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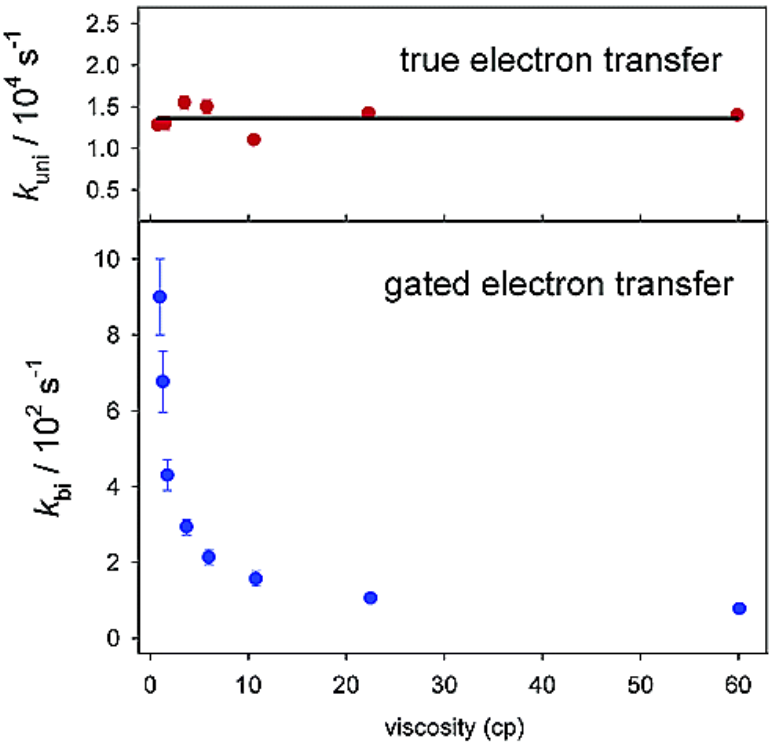

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Metalloprotein Association, Self-Association, and Dynamics Governed by Hydrophobic Interactions: Simultaneous Occurrence of Gated and True Electron-Transfer Reactions between Cytochrome *f* and Cytochrome c_6 from *Chlamydomonas reinhardtii*

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Abstract: Noninvasive reconstitution of the heme in cytochrome c_6 with zinc(II) ions allowed us to study the photoinduced electron-transfer reaction ^{3}Zncyt c_6 + cyt *f*(III) → Zncyt c_6^+ + cyt *f*(II) between physiological partners cytochrome c_6 and cytochrome *f*, both from *Chlamydomonas reinhardtii*. The reaction kinetics was analyzed in terms of protein docking and electron transfer. In contrast to various protein pairs studied before, both the unimolecular and the bimolecular reactions of this oxidative quenching take place at all ionic strengths from 2.5 through 700 mM. The respective intracomplex rate constants are k_{uni} (1.2 ± 0.1) × 10^4 s^{-1} for persistent and k_{bi} (9 ± 4) × 10^2 s^{-1} for the transient protein complex. The former reaction seems to be true electron transfer, and the latter seems to be electron transfer gated by a structural rearrangement. Remarkably, these reactions occur simultaneously, and both rate constants are invariant with ionic strength. The association constant K_a for zinc cytochrome c_6 and cytochrome *f*(III) remains (5 ± 3) × 10^5 M^{-1} in the ionic strength range from 700 to 10 mM and then rises slightly to (7 ± 2) × 10^6 M^{-1}, as ionic strength is lowered to 2.5 mM. Evidently, docking of these proteins from *C. reinhardtii* is due to hydrophobic interaction, slightly augmented by weak electrostatic attraction. Kinetics, chromatography, and cross-linking consistently show that cytochrome *f* self-dimerizes at ionic strengths of 200 mM and higher. Cytochrome *f*(III) quenches triplet state ^{3}Zncyt c_6, but its dimer does not. Formation of this unreactive dimer is an important step in the mechanism of electron transfer. Not only association between the reacting proteins, but also their self-association, should be considered when analyzing reaction mechanisms.

Introduction

Because electron-transfer reactions between metalloproteins are essential to life, it is important to understand their mechanisms. The overall redox process may consist of several steps, including protein−protein recognition, binding, and subsequent electron transfer.[1−3] Electrostatic and hydrophobic interactions govern the affinity and specificity in recognition and association.[4] Existence of electrostatic interactions is well documented,[5−15] but mechanism and dynamics of electrostatic association are poorly understood. In simple cases there is only one energetically favorable binding configuration, which is also the reactive configuration. In more interesting cases, an orientation that is optimal for binding is not optimal for electron transfer or there are multiple binding conformations with similar energy, only some of which are competent for electron transfer.[16]

Very recent studies have shown that hydrophobic interactions can additionally stabilize the electrostatic complex.[4,17−22] Al-

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though hydrophobic interactions are expected to be prominent at physiological conditions (ionic strength of chloroplast interior is estimated at 400 mM), evidence that redox metalloproteins associate solely by hydrophobic interaction is scarce. Here we study the unexplored electron-transfer reaction of cytochrome *f* and reconstituted cytochrome c_6 and document the importance of hydrophobic forces not only for protein docking but also for their reactivity.

Cytochrome *f* is the lumen-exposed part of the cytochrome b_6f complex. Its iron(II) form donates an electron to plastocyanin(II) (in plants and photosynthetic bacteria) or cytochrome c_6(III) (in cyanobacteria and some eukaryotic algae), the reduced form of which then reduces the cofactor $P700^+$ of photosystem I.[5,6,23,24]

Some algae and cyanobacteria synthesize plastocyanin or cytochrome c_6. Others produce either protein, depending on the (un)availability of copper ions in the growth medium.[25,26] The notion that plastocyanin replaced cytochrome c_6 in the higher plants and some algae has very recently been refuted when cytochrome c_6 was found in a higher plant.[27] Although plastocyanin and cytochrome c_6 are functionally equivalent electron carriers, their primary, secondary, and tertiary structures are completely different. These proteins, however, have similar distributions of acidic patches and hydrophobic surfaces.[25] How these differences and similarities influence the mechanisms by which these proteins oxidize the same partner, cytochrome *f*(II), remains unknown. Electron-transfer reaction between cytochrome *f* and plastocyanin[5−7,14,17,24,28] in vitro involves electrostatic attraction between the positively charged patch of lysine residues in the former protein and the negatively charged patch of acidic residues in the latter.[6−8,28,29]

Very little is known about the electron-transfer reaction between cytochrome *f* and cytochrome c_6. Ours is the first study of protein−protein interactions that govern the kinetics of electron transfer between them. Because both of them are heme proteins, whose absorption spectra overlap, it is almost impossible to follow spectroscopically the simultaneous oxidation of one heme group and the reduction of the other.[30] In this study, we overcame this difficulty by reconstituting cytochrome c_6 with zinc(II) ions and making the electron-transfer step photoinduced. Similar reconstitution of other cytochromes of type *c* does not perturb their conformation and interaction with their redox partners.[31,32] Although the photoinduced electron-transfer step in eq 1, which is followed by the reaction in eq 2, is not biological, the very high rate of this step allowed us to observe and quantify the association of proteins from the same organism, a process of great importance. Because the excited-state reaction in eq 1 and the subsequent ground-state reaction in eq 2 do not require any redox agents other than the interacting proteins, their association is not perturbed. Monitoring the electron-transfer reaction in eq 1 is our means of studying structural and dynamic aspects of metalloprotein association.

$$^3\text{Zncyt } c_6 + \text{cyt } f(\text{III}) \rightarrow \text{Zncyt } c_6^+ + \text{cyt } f(\text{II}) \qquad (1)$$

$$\text{Zncyt } c_6^+ + \text{cyt } f(\text{II}) \rightarrow \text{Zncyt } c_6 + \text{cyt } f(\text{III}) \qquad (2)$$

The results of this study are interesting and surprising. Cytochrome *f* from *Chlamydomonas reinhardtii* strongly associates not only with its physiological partner cytochrome c_6 but also with itself. The "cross"-association is driven predominantly, and the self-association entirely, by hydrophobic interaction. Self-association has been recognized as an important means of regulating enzymatic reactions.[33−38] To our knowledge, this is the first report of self-association affecting the kinetics of electron-transfer reaction.

Previous research in our and other laboratories showed that mobility within the diprotein complex is necessary for efficient electron transfer, as in eq 1.[39] Dynamics of electrostatic diprotein complexes has been studied in detail[2,9,39−47] but that of nonelectrostatic complexes has not, until now.

Materials and Methods

Chemicals and Buffers. Distilled water was demineralized to a resistivity greater than 17 MΩ cm by a Barnstead Nanopure II apparatus. Chromatographic resins and gels were purchased from Sigma Chemical Co.; hydrogen fluoride, from Matheson Gas Products, Inc.; nitrogen and ultrapure argon, from Air Products Co.; BCA protein assay reagent kit, from Pierce Co.; all other chemicals, from Fisher Chemical Co. All buffers were prepared from the solid salts $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ and had pH of 7.00 ± 0.05. For kinetic measurements, the ionic strengths higher than 10 mM were adjusted with solid NaCl, and for chromatography, buffers of particular concentration were made. Unless concentration is stated, the buffers are specified by their ionic strength.

Proteins. Cytochrome *f* from *C. reinhardtii*, expressed from *Escherichia coli*, was isolated and purified as described previously,[48] and was kindly provided by Professor William A. Cramer. Cytochrome c_6 from *C. reinhardtii* was isolated and purified by the published method.[25] Iron was removed, and the free-base protein was reconstituted with zinc(II) ions by a modification of the standard procedure. Zinc cytochrome c_6 was always kept in the dark. Concentrations of the two proteins were determined from their UV−vis spectra on the basis of

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known absorptivities: cytochrome *f*(II), $\Delta\epsilon_{552} = 26\ mM^{-1}\ cm^{-1}$; cytochrome c_6(II), $\Delta\epsilon_{552} = 20\ mM^{-1}\ cm^{-1}$; and zinc cytochrome c_6, $\epsilon_{421} = (2.3 \pm 0.1) \times 10^5\ M^{-1}\ cm^{-1}$. The last value was determined from absorption spectra and quantification of total protein using the BCA protein assay reagent kit. All proteins were stored in liquid nitrogen. Before each series of experiments, the buffer in protein stock solutions was replaced by the working buffer using so-called ultrafree-4 centrifugal filter obtained from Millipore Co.

Laser Flash Photolysis. Experiments were performed with the second harmonic (at 532 nm) of a Q-switched Nd:YAG laser; the instrument was described elsewhere.[49] Argon was passed first through water and then through the buffer solution. The required volume of buffer was deaerated in a 10-mm cuvette for at least 30 min before zinc cytochrome c_6 was added. After each addition of cytochrome *f*(III), the solution was gently deaerated for 10 to 15 min. In the titration experiments, concentration of zinc cytochrome c_6 was kept constant in the interval 0.70−3.0 μM, and concentration of cytochrome *f*(III) was varied between 0.20 and 12 μM. Decay of the triplet state was monitored at 460 nm, where the transient absorbance reaches the maximum. The concentration of the triplet depended on the intensity of the laser pulse and was always much lower than the concentration of cytochrome *f*(III). Pseudo-first-order excess of cytochrome *f*(III) was maintained in all experiments. Formation and disappearance of the cation radical were monitored at 675 nm, where the difference between the absorbances of this species and the triplet is greatest. To enhance signal-to-noise ratio, at least 100 shots were collected and averaged.

Viscosity. The kinetic effects of viscosity were studied in the 10 mM sodium phosphate buffer at pH 7.00 and (20 ± 1) °C. Glycerol was added incrementally to the solution containing 3.0 mM zinc cytochrome c_6 and 9.0 mM cytochrome *f*(III), up to the concentration of 80% w/v. The viscosity of the solution was determined from the tables.[50,51]

Cross-Linking of Cytochrome *f*. A solution containing 5.0 μM cytochrome *f* and 50-fold molar excess of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) in sodium phosphate buffer at pH 7.00 and ionic strength 700 mM was stirred at room temperature. After 4 h, the reaction mixture was subjected to size-exclusion HPLC.

HPLC Separations. Hewlett-Packard 1100 HPLC system contained an autosampler and a multiwavelength detector set to 215, 280, and 410 nm. Absorption at 215 nm is common to all peptides and proteins; that at 280 nm is due to aromatic residues; and that at 410 nm is diagnostic of heme. In the reversed-phase separations, an analytical Vydac C5 column 214TP54 (sized 150 × 4.6 mm, beads of 5 μm) was used. The eluting solvent A was 0.10% (v/v) trifluoroacetic acid in H_2O, and solvent B was 0.08% (v/v) trifluoroacetic acid in acetonitrile. In a typical run, the percentage of solvent B in the eluent was kept at 2.0% for 5 min after the injection of the sample and then raised gradually to 60% over a 35-min period. The flow rate was 1.0 mL/min.

The size-exclusion separations were made with a Superdex 75 HR 10/30 column, having optimal separation range from 3 to 70 kD. The solvent was a 100 mM (concentration) phosphate buffer, and the flow rate was 0.50 mL/min. The size-exclusion column was calibrated with bovine serum albumine (67 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), trypsin inhibitor (20 kD), and myoglobin (17 kD). The void volume of the column was determined using blue dextran 2000. In size-exclusion separations, the protein concentration was varied between 2.0×10^{-6} and 2.0×10^{-5} M for cytochrome *f* and between 1.0×10^{-6} and 5.0×10^{-5} M for cytochrome c_6.

Analytical Ultracentrifugation. Sedimentation equilibrium involved cytochrome c_6(II) dissolved in phosphate buffer having pH 7.00 and ionic strength 300 mM at room temperature. The protein concentration was varied from 2.0×10^{-6} to 8.0×10^{-6} M. A Beckman Optima XL-A analytical ultracentrifuge equipped with an An60-Ti rotor ran at 30 000 rpm. Absorbance detector was set at 410 or 550 nm, depending on protein concentration, so that absorbance stayed below 0.7. Data were collected at a spacing of 1.0×10^{-3} cm, with 10 averages, in a step-scan mode every 3 h. Equilibrium was reached when the absorbance stopped changing. The molecular mass of monomeric cytochrome c_6 was set to the value obtained from amino acid sequence, 9.8 kD.

Fittings of the Kinetic Data. The rate constants for the reaction in eq 1 were obtained from the analysis of the changes of absorbance at 460 and 675 nm with time. The former change corresponds to the decay of ^{3}Zncyt c_6 and is a sum of several exponential terms (eq 3). The latter change is caused by both the triplet and the cation radical and is described by eqs 4−7.[52] Contribution of the triplet to the absorbance change at 675 nm is given by eq 5, in which a_t is the instantaneous absorbance after the laser flash. The contribution of cation radical is fitted with eq 7.

$$\Delta A_{460} = \sum_i a_i \exp(-k_i t) + b \tag{3}$$

$$\Delta A_{675} = \Delta A_{\text{triplet}} + \Delta A_{\text{cation}} \tag{4}$$

$$\Delta A_{\text{triplet}} = a_t[\sum_i f_i \exp(-k_i t)] \tag{5}$$

$$f_i = a_i/(a_{\text{uni}} + a_{\text{bi}}) \quad i = \text{uni, bi} \tag{6}$$

$$\Delta A_{\text{cation}} = a_c\,[\exp(-k_{\text{fall}} t) - \exp(-k_{\text{rise}} t)] \tag{7}$$

Kinetic results were analyzed with the SigmaPlot v.5.0 from SPSS, Inc. The error margins for all rate constants (*k*) and amplitudes (*a*) obtained from the fitting of the transient absorbance changes include two standard deviations, i.e., they correspond to the confidence limit of 95%. In some plots, error bars for some of the points are large, but fortunately these points are not crucial for the fitting and do not alter the results and their discussion. Dependence of the observed rate constant, k_{obs}, for the slower phase on the concentration of free cytochrome *f*(III) was fitted with the improved steady-state equation (eq 8),[53] in tandem with eq 9, as in several previous studies from this laboratory.[12,20,43,44,54]

$$k_{\text{obs}} = \frac{k_{\text{on}} k_{\text{bi}}\,[\text{cyt } f(\text{III})]}{k_{\text{off}} + k_{\text{bi}} + k_{\text{on}}[\text{cyt } f(\text{III})]} \tag{8}$$

$$[\text{cyt } f(\text{III})] = [\text{cyt } f(\text{III})]_0 - 0.5\{[\text{Zncyt } c_6]_{\text{tot}} + [\text{cyt } f(\text{III})]_0 + (1/K_a) - (([\text{Zncyt } c_6]_{\text{tot}} + [\text{cyt } f(\text{III})_0] + [1/K_a])^2 - 4[\text{Zncyt } c_6]_{\text{tot}}[\text{cyt } f(\text{III})]_0)^{0.5}\} \tag{9}$$

Many attempts to obtain the association constant K_a from fitting experimental results to eq 8 with SigmaPlot software failed because a global minimum and several local minima of the sum of squares occurred with similar probabilities. Each of these occurrences yielded a different set of fitting parameters, k_{on} and k_{off}, the ratio of which is K_a. Unfortunately, methods of numerical analysis often fail when data are fitted to an equation that contains a product of a very small and very large number. A common case in kinetics is a product between

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concentration and rate constant, such as k_{on}[cyt *f*(III)] in eq 8. Rescaling equation parameters sometimes improves numerical behavior and produces reasonable fittings.

The occurrence of the "multiple minima" is not yet adequately addressed in SigmaPlot, but we managed to avoid this problem. We designed our experiments so that complex kinetic equations, ambiguous fittings, and analysis of possibly unreliable fitting parameters became unnecessary. The rate constants k_{uni} and k_{bi} were read directly from the plots in Figures 2 and 3, and the association constant K_a was obtained from the fractional contributions (eq 6) of the persistent (a_{uni}) and the transient (a_{bi}) complex to the transient absorbance change using eqs 10 and 11, which are explained elsewhere.[52,55]

$$f_{uni} = \frac{1}{2[\text{Zncyt } c_6]}\{[\text{Zncyt } c_6] + [\text{cyt } f(\text{III})] + 1/K_a - ([\text{Zncyt } c_6] + [\text{cyt } f(\text{III})] + 1/K_a)^2 - 4[\text{Zncyt } c_6][\text{cyt } f(\text{III})])^{0.5}\} \quad (10)$$

$$\frac{[\text{cyt } f(\text{III})]}{f_{uni}} = \frac{[\text{cyt } f(\text{III})]}{g} + \frac{1}{gK_a} \quad (11)$$

Results

Natural Decay of the Triplet State ^{3}Zncyt c_6. In the absence of a quencher, the natural decay of the triplet excited state of the porphyrin to its ground state is monoexponential (eq 12). The rate constant, k_{nd}, is 100 ± 10 s^{-1} at room temperature in phosphate buffer having pH 7.00 and is independent of protein concentration in the interval from 1.0 to 10 mM and of ionic strength in the interval from 2.5 to 700 mM.

$$\Delta A_{460} = a_{nd} \exp(-k_{nd}t) + b \quad (12)$$

Oxidative Quenching of the Triplet State ^{3}Zncyt c_6 by Cytochrome *f*(III) Is Biphasic at All Ionic Strengths. In the presence of cytochrome *f*(III), decay of the triplet is accelerated and is best described by a biexponential function (eq 13 and Figure 1a) at all tested ionic strengths, from 2.5 through 700 mM. The rate constant k_{uni}, for the faster of the two reactions, is independent of concentration of cytochrome *f*(III) and ionic strength, as Figure 2 shows. The rate constant k_{obs}, for the slower reaction, levels off at relatively high cytochrome *f*(III) concentrations. As ionic strength is raised, the approximate breaking point in the plots shifts toward higher ratios of the protein concentrations cyt *f*(III)/Zncyt c_6, that is, to a higher concentration of the cytochrome *f*(III) (Figure 3). The rate constants for the faster (k_{uni}) and slower (k_{bi}) reaction of the oxidative quenching are listed in Table 1. The rate constants for the

$$\Delta A_{460} = a_{uni} \exp(-k_{uni}t) + a_{bi} \exp(-k_{bi}t) + b \quad (13)$$

appearance and disappearance of the cation radical, shown in Figure 1b, are independent of the cytochrome *f*(III) concentration. The absorbance at 675 nm grows at the rate of (5.6 ± 0.6) × 10^4 s^{-1} and declines at the rate of (1.3 ± 0.1) × 10^4 s^{-1}. This latter rate constant is the same as the k_{uni}. The increase in the absorbance at 675 nm is due to the back reaction (eq 2), and its decrease is due to the forward reaction (eq 1).[20,51] This study concerns the forward reaction because this reaction gives the information about protein association.

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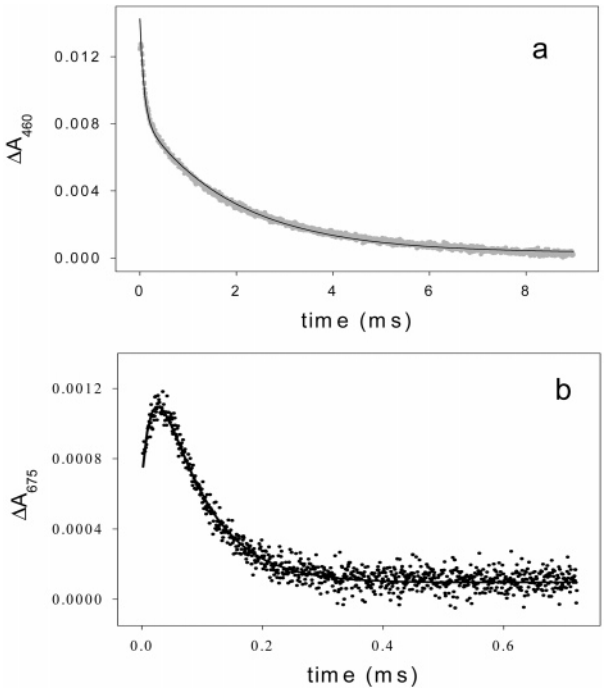

Figure 1. Transient absorbance changes in a solution initially containing 3.0 μM zinc cytochrome c_6 and 3.0 μM cytochrome *f*(III) in sodium phosphate buffer, at pH 7.00 and ionic strength of 300 mM at room temperature. (a) Disappearance of the triplet state ^{3}Zncyt c_6 monitored at 460 nm. The line is a fitting to eq 13. (b) Formation and disappearance of the cation radical Zncyt c_6^+ monitored at 675 nm. The line is a fitting to eqs 4−7.

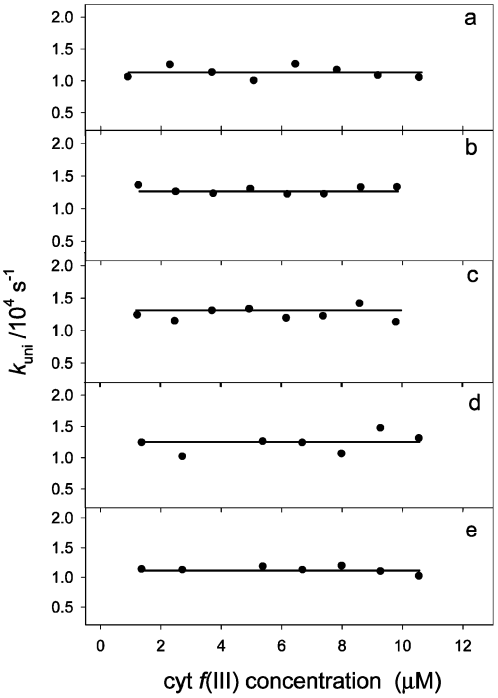

Figure 2. Independence of the microscopic rate constant k_{uni}, for the unimolecular reaction in Scheme 1, of the concentration of cytochrome *f*(III) in sodium phosphate buffer at pH 7.00, room temperature, and ionic strength of (a) 2.5, (b) 10, (c) 100, (d) 300, and (e) 700 mM. The k_{uni} values are listed in Table 1. Error bars are smaller than dots and invisible.

Kinetic Effects of Viscosity. The reaction in eq 1 was studied at ionic strength of 10 mM and temperature 20 ± 1 °C. The decay of ^{3}Zncyt c_6 remains biphasic throughout the viscosity

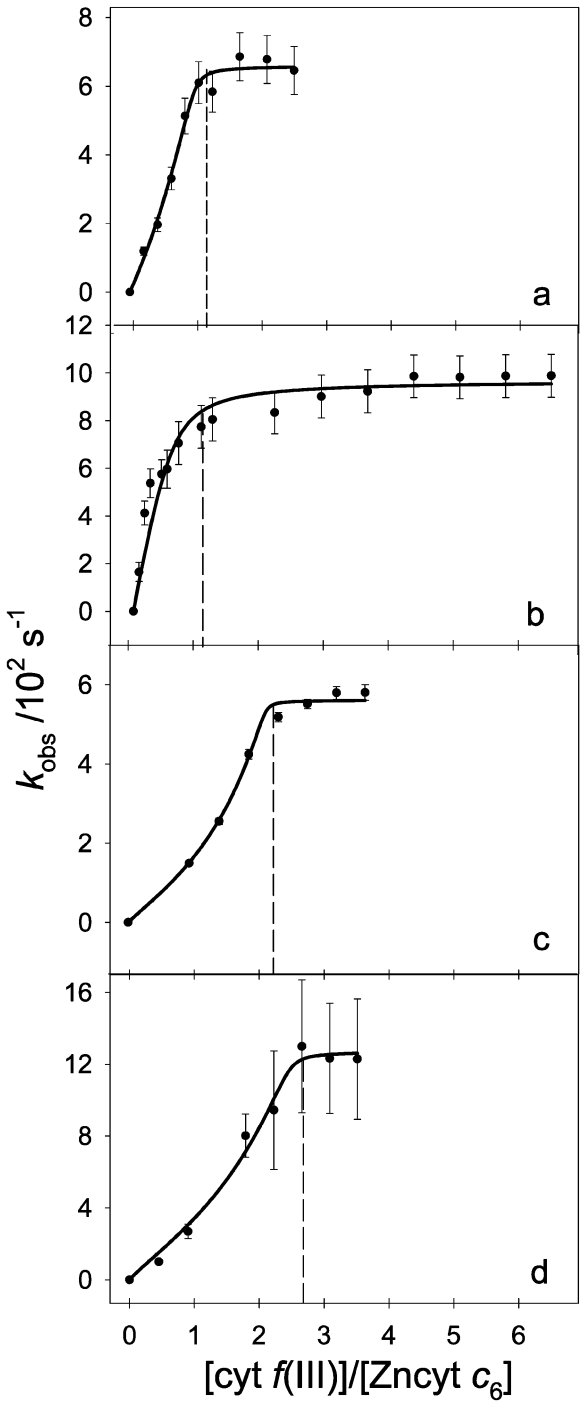

Figure 3. Dependence of the observed rate constant k_{obs} on the ratio of cyt *f*(III) and Zncyt c_6 concentrations, in sodium phosphate buffer at pH 7.00, room temperature, and ionic strengths of (a) 2.5, (b) 10, (c) 300, and (d) 700 mM. Note the shifting of the plateau onset with rising ionic strength. Solid lines are fittings to the mechanism in Scheme 1 and eq 14. Error bars smaller than dots cannot be seen.

range studied, from 1.005 through 60.1 cp. The amplitudes of both reactions are unaffected by viscosity, as Figure 4 shows. Figure 5 shows that the rate constant for unimolecular reaction, k_{uni}, does not depend on viscosity, but the rate constant for the bimolecular reaction, k_{bi}, does.

Evidence that Cytochrome c_6 Is Monomeric. In analytical centrifugation experiments, as the concentration of the protein is raised, the observed molecular mass stays constant and equal to that calculated from amino acid sequence, as Figure S4 shows.

Table 1. Rate Constants Obtained from Fitting the Results in Figures 2 and 3 to the Mechanism in Scheme 1 and Eq 14, and Association Constants Obtained from Eqs 10 and 11

ionic strength/ mM	$k_{uni}/10^4$ s^{-1} unimolecular reaction	$k_{bi}/10^2$ s^{-1} bimolecular reaction	$K_a/10^5$ M^{-1} from eq 10	$K_a/10^5$ M^{-1} from eq 11
2.5	1.1 ± 0.2	6.6 ± 0.2	70 ± 20	20 ± 10
10	1.3 ± 0.1	9 ± 2	4 ± 2	6 ± 2
300	1.2 ± 0.4	5.6 ± 0.2	4 ± 2	2 ± 1
700	1.1 ± 0.2	13 ± 5	8 ± 2	4 ± 2

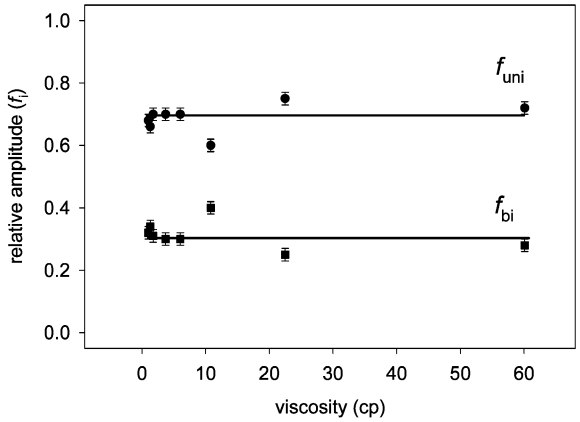

Figure 4. Independence of solution viscosity of the relative amplitudes f_{uni} and f_{bi}, respectively, of the unimolecular (●) and bimolecular (■) reaction in Scheme 1. Viscosity of the sodium phosphate buffer solution at pH 7.00, ionic strength 10 mM, and 20 ± 1 °C was adjusted with glycerol.

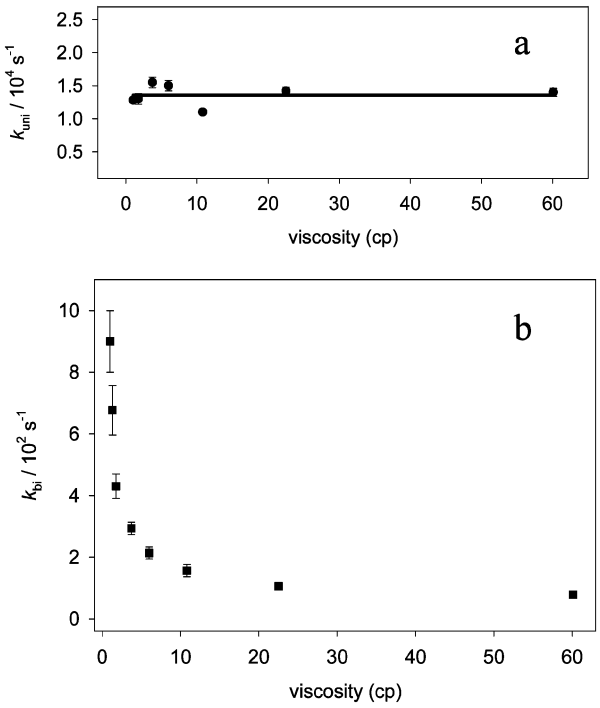

Figure 5. Dependence on the solution viscosity of the intracomplex rate constant for the (a) unimolecular and (b) bimolecular reactions in Scheme 1. Viscosity of the sodium phosphate buffer solution at pH 7.00, ionic strength 10 mM, and 20 ± 1 °C was adjusted with glycerol. Error bars smaller than dots cannot be seen.

In size-exclusion HPLC experiments, the elution time of 26 min corresponds to the molecular mass of the monomer (Figure S5).

Evidence for the Dimerization of Cytochrome *f*. Size-exclusion HPLC of cytochrome *f* solutions at ionic strengths 200 mM and higher (Figure 6a) shows an unsymmetric signal

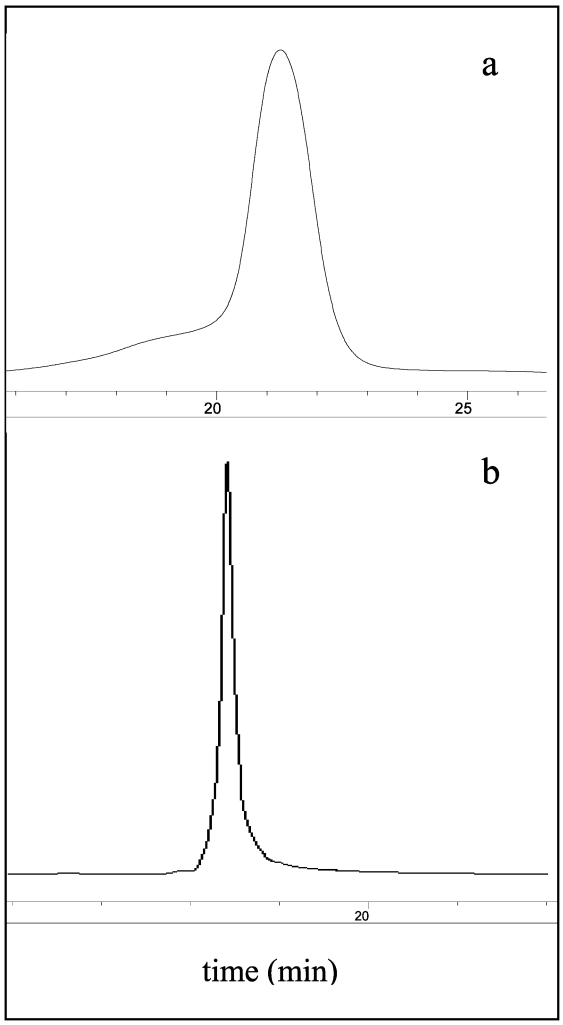

Figure 6. Chromatograms of a 5.0 μM cytochrome *f* from *C. reinhardtii*. (a) Size-exclusion HPLC, with a 100 mM (concentration) sodium phosphate buffer at pH 7.00. Note the shoulder preceding the main signal. (b) Reversed-phase HPLC with 0.10% (v/v) trifluoroacidic acid in water and 0.08% (v/v) trifluoroacidic acid in acetonitrile. Note the sharpness and symmetry of the signal.

that is broadened on the side of shorter elution times. Repeated chromatography of the main fraction, having elution time of 21 min, and of the shoulder preceding it yielded the same chromatogram: the main band at 21 min and a broad shoulder at shorter times. The UV−vis spectra of the main and shoulder fractions are identical to each other and to the spectrum of cytochrome *f* prior to the separation. The main fraction and the shoulder fraction, separated by size, gave the same reversed-phase chromatograms, shown in Figure 6b, consisting of one sharp, symmetrical signal.

Cross-Linking of Cytochrome *f*. Cross-linking of cytochrome *f* with the carbodiimide EDC yielded only two products, which eluted from the size-exclusion HPLC column in 18.3 and 21.1 min, as Figure 7 shows. The ratio of their molecular masses is 1.8. Evidently, the first and the second fraction, respectively, are dimer and monomer of cytochrome *f*.

Attempt at Oxidative Quenching of ^{3}Zncyt c_6 with Dimer of Cytochrome *f*(III). Attempts to oxidatively quench ^{3}Zncyt c_6 with the product of cross-linking of cytochrome *f* gave kinetic traces that were well fitted with the monoexponential function in eq 12. The rate constant obtained from these fittings is

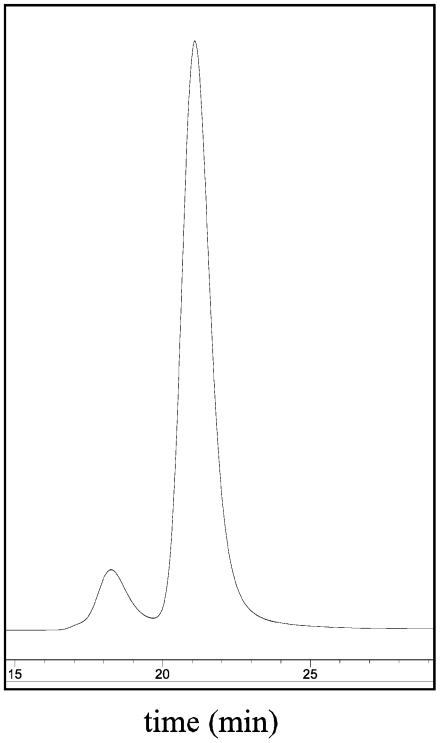

Figure 7. Size-exclusion HPL chromatogram of the reaction mixture containing cytochrome *f* from *C. reinhardtii* and a large excess of carbodiimide EDC. Elution solvent is 100 mM (concentration) sodium phosphate buffer at pH 7.00. The fractions eluting at 21.1 and 18.3 min are, respectively, cytochrome *f* and a cross-linked dimer of it.

$111 \pm 1\ s^{-1}$, the same as that for natural decay of the triplet state. Residuals of the fitting are presented in Figure S3.

Discussion

Interactions Between Metalloproteins. Our research group extensively studied the mechanism of electron-transfer reaction and dynamic aspects of docking between zinc cytochrome *c* and plastocyanin.[12,13,20,39−44,51,52,56−58] In this and similar systems,[59−63] where proteins have high association constants, the kinetics is biphasic at low ionic strength. At intermediate and high ionic strength, kinetics is monophasic, and the observed rate constant is directly proportional to the concentration of the reactant in excess. When the association constant for a metalloprotein pair is low even at low ionic strength, the observed rate constant linearly depends on concentration.[6,14−16,26,28,64−71] In some cases, kinetics of interprotein reaction may be

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monophasic throughout because so-called saturation occurs under different reaction conditions.

In various protein pairs, the rate constant for electron transfer and the association constant markedly decrease as ionic strength is raised because electrostatic attraction dominates protein−protein association. Hydrophobic interaction unaccompanied by electrostatic attraction, a phenomenon well-documented in enzyme−substrate binding,[34,72−74] has only begun to be noticed in association of redox proteins.[19,21,22,26] Cytochrome *f* and plastocyanin from *Phormidium laminosum* reportedly are held together mostly by hydrophobic forces.[4,75]

Cytochrome *f* and Cytochrome c_6. The water-soluble part of cytochrome *f* from *C. reinhardtii* is an elongated, β-barell protein, and the iron(III) form has a net charge of −2 at pH of 7.00 (assuming normal pK_a values). The detailed crystal structure of the membrane-bound cytochrome b_6f complex is unknown, and nearly all kinetic studies in vitro have been done with the truncated form of cytochrome *f*.[23] A cationic patch of lysine residues has been implicated in docking with plastocyanin.[6,7,14,28] The biological function of the hydrophobic outer face of the heme-binding pocket in cytochrome *f*, however, has barely been studied.[17] We are interested in this hydrophobic area because both acidic substituents (so-called propionate chains) and one of the vinyl substituents of the heme are accessible in this part of the protein surface, as shown in Figure 8a.

Cytochrome c_6(II) from *C. reinhardtii* has a net charge of −7 at pH 7.00 (assuming normal pK_a values). As Figure 8a shows, the heme is largely buried in the protein; only one carboxylic group and a porphyrin edge are exposed. The surface around these exposed parts is largely nonpolar, except for Lys29, Lys57, and Asp41 residues.[25] Anionic groups are mostly located on the opposite side from the exposed parts of the heme.[76]

On the basis of NMR spectroscopic experiments, it was vaguely suggested that electron-transfer reactions involving cytochrome *f*, cytochrome c_6, and plastocyanin may occur by different mechanisms, depending on the organism to which these proteins belong.[21] We study the reactions between homologous proteins, those belonging to the same organism.

Mechanism of ^{3}Zncyt c_6 Quenching by Cytochrome *f*. The excited state of zinc porphyrin, ^{3}Zncyt c_6, produced by a laser flash, is oxidatively quenched by cytochrome *f*(III), according to eq 1. The resulting cation radical, Zncyt c_6^+, returns to the initial ground state in a thermal back reaction shown in eq 2. This study deals with the photoinduced reaction in eq 1. Unexpectedly, this reaction is biphasic at all ionic strengths examined, not only at low ionic strength, as was the case in numerous studies cited above. (See Scheme 1.) The intramolecular rate constant, k_{uni}, for the faster reaction does not depend on the cytochrome *f*(III) concentration and ionic strength. Its values, given in Table 1, are read directly from the horizontal plots in Figure 2. The relative amplitude of this reaction (f_{uni}) increases with the concentration of the persistent complex as the cytochrome *f*(III) concentration is raised, as shown in Figure S1. This rate constant corresponds to the intracomplex reaction within the persistent diprotein complex Zncyt c_6/cyt *f*(III), which

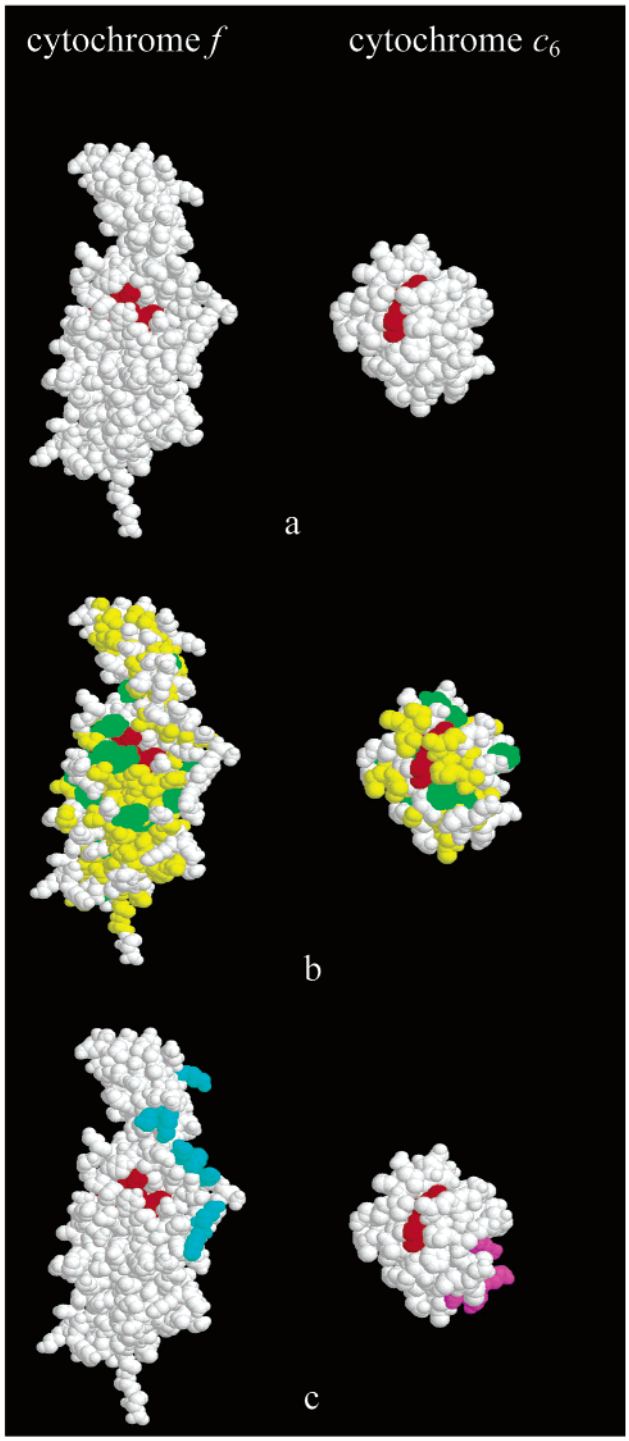

Figure 8. Surfaces of (truncated) cytochrome *f* and cytochrome c_6 from *C. reinhardtii*. Color code: blue, basic residues; magenta, acidic residues; yellow, hydrophobic residues; green, aromatic residues; and red, heme. (a) Exposed parts of the heme. (b) Hydrophobic residues surrounding the exposed part of the heme, the proposed docking sites. (c) Basic and acidic patches are assisting association through electrostatic attraction at low ionic strength.

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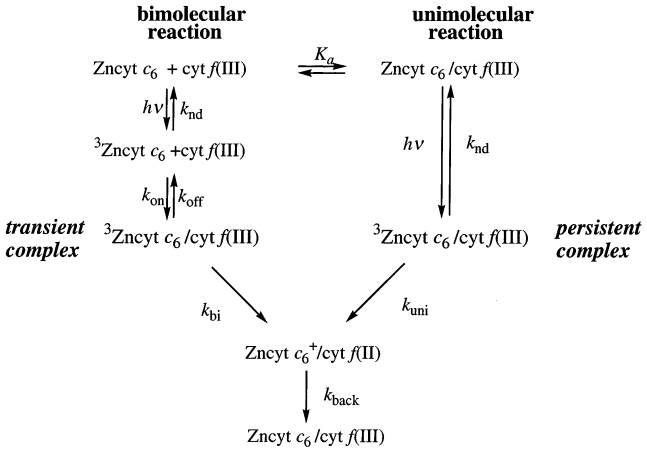
Scheme 1



already exists in solution before the laser flash. The observed rate constant for the slower reaction, k_{obs}, does depend on cytochrome *f*(III) concentration. Because this is the bimolecular reaction between free proteins that associate in a transient Zncyt c_6/cyt *f*(III) complex, the plateau in Figure 3 corresponds to the maximal value of k_{obs}, achieved when zinc cytochrome c_6 is completely associated with cytochrome *f*(III). Therefore, the intracomplex rate constant k_{bi}, for the transient diprotein complex, can be read from the leveled plot. Despite the large error bars for some of the points in Figure 3, the k_{bi} values are sufficiently precise for our discussion; these results also are given in Table 1. Direct detection of the cation radical Zncyt c_6^+ at both low and high ionic strength is evidence that quenching occurs by oxidation of the triplet state.

Association between Cytochrome *f* and Cytochrome c_6 from *C. reinhardtii* Is Mostly Due to Hydrophobic Interaction. The persistence of the two intracomplex reactions in Scheme 1 at ionic strength as high as 700 mM is evidence that electrostatic attraction plays a small, if any, role in association of these two proteins. We determined the association constants directly form the amplitudes of the two reactions shown in Figures 2 and 3, using eqs 10 and 11.[52,55] (Fitting of the amplitudes is shown in Figures S1 and S2.) In this treatment only the total concentration of cytochrome *f*(III) matters, and its state (free or bound) does not matter. These two straightforward methods are independent of the mechanism of the subsequent electron transfer. Indeed, as Table 1 shows, fittings to eqs 10 and 11 gave consistent results. Invariance of k_{uni}, k_{bi}, and K_a with ionic strength consistently shows that the interaction between cytochrome c_6 and cytochrome *f* is not electrostatic. Indeed, hydrophobic interaction is fully consistent with Figure 8b, which shows hydrophobic surfaces surrounding the exposed parts of the heme in both proteins. Further speculation about structural and other details of this complex is unwarranted. As Table 1 shows, the association constant K_a decreases a little when ionic strength is raised from 2.5 to 10 mM and stays almost unchanged at higher ionic strengths. This initial drop is a sign of weak electrostatic attraction, which augments the strong hydrophobic effect insensitive to ionic strength. This weak electrostatic interaction cannot be atributed to the net charges of cytochrome c_6(II) and cytochrome *f*(III), which are −7 and −2 at pH 7.00, respectively; local charges are relevant here. Indeed, Ullmann et al. identified a minor anionic (acidic) patch in cytochrome c_6 that may interact with the predominant cationic (basic) patch of lysine residues in cytochrome *f*;[77] these patches are shown in Figure 8c. This pair of redox metalloproteins is remarkable because association is dominated by hydrophobic interactions and only weakly enhanced by electrostatic interactions.

Multiple Configurations of Diprotein Complexes in General. Much evidence shows that the same pair of redox proteins may form multiple complexes, but little experimental evidence shows that these complexes may undergo essentially the same intracomplex electron-transfer reaction at different rates. This last notion is plausible and has been accepted even though experimental studies corroborating it are still few. Three recent studies have uniformly dealt with photoinduced reactions in which the triplet excited state of zinc cytochrome *c* is oxidatively quenched by heme proteins or a metal complex. Two simultaneous first-order reactions are detected in each case, but more than two complexes may perhaps be present.[45,46,51,78−83] The variety of kinetic results in these three studies shows the diversity of dynamic properties of fairly similar donor−acceptor systems, all of which are mostly held by electrostatic forces. No two of these systems show the same effects and noneffects of ionic strength and viscosity on the intracomplex rate constant. Clearly, general rules about the dynamic aspects of electron-transfer reactions within protein complexes still elude us.

Multiple Configurations of Diprotein Complexes of Zinc Cytochrome c_6 and Cytochrome *f*. By monitoring the decay of the triplet state ^{3}Zncyt c_6 we detected the persistent complex in Scheme 1, determined its association constant K_a, and precisely determined its rate constant k_{uni}. We also detected the transient complex and determined its rate constant, k_{bi}, reliably but less precisely because kinetic traces due to the cation radical Zncyt c_6^+ were relatively noisy. Fortunately, both rate constants are known with precision that is sufficient for the discussion that follows.

I. Kinetic Noneffects of Ionic Strength: Evidence for Hydrophobic Interaction. Table 1 shows that the intracomplex rate constants for the persistent (k_{uni}) and transient (k_{bi}) complexes in Scheme 1 differ as much as 20-fold and that both values are invariant with ionic strength over a wide range. This invariance shows, for the first time, that hydrophobic, and not only electrostatic, interaction between metalloproteins can give rise to structurally heterogeneous association. This heterogeneity in turn gives rise to multiphasic reactivity.

II. Kinetic Noneffects and Effects of Viscosity: Evidence for Simultaneous Occurrence of True and Gated Electron Transfer. In previous studies in our laboratory, changing solution viscosity was used to determine whether the electron-transfer reaction is gated.[12,13,39,44,52] An increase in viscosity slows down protein motion and rearrangement of the diprotein

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complex but does not affect the association constant and the rate constants of the so-called true and coupled electron-transfer reactions.[52]

Glycerol is noninvasive to proteins and even stabilizes them. Because hydrophobic interactions are essential for protein folding, and glycerol does not unfold proteins, this solvent evidently does not perturb hydrophobic interaction. Indeed, relative amplitudes of the persistent and transient complex are unaffected by glycerol (Figure 4). Therefore, the association constant K_a must also be unaffected.

Independence of the rate constant k_{uni} of solution viscosity, shown in Figure 5a, is evidence for *true electron transfer within the persistent diprotein complex*. Large and smooth dependence of the rate constant k_{bi} on solution viscosity, shown in Figure 5b, is diagnostic of *gated electron transfer within the transient (collisional) diprotein complex*. Electron transfer is the rate-limiting step in the unimolecular reaction because the persistent complex either is static or rearranges at a rate higher than 1.2×10^4 s^{-1}, but the transient complex rearranges at a rate of $(9 \pm 4) \times 10^2$ s^{-1} (the average of the four values of k_{bi} in Table 1). Because this latter process is slower than the (unobservable) electron-transfer step within this complex, we detected this structural process when we monitored electron transfer that is controlled by it.

Self-Association of Cytochrome *f*. Figure 3 shows that as the ionic strength is raised, a greater excess of cytochrome *f*(III) over zinc cytochrome c_6 is needed to reach a plateau in k_{obs}. The cyt *f*(III)/Zncyt c_6 ratio at the onset of the plateau increases from 1.2 to ca. 2.7. Not all cytochrome *f*(III) put in solution seems available for association (and subsequent reaction) with cytochrome c_6. The asymmetric chromatogram of cytochrome *f* in Figure 6a shows a main signal and a prominent shoulder on the side corresponding to a larger molecular mass. Repeated experiments with separate samples taken from the main fraction and from the incompletely resolved fraction preceding it consistently yielded this same pattern, shown in Figure 6a. The width of the shoulder precluded accurate determination of the elution time of the aggregate and of its molecular mass, but we estimated its mass to be approximately twice the nominal value. Cytochrome *f* and each of its two incompletely separated fractions in Figure 6a all consistently gave the same narrow and symmetric chromatogram in Figure 6b.

The size-exclusion pattern in Figure 6a is characteristic of self-association equilibrium that is fast on the chromatography time scale of minutes.[84] The reversed-phase pattern in Figure 6b, however, proves that both fractions in Figure 6a contain the same protein: cytochrome *f*. If those fractions had contained different proteins of similar molecular masses, these proteins would have eluted separately. This did not happen. Instead, when all noncovalent interactions between the cytochrome *f* molecules were disrupted by denaturation in the reversed-phase experiment, the sample became homogeneous in terms of polarity.

Covalent cross-linking, followed by size-exclusion chromatography, is the standard method for detecting protein association.[85] An existing oligomer is captured by specific cross-linking, and a distinct signal appears in the chromatogram.[33,85,86] Random cross-linking gives a multitude of chromatographic features, which may be smeared.[85] As Figure 7 shows, cross-linked cytochrome *f* gave one distinct new fraction, whose molecular mass is approximately twice that of the protein (monomer). Although EDC was present in large excess over the protein, higher oligomers were absent. Evidently, cross-linking captures a dimer that already exists in solution. Because at the ionic strength of 700 mM all electrostatic interactions are absent, we conclude that *the protein dimer is held by hydrophobic forces*.

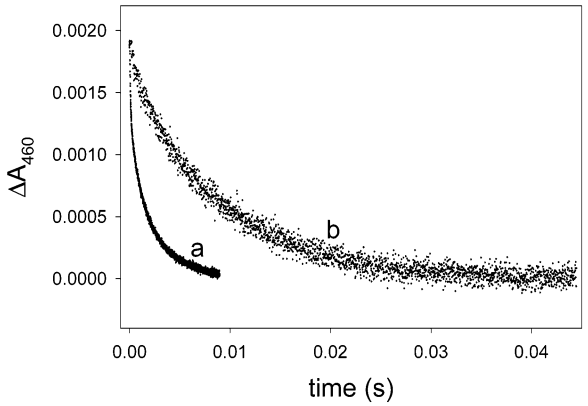

Figure 9. Disappearance of the triplet state ^{3}Zncyt c_6, monitored at 460 nm, in the solution initially containing 3.0 μM zinc cytochrome c_6 and (a) 3.0 μM cytochrome *f*(III) or (b) 3.0 μM cross-linked dimer of cytochrome *f*(III). The solvent in both cases is a sodium phosphate buffer at pH 7.00 and ionic strength of 300 mM. The triplet state is quenched in (a) but not in (b).

Kinetic Consequences of Cytochrome *f* Dimerization. As Figure 9 shows, monomeric cytochrome *f*(III) does, but the cross-linked dimer of cytochrome *f*(III) does not, quench the triplet state ^{3}Zncyt c_6. The observed rate constant in the latter case is that for the natural decay of the triplet. Because the cytochrome *f* dimer is formed owing to hydrophobic interactions (and is only reinforced by cross-linking), the protein molecules likely cover each other's nonpolar surfaces (Figure 8b), and the heme edge is no longer accessible to zinc cytochrome c_6.

Attempts to fit the results in Figure 3 to Scheme 1 with an improved steady-state approximation (eq 8) failed. This fitting method, which had succeeded in treatments of other diprotein complexes that associate and react by parallel unimolecular and bimolecular reactions,[12,13,51,56,61] is inapplicable to cytochrome c_6 and cytochrome *f*. The mechanism involving only association between the reactants (i.e., Scheme 1 alone) proved to be inadequate.

When our size-exclusion and reversed-phase HPLC separations and cross-linking experiments (discussed above) clearly showed that cytochrome *f* forms a homodimer in solution, we had to add the

$$2 \text{ cyt } f(\text{III}) \rightleftarrows \text{cyt } f(\text{III})/\text{cyt } f(\text{III}) \qquad (14)$$

equilibrium in eq 14 to Scheme 1. The fittings then became very good to excellent, as Figure 3 shows. The most surprising feature in Figure 3, namely the shifting of the "plateau point" to the increasing values of the [cyt *f*(III)]/[Zncyt c_6] ratio, was faithfully reproduced. *This ratio is greater than unity*, not because larger hetero-oligomers of zinc cytochrome c_6 and cytochrome *f*(III) form, but *because a redox-inactive homodimer of cytochrome f(III) forms*. As the concentration of this protein

(84) *Aqueous Size-Exclusion Chromatography;* Dubin, P. L., Ed.; Journal of Chromatography Library, Vol. 40; Elsevier: New York, 1988.
(85) Loster, K.; Josic, D. *J. Chromatogr., B: Biomed. Sci. Appl.* **1997**, *699*, 439−461.
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is raised, it increasingly associates not only with zinc cytochrome c_6, but also with itself, thus inhibiting the reaction in Scheme 1.

We can only conjecture about the significance in vivo of this process, which we documented in vitro. Dimerization of cytochrome *f* in the crystal has recently been considered.[23] The dimerization of cytochrome *f* from *C. reinhardtii* in aqueous solution is interesting because cytochrome b_6f is claimed to be a dimer, but its structure is unknown.[6,87−93] We detected dimerization at ionic strengths of both 300 and 700 mM, conditions relevant to the ionic strength in vivo, which is 300 mM.[24] We do not know of any other reports that kinetics of interprotein electron-transfer reaction is modulated by protein self-association, and we intend to study this mechanistic phenomenon.

Comparison of Cytochrome c_6 and Plastocyanin from *C. reinhardtii* in Their Association and Electron Transfer with Cytochrome *f* from *C. reinhardtii*. *C. reinhardtii* is the first organism known to biosynthesize both plastocyanin (when copper ions are available) and cytochrome c_6 (when they are unavailable) and to use either of these proteins as an electron carrier from cytochrome *f*(II) to $P700^+$ in photosystem I.[25] The reaction of each carrier with $P700^+$ is biphasic. Since the rate of the unimolecular process (the faster phase) is the same for copper(I) plastocyanin and cytochrome c_6(II), these two proteins probably interact similarly with photosystem I.[62] Association and reaction between cytochrome *f* and plastocyanin have been much studied[4−8,14,17,18,22,24,26,29,55,94,95] but mostly with heterologous proteins. Their association is largely electrostatic.[5−8,14,26,29,55]

This study, however, showed that *homologous proteins cytochrome f and cytochrome c_6 from C. reinhardtii associate by hydrophobic interactions*. Although the persistent and transient complexes differ in reactivity, their different intracomplex rate constants are similarly invariant with ionic strength.

Conclusions

Photoinduced electron-transfer reactions are "clean" and fast and therefore suitable for the study of protein association. Because electrostatic interactions are relatively easily detected and adjusted (by changing ionic strength), they have been much studied lately. Metalloprotein association, however, can be governed also by hydrophobic interactions, and resulting complexes can be as stable as typical electrostatic complexes. Persistent and transient complexes held by hydrophobic interactions differ so much in the interplay between electron-transfer step and configurational rearrangement that the intracomplex reaction is true electron transfer in the former and gated electron transfer in the latter. Remarkably, these reactions occur simultaneously in the same diprotein system. Not only association between electron donor and the electron acceptor, but also their self-association, should be kept in mind when analyzing complex reaction mechanisms and finding unexpected kinetics.

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Supporting Information Available: One figure showing the dependence of the relative amplitudes of the unimolecular reaction on cytochrome *f*(III) concentration, one figure showing the dependence of the ratio of cytochrome *f*(III) concentration and relative amplitude for the unimolecular reaction on the cytochrome *f*(III) concentration, one figure showing residuals for the fitting of traces in Figure 9 to the monoexponential function in eq 12, one figure showing the study of cytochrome c_6 by ultracentrifugation, and one figure showing size-exclusion chromatograms of cytochrome c_6 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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