Redox Reactivity and Reorganization Energy of Zinc Cytochrome *c* Cation Radical

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Received August 31, 1999

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

Introduction

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

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

$$\mathrm{Zncyt}^+ + [\mathrm{M(CN)}_n]^{4-} \xrightarrow{k_r} \mathrm{Zncyt} + [\mathrm{M(CN)}_n]^{3-} \qquad (1)$$

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

Experimental Section

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

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10.1021/ic9910514 CCC: $19.00

purchased from Alfa Æsar. All other chemicals were purchased from Fisher Chemical Co.

Synthesis and Purification of Cyano Complexes. The following complex salts were synthesized by standard methods: $K_4[Mo(CN)_8]\cdot 2H_2O$;[14] $K_4[Os(CN)_6]\cdot 3H_2O$;[15] $K_4[W(CN)_8]\cdot 2H_2O$;[16] and $K_3[W(CN)_8]\cdot 2H_2O$.[17] The salts were dissolved in several milliliters of water, and methanol was added until precipitation started.[18] The solution was cooled to 4 °C and left for 1 day. Translucent and opalescent crystals were filtered and rinsed twice with ethanol. The purification was done in the dark to avoid complex decomposition.[19]

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

Proteins. Horse-heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co. The iron-free form was made, purified, and reconstituted with zinc(II) ions by a modification[20] of the original procedure.[21] The product, zinc cytochrome *c*, was handled at 4 °C in the dark. The criteria of its purity were the absorbance ratios, $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$, and the rate constant of natural decay of the triplet state, less than 120 s^{-1}.[22] Concentration of Zncyt was determined spectrophotometrically on the basis of its molar absorptivity $\epsilon_{423} = 2.43 \times 10^5\ M^{-1}\ cm^{-1}$.[22]

Recombinant spinach plastocyanin was prepared according to the previously published method for overexpression in *Escherichia coli*.[23] The protein was purified first with a DE32 column and then with a 26/10 Q Sepharose high-performance fast protein liquid chromatography (FPLC) column from Amersham Pharmacia Biotech, Inc. Plastocyanin was oxidized with an excess of $K_3[Fe(CN)_6]$ and reduced with a small excess of ascorbic acid. The protein was concentrated, and the buffer was exchanged, with Centricon (Amicon, Millipore Corporation) and Macrosep (Filtron Technology Co.) concentrators. The criterion for purity was $A_{278}/A_{597} < 1.2$. The concentration of the protein was determined spectrophotometrically, on the basis of its molar absorptivity $\epsilon_{597} = 4700\ M^{-1}\ cm^{-1}$. The reduction potential of plastocyanin was taken to be $E_{1/2} = 0.384$ V.[23]

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

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

Cyclic voltammograms of metal complexes were recorded with a RDE4 bipotentiostat and a disk electrode having a surface area of 0.165 cm^2 from Pine Instrument, Co. A three-compartment electrochemical cell separated the working, reference, and counter electrodes. A saturated calomel electrode from Fisher Scientific Co. provided the reference potential. The counter electrode was a coiled platinum wire with a surface area of ca. 5 cm^2. The supporting electrolyte was a phosphate buffer at pH 7.00 with an ionic strength of 1.00 M. Data were collected with a personal computer equipped with an AT-MIO-16XE-50 board from National Instruments and with LabView software. The scan rate was 50 mV s^{-1} for $Ru(CN)_6^{4-}$ because of the high background current at potentials greater than 1.2 V and was 100 mV s^{-1} for the other complexes.

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

The second-order rate constant k_b, for the reaction of zinc cytochrome *c* cation radical and cuproplastocyanin, was determined by addition of as much as 30 μM cuproplastocyanin to the reaction mixture in which the concentration of Zncyt$^+$ was ca. 1 μM. The pseudo-first-order conditions were achieved again.

Kinetic Calculations. Kinetic traces from separate flashes were fitted, and results statistically analyzed, with SigmaPlot, version 3.06, software, using the least-squares averaging method. The correlation coefficients R^2 for the linear plots that provided the rate constants were greater than 0.990. The error margins for all results include two standard deviations and correspond to a confidence limit greater than 95%. The work terms, w_{ij}, and correction factors, W_{12} and f_{12}, were calculated with Microsoft Excel 97. The rate constant for diffusion and for activation-controlled electron transfer were calculated with modifications of the program kindly provided by Professor Claudia Turro.[25]

Results

Synthesis and Characterization of Cyano Complexes. Absorptivities at 190 nm of $K_4[Mo(CN)_8]\cdot 2H_2O$, $K_4[Os(CN)_6]\cdot 3H_2O$, and $K_3[W(CN)_8]\cdot 2H_2O$ were greater than the published value[26,27] because KCN, used in syntheses, was in excess and cocrystallized with the complex salts. For example, the salt $K_4[Os(CN)_6]\cdot 3H_2O$ showed two ^{13}C NMR resonances, at 142.3 and 165.1 ppm. The former is due to the cyano ligand and the latter to the free CN^- ion.[28] Recrystallization of crude salts from water failed because both they and KCN are very soluble in water.[29] Fractional crystallization from aqueous methanol succeeded. Absorption spectra of pure salts agreed with the published ones; ^{13}C NMR spectra of pure salts contained only one resonance due to the cyano ligands.

Because of a discrepancy in one report[30] about the reduction potentials of $Os(CN)_6^{4-/3-}$ and $Fe(CN)_6^{4-/3-}$, we determined the $E_{1/2}$ values of all the complex anions. Phosphate buffer at pH 7.00 and with an ionic strength of 1.00 M was used to record background current. Cyclic voltammograms of each complex had two well-resolved peaks corresponding to oxidation and reduction by one electron. The results in Table 1 completely agree with the results of other reports.[31−34]

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Table 1. Properties of the Cyano Complexes and Other Quantities Dependent on These Properties: Reduction Potential ($E_{1/2}$), Driving Force for the Reaction in Eq 1 (ΔG_r^0), Rate Constant for the Self-Exchange Reaction in Eq 18 (k_{22}), So-called Work Terms in the Marcus Theory (w_{12}, w_{21}, w_{22}, and W$_{12}$), the Factor f_{12}, Rate Constant for the Reaction in Eq 9 (k'_f), and Rate Constant for Reaction 1 (k_r)

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

[a] From ref 28.

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

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

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

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

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

When cupriplastocyanin, pc(II), is present at an ionic strength of 1.00 M, the triplet state disappears monoexponentially, as shown in Figure 1a. This quenching is the so-called forward electron-transfer reaction[9] in eq 4, the rate of which is linearly proportional to the concentration of pc(II). The change in absorbance at 675 nm (Figure 1b, upper trace) indicates the formation of the cation radical Zncyt$^+$.[5,9] Traces at 675 nm were fitted to

$$\Delta A = -a_1\, e^{-k_1 t} + a_b \frac{a_0}{1 + a_0 k_b t} \tag{5}$$

in which a_1 and a_b are the amplitudes for appearance and disappearance of Zncyt$^+$, a_0 is the concentration of Zncyt$^+$, k_1 is the pseudo-first-order rate constant for appearance of Zncyt$^+$, and k_b is the second-order rate constant for the thermal back-reaction in

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

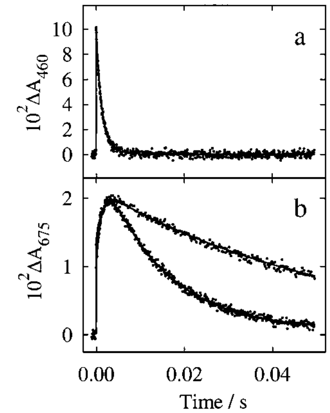

Figure 1. Transient absorbance changes in a solution containing 10 μM zinc cytochrome *c* and 30 μM cupriplastocyanin in a phosphate buffer at pH 7.00, with the ionic strength adjusted to 1.00 M with NaCl, and at 293 K: (a) triplet state, ^{3}Zncyt; (b) cation radical, Zncyt$^+$, in the absence of cyano complexes (upper trace) and in the presence of 4.0 μM $[Os(CN)_8]^{4-}$ complex. The upper trace was recorded for 0.200 s; it continues to decline but is truncated at 0.050 s to fit in the figure.

The change in absorbance was ca. 5 times smaller at 675 nm than at 460 nm. To improve accuracy in fittings of the smaller signals to eq 5, we determined k_1 precisely from the strong signals for the disappearance of ^{3}Zncyt at 460 nm, fixed this k_1 value, and then allowed a_1, a_b, and a_0 to vary. As more pc(II) was added, the maximum transient concentration of Zncyt$^+$ increased as well. In the thermal (so-called back) reaction (eq 6), the direction of electron transfer is the reverse of that in the forward reaction (eq 4), and the system returns to the state prior to the laser pulse, namely, Zncyt and pc(II).

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

Reduction of Zncyt$^+$ by Cyano Complexes and Side Reactions. When $[Os(CN)_6]^{4-}$, $[Mo(CN)_8]^{4-}$, or $[Ru(CN)_6]^{4-}$ complex is added to the reaction in eq 4, the disappearance of Zncyt$^+$ becomes faster, as the lower trace in Figure 1b shows. These kinetic traces were fitted to

$$\Delta A = -a_1\, e^{-k_1 t} + a_b \frac{a_0}{1 + a_0 k_b t} + a_2\, e^{-k_2 t} \tag{7}$$

in which the last term corresponds to the reaction of interest, that is, eq 1. The first-order rate constant k_2 is linearly

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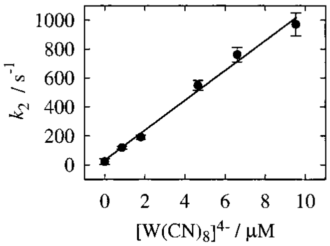

Figure 2. Rate constant k_2 for the disappearance of the cation radical Zncyt^{+} as a function of the concentration of the $[W(CN)_8]^{4-}$ complex in a phosphate buffer at pH 7.00, with the ionic strength adjusted to 1.00 M with NaCl, and at 293 K.

proportional to the concentration of the cyano complex, as Figure 2 shows. The k_2 values are given in Table S1 in the Supporting Information. Since the cyano complexes were present in excess over Zncyt^{+}, the pseudo-first-order conditions were satisfied. Plots of k_2 versus the concentration of the cyano complexes gave the second-order rate constants k_r for the reaction of interest (eq 1), which are given in Table 1.

With $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ complexes the first-order kinetic plots showed slight curvature. Moreover, the rate of ^{3}Zncyt quenching in the presence of pc(II) increased with addition of these two complexes. To check whether the triplet was directly quenched by the cyano complexes, we separately treated ^{3}Zncyt with relatively high concentrations (up to 40 μM) of all five 4− complexes in the absence of pc(II). None of the $[M(CN)_n]^{4-}$ complexes quenched ^{3}Zncyt. We concluded that the observed quenching in the complete reaction mixtures containing $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ is due to the $[Fe(CN)_6]^{3-}$ and $[W(CN)_8]^{3-}$ complexes, products of oxidation of the corresponding 4− complexes by pc(II). To test this hypothesis, we treated a 30 μM solution of pc(II) with various concentrations of $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ ions and easily detected bleaching of the blue color (reduction of the protein) by UV−vis spectrophotometry. This side reaction is shown in

$$\mathrm{pc(II)} + [\mathrm{M(CN)}_n]^{4-} \overset{K_e}{\rightleftharpoons} \mathrm{pc(I)} + [\mathrm{M(CN)}_n]^{3-} \tag{8}$$

in which M is Fe or W and n is 6 or 8. The equilibrium constant, K_e, is 21 ± 5 for $[Fe(CN)_6]^{4-/3-}$ and 1200 ± 200 for $[W(CN)_8]^{4-/3-}$. Those results agree with the K_e values determined from the reduction potentials, which are 17 and 1070.

The 3− cyano complexes formed in one side reaction (eq 8) undergo another side reaction, shown in

$$^3\mathrm{Zncyt} + [\mathrm{M(CN)}_n]^{3-} \xrightarrow{k_f'} \mathrm{Zncyt}^{+} + [\mathrm{M(CN)}_n]^{4-} \tag{9}$$

In two series of control experiments, we separately determined the rate constants k_f' listed in Table 1. Because reaction 9 (k_f') competes with reaction 4 (k_f) and because in both of them electron transfer occurs in the forward direction, the corresponding rate constants have similar symbols.

We can quantitatively determine the contributions of reactions 4 (k_f) and 9 (k_f') to the observed rate of ^{3}Zncyt quenching in the presence of both pc(II) and the $[M(CN)_6]^{3-}$ complex of Fe(III) or W(V). For example, the rate constant of ^{3}Zncyt quenching by 30 μM pc(II) is $k_1 = 600 \pm 20$ s^{-1}. Upon addition of 10 μM $[W(CN)_8]^{4-}$ the rate constant becomes $k_1 = 1200 \pm 100$ s^{-1}, an increase of ca. 600 s^{-1}. The concentration of $[W(CN)_8]^{3-}$, calculated from the given concentrations of the reactants and the known K_e in eq 8, is 0.47 μM. Therefore, the contribution to k_1 from the side reaction in eq 9 is $(1.15 \times 10^9\ \mathrm{M^{-1}\ s^{-1}})(4.7 \times 10^{-7}\ \mathrm{M}) = 540\ \mathrm{s^{-1}} \approx 600\ \mathrm{s^{-1}}$. The additional quenching of ^{3}Zncyt by the 3− complex is accounted for. Because the 4− complexes of Os(II), Mo(IV), and Ru(II) have reduction potentials that are too high to be oxidized by pc(II), i.e., they do not undergo the side reaction in eq 8, the corresponding 3− complexes are not formed, and the side reaction in eq 9 does not occur. Although the side reactions in eqs 8 and 9 with the iron and tungsten complexes are unavoidable, we studied their kinetics in detail and separated these reactions from the reaction of interest, reaction 1 (k_r).

Concentrations of the 4− complexes of Fe(II) and W(IV) in the reaction mixture could not be determined simply from the amounts added to the mixture because of the side reaction in eq 8; we calculated these concentrations on the basis of K_e. In the cases of Os(II), Mo(IV), and Ru(II), the concentrations of the 4− complexes could be determined simply. Plots of first-order rate constants k_2 vs concentration of the $[M(CN)_n]^{4-}$ complexes were linear. The slopes of these plots yielded the second-order rate constants k_r for the reduction of the cation radical Zncyt^{+} by $[M(CN)_n]^{4-}$ complexes (eq 1).

Reorganization Energy of Zncyt^{+}. The bimolecular reaction in eq 1 occurs in two consecutive steps; diffusion of the two reactants is followed by activated electron transfer between them, hence, the two contributions, k_{diff} and k_{act}, to the rate constant k_r in the following equation:[35,36]

$$k_r = \frac{k_{\mathrm{act}} k_{\mathrm{diff}}}{k_{\mathrm{act}} + k_{\mathrm{diff}}} \tag{10}$$

To calculate k_{diff}, we treat cytochrome c and the cyano complexes as charged spheres[36] and we treat the intermolecular potential between them, U, as predominantly electrostatic. In

$$k_{\mathrm{diff}} = \frac{4\pi N\mathrm{D}}{1000\int_r^{\infty} \mathrm{d}r\, r^2 \exp\left(-\frac{U}{k_B T}\right)} \tag{11}$$

and

$$U = \frac{z_1 z_2\, e^2}{D_s r(1 + \beta r\sqrt{\mu})} \tag{12}$$

$D = 8.08 \times 10^{-10}$ m^2 s^{-1} is the sum of diffusion coefficients for cytochrome c and $[M(CN)_n]^{4-}$, $D_s = 8.89 \times 10^{-9}$ F m^{-1} is the static dielectric constant of the solvent, $k_B = 1.38 \times 10^{-23}$ J K^{-1} is the Boltzman constant, and $\beta = 1.05 \times 10^8$ M$^{-1/2}$ m^{-1} is a factor at 293 K, defined in

$$\beta = \sqrt{\frac{8\pi N e^2}{D_s k_B T}} \tag{13}$$

The result is $k_{\mathrm{diff}} = 1.8 \times 10^{10}$ M^{-1} s^{-1}. This value is marked with a horizontal line in Figure 3. We also calculated k_{diff} according to van Leeuwen theory, taking into account the dipole moment of cytochrome c. The result is $k_{\mathrm{diff}} = 2.5 \times 10^{10}$ M^{-1} s^{-1}, consistent with the previous one. The k_{diff} value is reasonable and will be justified in the Discussion.

When the reactants come sufficiently close to one other, an electron can be transferred over a range of distances, d, between the nearest atoms. Therefore, the rate constant k_{act} is obtained by integrating the rate constant for the electron transfer, k_{et}, from

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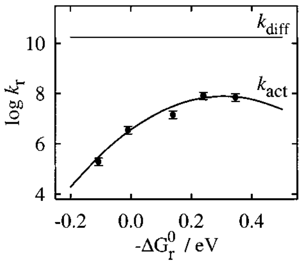

Figure 3. The curve is the so-called Marcus plot for reaction 1, the best fit to eqs 10 and 14−16. The plotted quantities are in Table 1. The horizontal line is at k_{diff}, the result of the calculation according to eqs 11−13.

the contact distance to infinity. The driving force, ΔG_r^0, for the reaction in eq 1 was calculated from the reduction potentials of Zncyt$^+$ and the $[M(CN)_n]^{4-}$ complexes and was corrected for the work required to bring the reactants and the products together.[36] The rate constants k_r and ΔG_r^0, listed in Table 1, were fitted to eqs 10 and 14−16.[25,36]

$$k_{act} = \frac{4\pi N}{1000}\int_r^\infty dr\, r^2 k_{et} \exp\left(-\frac{U}{k_B T}\right) \tag{14}$$

$$k_{et} = \frac{4\pi^2 {H_{DA}}^2}{hN\sqrt{4\pi\lambda RT}} \exp\left(-\frac{(\Delta G_r^0 + \lambda)^2}{4\lambda RT}\right) \tag{15}$$

$$H_{DA} = (200\ \mathrm{cm}^{-1}) \exp\left\{-\frac{(1.2\ \text{Å}^{-1})[d - (3\ \text{Å})]}{2}\right\} \tag{16}$$

In eq 16 the electronic coupling between the electron donor and the electron acceptor (so-called tunneling matrix element, H_{DA}) is taken to be 200 cm^{-1} when $d = 3$ Å, and it decreases with distance by the factor 1.2 Å^{-1}; these are accepted values.[36−38] Fitting to Marcus theory usually is done with different forms of the same equations, in which quantities have various units. Because there are many ways to do this task correctly, we briefly explain our fittings to avoid confusion. In eq 15 $h = 6.625 \times 10^{-34}$ J s, $N = 6.023 \times 10^{23}$ mol^{-1}, λ is the reorganization energy in J mol^{-1}, $R = 8.314$ J K^{-1}mol^{-1}, T is in K, and ΔG_r^0 is in J mol^{-1}. In the fittings of ΔG_r^0 and k_r to eqs 10 and 14−16 there were only two variable parameters; all the other quantities were known. The best fit, shown in Figure 3, yielded $d = 3.8$ Å and $\lambda = 0.35 \pm 0.05$ eV.

Calculation of Self-Exchange Rate Constant for Zncyt$^+$ and Zncyt. The self-exchange redox reaction in eq 17 cannot be studied directly, but the reaction in eq 1 can. We used Marcus cross-relation (eq 19) to estimate the rate constant k_{11}. The rate constant k_r (in the general case it is k_{12}) is proportional to the self-exchange rate constants k_{11},

$$\mathrm{Zncyt} + \mathrm{Zncyt}^+ \xrightarrow{k_{11}} \mathrm{Zncyt}^+ + \mathrm{Zncyt} \tag{17}$$

and k_{22},

$$[M(CN)_n]^{4-} + [M(CN)_n]^{3-} \xrightarrow{k_{22}} [M(CN)_n]^{3-} + [M(CN)_n]^{4-} \tag{18}$$

k_{11} is given by

$$k_{11} = \frac{{k_r}^2}{k_{22} K_r f_{12} {W_{12}}^2} \tag{19}$$

The equilibrium constant K_r (in the general case it is K_{12}) for the reaction of interest (eq 1), the factor f_{12}, and the work W_{12} required to bring two reactants together are unitless numbers calculated from the following equations (see Table 1):

$$K_r = \exp\left(-\frac{zF\Delta E_{1/2}}{RT}\right) \tag{20}$$

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

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

$$w_{ij} = \frac{z_i z_j e^2\left(\frac{\exp(\beta\sigma_i\sqrt{\mu})}{1 + \beta\sigma_i\sqrt{\mu}} + \frac{\exp(\beta\sigma_j\sqrt{\mu})}{1 + \beta\sigma_j\sqrt{\mu}}\right)}{2D_s r \exp(\beta r\sqrt{\mu})} \tag{23}$$

The other quantities are as follows: z is the number of electrons exchanged (one in our case), $\Delta E_{1/2}$ is in volts, $F = 96485$ F mol^{-1}, $R = 8.314$ J K^{-1} mol^{-1}, $T = 293$ K, and the collision frequency $Z = 1 \times 10^{11}$ M^{-1} s^{-1}.[36,39]

When the reactants bear opposite charges, the Marcus cross-relation may be inapplicable.[36] The problems can be minimized by raising ionic strength, as we did. When the reactants are approximately spherical and the work required to bring them together is predominantly Coulombic, the driving force can be corrected with the so-called work terms, w_{ij} (eq 23). We did this for reactions 1, 17, and 18.

In eq 23, σ_i and σ_j are the sums of radii (in meters) of reacting ions and predominant counterions: Zncyt and Zncyt$^+$ (17.5×10^{-10} m) with Cl$^-$ (1.81×10^{-10} m) and $[Fe(CN)_6]^{4-}$ (4.50×10^{-10} m), $[Ru(CN)_6]^{4-}$ (4.70×10^{-10} m), $[W(CN)_8]$,$^{4-}$ $[Os(CN)_6]^{4-}$, and $[Mo(CN)_8]^{4-}$ (4.80×10^{-10} m each) with Na$^+$ (1.10×10^{-10} m).[34] Also, in eq 23 μ is the ionic strength in mol m^{-3}, charges z_i and z_j are +7 for Zncyt$^+$, +6 for Zncyt, −4 for $[M(CN)_n]^{4-}$, and −3 for $[M(CN)_n]^{3-}$, the elemental charge is $e = 1.6 \times 10^{-19}$ C, the dielectric constant of the medium is $D_s = 4\pi\epsilon\epsilon_0 = 8.89 \times 10^{-9}$ F m^{-1}, and the radius of the transient complex, r, is the sum of the Zncyt$^+$ and $[M(CN)_n]^{4-}$ radii in meters.

Since the self-exchange rate constant k_{11} (eq 17) is needed for the calculation of f_{12}, we used the experimental result[40] for cytochrome *c*, $k_{11} = 5 \times 10^4$ M^{-1} s^{-1}, as an approximate starting value in our calculations. The self-exchange rate constant of interest, k_{11}, can be determined from Figure 4 and from the equation

$$k_{11} = \exp[2(\text{intercept})] \tag{24}$$

The intercept with the vertical axis was calculated iteratively, with the k_{11} value from the previous cycle as input for the next.

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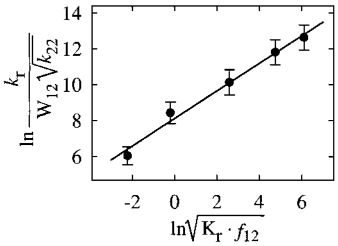
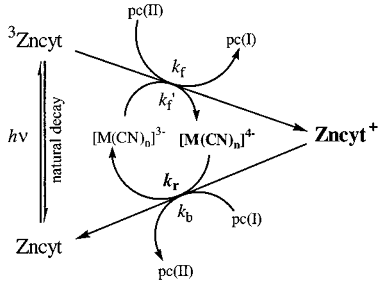


Figure 4. Determination of the rate constant k_{11} for the self-exchange reaction between Zncyt$^+$ and Zncyt (eq 17) based on the kinetics of the reaction in eq 1 involving five $[M(CN)_n]^{4-}$ complexes. The straight line is the best fit of these kinetic results to eqs 19−24.

Scheme 1



The iterations converged, and the self-consistent result was obtained: $k_{11} = (1.0 \pm 0.5) \times 10^7$ M^{-1} s^{-1}.

Discussion

Chemical Reactions. All the reactions occurring in this intricate system are shown in Scheme 1. Excitation (*hν*) of the porphyrin chromophore in Zncyt indirectly produces the long-lived triplet state, ^{3}Zncyt. In the absence of a quencher, this state decays to the ground state. The blue copper protein cupriplastocyanin, pc(II), quenches ^{3}Zncyt in a reaction that is much faster than natural decay; the rate constant of this forward electron-transfer reaction is designated k_f. The copper site becomes reduced into pc(I), and the porphyrin becomes oxidized into Zncyt$^+$. This cation radical is short-lived because it undergoes the back-electron-transfer reaction whose rate constant is designated k_b. In this thermal reaction, an electron moves from the copper site to the porphyrin; the Zncyt$^+$ is reduced and converted back to Zncyt. The cycle is completed.

The transient species Zncyt$^+$ is the main object of our study. We separately add five cyano complexes $[M(CN)_n]^{4-}$ to reduce Zncyt$^+$, in competition with pc(I). The fraction of Zncyt$^+$ reduced by $[M(CN)_n]^{4-}$ depends on the concentration of the cyano complex and the relative magnitudes of k_r and k_b.[41] Reduction of Zncyt$^+$ by $[M(CN)_n]^{4-}$ produces Zncyt and $[M(CN)_n]^{3-}$. This last reaction is irreversible,[5] and the 3− complex in turn oxidizes pc(I) to regenerate the 4− complex and pc(II), according to eq 8. Again, the cycle is completed.

Kinetics becomes further complicated if a fraction of the $[M(CN)_n]^{4-}$ complex is oxidized to $[M(CN)_n]^{3-}$ by pc(II). This was the case with $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$, the only two complexes thermodynamically capable of this reaction. Because this equilibrium (shown in eq 8) is established prior to the laser pulse, it is omitted from Scheme 1. The important consequence of this side reaction is that, in addition to Zncyt, pc(II), and $[M(CN)_n]^{4-}$, which are added to the solution, the reaction mixture contains also some $[M(CN)_n]^{3-}$. This is a new species, which undergoes reaction 9 (k_f'). The equilibrium process in eq 8 also causes a small conversion of pc(II) to pc(I). We quantified this conversion in all experiments and adjusted the concentrations of both forms of plastocyanin in kinetic calculations concerning the reactions 4 and 6 (k_f and k_b, respectively). The measured rate constant k_b used in these calculations is $(3.6 \pm 0.2) \times 10^6$ M^{-1} s^{-1}.

Requirements of the Reductants for Zncyt$^+$. To serve as a reducing agent in the reaction of interest (eq 1 and k_r in Scheme 1), a compound should meet several requirements. Because Zncyt$^+$ bears a charge +7 at pH 7.00, its reaction partner should bear a negative (preferably high) charge, for favorable association. The compound should undergo only one-electron oxidation to match the oxidizing capacity of Zncyt$^+$. The compound should be stable in both the reduced and the oxidized form, that is, as both the reactant and the product in eq 1. Because we trigger the thermal redox reaction photochemically, the reductant and its oxidized form must not absorb in the wavelength ranges in which Zncyt is excited (by the laser pulse) and in which ^{3}Zncyt and Zncyt$^+$ are monitored (by the spectrophotometric beam). The five transition-metal cyano complexes in Table 1 meet all of these requirements. Moreover, they have similar, highly symmetrical shapes and the same charge. Because of this uniformity, we did not have to be concerned with the effects of binding affinity (i.e., the association constant) on the bimolecular rate constant k_r.[42] These five complexes constitute an ideal series of reductants with which to test the reactivity of Zncyt$^+$ as an oxidant.

To reduce Zncyt$^+$, an agent also must be a member of a redox couple with a reduction potential of ca. 0.80 V or lower. To avoid reducing pc(II), whose reduction potential is 0.384 V, the reduction potential of the suitable couple must be ca. 0.40 V or higher. The values near this limit posed a problem; both $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ ions partially reduce pc(II). We overcame this problem by careful studies of reactions 8 and 9. Knowing the exact concentration of $[Fe(CN)_6]^{3-}$ and $[W(CN)_8]^{3-}$, we were able to account for contributions to the ^{3}Zncyt quenching from both rate constants k_f' and k_r. Because the complexes $[Mo(CN)_8]^{4-}$ and $[Ru(CN)_6]^{4-}$ belong to redox couples with reduction potentials slightly greater than 0.80 V, their reactions involving k_r occur slightly “uphill” in the thermodynamic sense, and the 4− complexes had to be used in great excess over Zncyt$^+$. The small amount of 3− complex formed in reaction 1 (k_r) was rapidly removed from the reaction mixture by pc(I), according to eq 8. In this way, the reduction of Zncyt$^+$, even with these two complexes, was irreversible.

Because the 4− cyano complex has to compete with pc(I) for reduction of Zncyt$^+$, reaction 1 (k_r) has to be faster than reaction 6 (k_b). Fortunately, this was the case with the complexes $[Mo(CN)_8]^{4-}$ and $[Ru(CN)_6]^{4-}$. Because weaker reducing reagents would not necessarily meet this kinetic requirement, we could not go beyond the reduction potential of 0.907 V.

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

Side Reactions. When the reaction mixture contained $[Fe(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$, quenching of ^{3}Zncyt by pc(II), which we initially considered the only quencher, was faster than we expected on the basis of the rate constant k_f. To study the

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reaction of interest, eq 1 (k_r), we had to use relatively high concentrations of pc(II), a condition that favored the side reaction in eq 8. The products of this side reaction, complexes $[Fe(CN)_6]^{3-}$ and $[W(CN)_8]^{3-}$, also turned out to quench ^{3}Zncyt, as in eq 9. We prepared these two 3− complexes, unexpected ingredients of the reaction mixtures, and determined the equilibrium constants K_e (eq 8) and the rate constants k_f' (eq 9). Both quenching reactions 4 (k_f) and 9 (k_f') are shown in Scheme 1. Because k_f' is 6 times greater in the case of $[Fe(CN)_6]^{3-}$ and 100 times greater in the case of $[W(CN)_8]^{3-}$ than k_f, even the low concentrations of these 3− complexes in the reaction mixture markedly enhanced the observed quenching of ^{3}Zncyt. Knowing both k_f and k_f' values, we quantitatively accounted for this enhanced rate. Then we returned to our main purpose, the study of reaction 1 (k_r) and of the properties of Zncyt$^+$ that govern its redox reactions.

Formation and Lifetime of Zncyt$^+$. This cation radical is formed by oxidative quenching of the triplet state, ^{3}Zncyt, in reactions 4 and 6 (k_f and k_f', respectively). To prolong the lifetime of Zncyt$^+$ so that we can study its reactivity (eq 1), we had to disfavor back-reaction 6 (k_b).

At first, we tried to keep the reaction mixture at pH 4.00 and at an ionic strength of 40 mM, at which plastocyanin is easily reduced, so that reaction 4 (k_f) would occur, but not reoxidized, so that reaction 6 (k_b) would be hampered.[43] These experiments failed because of partial denaturation of pc(I) in acidic solution.

The reactants Zncyt$^+$ and pc(I) bear charges of +7 and −9 at pH 7.00. To disfavor the undesirable back-reaction 6, k_b in Scheme 1, we added NaCl to the buffer at pH 7.00. By keeping the ionic strength at 1.00 M, we were able to prolong the lifetime of Zncyt$^+$ and make it accessible to the cyano complexes $[M(CN)_n]^{4-}$.

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

The leveling off, predicted by Marcus theory,[36] could be due to two causes. First, the reaction could have became diffusion-limited ($k_{act} \gg k_{diff}$ in eq 10) and therefore independent of the driving force (ΔG_r^0). In this case, the diffusion limit would have to be approximately equal to, or a little greater than, 7×10^7 M^{-1} s^{-1}, the value at which the plot in Figure 3 levels off. Second, the reaction could be a case of true electron transfer ($k_{act} \ll k_{diff}$ in eq 10) that became nearly activationless ($\Delta G_r^0 \approx \lambda$ in eq 15) with the $[Fe(CN)_6]^{4-}$ as the reductant. In this case the diffusion limit would be grater than 1×10^8 M^{-1} s^{-1}.

To distinguish between these two cases, we calculated the rate constant for diffusion because the experimental determination has proven to be very difficult.[36,44] To calculate the rate constant for diffusion, k_{diff}, we applied the Smoluchowski equation[45] to Zncyt$^+$ and $[Fe(CN)_6]^{4-}$. The product of their charges is $(+7)(-4) = -28$; the rate constant for diffusion is $k_{diff} = 1.8 \times 10^{10}$ M^{-1} s^{-1}. This result generally agrees with the experimental value of 4.2×10^9 M^{-1} s^{-1}, obtained for a somewhat similar pair of reactants: pc(II) with a net charge of −8 and $[Ru(bpy)_3]^{2+}$ in a reaction with a high driving force ($\Delta G = 1.2$ eV) that is believed to be diffusion-controlled.[36] The calculated rate constant for diffusion from eqs 11−13 for pc(II) and $[Ru(bpy)_3]^{2+}$ is 6×10^9 M^{-1} s^{-1}. Electrostatic effects are slight at an ionic strength of 1.00 M, but a greater (negative) product of charges still corresponds to a 3 times faster diffusion, which is in excellent agreement with our calculated value. We may tentatively conclude that the diffusion limit lies well above the plateau in Figure 3, i.e., that reaction 1 (k_r) has not become diffusion-controlled in the case of $[Fe(CN)_6]^{4-}$.

Because the Smoluchowski equation tends to overestimate k_{diff} (while still realistically predicting its relative values for related systems),[46] we further tested the nature of the reaction in eq 1. We know the experimental rate constant, $k_f' = 7.7 \times 10^8$ M^{-1} s^{-1}, for reaction 9, which involves essentially the same reactants except with different charges, whose product is $(+6)(-3) = -18$. If reaction 9 (k_f') were diffusion-controlled, then we could estimate the rate constant of interest, k_r. The product of charges, −28, would correspond to $k_r = 1.2 \times 10^9$ M^{-1} s^{-1}, a value greater than k_f'. The experimental result, $k_r = 7 \times 10^7$ M^{-1} s^{-1} at the plateau, is much lower than the estimate and the known k_f'. Clearly, it is not onset of diffusion control that causes the plateau in Figure 3. We conclude that the reaction in eq 1 is a case of true electron transfer, which is approaching or has reached the activationless regime. Fitting to the Marcus equation is justified.

Fitting to Marcus Equation and Reorganization Energy of Zncyt$^+$. Because the five cyano complexes in eq 1 have similar structures and the same charge, the fitting in Figure 3 is meaningful. One of its results is the edge-to-edge distance between the heme and cyano complex, which is $d = 3.9 \pm 0.2$ Å. This result is in excellent agreement with the previously determined reaction distances for cytochrome *c* and anionic complexes,[47] but the negatively charged cyano complexes seem to react at a closer distance than the positively charged Ru^{2+} complexes.[25] We conclude that the electron is transferred at the exposed-heme edge and that the significant localization of the unpaired spin on the meso carbon atoms[48,49] may render the singly occupied a_{2u} orbital accessible to the $[M(CN)_n]^{4-}$ complex. Because of inherent errors associated with the estimate of reaction distance,[47] we abstain from further discussion.

The other, and more interesting, result of our finding is the total reorganization energy, $\lambda = 0.35 \pm 0.05$ eV. As the equation

$$\lambda \approx \frac{\lambda_{Zncyt^+} + \lambda_{[M(CN)_n]^{4-}}}{2} \tag{25}$$

shows, this quantity is approximately equal to the average reorganization energy of the two reactants.[50] Each of these energies is made up of inner-sphere and outer-sphere terms. For the cyano complexes these separate terms have been estimated at 0.031 and 0.29 eV, respectively:[28]

$$\lambda_{[M(CN)_n]^{4-}} = \lambda_{in} + \lambda_{out} \tag{26}$$

Evidently, the 4− and 3− forms of the complexes have very similar structures (bond lengths) but are solvated to different extents.

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Given $\lambda_{[\mathrm{M(CN)}_n]^{4-}} = 0.32$ eV, from eq 25 we estimate $\lambda_{\mathrm{Zncyt}^+} = 0.38$ eV. This result can be compared with the reorganization energy of native cytochrome *c* (in ferric and ferrous oxidation states). Estimates of this quantity vary widely, between 0.5[36] and 1.2 eV.[51] The experimental study of the self-exchange redox reaction gave the result of 0.72 eV.[40] Recent theoretical studies yielded the values of 0.69,[52] 0.87,[53] and 0.68 eV.[54] The last four results are fairly consistent, and we take the reorganization energy of native cytochrome *c* to be their average, or 0.74 eV.

Oxidation and reduction of the iron protein occur predominantly at this transition metal, on which are largely localized the redox orbitals of the heme.[5] Particularly affected is the Fe−S bond involving the axial ligand Met80, which changes by 0.08 Å.[55] Because the protein surface is not significantly perturbed by the replacement of the metal ion,[8] solvation, the chief outer-sphere factor, may be considered unchanged. Oxidation and reduction of the zinc protein occur at the porphyrin ring. Changes in the occupancy of the delocalized redox orbital are expected to have only a slight effect on the porphyrin geometry. The zinc(II) ion seems to be five-coordinate, detached from Met80.[20] Without this bond, Met80 probably is not affected by the oxidation state of the heme; yet another source of inner-sphere reorganization energy is absent in zinc cytochrome *c*.

Conformational and other structural differences between the iron(II) and iron(III) forms of cytochrome *c* are still being debated,[56−61] and their contributions to the reorganization energy are difficult to estimate.[62] Without the firm knowledge about the native protein, we can only suppose that the decrease in total reorganization energy from 0.74 to 0.38 eV is due mainly to a decrease in the internal structural rearrangement from native to zinc-substituted cytochrome *c*.

Electron Self-Exchange between Zncyt and Zncyt$^+$. The rate constant k_{11} (eq 17) is 200 times greater than the corresponding rate constant for native cytochrome *c*, at an ionic strength of 1.00 M. We attribute this greater intrinsic reactivity to the greater accessibility of the redox orbital and lower reorganization energy, properties discussed in the preceding subsection.

Conclusion

The iron and zinc forms of the same protein, cytochrome *c*, differ markedly in their properties as redox agents. For meaningful fittings to Marcus theory, kinetic results obtained with heme proteins and their reconstituted forms should not be used in the same plot.[5,63] Ground-state and excited-state redox reactions should not be treated together, as they sometimes are in studies of this kind.

Acknowledgment. We thank Dr. Mikael Ejdebäck, of Göteburg University (now Skövde University), for reconstituted spinach plastocyanin, Dr. Nataša D. Popović for electrochemical measurements, Dr. Natalia Kaminskaia for NMR measurements, Professor Claudia Turro of Ohio State University for the program for calculating the rate constant of diffusion, Dr. Tatjana N. Parac for sharing her preliminary kinetics results with us; Mr. Nebojša M. Milović for help with the manuscript, and an anonymous reviewer for helpful advice about kinetic treatment. This study was funded by the National Science Foundation, through Grant MCB-9808392.

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

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