{a} = (1.2 \pm 1.3) \times 10^5$ M^{-1}. We then fixed these two parameters in a global fitting of all 12 curves and obtained an improved fit ($R^2 = 0.954$). The values of the association constants K^{b}_{a} and K^{t}_{a} are consistent with those obtained from our kinetic data. This consistency supports the conclusion from the reverse titration, namely that two molecules of cytochrome *c* can bind to the surface of fern plastocyanin. Because these proteins can form a ternary complex, they likely can form at least two binary complexes.

We were able to assign several of those NMR signals in cuproplastocyanin that were significantly shifted during the titration with ferrocytochrome *c*. (See Table S1 in the Supporting Information.) Among the identified residues, three are exposed on the surface: His90 and Phe12 lie on the north side of the protein, whereas Lys56 lies on the east side. These residues are located too far apart on the surface to belong to the same binding site for cytochrome *c*. Interestingly, Ile39, in the hydrophobic core of the protein, is also affected by the association. This finding agrees with a previous study, which showed structural changes deep inside the high-plant plastocyanin upon its association with cytochrome *c*.[9]

Intracomplex Electron-Transfer Reactions Are Gated by Rearrangement of the Diprotein Complexes. In this study, we directly observe two intracomplex reactions, corresponding to the unimolecular rate constants k_1 and k_2. As in previous studies of the intracomplex electron-transfer reactions, we can examine the processes following protein association. But now, for the first time, we can directly compare two different electron-transfer reactions within the same pair of proteins. We examine the effects of viscosity, temperature, and ionic strength on the unimolecular rate constants k_1 and k_2 and show that both of these reactions are gated by structural rearrangement of the respective diprotein complexes.

I. Kinetic Effects of Viscosity. In previous studies in our laboratory, solution viscosity was introduced as an experimental variable to determine whether the intracomplex electron-transfer reaction is gated.[11,13,16−18,25] Solution viscosity impedes protein

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motion and slows down rearrangement of the diprotein complex, but does not affect so-called true and coupled electron-transfer reactions.[18]

Dependence of the rate constant k_1 on viscosity, shown in Figure 7, was fitted to the empirical eq 21,[82] in which η is solvent viscosity; $\Delta G^{\ddagger}$ is the free energy of activation for the rearrangement; and δ is a parameter related to the protein friction. The results of the fittings are listed in Table 2.

$$k = \frac{k_B T}{h} \eta^{-\delta} \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right) \qquad (21)$$

The parameters $\Delta G^{\ddagger}$ and δ for the faster of the two intracomplex reactions of fern plastocyanin are similar to these parameters for the only intracomplex reaction of high-plant plastocyanins. Because solution viscosity similarly affects their dynamics, the rearrangements involving these two variants of plastocyanin probably are similar in kind: zinc cytochrome *c* explores the surface of plastocyanin in the vicinity of the initial binding site, to improve the electronic coupling between the heme and the copper site. Because δ depends on the protein surfaces involved in the rearrangement,[13,18] and given the structural similarities between plastocyanins from fern and high plant,[31] we propose that this rearrangement occurs essentially at the same part of the plastocyanin surface—at or near the acidic residues 59−61, which correspond to the upper acidic cluster in high-plant plastocyanins.

II. Kinetic Effects of Temperature. Quenching of the triplet ^{3}Zncyt by fern cupriplastocyanin remained triphasic at all temperatures. The distribution parameter *n* obtained in fitting to eq 18 is unaffected by temperature. (See Figure S7 in the Supporting Information.) If the number of the protein complexes remains the same in the temperature range examined, analysis of the temperature effects on the individual rate constants k_1 and k_2 is justified.

As Table 2 shows, the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for both reactions k_1 and k_2 of fern plastocyanin are similar in sign to the parameters for high-plant plastocyanins: $\Delta H^{\ddagger} > 0$ and $\Delta S^{\ddagger} < 0$. The former parameter is related to the change in the character of the exposed surfaces in the rearrangement.[12] The latter parameter reflects tightening of the diprotein complex, required for the efficient electron transfer. The activation parameters for the faster unimolecular reaction (k_1) of fern plastocyanin and for the only unimolecular reaction of two high-plant plastocyanins are equal, within the error bounds. This equality supports our proposal that these reactions occur essentially at the same region of the plastocyanin surface. The $\Delta H^{\ddagger}$ value for the slower unimolecular reaction (k_2) of fern plastocyanin is larger than that for the reaction k_1. If the reaction k_2 is also gated by structural rearrangement, this difference would mean that this protein motion is more impeded in the complex undergoing the reaction k_2 than the complex undergoing k_1. As the discussion below will show, both of these intracomplex reactions of fern plastocyanin are gated.

Analysis by Marcus theory of the temperature effects on an electron-transfer reaction allows a tentative diagnosis whether the reaction is gated.[21] The thermodynamic driving force, $\Delta G°$, for the unimolecular reactions in eq 1 is the sum of the potentials for the oxidation of ^{3}Zncyt to Zncyt$^+$ (0.88 V) and for the reduction of cupriplastocyanin to cuproplastocyanin (0.39 V). We fit the temperature dependencies of the rate constants k_1 and k_2 to eqs 22 and 23,[60] in which the symbols have their usual meanings: H_{AB} is the electronic coupling between the heme and the copper site; λ is the reorganizational energy; *h* is the Planck's constant; *R* is the gas constant; 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 over distance (1.4 Å^{-1}); and *r* is the donor−acceptor distance.

$$k = \frac{4\pi^2 {H_{AB}}^2}{h\sqrt{4\pi\lambda RT}} \exp\left[\frac{-(\Delta G° + \lambda)^2}{4\lambda RT}\right] \qquad (22)$$

$$k = k_0 \exp[-\beta(r - r_0)] \exp\left[\frac{-(\Delta G° + \lambda)^2}{4\lambda RT}\right] \qquad (23)$$

Fittings to eq 22 gave the following respective values of λ and H_{AB}: 265 ± 10 kJ/mol (or 2.7 eV) and 5×10^{-23} J (or 2.5 cm^{-1}) for the more reactive complex (reaction k_1), and 320 ± 30 kJ/mol (or 3.3 eV) and 8×10^{-23} J (or 4.0 cm^{-1}) for the less reactive complex (reaction k_2). Fittings to eq 23 gave the following respective values of λ and *r*: 259 ±10 kJ/mol (or 2.7 eV) and 10.2 ± 0.6 Å for the more reactive complex, and 314 ±30 kJ/mol (or 3.3 eV) and 9.3 ± 2.0 Å for the less reactive complex. The reorganizational energies (λ) for both complexes fall out of the interval 0.8−2.3 eV for the electron-transfer reactions involving pairs of metalloproteins.[83−87] Fittings to eqs 22 and 23 in the case of the triplet ^{3}Zncyt and high-plant cupriplastocyanin, the reaction proven to be gated,[13,17] gave results similar to those just given above for fern plastocyanin. The exceptionally high λ values give further evidence that the faster intracomplex reaction is gated and suggest that the slower intracomplex reaction also is gated.

III. Kinetic Effects of Ionic Strength. Neither the rate constant k_1 nor k_2 depends on ionic strength. We conclude that both of these intracomplex rearrangements are local configurational fluctuations within electrostatically stabilized diprotein complexes. Tight binding at low ionic strength precludes the larger migration of the associated proteins.

Preliminary Identification of the Binding Sites. In the conventional orientation of plastocyanin, shown in Figure 1, residues His90 and Tyr86 of the fern protein mark the "north" and the "east" sides, respectively. The corresponding residues in high-plant proteins are His87 and Tyr83.

Monte Carlo calculations support our conclusion that fern plastocyanin has more than one binding site. Numerous relatively stable configurations are found; a very broad area on the surface seems to be involved in binding of cytochrome *c*. As Figure 9 shows, the binding occurs on both northeast and northwest sides and also on the "back" of plastocyanin. In the NMR experiments we find that residues affected by the protein association lie in this general area of plastocyanin. The calculations support our proposal from kinetics, namely that the northeast area participates in binding of zinc cytochrome *c* and electron transfer. We assign the faster intracomplex reaction, the rearrangement k_1, to this area. Binding to the northwest region of the plastocyanin surface provides the greatest electrostatic stabilization. This is the region that is acidic (negatively charged) in the fern protein, but not in the high-plant proteins. This region, however, is distant from His90 and Tyr86, and the surface topography would present obstacles to the movement

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of zinc cytochrome *c*. We implicate this site in the slower intracomplex reaction, the rearrangement k_2.

Conclusions

One well-characterized protein molecule, cytochrome *c*, can be used to explore the binding surface of another, a new plastocyanin from fern. Electrostatic attraction between them results in multiple binary complexes that are trapped at low ionic strength. We detect two different complexes that markedly differ in electron-transfer reactivity. We directly observe unimolecular reactions in these complexes and analyze dynamic processes possible because of multiple binding sites. As ionic strength increases and the binary complexes interconvert, fern plastocyanin becomes intrinsically more reactive. Effects of viscosity and temperature at low ionic strength, when large migration of the proteins is prohibited by electrostatic attractions, showed that each of the two identified binary complexes rearranges before electron transfer. Local configurational fluctuations may be needed to optimize the electron-transfer reaction in both complexes. This dynamic mobility may be a general feature of redox reactions in proteins, whether these reactions are intermolecular (as between a mobile electron carrier and its membrane-bound partner)[22,58,69,74,89] or intramolecular (as between subunits of an oligomeric redox enzyme).[90] Because the distinction between these two types of reactions fades in biological systems, it is more useful to consider whether an electron-transfer reaction involves an interface between the proteins. Dynamic processes at this interface may affect the reaction in ways that remain unknown.

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Supporting Information Available: One table showing assignments of several plastocyanin resonances; seven figures showing the residuals for the biexponential fit of a typical kinetic trace; the dependence of the relative ampitudes of the three kinetic phases on cupriplastocyanin concentration; the dependence of the relative ampitudes on ionic strength; the dependence of the amplitude a_1 on the glycerol concentration; the dependencies on temperature of the rate constants k_1 and k_2, fitted to the Eyring equation; temperature dependence of the relative amplitudes; and temperature dependence of the distribution parameter *n* (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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