compounds react readily photolytically. Such an unusual order of reactivity of nitrobenzyl alcohols can be nicely interpreted in terms of the results presented in this paper. Furthermore, from the point of view of the appearance of nodal planes and less significant charge transfer in the excited state of 3,5-$A_2$-ph-D systems, one would predict that (3,5-dinitrophenyl)acetylene should undergo no or inefficient photohydration reaction.[21] The photophysics and photochemistry of such systems are currently under investigation in our laboratory.

Finally, Figure 4 summarizes the relative directions of $\hat{p}$, $\mu_g$, and $\mu_e$ for all three different substituted benzene systems studied here. Even though these directions are definitively assigned for *p*-A-ph-D and 3,5-$A_2$-ph-D systems, similar assignment is less clear in the case of corresponding meta isomers. However, the experimental values in Table II suggest that depending on the nature of the substituents the angle $\theta$ varies between 0° and 90° ($0° < \theta < 90°$).

Both (*p*- and (*m*-nitrophenyl)acetylene (compounds VII and VIII) are known to undergo fast and efficient photohydration from the first excited triplet state.[21] The enhancement of reactivity in both singlet and triplet state photohydrations has been explained as due to a high degree of charge transfer in the respective states, which facilitates the protonation step. $\Delta\mu$ values of (*p*- and (*m*-nitrophenyl)acetylene, which are quite large, provide direct evidence for extensive charge transfer in the excited state of such systems. Though the $\Delta\mu$ values represent the charge transfer in the excited singlet $\pi,\pi^*$ state, there is no reason to invalidate a similar magnitude of charge transfer in the triplet $\pi\pi^*$ state of VII and VIII, from which the reaction proceeds in the above systems. In fact, semiempirical MO calculations on simple nitro compounds show very little difference in the calculated dipole moments of corresponding singlet and triplet states, which supports the above argument.[22]

## Experimental Section

*p*-Nitroaniline (I), *m*-nitroaniline (II), 3,5-dinitroaniline (III), *p*-nitroanisole (IV), *m*-nitroanisole (V), and 3,5-dinitroanisole (VI) were obtained from Aldrich Chemicals and recrystallized from appropriate solvents prior to use. (*p*-Nitrophenyl)acetylene (VII) and (*m*-nitrophenyl)acetylene (VIII) were synthesized by methods reported in the literature.[23] Spectrograde dioxane, from Aldrich Chemicals, was used as solvent without further purification. The solution was continuously circulated by a micropump into the electric field cell from a reservoir to avoid any significant photochemical decomposition.

## Conclusion

Variation of nature or position of substituents on the benzene ring has a profound effect on the charge-transfer characteristics of the molecule, particularly in the excited state. The simple additive nature of group moments, which is true for the ground state, is found to be inapplicable in explaining the observed dipole moment of the excited state. This indicates quite different substituent effects in the excited state. Depending on the position of the substituents, the direction of the transition moment varies between 0° and 90° with respect to the direction of $\mu_g$, rendering the HOMO-to-LUMO transition either $^1L_a$ or $^1L_b$ type in the extreme cases. Occurrence of extensive charge transfer in the excited states and the presence of a nodal plane through the reaction center are found to be the key factors in many photochemical reactions.

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# Kinetics of Static and Diffusive Electron Transfer between Zinc-Substituted Cytochrome *c* and Plastocyanin. Indications of Nonelectrostatic Interactions between Highly Charged Metalloproteins

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**Abstract:** Cupriplastocyanin, pc(II), quenches the triplet state of zinc cytochrome *c*, $^3$Zn(cyt), by electron transfer as shown in Scheme I. All the experiments are done at pH 7.0. Nonredox modes of quenching are ruled out by detection of the cation radical Zn(cyt)$^+$ and by experiments in which redox quenching is precluded. At the ionic strengths of 0.100 M and higher, the electron-transfer reaction occurs bimolecularly, via the encounter complex $^3$Zn(cyt)/pc(II); $k_f = (2.8 \pm 0.6) \times 10^5$ s$^{-1}$, and the equilibrium constant $K_a$ depends on ionic strength. At the ionic strengths of 10 mM and lower, the reaction can be made to occur predominantly unimolecularly, within the preformed complex $^3$Zn(cyt)/pc(II); $k_F = (2.5 \pm 0.4) \times 10^5$ s$^{-1}$ within $^3$Zn(cyt)/pc(II), and $k_B = (1.1 \pm 0.5) \times 10^6$ s$^{-1}$ within Zn(cyt)$^+$/pc(I). The rate constant $k_f$ is independent of ionic strength (in the range from 10 to 100 mM) and so is the rate constant $k_F$ (below 20 mM). The equality of $k_f$ and $k_F$ shows either that the encounter complex and the preformed complex have structures with equal electronic couplings and activation energies for electron transfer or that both complexes can reach such structures by fast rearrangement before the electron-transfer step. The estimated association constant $K_a$ for zinc cytochrome *c* and cupriplastocyanin at zero ionic strength is $(2 \pm 1) \times 10^7$ M$^{-1}$. Cytochrome *c* interacts similarly with various anionic metalloproteins, and replacement of iron with zinc does not noticeably alter these docking interactions. As the ionic strength increases, the efficiency of charge separation in the bimolecular reaction first increases and then decreases. At high ionic strength, even these charged proteins perhaps attract each other by hydrophobic or other nonelectrostatic forces.

## I. Introduction

Metalloproteins participate in various biological oxidoreduction processes, and it is important to understand kinetics and mechanisms of their electron-transfer reactions. Many studies have dealt with bimolecular reactions between proteins and, lately, also with unimolecular reactions within modified proteins[1-12] and within

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diprotein complexes.[13-35] Both unimolecular and bimolecular reactions occur in photosynthetic and respiratory electron-transport chains; when a reaction involves associated proteins, e.g., subunits of an oligomeric enzyme, the distinction between the two mechanisms becomes blurred.[36]

The heme protein cytochrome $c$[37,38] (designated cyt) and the blue copper protein plastocyanin[39] (designated pc) are well suited to kinetic and mechanistic studies. Their structures are known in detail, their properties have been thoroughly examined by spectroscopic and electrochemical methods, and their reactions with various redox agents have been examined by kinetic methods. Previous studies in this laboratory[28,29] concerned the unimolecular electron-transfer reaction, with a small thermodynamic driving force, between the associated native proteins in their ground electronic states. This intracomplex reaction apparently requires rearrangement of the diprotein complex from a stable but unreactive configuration to a reactive one.

The present study concerns both the ground-state and the excited-state electron-transfer reactions, with large driving forces, between zinc cytochrome $c$ and plastocyanin. Our goal is to examine both the bimolecular diffusion-controlled processes involving the separate proteins and the unimolecular electron-transfer reactions between the associated proteins. To our knowledge, such a study with metalloproteins has not been reported before. It proved possible to change the overall mechanism from bimolecular to predominantly unimolecular to a combination of the two by adjusting ionic strength, to compare the electron-transfer reactions in the persistent and in the transient diprotein complexes, and to address the question of nonelectrostatic attraction between these two highly charged proteins.

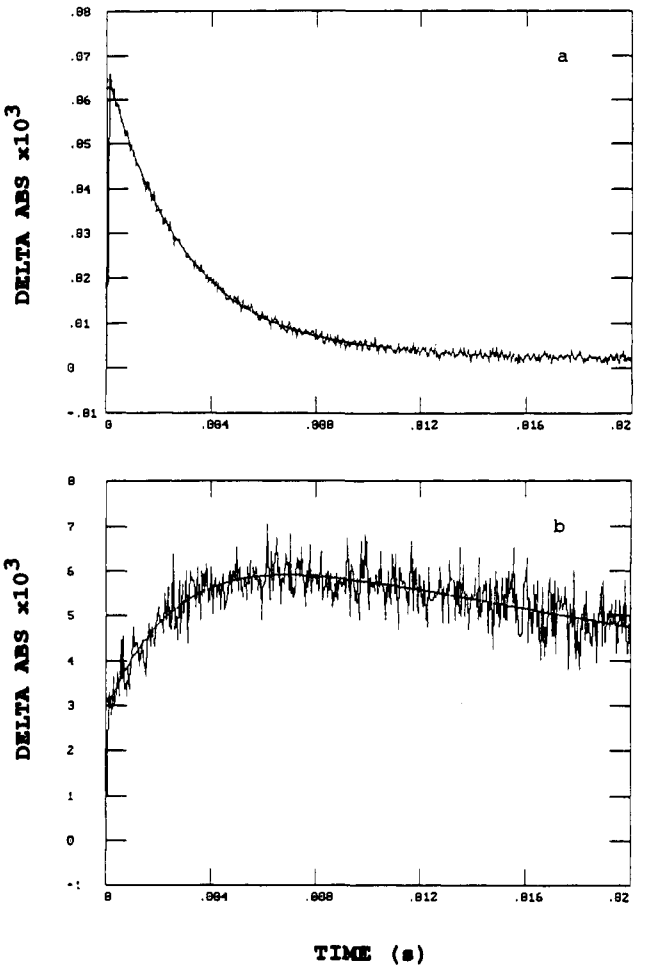


Figure 1. Transient absorbance changes in a solution containing 10.0 $\mu$M zinc cytochrome $c$ and 20.0 $\mu$M cupriplastocyanin in phosphate buffer at pH 7.0, ionic strength of 1.00 M, and 25 °C. Each solid line shows fitting to two exponentials. (a) The triplet state $^3$Zn(cyt) monitored at 460 nm. The solid line shows fitting to one exponential. (b) The cation radical Zn(cyt)$^+$ monitored at 675 nm.

## II. Materials and Methods

**IIA. Chemicals.** The free-base form of horse-heart cytochrome $c$ (Sigma Chemical Co., type III) was prepared, purified, and reconstituted with zinc by standard procedures, in the dark.[40,41] Zinc cytochrome $c$ (designated Zn(cyt)) was always handled in the dark. French-bean plastocyanin was isolated by standard methods[42] and purified repeatedly by gel-filtration chromatography on Sephadex G-25 and G-75 columns and by anion-exchange chromatography on a Sephadex DEAE A-25 column until the absorbance quotient $A_{278}/A_{597}$ became less than 1.20. Apoplastocyanin and cobalt(II) plastocyanin were prepared and purified by standard methods.[43] Distilled water was demineralized to a resistance

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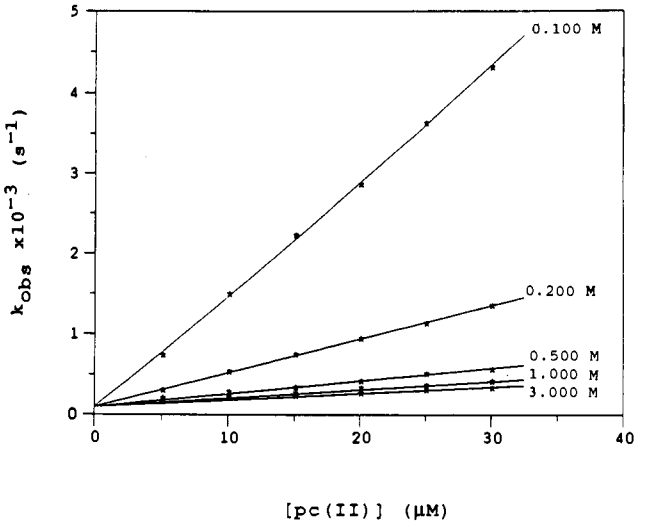


**Figure 2.** Rate of decay of the triplet state $^3$Zn(cyt) as a function of cupriplastocyanin concentration in phosphate buffer at the indicated ionic strengths (M), pH 7.0, and 25 °C. Solid lines are fittings to eq 2.

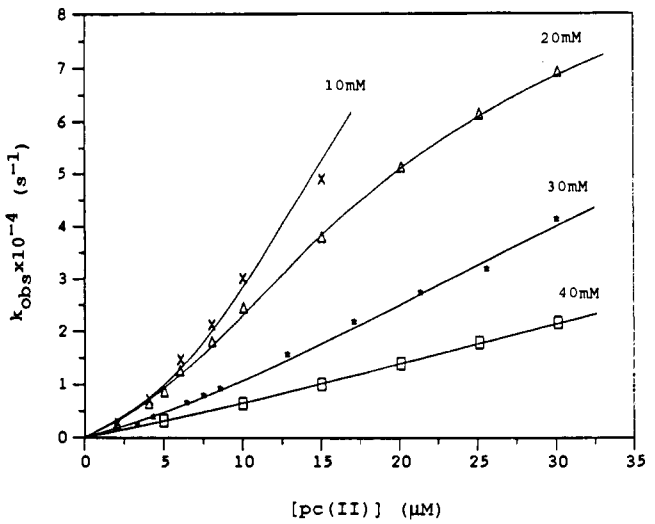


**Figure 3.** Rate of decay of the triplet state $^3$Zn(cyt) as a function of cupriplastocyanin concentration in phosphate buffer at the indicated ionic strength (mM), pH 7.0, and 25 °C. The upper two plots are for the slower of the two exponential processes, and the lower two plots are for the exponential process. Solid lines are fittings to eq 2.

greater than 10 MΩ cm. Potassium phosphate buffer had pH 7.0 and ionic strength of 10 mM; ionic strength was raised with NaCl and lowered by dilution. Viscosity was raised by addition of ethylene glycol. Ultrafiltration was done in Amicon cells, with YM-5 membranes, at 4 °C, under pressure of pure nitrogen.

**IIB. Kinetics.** Flash kinetic spectrophotometry on the microsecond scale was done with a standard apparatus. The sample solution in a 10-mm curvette was thoroughly deaerated by gentle flushing with ultrapure argon (supplied by Air Product Co.). A Phase-R DL1100 laser contained a 50 μM solution of rhodamine 590 in methanol and delivered 0.4-μs pulses of excitation light. The monochromatic monitoring beam from a tungsten–halogen lamp was perpendicular to the excitation beam. The absorbance–time curves were analyzed with kinetic software from On Line Instrument Systems, Inc. Flash kinetic spectrophotometry on the submicrosecond scale was done with a Q-switched Nd-YAG laser Quantal YG 571-C10, which delivered 10-ns pulses at 532 nm; this instrument was described elsewhere.[44] The signals were averaged over 20 pulses. Formation and decay of the triplet state of zinc cytochrome *c* (designated $^3$Zn(cyt)) were monitored at 460 nm, where this transient absorbance reaches a maximum. Appearance and disappearance of the cation radical of zinc cytochrome *c* (designated Zn(cyt)$^+$) were monitored at 675 nm, where the difference between the cation and the triplet absorbances is greatest. The concentration of zinc cytochrome *c* was 10.0 μM. The concentration of the triplet $^3$Zn(cyt) depended on the excitation power but was always kept well below the concentrations of the quenchers cupriplastocyanin, apoplastocyanin, and cobaltoplastocyanin, which were varied between 2.00 and 40.0 μM. Ionic strength of the phosphate buffer at pH 7.0 was varied between 2.5 mM and 3.00 M. The temperature was 25 ± 2 °C.

**IIC. Yield of Electron-Transfer Products from $^3$Zn(cyt) Quenching.** The difference in absorptivities (molar extinction coefficients) at 460 nm between the triplet ($^3$Zn(cyt)) and the ground (Zn(cyt)) states of zinc cytochrome *c* was determined by the method of total conversion.[45] The yield of electron-transfer products was determined in the presence of a very high excess (concentrations 150 μM and greater) of cupriplastocyanin, when the triplet was quenched more than 97%. The yield of the cation radical Zn(cyt)$^+$ was estimated according to eq 1, in which $\Delta A_{675}$ is the maximum change, and $\Delta A_{460}$ is the change coincident with the pulse (at time zero), in absorbance at the specified wavelength.

$$Y_{el} = \frac{[\mathrm{Zn(cyt)^+}]}{[^3\mathrm{Zn(cyt)}]} = \frac{\Delta A_{675}}{\Delta A_{460}} \frac{\Delta \epsilon_{460}}{\Delta \epsilon_{675}} \quad (1)$$

## III. Results

**IIIA. Decay of the Free $^3$Zn(cyt).** The triplet excited state of free zinc cytochrome *c* decays with the rate constant of 100 ± 10 s$^{-1}$ in the entire range of ionic strength from 3.00 M to 2.5 mM. This value falls in the middle of a narrow range, 70–140 s$^{-1}$, spanned by the values reported before.[9,46,47] The slight variation in the rate constant perhaps is caused by small differences in the protein preparation, deoxygenation procedure, buffer, and temperature. The variation is far too small to affect the kinetic arguments and conclusions.

**Table I.** Fractional Contribution of the Faster Process to Decay of the Triplet State of Zinc Cytochrome *c*, $^3$Zn(cyt), as a Function of Cupriplastocyanin Concentration and Ionic Strength at pH 7.0 and 25 °C

| | μ, mM | | |
|---|---|---|---|
| [pc(II)], μM | 2.5 | 10 | 20 |
| 4.00 | 0.08 | | |
| 6.00 | 0.30 | 0.10 | |
| 8.00 | 0.53 | 0.14 | |
| 10.0 | 0.73 | 0.29 | |
| 15.0 | 0.86 | 0.46 | |
| 20.0 | 0.87 | 0.67 | |
| 25.0 | 0.87 | 0.71 | 0.08 |
| 30.0 | | 0.74 | 0.17 |

**IIIB. Redox Quenching of $^3$Zn(cyt) by Pc(II) at High and Intermediate Ionic Strengths.** In the presence of cupriplastocyanin, at ionic strengths from 3.00 M to 0.100 M, the triplet decay and the cation radical Zn(cyt)$^+$ formation are exponential processes. Their rate constants are equal within the error bounds. Typical traces are shown in Figure 1. The pseudo-first-order rate constant is linearly proportional to the quencher concentration and dependent on ionic strength, as Figure 2 shows.

**IIIC. Redox Quenching of $^3$Zn(cyt) by Pc(II) at Low Ionic Strengths.** In the presence of cupriplastocyanin, as ionic strength is lowered further, the decay of the triplet dramatically accelerates and the kinetics is no longer simple. At the ionic strength of 30 mM, the decay is still exponential but the rate constant is no longer linearly proportional to the quencher concentration; slight curvature is evident in Figure 3. At the ionic strengths from 20 to 2.5 mM, the triplet decay becomes biexponential, as Figure 4a shows. The faster and the slower components of this biexponential decay will be presented separately.

The rate constant for the faster component is (2.5 ± 0.4) × 10$^5$ s$^{-1}$ regardless of the cupriplastocyanin concentration and of

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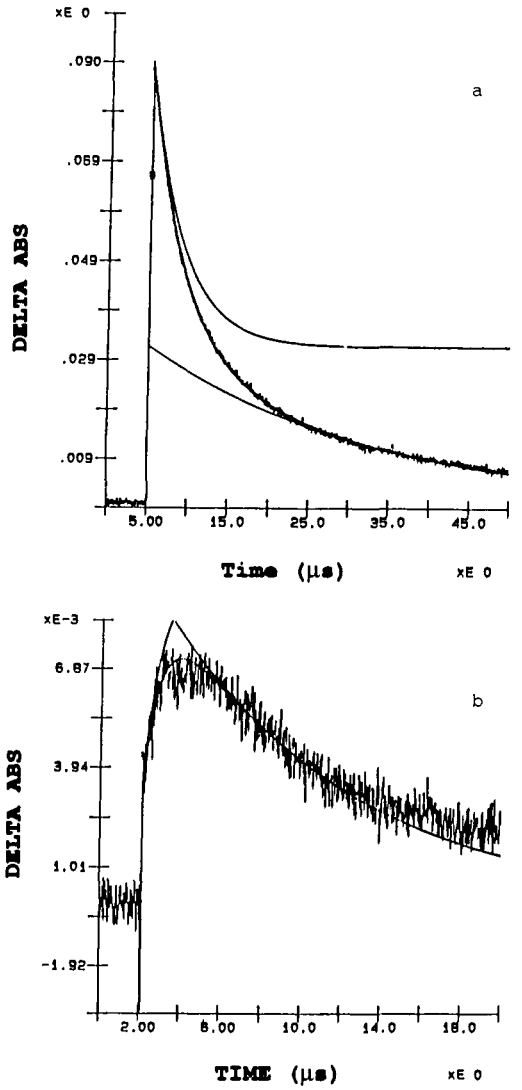



**Figure 4.** Transient absorbance changes in a solution containing 10.0 $\mu$M zinc cytochrome *c* and 20.0 $\mu$M cupriplastocyanin in phosphate buffer at pH 7.0, ionic strength of 10 mM, and 25 °C. Solid lines are fittings with two exponentials and their composites. (a) Disappearance of the triplet state $^3$Zn(cyt) monitored at 460 nm. (b) Appearance and disappearance of the cation radical Zn(cyt)$^+$ monitored at 675 nm.

the (low) ionic strength. Table I shows that the relative amplitude of this process, i.e., its fraction of the total decay, increases as the quencher concentration increases and as the ionic strength decreases. To this faster component of the triplet $^3$Zn(cyt) decay, after the instantaneous vertical signal, corresponds a biexponential change of the cation radical Zn(cyt)$^+$ concentration, shown in Figure 4b. The absorbance grows at the rate of (1.1 ± 0.5) × $10^6$ $s^{-1}$ and declines at the rate of (2.5 ± 0.8) × $10^5$ $s^{-1}$. Both of these rate constants are independent of the cupriplastocyanin concentration.

The rate constant for the slower component of the triplet decay depends on the cupriplastocyanin concentration, as shown in Figure 3 for the ionic strengths of 20 and 10 mM. At high quencher concentrations and ionic strengths of 20 mM and lower, a saturation trend is evident. The relative amplitude of the slower process decreases as the quencher concentrationn increases and as the ionic strength decreases. At 10 mM, this amplitude is large enough for accurate determination only at low and intermediate quencher concentrations, hence one shorter curve in Figure 3. The amplitudes of the slower process are simply complements to unity of the amplitudes of the faster process, listed in Table I.

**IIID. Nonredox Quenching of $^3$Zn(cyt).** In control experiments, native cupriplastocyanin as a quencher was replaced by two of its derivatives. Acceleration of the $^3$Zn(cyt) decay (with respect to the value of 100 ± 10 $s^{-1}$) was measured at the ionic strength

**Table II.** Effect of Ionic Strength on the Yield of Cation Radical Zn(cyt)$^+$ Formed in the Slower Process of Quenching of the Triplet State of Zinc Cytochrome *c*, $^3$Zn(cyt), by Cupriplastocyanin at pH 7.0 and 25 °C

| $\mu$, mM[a] | $Y_{et}$, % | viscosity relative to $H_2O$[b] |
|---|---|---|
| 20 | 6.9 | 1.00 |
| 40 | 25 | 1.00 |
| 100 | 53 | 1.01 |
| 200 | 53 | 1.02 |
| 500 | 51 | 1.05 |
| 1000 | 46 | 1.10 |
| 3000 | 42 | 1.38 |

[a] Adjusted with NaCl. [b] From *Handbook of Chemistry and Physics*, 66th ed.; CRC Press: Boca Raton, FL, 1986.

**Table III.** Effect of Viscosity on the Yield of Cation Radical Zn(cyt)$^+$ Formed in the Slower Process of Quenching of the Triplet State of Zinc Cytochrome *c*, $^3$Zn(cyt), by Cupriplastocyanin at pH 7.0, 25 °C, and Ionic Strength of 0.100 M

| ethylene glycol, % by wt | viscosity relative to $H_2O$[a] | $Y_{et}$, % |
|---|---|---|
| 5.0 | 1.12 | 54 |
| 8.0 | 1.21 | 51 |
| 12 | 1.34 | 51 |
| 29 | 2.10 | 50 |

[a] From *Handbook of Chemistry and Physics*, 66th ed.; CRC Press: Boca Raton, FL, 1986.

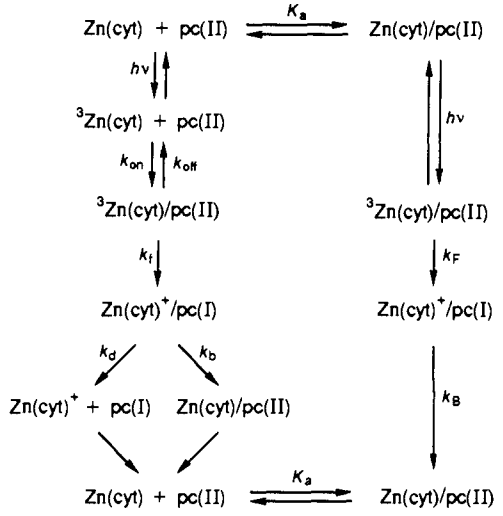
of 2.5 mM. The triplet decays at the rate of 200 ± 20 $s^{-1}$ (an acceleration of 100 ± 20 $s^{-1}$) in the presence of apoplastocyanin and at the rate of 250 ± 20 $s^{-1}$ (an acceleration of 150 ± 20 $s^{-1}$) in the presence of cobaltoplastocyanin. Both rate constants are independent of the wavelength monitored and of the quencher concentration in the range from 10.0 to 40.0 $\mu$M. The cation radical Zn(cyt)$^+$ was undetectable in either case.

**IIIE. Yield of Electron-Transfer Products from $^3$Zn(cyt) Quenching.** The absorptivity at 460 nm increases by (3.5 ± 1.0) × $10^4$ $M^{-1}$ $cm^{-1}$ as Zn(cyt) is excited to $^3$Zn(cyt). The difference in absorptivities at 675 nm between Zn(cyt)$^+$ and Zn(cyt) is (1.40 ± 0.05) × $10^4$ $M^{-1}$ $cm^{-1}$.[9] The yield of the cation radical Zn(cyt)$^+$ was estimated according to eq 1. Because of the limits of experimental accuracy, the estimated relative error in the yields $Y_{et}$ for the slower component of the triplet decay is ca. 10%. Therefore, the dependence of this yield on ionic strength, shown in Table II, is real, and so is the lack of dependence on viscosity, shown in Table III. The yield of Zn(cyt)$^+$ formed in the faster component of the triplet decay is estimated at 0.5 ± 0.2. This value reflects additional uncertainties in the expansion coefficients of the two exponentials and also the effects of fluorescence, of possible redox quenching of the singlet state $^1$Zn(cyt), and of the slower component of the triplet decay.

## IV. Discussion

**IVA. Mechanistic Scheme.** The various processes involving zinc cytochrome *c* and native (copper-containing) plastocyanin are shown in Scheme I. The Roman numerals are the oxidation states of copper. Because of the overall charges of zinc cytochrome *c* and cupriplastocyanin at pH 7.0 are +6 and −8, respectively, the proteins readily associate. Because the extent of this association depends on ionic strength, the kinetic regime can be adjusted to bimolecular (the left-hand side) at high ionic strengths, to predominantly unimolecular (the right-hand side) at low ionic strengths, or to a combination of the two (the entire Scheme I) at intermediate ionic strengths. The unimolecular mechanism is conceptually simple: Excitation of zinc cytochrome *c* associated with cupriplastocyanin is followed by charge separation involving the triplet state $^3$Zn(cyt) (the "forward" electron transfer, $k_F$) and by charge recombination involving the cation radical Zn(cyt)$^+$ (the "back" electron transfer, $k_B$). In the bimolecular mechanism, the counterparts of these two electron-transfer processes—they too are unimolecular—are designated $k_f$ and $k_b$. But now there are also the diffusion-controlled processes of reactant association

**Scheme I**




($k_{on}$), reactant dissociation ($k_{off}$), and product dissociation ($k_d$). The unimolecular mechanism involves the preformed diprotein complex, and the bimolecular mechanism involves the encounter diprotein complex. Although both of them are designated $^3$Zn(cyt)/pc(II), their properties and reactivity need not be identical. One of the goals in this study is to find out if they are by comparing the rate constants $k_F$ and $k_f$.

**IVB. Biexponential Quenching of $^3$Zn(cyt) by Pc(II).** The triplet decay at low ionic strength (Figure 4a) is biphasic because zinc cytochrome *c* exists in two forms—free and associated with curpriplastocyanin. These two exponential processes are experimentally separable and will be discussed separately.

**IVC. The Slower Process.** This is the only mode of $^3$Zn(cyt) quenching by cupriplastocyanin at the ionic strengths of 0.100 M and higher. Because the triplet disappears (the declining portion of Figure 1a) and the cation radical appears (the rising portion of Figure 1b) at the same rate, this quenching occurs by electron transfer. (Further evidence will be given in section IVE.) Linear dependence of $k_{obs}$ on cupriplastocyanin concentration, shown in Figure 2, shows this quenching to be bimolecular (diffusive); the rate constant decreases as ionic strength increases because the two proteins overall, and the domains through which they specifically bind to each other, are oppositely charged. At the ionic strengths from 40 to 10 mM (Figure 3), the quenching is still predominantly diffusive. The observed rate constants obey eq 2,[48] in which [pc(II)$_q$] is concentration of the free (unassociated)

$$k_{obs} = \frac{k_{on}K_a k_f[\mathrm{pc(II)_q}]}{k_{on} + K_a k_f + k_{on}K_a[\mathrm{pc(II)_q}]} \qquad (2)$$

quencher; its dependence on the total concentrations [pc(II)] and [Zn(cyt)] is given by eq 3. The association constant is defined $K_a = k_{on}/k_{off}$. It applies to both the encounter and the preformed complexes because the interactions between the protein surfaces should not be appreciably affected by the electronic state (ground or excited) of the zinc porphyrin inside cytochrome *c*. In principle,

$$[\mathrm{pc(II)_q}] = [\mathrm{pc(II)}] - [[\mathrm{Zn(cyt)}] + [\mathrm{pc(II)}] + K_a^{-1} - \{([\mathrm{Zn(cyt)}] + [\mathrm{pc(II)}] + K_a^{-1})^2 - 4[\mathrm{Zn(cyt)}][\mathrm{pc(II)}]\}^{1/2}/2] \qquad (3)$$

changes in the electron distribution upon porphyrin excitation may affect the surrounding protein, but significant structural changes are very unlikely. The rate constants at the ionic strengths from 3.00 M to 20 mM conform very well to eq 2, and Figures 2 and 3 show overlap of experimental and calculated values. But only the curved lines in Figure 3 truly verify eq 2; the straight ones in Figure 2 do not. The results of this three-parameter fitting are listed in Table IV. Because $k_f = (2.8 \pm 0.6) \times 10^5$ s$^{-1}$ over the entire range of ionic strength, the fitting was repeated with this $k_f$ value fixed. The values of $k_{on}$ and $K_a$ from the three-parameter and the two-parameter fittings generally differed by less than 50%. Such calculations are always more or less uncertain. In this case, however, the value of $k_f$ is confirmed by direct measurements that will be discussed next in section IVD, and the values of $k_{on}$ converge to a reasonable value.[49]

**Table IV.** Kinetic Parameters[a] Obtained by Fitting of Observed Rate Constants and Plastocyanin Concentrations to Equation 2

| $\mu$, mM | $k_f$, s$^{-1}$ | $K_a$, M$^{-1}$ | $k_{on}$, M$^{-1}$ s$^{-1}$ |
|---|---|---|---|
| 10 | $2.6 \times 10^5$ | $6.0 \times 10^5$ | $9.5 \times 10^9$ |
| 20 | $1.5 \times 10^5$ | $4.4 \times 10^5$ | $8.9 \times 10^9$ |
| 30 | $3.3 \times 10^5$ | $1.6 \times 10^5$ | $2.1 \times 10^9$ |
| 40 | $2.4 \times 10^5$ | $7.6 \times 10^4$ | $1.1 \times 10^9$ |
| 100 | $3.3 \times 10^5$ | $1.4 \times 10^4$ | $1.6 \times 10^8$ |

[a] Defined in Scheme I.

**IVD. The Faster Process.** As Table I shows, the contribution of the faster process to the overall electron-transfer reaction (i.e., its relative amplitude) is proportional to the fraction of associated zinc cytochrome *c*. The rate of the faster process, $(2.5 \pm 0.4) \times 10^5$ s$^{-1}$, is determined directly from traces such as the one shown in Figure 4a. This rate constant is independent of cupriplastacyanin concentration, as expected of static quenching. On the basis of these facts, we attribute the faster process to unimolecular electron transfer within the preformed diprotein complex, $k_F$ in Scheme I. The independence of this rate constant on ionic strength in the range (from 2.5 to 20 mM) over which the process is observable indicates that ionic strength affects only the abundance but not the structure or other electron-transfer properties of the $^3$Zn(cyt)/pc complex. Both our kinetic assignment of the reaction $k_F$ and the independence of this rate constant of ionic strength agree with detailed, recent findings for a similar electron-transfer system.[50,51]

**IVE. Mechanism of $^3$Zn(cyt) Quenching by Pc(II).** There are three possible causes of dramatic acceleration, from $100 \pm 10$ to $(2.5 \pm 0.4) \times 10^5$ s$^{-1}$, of the triplet decay in the presence of cupriplastocyanin: enhancement of radiationless decay upon association with plastocyanin, energy transfer to plastocyanin, and electron transfer to plastocyanin. The control experiments with apoplastocyanin straightforwardly show the first cause to be negligible. Our finding of acceleration by $100 \pm 10$ s$^{-1}$ is comparable to the value of 61 s$^{-1}$, reported for a complex between $^3$Zn(cyt) and apocytochrome *c* peroxidase.[52] The small enhancement of the triplet decay was attributed, without evidence, to slight conformational change in zinc cytochrome *c* upon association with another protein.[52] The rates of excited-state decay generally reflect such a multitude of factors that small changes cannot easily be ascribed to particular causes.

The experiments with cobaltoplastocyanin hardly require interpretation. The observed acceleration of $150 \pm 20$ s$^{-1}$ is a joint effect of enhanced radiationless decay ($100 \pm 10$ s$^{-1}$) and energy transfer ($50 \pm 10$ s$^{-1}$); electron transfer is precluded by the oxidation state II of cobalt. Because the rate constant for dipole–dipole energy transfer is proportional to overlap between the emission spectrum of $^3$Zn(cyt) ($\lambda_{max}$ = 730 nm) and the absorption spectrum of the quencher, and because native plastocyanin absorbs at most 10 times more strongly than cobaltoplastocyanin in the region between 650 and 810 nm, the experimental rate constant of $50 \pm 10$ s$^{-1}$ should be raised to ca. 500 s$^{-1}$. Even this adjusted value is negligible in comparison with the observed rate constant of $(2.5 \pm 0.4) \times 10^5$ s$^{-1}$.

These control experiments show that $^3$Zn(cyt) is quenched by electron transfer to cupriplastocyanin, i.e., that the diprotein complex is not a dead-end product. This conclusion was confirmed by actual detection of the cation radical Zn(cyt)$^+$ (Figure 4b).

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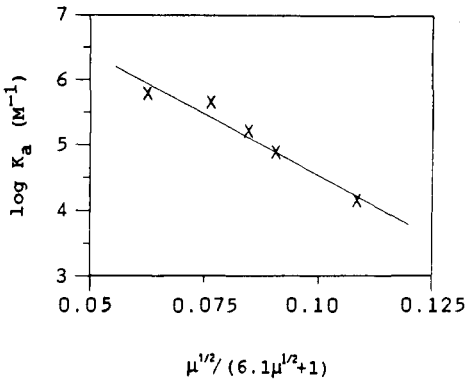


**Figure 5.** Association constant for zinc cytochrome *c* and cupriplastocyanin as a function of ionic strength (0.010, 0.020, 0.030, 0.040, 0.100 M) in phosphate buffer at pH 7.0 and 25 °C. The line is a linear least-squares fit.

The rate constant of $(2.5 \pm 0.8) \times 10^5$ s$^{-1}$ for the declining portion of the trace in Figure 4b is identical with the rate constant $k_F = (2.5 \pm 0.4) \times 10^5$ s$^{-1}$ for the faster process of $^3$Zn(cyt) decay in Figure 4a. The kinetic profile of Zn(cyt)$^+$ in the preformed complex is consistent with Scheme I if the unimolecular step $k_F$ is slower than the unimolecular step $k_B$.[53] The rising portion of the trace in Figure 4b therefore corresponds to $k_B = (1.1 \pm 0.5) \times 10^6$ s$^{-1}$. Evidently, the cation radical Zn(cyt)$^+$ and the triplet $^3$Zn(cyt) (whose formation corresponds to the vertical signal at time zero) participate in the same process $k_F$. Because $k_B$ is greater than $k_F$, the transient intermediate Zn(cyt)$^+$/pc(I) eluded detection. Such problems have plagued studies of photoinduced reactions between metalloproteins,[13] but this direct evidence of electron-transfer quenching is sometimes obtained.[9,10,19,20,22,50,51] In this case, the intermediate proved detectable because $k_B$ is only ca. 4 times greater than $k_F$.

**IVF. The Diprotein Complex.** Chemical modification,[54-58] electrochemistry,[59] competitive inhibition,[60] cross-linking,[61] and NMR spectroscopy[62,63] showed that cytochrome *c* and plastocyanin associate via the positively charged domain around the exposed heme edge in the former and the negatively charged domain on the "east" (in the conventional orientation) side in the latter protein. These domains are approximately defined by the lysine residues nos. 13, 25, 27, 86, and 87 in the horse cytochrome *c* and by aspartate residues nos. 43 and 45 and glutamate residues nos. 44, 46, 60, 61, and 62 in the bean plastocyanin. Substitution of zinc for iron does not noticeably alter the conformation of cytochrome *c*[64] and its association with other proteins.[40,41] Little is known about effects of ionic strength on the structure of cytochrome *c* and plastocyanin. Because very similar interatomic distances are deduced from crystal structures (at extremely high ionic strength in solid) and from NMR spectra (at intermediate and low ionic strength in solution), these effects cannot be great. Indeed, the rate of $^3$Zn(cyt) decay is constant over a full range of ionic strength (section IIIA), and recent spectroscopic studies of cytochrome *c* showed no alterations of its secondary structure.[65,66] A recent kinetic study of plastocyanin showed that potential effects of ionic strength do not appear in the rate laws for its electron-transfer reactions with metal complexes.[67]

The association constant $K_a$ for the encounter complex $^3$Zn(cyt)/pc(II) greatly depends on ionic strength, as Table IV shows, because the interaction domains in the two proteins and the whole proteins bear large opposite charges. Although Debye–Hückel theory does not apply rigorously to macromolecules,[48] it is useful in studies of small, globular metalloproteins. Plastocyanin is a little smaller than cytochrome *c*, but use of the cytochrome *c* radius for both proteins simplified our fitting of the association constant to a Brønsted–Debye–Hückel equation.[67,68] The slope of the graph in Figure 5 is $-(37 \pm 5)$, whereas the product of the overall protein charges is −48. Plotting of log $K_a$ versus $\mu^{1/2}/(1 + \mu^{1/2})$ gave a bad fit and an unrealistic slope, probably because this latter treatment embodies an assumption that the Coulombic radius is 3 Å. This incorrect plot may, however, be useful for comparative purposes. Extrapolation of its linear portion yielded $K_a \approx (2 \pm 1) \times 10^7$ M$^{-1}$ at zero ionic strength. This estimate agrees with similar estimates for complexes that cytochrome *c* forms with cytochrome $b_5$[69] and with cytochrome *c* peroxidase.[70] Cytochrome *c* seems to interact similarly with various anionic metalloproteins, and replacement of iron with zinc does not perturb these docking interactions.

The present $K_a$ values, obtained by fitting of flash-photolysis rate constants, agree well with the values obtained in three other studies of cytochrome *c* and plastocyanin. Fitting of pulse-radiolysis rate constants yielded $8.4 \times 10^4$ M$^{-1}$ at the ionic strength of 40 mM,[28] and direct NMR spectroscopic determinations yielded $1.5 \times 10^4$ M$^{-1}$ at the ionic strength of 0.100 M.[62,63] This agreement justifies our assumption, in section IVC, that the same association constant $K_a$ applies to the encounter complex and the preformed complex and verifies the fittings to eq 2.

Theoretical calculations of Brownian dynamics showed that cytochrome *c* and cytochrome *c* peroxidase can form a multitude of complexes and that these complexes can interconvert as the proteins diffuse two-dimensionally on each other's surface.[71-73] There is also evidence, mainly from theoretical studies, for flexibility of other complexes composed from oppositely charged redox proteins.[13] A rearrangement on a nanosecond or a picosecond scale cannot be observed directly by microsecond kinetic methods. But the rearrangement in the complex between native cytochrome *c* and plastocyanin was detected indirectly, by comparing the electrostatic and covalent complexes of these two proteins.[28,29] The intracomplex electron transfer from iron(II) to copper(II), the ground-state analogue of the reactions $k_f$ and $k_F$, is fast in the electrostatic complex ($1300 \pm 200$ s$^{-1}$) but undetectably slow (less than 0.2 s$^{-1}$) when the electrostatic complex is reinforced by noninvasive cross-links between the two proteins. This electron-transfer reaction apparently requires a migration of cytochrome *c* from the negatively charged (east) domain on the plastocyanin surface that lies 14–19 Å away from the copper atom, to the neutral, hydrophobic ("north") domain that lies only 3–9 Å away from the copper atom. This migration is impededed by covalent cross-linking. The equality of $k_f$ and $k_F$ is unexpected because cytochrome *c* and plastocyanin associate strongly and specifically, but this finding is consistent with the previous findings and can

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be explained in two ways. First, according to a recent analysis,[67] a strong reductant may transfer an electron to the cupric atom even from the remote anionic domain in the plastocyanin surface. If so, the triplet excited state of zinc cytochrome *c*, which is a much stronger reductant ($E^\circ$ = −0.88 V vs NHE)[74] than the ground state of ferrocytochrome *c* ($E^\circ$ = 0.26 V vs NHE), may not need the benefits of a short distance and of favorable electron-transfer pathways,[75] provided by the proximate hydrophobic domain. Second, both the encounter and the precursor complexes $^3$Zn-(cyt)/pc(II) can rearrange into the same reactive conformation, and therefore $k_f$ and $k_F$ are equal. This rearrangement presumably involves rotational diffusion, which is much faster than the subsequent electron transfer. These two possible explanations are being tested by examining the effect of covalent cross-linking of zinc cytochrome *c* and plastocyanin on the electron-transfer reaction between them.[76]

The rate constants for the ground-state[28,29] and the excited-state reactions are 1300 ± 200 and (2.5 ± 0.4) × $10^5$ $s^{-1}$, and the driving forces are 0.10 and 1.2 eV, respectively. If the reorganization energy is 0.9 eV, the acceleration of the reaction can be attributed to the increase in the driving force. This assumed reorganization energy agrees well with the values around 1.0 eV, consistently obtained in quantitative treatments by Marcus theory[48] of various metalloprotein electron-transfer reactions.[13] It is not known whether reorganization energy depends on ionic strength. We plan to vary further the driving force for the reactions between cytochrome *c* and plastocyanin.

**IVG. Nonelectrostatic Interactions and the Efficiency of Charge Separation.** When the triplet $^3$Zn(cyt) is completely quenched by cupriplastocyanin as an oxidant—this mechanism was proved in section IVF—the yield of electron-transfer products $Zn(cyt)^+$ and pc(I) depends on the competition between the reactions $k_d$ and $k_b$ according to eq 4. Table II shows an interesting trend—the

$$Y_{et} = \frac{k_d}{k_d + k_b} \quad (4)$$

efficiency of charge separation increases, levels off, and decreases as the salt concentration increases over a wide range. Table III shows that it is ionic strength, and not simply viscosity of the solution, that alters the yield of charge separation. Because the rate constant $k_f$ is independent of ionic strength, the rate constant $k_b$ too probably is invariant over the same range of ionic strength. There is no direct evidence for this plausible assumption, but an alternative—that the driving force of the back reaction, and therefore the rate constant $k_b$, depends on ionic strength—appears unlikely. The component of the driving force that depends on electrostatic interactions[77] between associated proteins is very small, especially at the relatively high ionic strengths involved, because the redox sites are more than 10 Å apart and their charges change by only one unit. The reduction potential of native cytochrome *c* depends only slightly on ionic strength,[78] and presumably so do the reduction potentials of the proteins used in this study. The trend in Table II can therefore be discussed in terms of $k_d$ alone. From the electrostatic point of view, an increase in ionic strength should facilitate dissociation of the diprotein complex until the partner proteins are fully separated; further increase should have no effect thereafter. The yield $Y_{et}$ behaves as expected up to the ionic strength of 0.500 M, but it then decreases significantly at 1.00 and 3.00 M. When the charged sites on the protein surfaces are completely screened by counterions from solution, nonelectrostatic attractive forces may manifest themselves. Hydrophobic interactions are well documented in protease–inhibitor and antibody–antigen complexes,[79] but their possible contribution to association between highly charged redox complexes is more difficult to assess. The study of electron-transfer reactions at very high ionic strength may prove profitable in this regard.

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