Gating of Photoinduced Electron Transfer from Zinc Cytochrome *c* and Tin Cytochrome *c* to Plastocyanin. Effects of Solution Viscosity on Rearrangement of the Metalloprotein Complex

Jian S. Zhou and Nenad M. Kostić*

Contribution from the Department of Chemistry, Iowa State University, Ames, Iowa 50011

Received June 21, 1993®

Abstract: Oxidative quenching by pc(II) of the triplet excited states ^{3}Zn(cyt) and ^{3}Sn(cyt) is studied by laser flash photolysis. The quenching is biphasic at low ionic strength. The faster component corresponds to the unimolecular electron-transfer reactions within the electrostatic complexes ^{3}M(cyt)/pc(II), in which M is Zn(II) or Sn(IV); these reactions are the main subject of this study. The slower component corresponds to the bimolecular reactions between the unassociated ^{3}M(cyt) and pc(II). The relative amplitudes of the two components depend on ionic strength and pc(II) concentration in a predictable way. The unimolecular reaction is studied in solutions whose viscosity is adjusted with glycerol, ethylene glycol, and D-glucose. In the electrostatic complex the positively charged (basic) patch around the exposed heme edge in cyt abuts the negatively charged (acidic) patch remote from the copper atom in pc. In the covalent complex this orientation is reinforced by noninvasive, tight cross-links. The intracomplex rate constant in the electrostatic complexes ^{3}M(cyt)/pc(II), which are flexible, does not depend on ionic strength (in the range from 2.5 to 20 mM) but decreases smoothly from $(2.5 \pm 0.4) \times 10^5$ s^{-1} when M is Zn(II) and from $(1.9 \pm 0.4) \times 10^5$ s^{-1} when M is Sn(IV) as the viscosity is raised. The intracomplex rate constant in the covalent complex ^{3}Zn(cyt)/pc(II), which is rigid, is $(2.4 \pm 0.2) \times 10^4$ s^{-1} and is invariant with viscosity. The viscosity effects on the electrostatic complexes are fitted well to a simple mechanism involving two conformations of the electrostatic complex—the initial one that optimizes docking but not the intracomplex electron-transfer reaction and the rearranged one that optimizes this reaction. The fitted rate constant within the initial conformation of the electrostatic complex equals the observed rate constant within the covalent complex. At very high viscosity the rate constant within the electrostatic complex converges down to the rate constant within the covalent complex. These facts show that viscous solvents and cross-links act similarly in stabilizing and capturing the initial conformation of the electrostatic complex. The two limiting conformations of ^{3}M(cyt)/pc(II) interconvert by a process, probably conformational fluctuation, for which the fitted rate constants are 2.2×10^5 and 1.8×10^5 s^{-1} when M is Zn(II) and Sn(IV), respectively. Because the small difference between these values probably is insignificant, conformational fluctuation does not seem to depend on the protein overall charge as long as the surface charge is unaltered. The photoinduced reactions in ^{3}Zn(cyt)/pc(II) and ^{3}Sn(cyt)/pc(II), with the respective driving forces of 1.2 and 0.8 eV, are gated because the electron-transfer step in the rearranged complexes is faster than the rearrangement. The thermal reaction in cyt(II)/pc(II), with a driving force of 0.10 eV, is not gated because the electron-transfer step (1300 ± 200 s^{-1}) is slower than this rearrangement. Similar electron-transfer reactions between proteins can be gated or not gated, depending on the driving force.

Introduction

Redox Reactions of Metalloproteins. Metalloproteins are essential to various biological oxidoreduction processes. Research into the kinetics and mechanism of metalloprotein electron-transfer reactions continues to grow.[1-9] Our goal is to understand, at a molecular level, the twin requirements for sufficiently high rate and for protein specificity in these reactions. Both of these factors depend on protein–protein orientation. The simplest systems in which this orientation and its modulation by dynamic processes can be studied are diprotein complexes.[1-4,7-9] A pair of metalloproteins can form multiple complexes in solution,[9-16] and an orientation that is optimal for recognition and binding need not be optimal for the subsequent reaction. When the protein complex rearranges among different configurations, the rate of the electron-transfer reactions may be limited by the rate of this structural change. In such a case the reaction is said to be gated.[9,16-25] The phenomenon of gating is not limited to electron-transfer reactions, and it may be common in proteins.

* Abstract published in *Advance ACS Abstracts*, October 15, 1993.

(1) Hoffman, B. M.; Natan, M. J.; Nocek, J. M.; Wallin, S. A. *Struct. Bonding* **1991**, *75*, 86.
(2) McLendon, G. *Struct. Bonding* **1991**, *75*, 160.
(3) McLendon, G. *Met. Ions Biol. Syst.* **1991**, *27*, 183.
(4) McLendon, G.; Hake, R. *Chem. Rev.* **1992**, *92*, 481.
(5) Therien, M. J.; Chang, J.; Raphael, A. L.; Bowler, B. E.; Gray, H. B. *Struct. Bonding* **1991**, *75*, 110.
(6) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369.
(7) Mauk, A. G. *Struct. Bonding* **1991**, *75*, 131.
(8) Kostić, N. M. *Met. Ions Biol. Syst.* **1991**, *27*, 129.
(9) Willie, A.; Stayton, P. S.; Sligar, S. G.; Durham, B.; Millett, F. *Biochemistry* **1992**, *31*, 7237.
(10) Northrup, S. H.; Boles, J. O.; Reynolds, J. C. L. *Science* **1988**, *241*, 67.
(11) Wendoloski, J. J.; Matthew, J. B.; Weber, P. C.; Salemme, F. R. *Science* **1987**, *238*, 794.
(12) Rodgers, K. K.; Pochapsky, T. C.; Sligar, S. G. *Science* **1988**, *240*, 1657.
(13) Burch, A. M.; Rigby, S. E. J.; Funk, W. D.; MacGillivray, R. T. A.; Mauk, M. R.; Mauk, A. G.; Moore, G. R. *Science* **1990**, *247*, 831.
(14) Wallin, S. A.; Stemp, E. D. A.; Everest, A. M.; Nocek, J. M.; Netzel, T. L.; Hoffman, B. M. *J. Am. Chem. Soc. 113*, 1842.
(15) McLendon, G.; Zhang, Q.; Wallin, S. A.; Miller, R. M.; Billstone, V.; Spears, K. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1993**, *115*, 3665.
(16) Nocek, J. M.; Stemp, E. D. A.; Finnegan, M. G.; Koshy, T. I.; Johnson, M. K.; Margoliash, E.; Mauk, A. G.; Smith, M.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 6822.
(17) McLendon, G.; Pardue, K.; Bak, P. *J. Am. Chem. Soc.* **1987**, *109*, 7540.
(18) Feitelson, J.; McLendon, G. *Biochemistry* **1991**, *30*, 5051.
(19) Walker, M. C.; Tollin, G. *Biochemistry* **1991**, *30*, 5546.
(20) Walker, M. C., Tollin, G. *Biochemistry* **1992**, *31*, 2798.
(21) Sullivan, E. P., Jr.; Hazzard, J. T.; Tollin, G.; Enemark, J. H. *J. Am. Chem. Soc.* **1992**, *114*, 9662.
(22) Moser, C. C.; Dutton, P. L. *Biochemistry* **1988**, *27*, 2450.

0002-7863/93/1515-10796$04.00/0

Plastocyanin and Cytochrome *c*. The blue copper protein plastocyanin[26,27] and the heme protein cytochrome *c*,[28,29] which are designated pc and cyt, are well suited to studies of protein–protein orientation because their three-dimensional structures in both oxidized and reduced states are known in detail. Plastocyanin is notable for it contains two distinct surface patches through which it can exchange electrons with redox partners. The broad, negatively charged acidic patch is remote from the copper atom, whereas the electroneutral hydrophobic patch is proximate to this atom. The β-barrel structure of plastocyanin seems to provide relatively strong electronic coupling of the copper atom to the strands to which it is ligated but not to other strands. Both the acidic patch and the hydrophobic patch consist of the side chains in the ligated strands.[26,27,30] The channel via the hydrophobic patch seems to afford stronger electronic coupling, but the channel via the acidic patch allows electrostatic binding of the positively charged redox agent to the plastocyanin surface.[31,32] The existence of multiple docking sites and of multiple electron-transfer paths makes plastocyanin suitable to the study of rearrangement of a diprotein complex.

Because the positively charged (basic) patch of lysine residues surrounding the exposed heme edge in cytochrome *c* is the only surface site for efficient electron transfer, this protein is well suited to probing the surface of plastocyanin. Replacement of iron(II) by zinc(II) and tin(IV) does not perturb the conformation of cytochrome *c* and its association with other proteins,[33-35] and it makes the excited triplet state of the porphyrin long-lived and suitable for kinetic studies. Whereas native ferrocytochrome *c* is a weak reductant ($E°$ = 0.26 V vs NHE), the triplet state of the reconstituted protein is a stronger one: E° is –0.88 and –0.4 V for the zinc(II)[36-38] and tin(IV)[39] derivatives, respectively. Cupriplastocyanin ($E°$ = 0.36 V) can readily oxidize either of them. (All the reduction potentials are at pH 7.0.).

Because ferrocytochrome *c* and cupriplastocyanin bear respective net charges of +6 and –8 at pH 7.0, and because they contain oppositely charged surface patches, these two proteins associate in solution at low ionic strength. Much evidence shows that in the complexes cyt/pc and Zn(cyt)/pc the positive patch around the exposed heme edge is docked against the broad negative patch in plastocyanin.[40-42] This configuration is noninvasively reinforced by covalent cross-linking in the presence of a carbodiimide.[43-46] Besides efficiently cross-linking the proteins, carbodiimide converts certain carboxylate groups into neutral *N*-acylurea groups,[46,47] so that cation-exchange chromatography yields eight derivatives of the complex Zn(cyt)/pc. These derivatives differ in location and number of the neutralized side chains but not in the general protein–protein orientation. In all the derivatives the cross-links join the basic heme patch in zinc cytochrome *c* and the acidic remote patch in plastocyanin.[46] This configuration, however, need not be the reactive one. Studies in our laboratory and other laboratories of the unimolecular reaction in eq 1[44,45,48,49] and of the bimolecular reaction in eq 2[50-52] showed

$$\text{cyt(II)/pc(II)} \rightarrow \text{cyt(III)/pc(I)} \quad (1)$$

$$\text{cyt(II)} + \text{pc(II)} \rightarrow \text{cyt(III)} + \text{pc(I)} \quad (2)$$

that the weak reductants ferrocytochrome *c* and ferrocytochrome *f* reduce cupriplastocyanin from the acidic patch but not from the initial binding site within this broad patch. Our studies[53-55] of the corresponding reactions in eqs 3 and 4 showed that the strong

$$^3\text{Zn(cyt)/pc(II)} \rightarrow \text{Zn(cyt)}^+\text{/pc(I)} \quad (3)$$

$$^3\text{Zn(cyt)} + \text{pc(II)} \rightarrow \text{Zn(cyt)}^+ + \text{pc(I)} \quad (4)$$

reductant zinc cytochrome *c* in the triplet state can reduce cupriplastocyanin from the initial binding site within the acidic patch when rearrangement is prevented by cross-linking. But when the rearrangement is possible, in the electrostatic complex ^{3}Zn(cyt)/pc(II), reduction seems to occur faster. Both before and after the rearrangement, however, the heme edge seems to abut the acidic patch.

Now we study the kinetics of this rearrangement in the reactions in eqs 3 and 5 and investigate the interplay between structural rearrangement and the electron-transfer event. We also observe

$$^3\text{Sn(cyt)/pc(II)} \rightarrow \text{Sn(cyt)}^+\text{/pc(I)} \quad (5)$$

$$^3\text{Sn(cyt)} + \text{pc(II)} \rightarrow \text{Sn(cyt)}^+ + \text{pc(I)} \quad (6)$$

the reactions in eqs 4 and 6 and briefly comment on them. This complete study of the two reactions follows our preliminary study of the reaction in eq 3.[54]

Viscosity Effects. The role of solvent in electron-transfer reactions is the subject of various theories at the molecular level.[56-58] One of the solvent effects is to modulate protein motion. Investigation of kinetic effects of solvent viscosity revealed much about the mechanism and dynamics of bimolecular reactions,[59] unimolecular reactions,[60] motion of small molecules inside

(23) (a) Hoffman, B. M.; Ratner, M. A. *J. Am. Chem. Soc.* **1987**, *109*, 6237. (b) See also: Hoffman, B. M.; Ratner, M. A. *J. Am. Chem. Soc.* **1988**, *110*, 8267.
(24) Hoffman, B. M.; Ratner, M. A.; Wallin, S. A. *Adv. Chem. Ser.* **1990**, *226*, 125.
(25) Brunschwig, B. S.; Sutin, N. *J. Am. Chem. Soc.* **1989**, *111*, 7454.
(26) Sykes, A. G. *Struct. Bonding* **1991**, *75*, 177.
(27) Sykes, A. G. *Adv. Inorg. Chem.* **1991**, *36*, 377.
(28) Pettigrew, G. W.; Moore, G. R. *Cytochromes c: Biological Aspects*; Springer-Verlag: Berlin, 1987.
(29) Moore, G. R.; Pettigrew, G. W. *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects*; Springer-Verlag: Berlin, 1990.
(30) Betts, J. N.; Beratan, D. N.; Onuchic, J. N. *J. Am. Chem. Soc.* **1992**, *114*, 4043.
(31) Christensen, H. E. M.; Conrad, L. S.; Mikkelsen, K. V.; Nielsen, M. K.; Ulstrup, J. *Inorg. Chem.* **1990**, *29*, 2808.
(32) Christensen, H. E. M.; Conrad, L. S.; Ulstrup, J.; Mikkelsen, K. V. *Met. Ions Biol. Syst.* **1991**, *27*, 57.
(33) Vanderkooi, J. M.; Erecińska, M. *Eur. J. Biochem.* **1975**, *60*, 199.
(34) Vanderkooi, J. M.; Adar, F.; Erecińska, M. *Eur. J. Biochem.* **1976**, *64*, 381.
(35) Moore, G.; Williams, R. J. P.; Chien, J. C. W.; Dickinson, L. C. *J. Inorg. Biochem.* **1980**, *13*, 1.
(36) Magner, E.; McLendon, G. *J. Phys. Chem.* **1989**, *93*, 7130.
(37) Conklin, K. T.; McLendon, G. *J. Am. Chem. Soc.* **1988**, *110*, 3345.
(38) Nocek, J. M.; Liang, N.; Wallin, S. A.; Mauk, A. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1990**, *112*, 1623.
(39) Estimated from the redox potential of tin(IV) porphyrin: Fuhrhop, J. H.; Mauzerall, D. *J. Am. Chem. Soc.* **1969**, *91*, 4174.
(40) King, G. C.; Binstead, R. A.; Wright, P. E. *Biochim. Biophys. Acta* **1985**, *806*, 262.
(41) Bagby, S.; Driscoll, P. C.; Goodall, K. G.; Redfield, C.; Hill, H. A. O. *Eur. J. Biochem.* **1990**, *188*, 413.
(42) Roberts, V. A.; Freeman, H. C.; Getzoff, E. D.; Olson, A. J.; Tainer, J. A. *J. Biol. Chem.* **1991**, *266*, 13431.
(43) Geren, L. M.; Stonehuerner, J.; Davis, D. J.; Millett, F. *Biochim. Biophys. Acta* **1983**, *724*, 62.
(44) Peerey, L. M.; Kostić, N. M. *Biochemistry* **1989**, *28*, 1861.
(45) Peerey, L. M.; Brothers, H. M., II; Hazzard, J. T.; Tollin, G.; Kostić, N. M. *Biochemistry* **1991**, *30*, 9297.
(46) Zhou, J. S.; Brothers, H. M., II; Neddersen, J. P.; Cotton, T. M.; Peerey, L. M.; Kostić, N. M. *Bioconjugate Chem.* **1992**, *3*, 382.
(47) Timkovich, R. *Anal. Biochem.* **1977**, *79*, 135.
(48) Qin, L.; Kostić, N. M. *Biochemistry* **1993**, *32*, 6073.
(49) Meyer, T. E.; Zhao, Z. G.; Cusanovich, M. A.; Tollin, G. *Biochemistry* **1993**, *32*, 4552.
(50) Modi, S.; He, S.; Gray, J. C.; Bendall, D. S. *Biochim. Biophys. Acta* **1992**, *1101*, 64.
(51) Modi, S.; Nordling, M.; Lundberg, L. G.; Hansson, Ö.; Bendall, D. S. *Biochim. Biophys. Acta* **1992**, *1102*, 85.
(52) Qin, L.; Kostić, N. M. *Biochemistry* **1992**, *31*, 5145.
(53) Zhou, J. S.; Kostić, N. M. *J. Am. Chem. Soc.* **1991**, *113*, 7040.
(54) Zhou, J. S.; Kostić, N. M. *J. Am. Chem. Soc.* **1992**, *114*, 3562.
(55) Zhou, J. S.; Kostić, N. M. *Biochemistry* **1993**, *32*, 4539.
(56) Heitele, H. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 359.
(57) Weaver, M. J. *Chem. Rev.* **1992**, *92*, 463.
(58) Fleming, G. R.; Wolynes, P. G. *Phys. Today* **1990**, *5* (May), 36.
(59) Rice, S. A. *Diffusion-Limited Reactions: Comprehensive Chemical Kinetics* Vol. 25; Elsevier: Amsterdam, 1985.
(60) Hänggi, P.; Talkner, P.; Borkovec, M. *Rev. Mod. Phys.* **1990**, *62*, 251.

proteins,[61] enzyme catalysis,[62] protein folding,[63] and protein conformational changes.[64] We know of only one prior study of metalloprotein electron-transfer reactions in which viscosity was varied[38] and of none in which this variation is quantitatively analyzed as in the present work.

Materials and Methods

Chemicals. Horse-heart cytochrome *c* (Type III) was obtained from Sigma Chemical Co. Iron was removed, the free-base protein was purified, and zinc(II) was inserted by published procedures.[33,34] Reconstituted cytochrome *c* was always handled in the dark. French-bean plastocyanin was isolated[65] and purified[55] by standard methods until the absorbance quotient A_{278}/A_{597} became less than 1.20. Distilled water was demineralized to a resistance greater than 15 MΩ cm. Ultrafiltration was done in Amicon cells, with YM-5 membranes, at 4 °C, under pressure of purified nitrogen.

Sn(cyt). Insertion of tin(IV) was carried out by a modification[66] of the published procedure.[34,67] Everything was done in the dark. A 0.1 mM solution of the free-base protein in a 5 mM phosphate buffer at pH 7.0 was adjusted to pH 2.5 with glacial acetic acid. The final solution contained about 10% by volume of acetic acid. An equivalent amount of $SnCl_2$, as a 100 mM solution at pH 2.5, was added slowly, in small drops. The reaction mixture was kept at 50 °C, in the dark, and the reconstitution was monitored by recording UV–vis spectra of aliquots. The nonprotein solutes were removed by ultrafiltration and by a column of Sephadex G-25 equilibrated with an 85 mM phosphate buffer at pH 7.0; the same buffer was the eluent. The major band was concentrated by ultrafiltration and purified again, on a column of CM-52 sized 2.5 × 40 cm that was equilibrated with a 10 mM phosphate buffer at pH 7.0; an 85 mM phosphate buffer at pH 7.0 was the eluent. The major band was used in further experiments.

Covalent Complex Zn(cyt)/pc. Cross-linking experiments followed the published procedure,[46,68] and everything was done in the dark. The reaction mixture was 80 μM in each protein and 1.0 mM in the carbodiimide EDC, the solvent was a 5.0 mM MOPS buffer at pH 6.5, and the incubation lasted for 24 h at room temperature. The diprotein complex was dialyzed and concentrated by ultrafiltration with an 85 mM phosphate buffer at pH 7.0. It was purified first on a column of 50-mesh Sephadex G-75 sized 1.0 × 75 cm with the same phosphate buffer as the eluent and next on a column of CM-52 sized 2.5 × 30 cm. The first two fractions were eluted with a 5.0 mM phosphate buffer at pH 7.0, and the next six fractions required a shallow gradient from an 8.5 mM to a 40 mM phosphate buffer at pH 7.0.

Kinetics. Laser kinetic spectroscopy (so-called laser flash photolysis) at microsecond resolution was done with a standard apparatus, described in an earlier publication.[55] Potassium phosphate buffer for kinetic experiments had pH 7.0 and an ionic strength of 10 mM; the ionic strength was raised with NaCl and lowered by dilution. Decay of the triplet states ^{3}Zn(cyt) and ^{3}Sn(cyt) was monitored at 460 nm because transient absorbances reach their maxima at this wavelength. After each laser pulse, 500 data points were collected, and each signal was an average of six pulses. Formation and decay of the cation radical $Zn(cyt)^+$ were monitored at 675 nm, where the difference in absorbance between the cation radical and the triplet state is greatest. Again, after each pulse, 500 data points were collected, but now each signal was an average of 20 pulses. The electrostatic complexes Zn(cyt)/pc(II), Zn(cyt)/pc(I), and Sn(cyt)/pc(II) were preformed in the phosphate buffer at pH 7.0, at the ionic strength of 2.5–20 mM. In many experiments, the viscosity of the solutions was adjusted by adding buffered solutions, at the same ionic strength, of glycerol, ethylene glycol, or D-glucose; in some cases pure ethylene glycol was added. In other experiments, dielectric properties and polarities of the solutions were adjusted by adding buffered solutions, at the same ionic strength, of methanol or *p*-dioxane. The covalent complex Zn(cyt)/pc was dissolved in the phosphate buffer at pH 7.0, at the ionic strength of 2.5 mM–1.00 M. Further details are given in the table headings and figure captions.

Table I. Relative Increase in the Soret Absorbance upon Addition of Organic Solvents to the Protein Solution in the Phosphate Buffer at pH 7.0 and 25 °C

	$\Delta A/A$	
wt % org solv	Zn(cyt)	electrostatic complex Zn(cyt)/pc(II)
25% dioxane	0.051	0.0024
30% methanol	0.124	0.043
60% ethylene glycol	0.090	0.0048
80% glycerol	0.038	0.0020

Table II. Rate Constants for Exponential Decay of the Triplet Excited State of Reconstituted Cytochrome *c* in Phosphate Buffer and in Buffered Mixtures, All at pH 7.0 and 25 °C

	rate constant, s^{-1}		
wt % org solv	^{3}Zn(cyt)	^{3}Sn(cyt)	electrostatic complex ^{3}Zn(cyt)/pc(I)
0%	120 ± 10	550 ± 20	200 ± 20
25% dioxane	160 ± 10		200 ± 20
30% methanol	170 ± 10		210 ± 20
60% ethylene glycol	110 ± 10	540 ± 20	180 ± 20
80% glycerol	83 ± 8	530 ± 20	170 ± 20

Results

Ultraviolet–Visible Spectra of the Proteins. The characteristic blue band of cupriplastocyanin remained at 597 nm when the phosphate buffer at pH 7.0 was made 25% in *p*-dioxane, 30% in methanol, 60% in ethylene glycol, or 80% in glycerol. (All of these are percentages by weight.) The absorbance at this band increased slightly upon addition of these buffered organic solvents to the pure buffer—by 7.0% in the case of methanol and by less than 1.0% in the case of ethylene glycol and glycerol—and remained constant afterward. The blue absorbance decreased upon addition of *p*-dioxane and continued decreasing afterward, by 12% in 1 h and by 34% in 4 h.

The characteristic Soret band of zinc cytochrome *c* did not shift in any of the four mixtures mentioned above. Its absorbance increased in going from the pure buffer to the mixtures but did not change with time. The electrostatic complex Zn(cyt)/pc(II) behaved like zinc cytochrome *c* alone, with two exceptions. First, the increase in absorbance was much smaller, as Table I shows. Second, the absorbances of the blue and Soret bands in the 25% *p*-dioxane solution changed with time. The spectra of the other three solutions remained unchanged with time.

Decay of the Triplet State in the Free ^{3}Zn(cyt) and ^{3}Sn(cyt). The triplet excited state of each reconstituted cytochrome *c* in the absence of plastocyanin decays exponentially. The rate constants are given in Table II.

Decay of the Triplet State in the Electrostatic Complex ^{3}Zn(cyt)/pc(I). This electrostatic diprotein complex was formed in solutions containing 10 μM zinc cytochrome *c* and 20–30 μM cuproplastocyanin in a 2.5 mM phosphate buffer at pH 7.0. The decay of the triplet state was exponential, and the rate constants are given in Table II. The absorbance at 435 nm did not change.

Quenching of the Triplet State ^{3}Zn(cyt) by pc(II) and Solvent Effects on Kinetics. Typical concentrations of Zn(cyt) and pc(II) were 10 and up to 30 μM, respectively. Only a small fraction of the former was excited into the triplet state. Decay of ^{3}Zn(cyt) in the presence of cupriplastocyanin at low ionic strength (2.5–20 mM) is biphasic. All solutions were buffered with phosphate at pH 7.0. The trace in Figure 1a could not be fitted to a single exponential but was fitted very well to two exponentials, according to eq 7. The early or fast component and the late or slow

$$F(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) \qquad (7)$$

(61) Beece, D.; Eisenstein, L.; Frauenfelder, H.; Good, D.; Marden, M. C.; Reinisch, L.; Reynolds, A. H.; Sorensen, L. B.; Yue, K. T. *Biochemistry* **1980**, *19*, 5147.
(62) Khoshtariya, D. E.; Hammerstad-Pedersen, J. M.; Ulstrup, J. *Biochim. Biophys. Acta* **1991**, *1076*, 359.
(63) Chrunyk, B. A.; Matthews, C. R. *Biochemistry* **1990**, *29*, 2149.
(64) Ansari, A.; Jones, C. M.; Henry, E. R.; Hofrichter, J.; Eaton, W. A. *Science* **1992**, *256*, 1796.
(65) Milne, P. R.; Wells, J. R. E. *J. Biol. Chem.* **1970**, *245*, 1566.
(66) Vanderkooi, J. M. Personal communication.
(67) Erecińska, M.; Vanderkooi, J. M. *Methods Enzymol.* **1978**, *53*, 165.
(68) Zhou, J. S.; Kostić, N. M. *Biochemistry* **1992**, *31*, 7543.

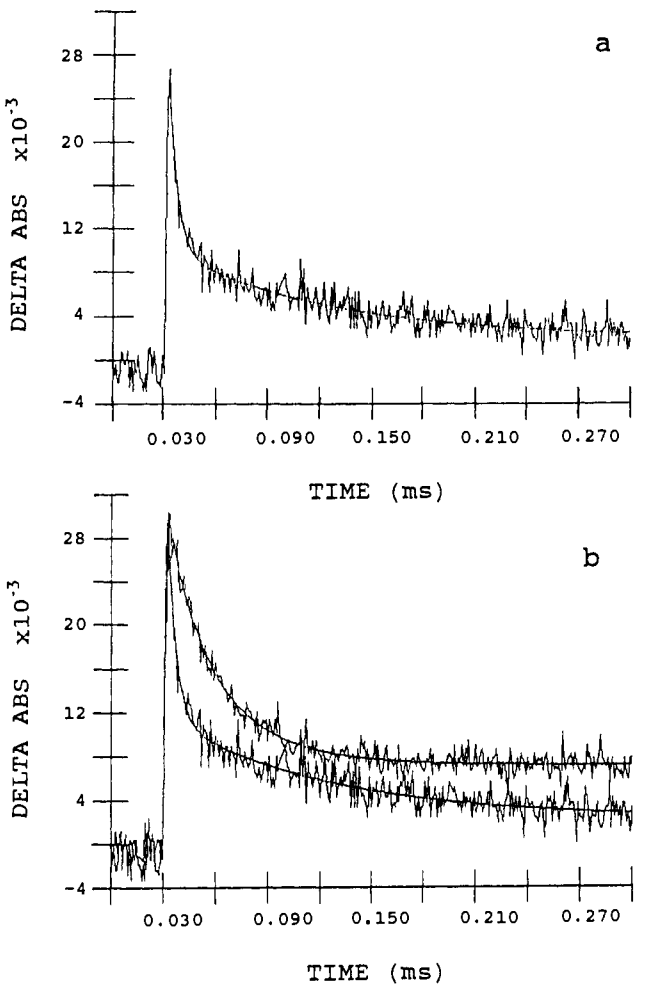


Figure 1. Redox quenching of the triplet state ^{3}Zn(cyt), monitored at 460 nm. The solid lines are biexponential fits. (a) The solution contains 10 μM zinc cytochrome *c* and 20 μM cupriplastocyanin in a phosphate buffer at an ionic strength of 2.5 mM and pH 7.0. (b) Solutions are as in part a but contain 60% (upper trace) and 80% (lower trace) glycerol by weight.

component of the decay are designated with subscripts 1 and 2, respectively. The symbols A and k stand for amplitude and first-order rate constant. The decay of the triplet state remained biphasic upon addition to the buffer of the five organic compounds, one at a time. The mixtures containing glycerol were investigated especially thoroughly—its concentration was varied from 28 to 80% by weight, and the ionic strength of the mixed solvent was varied from 2.5 to 20 mM. Some typical results are shown in Figure 1b. Mixtures containing the other four organic compounds were kept at the ionic strength of 2.5 mM, and the concentrations (percentages by weight) were as follows: 20–60% ethylene glycol, 22–52% D-glucose, 30% methanol, and 25% *p*-dioxane.

The solutions in the pure phosphate buffer and solutions containing added glycerol were adjusted also to ionic strengths from 30 mM to 3.00 M. In both of these solvents, at these higher ionic strengths, the decay of the triplet state ^{3}Zn(cyt) in the presence of cupriplastocyanin was monoexponential, and the observed rate constant increased linearly with increasing cupriplastocyanin concentration.

We will describe separately the two components of the biphasic quenching of ^{3}Zn(cyt) by cupriplastocyanin. The slow component behaves simply—the rate constant k_2 is directly proportional to concentration of cupriplastocyanin. This was found in all solvents, that is, with or without the organic additive in the buffer and at all concentrations of these additives.

The fast component of ^{3}Zn(cyt) quenching behaved in a more interesting way. The rate constant k_1 is independent of cupriplastocyanin concentration and of ionic strength in all solvents, with or without the organic additive in the buffer and at all concentrations of these additives. The relative amplitude of the fast component, $A_1/(A_1 + A_2)$, increases with increasing cupriplastocyanin concentration and decreases with increasing ionic strength. Most interestingly, k_1 decreases as the concentration of glycerol, ethylene glycol, or D-glucose in the solvent increases. Typical results are given in Tables III–VI. Both the rate constants and the relative amplitudes remained unchanged, within the margins of error, after the protein solutions in these mixed solvents were left for several hours. The buffered solutions at the ionic

Table III. Rate Constant, k_1, at 25 °C for the Fast Component of the Decay of ^{3}Zn(cyt) (10 μM) in the Presence of Cupriplastocyanin (20 μM) in the Phosphate Buffer at pH 7.0, at Different Ionic Strengths and Different Concentrations (Percentages by Weight) of Glycerol

	$10^{-4}k_1$, s^{-1}				
μ, mM	0%	44%	60%	72%	80%
2.5	25.0	15.7	10.6	6.1	4.0
5.0	24.5	15.8	9.8	6.4	3.8
10	26.0	14.6	9.9	5.7	3.8
15	26.5	14.3	9.6	5.4	4.4
20[a]	26.1	15.5	10.4	6.0	3.4

[a] 40 μM cupriplastocyanin.

Table IV. Relative Amplitude $A_1/(A_1 + A_2)$, for the Fast Component of the Decay of ^{3}Zn(cyt) (10 μM) in the Presence of Different Concentrations of Cupriplastocyanin, in Phosphate Buffer at pH 7.0 and 25 °C, at Different Ionic Strengths and Different Concentrations (Percentages by Weight) of Glycerol

		pc(II) conc, μM						
μ, mM	wt % glycerol	5.0	10	15	20	25	30	40
2.5	0	0.30	0.73	0.86	0.87	0.87		
	60	0.34	0.68	0.77	0.79			
5.0	0		0.48	0.60	0.70	0.74		
	60		0.52	0.59	0.68	0.72		
10	0		0.29	0.46	0.67	0.71	0.74	
	60			0.42	0.56	0.66	0.70	
15	0				0.25	0.39	0.46	
	60				0.26	0.36	0.44	
20	0					0.08	0.17	
	60						0.18	0.31

Table V. Rate Constant, k_1, at 25 °C for the Fast Component of the Decay of ^{3}Zn(cyt) (10 μM) or ^{3}Sn(cyt) (20 μM) in the Presence of Cupriplastocyanin (20 μM) in a 2.5 mM Phosphate Buffer at pH 7.0, at Different Concentrations (Percentages by Weight) of Added Organic Solutes

	$10^{-4}k_1$, s^{-1}				
	glycerol		ethylene glycol		D-glucose
wt %	M is Zn	M is Sn	M is Zn	M is Sn	M is Zn
0	25.0	19.0	25.0	19.0	25.0
20	22.3	15.2	23.6	15.0	
22					21.0
28	19.2	13.6			
32			19.6	13.7	18.4
36		11.8			
40					15.2
44	15.7	9.9	17.1	11.5	13.0
48		8.5			11.7
50					8.9
52	13.6	7.5	16.3	10.4	8.9
56	11.7	6.8	15.6		
60	10.6	5.3	14.0	9.3	
64	8.6	4.5			
68	7.4	3.3			
72	6.1	3.1			
76	5.1	2.0			
80	4.0	1.5			

Table VI. Rate Constant, k_1, and Relative Amplitude, $A_1/(A_1 + A_2)$, for the Fast Component of the Decay of ^{3}Zn(cyt) (10 μM) in the Presence of Different Concentrations of Cupriplastocyanin in a 2.5 mM Phosphate Buffer at pH 7.0 Containing 30% by Weight of Methanol, at 25 °C

pc(II) conc, μM	$10^{-5}k_1$, s^{-1}	$A_1/(A_1 + A_2)$
5.0	2.32	0.47
10	2.25	0.69
15	2.32	0.79
20	2.28	0.81

strength of 2.5 mM containing 25% by weight of *p*-dioxane behaved like all the other solutions for several hours. As long as the fast component of the ^{3}Zn(cyt) decay was present, its rate constant and relative amplitude behaved as described above. In the solutions containing *p*-dioxane, however, the absolute amplitude A_1 decreased slowly with time. After more than 24 h, this fast component of the ^{3}Zn(cyt) decay disappeared, the cupriplastocyanin absorbance at 597 nm disappeared, and the decay became monoexponential, as for free ^{3}Zn(cyt) under identical conditions.

Quenching of the Triplet State ^{3}Sn(cyt) by pc(II) and Solvent Effects on Kinetics. These experiments were completely analogous to those in the previous subsection except that the tin derivative replaced the zinc derivative of cytochrome *c*. The ionic strength of the phosphate buffer at pH 7.0 was varied between 2.5 and 20 mM, the concentration of tin cytochrome *c* was 20 μM, and the cupriplastocyanin concentration was raised to 30 μM. Again, the decay of the triplet state was biphasic, the rate constant k_2 was directly proportional to cupriplastocyanin concentration, the rate constant k_1 was independent of this concentration and of ionic strength, and the relative amplitude of the fast component of the triplet decay increased with increasing quencher concentration and decreased with increasing ionic strength. All of these characteristics remained when glycerol or ethylene glycol were added to the protein solution. Again, the rate constant k_1 decreased as the concentration of these organic solutes increased and remained independent of the cupriplastocyanin concentration. The kinetic results did not change after the protein solutions in these mixed solvents were incubated for 4 h. The most important results are given in Table V.

Quenching of the Triplet State ^{3}Zn(cyt) in the Covalent Complex Zn(cyt)/pc. The concentration of the covalent complex was varied between 5.0 and 30 μM, and the ionic strength of the solutions buffered with phosphate at pH 7.0 was varied between 10 mM and 1.00 M. The eight fractions of the covalent complexes from the cation exchanger CM-52 were examined separately. All of these chromatographic fractions of Zn(cyt)/pc(I), which contains cuproplastocyanin, decay with the same rate constant of 190 ± 10 s^{-1}. This rate constant is independent of the complex concentration and of ionic strength.

Complete oxidation of plastocyanin in this covalent complex requires an excess of $[Fe(CN)_6]^{3-}$ in solution. Because this oxidant had to be removed lest it quench ^{3}Zn(cyt), oxidation of plastocyanin was only partial. The eight fractions of the covalent complex Zn(cyt)/pc containing both pc(I) and pc(II) showed biexponential decay of the triplet state. The two exponentials were completely separable in time. The rate constant of the faster process does not, whereas that of the slower process does, depend on the complex concentration and ionic strength. The relative amplitudes of the two exponentials match the proportion of cupriplastocyanin and cuproplastocyanin in the covalent complex, determined by UV–vis spectrophotometry. We chose for further experiments the chromatographic fractions numbers 1 and 5 in the order of elution from the CM-52 column. The rate constant of the slower decay component decreased as the concentration of glycerol in the phosphate buffer at pH 7.0 increased. The rate constant k_1 and the relative amplitude $A_1/(A_1 + A_2)$ of the faster decay component were unaffected by the addition of glycerol and of ethylene glycol to the buffer. This independence of the added solutes makes the results for the covalent complex, in Table VII, different from the results for the electrostatic complex, in Table V.

Table VII. Rate Constant, k_1, at 25 °C for the Fast Component of the Decay of the Triplet State in a 10 μM Solution of the Covalent Complex Zn(cyt)/pc(II) in a 2.5 mM Phosphate Buffer at pH 7.0, at Different Concentrations (Percentages by Weight) of Glycerol

	$10^{-4}k_1$, s^{-1}	
wt % glycerol	fraction 1[a]	fraction 5[a]
0	2.45	2.50
28	2.68	2.55
44		2.21
60	2.56	2.63
72		2.15
80		2.17

[a] Elution order from CM-52.

Discussion

Effects of Solvents on the Proteins. Experiments with proteins are commonly done in buffered aqueous solutions that contain no additional solutes. For some purposes, however, organic solvents and other solutes are added. Glycerol, ethylene glycol, and glucose are widely used because of their ability to stabilize proteins and because of their favorable properties at low temperatures.

The rate constant of 120 ± 10 s^{-1} in Table II falls in the middle of a narrow range, 70–140 s^{-1}, of the values for free ^{3}Zn(cyt) reported before.[69-73] The rate constant of 550 ± 20 s^{-1} in the absence of organic solutes is somewhat greater than the value of 340 s^{-1}, reported before for free ^{3}Sn(cyt).[74] The slight variation in the rate constants 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.

The UV–vis spectra and triplet lifetimes indicate that 80% glycerol and 60% ethylene glycol can be considered noninvasive, that 30% methanol is slightly invasive, and that 25% *p*-dioxane is invasive. Because a classification of this kind cannot be exact, it requires an explanation. A mere difference in solvation, as manifested in a small but constant change in the UV–vis spectrum, without a significant increase in the rate constant for the triplet decay is not considered a symptom of structural change, and solvents that cause it are regarded as noninvasive. A progressive change in the UV–vis spectrum and a significant increase in the rate constant for the triplet decay, which may reflect a slight opening of the heme crevice, are considered symptoms of structural change. Only the solvents that cause such effects are regarded as invasive. This definition of ours is more cautious than the criterion of invasiveness used in other studies. It was claimed that methanol and *p*-dioxane do not unfold horse cytochrome *c*,[75] and spectroscopic experiments indicated that methanol alters the conformation of this protein only at concentrations greater than 33% by weight.[76] The crucial experiments in this study were done with glycerol and ethylene glycol, which certainly are

(69) Elias, H.; Chou, M. H.; Winkler, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 429.
(70) Vos, K.; Lavalette, D.; Visser, A. J. W. G. *Eur. J. Biochem.* **1987**, *169*, 269.
(71) Dixit, S. N.; Waring, A. J.; Vanderkooi, J. M. *FEBS Lett.* **1981**, *125*, 86.
(72) Horie, T.; Maniara, G.; Vanderkooi, J. M. *FEBS Lett.* **1985**, *177*, 287.
(73) Dixit, B. P. S. N.; Moy, V. T.; Vanderkooi, J. M. *Biochemistry* **1984**, *23*, 2103.
(74) Koloczek, H.; Horie, T.; Yonetani, T.; Anni, H.; Maniara, G.; Vanderkooi, J. M. *Biochemistry* **1987**, *26*, 3142.
(75) Kaminsky, L. S.; Davison, A. J. *Biochemistry* **1969**, *8*, 4631.
(76) Kaminsky, L. D.; Yong, F. C.; King, T. E. *J. Biol. Chem.* **1972**, *254*, 4110.

Table VIII. Properties of Pure Solvents at 20 °C[a]

solvent	ϵ[b]	Z, kcal·mol^{-1} [c]	E, kcal·mol^{-1} [d]	η/η_0[e]
water	80.2	94.6	63.1	1.00
glycerol	42.5			1412
ethylene glycol	37.7	85.1	56.6	19.9
methanol	32.7	83.6	55.5	0.55
p-dioxane	2.21	59.1	36.0	1.44

[a] Sources: Murov, S. L. *Handbook of Photochemistry*; Marcel Dekker: New York, 1973; Table 9-1. Scaiano, J. C., Ed. *CRC Handbook of Organic Photochemistry*; CRC Press: Boca Raton, FL, 1989; Vol. II, Chapter 14, Table 1. [b] Static dielectric constant. [c] Kosower's polarity parameter. [d] Dimroth's polarity parameter. [e] Viscosity relative to water.

noninvasive. Methanol and *p*-dioxane in relatively low concentrations were cautiously used in control experiments.

The electrostatic complex Zn(cyt)/pc was prepared in pure phosphate buffer, at low ionic strength. As Table I shows, effects of differential solvation upon addition of organic solvents are far smaller for zinc cytochrome *c* in the complex than for free zinc cytochrome *c*. This finding is yet another piece of evidence for the protein–protein orientation in the complex, discussed above; it shows that the exposed heme edge is largely covered by the plastocyanin molecule.[46]

As was the case with separate proteins, the only solvent that perturbs the associated proteins in Zn(cyt)/pc(II) is 25% *p*-dioxane. Being the least polar and the most hydrophobic (Table VIII), this organic compound is the most effective in penetrating into the proteins, especially plastocyanin. Although *p*-dioxane unfolds plastocyanin and thus breaks up the complex, this process is sufficiently slow to allow reliable control experiments to be done with fresh solutions, in a relatively short time.

Inefficient Nonredox Quenching of the Triplet State in the Electrostatic Complex ^{3}Zn(cyt)/pc(I). The triplet state of zinc cytochrome *c* can, in principle, both donate ($E°$ = –0.88 V vs NHE)[36] and accept ($E°$ = 0.40 V)[37] electrons. The slight enhancement of the triplet decay upon association with cuproplastocyanin, shown in Table II, probably is not due to the reductive quenching in eq 8. The anion radical Zn(cyt)$^-$ would

$$^3\text{Zn(cyt)/pc(I)} \nrightarrow \text{Zn(cyt)}^-\text{/pc(II)} \qquad (8)$$

be expected to absorb light at 435 nm, the isosbestic point for the ground and triplet states of zinc cytochrome *c*. The lack of absorbance change at this wavelength militates against the involvement of the anion radical. Neither we nor others observed it in this and other diprotein systems. The reaction in eq 8, with the driving force of only ca. 0.03 eV, cannot compete with the natural decay. The slight enhancement of the decay cannot be due to energy transfer, either, because the emission spectrum of the triplet state and the absorption spectrum of cuproplastocyanin (which lacks the blue band) do not overlap. Indeed, we have previously found a similar slight enhancement of the ^{3}Zn(cyt) decay upon its association with apoplastocyanin, which has essentially the same conformation as holoplastocyanin.[77] All the evidence indicates that the increase from 120 ± 10 to 200 ± 20 s^{-1} can be attributed to a slight conformational change in zinc cytochrome *c* upon docking with plastocyanin. Others reached the analogous conclusion in a study involving cytochrome *c* peroxidase.[74]

Efficient Redox Quenching of the Triplet States in the Electrostatic Complexes ^{3}Zn(cyt)/pc(II) and ^{3}Sn(cyt)/pc(II). Cupriplastocyanin can oxidatively quench the triplet state, as the great increase in k_1 from Table II (200 and 550 s^{-1}) to Table III (2.5×10^5 and 1.9×10^5 s^{-1}, respectively) clearly shows. This quenching is biphasic because, at low ionic strength, the (reconstituted) cytochrome *c* exists in two forms—free and associated with the quencher. Consequently, the laser pulse creates two populations of the triplet state, which are quenched by unimolecular (eqs 3 and 5) and bimolecular (eqs 4 and 6) reactions, which have the respective rate constants k_1 and k_2. These two exponential processes are experimentally separable and will be discussed separately. This short analysis, concerning primarily kinetics at low ionic strength, completely agrees with our previous detailed analysis[77] of kinetics over a wide range of ionic strengths.

In an earlier study,[77] we observed the formation and decay of the cation radical Zn(cyt)$^+$ in both the slower and the faster components of the biphasic quenching. This evidence and comparisons among copper(II), copper(I), cobalt(II), and apo forms of plastocyanin as quenchers for ^{3}Zn(cyt) all showed that quenching by cupriplastocyanin occurs almost exclusively by electron transfer. Contribution of energy transfer to the rate constant is less than 500 s^{-1}. Because the absorption spectrum of cupriplastocyanin overlaps the emission spectra of ^{3}Zn(cyt) and of ^{3}Sn(cyt) to similar extents and because the natural decay of ^{3}Sn(cyt) (550 s^{-1}) is approximately 5 times faster than that of ^{3}Zn(cyt) (120 s^{-1}), the contribution of energy transfer to quenching in the ^{3}Sn(cyt)/pc(II) system should be approximately 5 times greater than in the ^{3}Zn(cyt)/pc(II) system. But even the contribution of ca. 2500 s^{-1} amounts to only ca. 1% of the observed rate constant of 1.9×10^5 s^{-1}, in the ^{3}Sn(cyt)/pc(II) system.

The slower component of the biphasic quenching corresponds to the bimolecular reactions in eqs 4 and 6. Indeed, the rate constant k_2 is linearly proportional to the concentration of free cupriplastocyanin. At ionic strengths of 30 mM and greater, at which the electrostatic complex no longer exists in appreciable concentration, the quenching becomes monoexponential, with the rate constant k_2.

The faster component of the biphasic quenching corresponds to the unimolecular reaction in eq 3 (and eq 5), the main subject of this study. As Tables IV an VI show, the contribution of this reaction to the overall electron-transfer reaction (i.e., its relative amplitude) is proportional to the fraction of associated zinc cytochrome *c*. This fraction increases with increasing cupriplastocyanin concentration and decreases with increasing ionic strength, as expected for the equilibrium between oppositely charged proteins. The fraction is not appreciably altered by addition of the noninvasive solutes glycerol (Table IV), methanol (Table VI), and ethylene glycol. In the presence of *p*-dioxane, however, the diprotein complex gradually breaks up and the triplet decay eventually becomes monoexponential, as for free zinc cytochrome *c*.

The rate constant k_1 is independent of the absolute amplitude A_1 and of the relative amplitude $A_1/(A_1 + A_2)$, as expected for this unimolecular reaction within the diprotein complex. The values of this rate constant are given in Table V.

Independence of the Intracomplex Rate Constant on Ionic Strength. As Table III shows, the rate constant for the reaction in eq 3 does not vary in the ionic strength interval from 2.5 to 20 mM. Ionic strength affects the degree of protein association, i.e., the concentration of the electrostatic complex, but it does not seem to affect electron-transfer properties of this complex. The same was found in our laboratory[44,45] and other laboratories[49] for the reaction in eq 1. A simple explanation of these consistent findings would be that the protein–protein docking (orientation) in the electrostatic complexes does not depend on ionic strength in the range examined because water is excluded from the interface.

In studies of various protein pairs the dependence of the observed rate constant on ionic strength, sometimes at only two ionic strengths, has been taken as evidence for protein rearrangement within the electrostatic complex.[8] This conclusion may well be correct in particular cases, but a note of caution seems warranted. The intuitive notions of "tight" or "stable" versus "loose" or "unstable" electrostatic protein complexes require scrutiny from thermodynamic and kinetic points of view. Positions of equilibria

(77) Zhou, J. S.; Kostić, N. M. *J. Am. Chem. Soc.* **1991**, *113*, 6067.

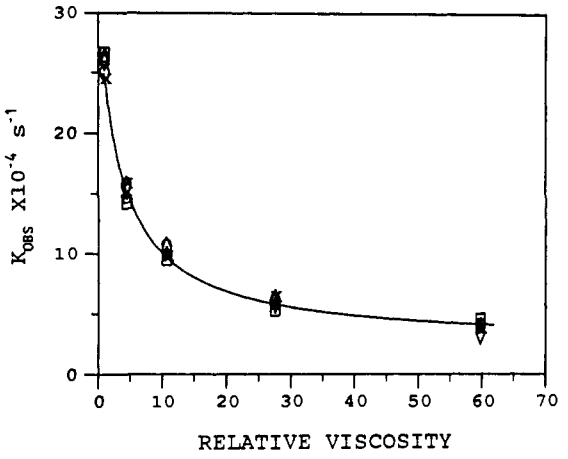


Figure 2. Rate of the unimolecular electron-transfer reaction within the electrostatic complex ^{3}Zn(cyt)/pc(II), in a phosphate buffer at pH 7.0 whose viscosity was adjusted with glycerol. The values at ionic strengths of 2.5 (○), 5.0, (×), 10 (△), 15 (□), and 20 mM (◇) unavoidably overlap. The error bars are smaller than the symbols. The solid line is the best fit to eqs 11 and 12.

among the free protein molecules and their complex require one kind of analysis, and rates at which these equilibria are established require another. More theoretical and experimental studies are needed of the effects of ionic strength on the energy of electrostatic and nonelectrostatic bonds in a diprotein complex. Because the degree of protein association (or dissociation) varies with ionic strength, the observed rate constant may reflect not only the unimolecular reaction within the diprotein complex but also bimolecular reactions between free protein molecules and bimolecular reactions between the diprotein complex and the free protein molecules. The reactions of the last type, which involve ternary protein systems, are amenable to kinetic analysis.[68,78] Finally, different diprotein complexes may undergo different rearrangements and other dynamic processes. Reversible breakup and reformation of the protein complex should depend on ionic strength, but purely unimolecular processes may not depend on it. These various dynamic processes need to be differentiated.

The present study illustrates the need for skeptical analysis. Although the intracomplex reaction does not depend on ionic strength, there is clear evidence for rearrangement of the electrostatic complexes Zn(cyt)/pc(II) and Sn(cyt)/pc(II), which limits the rates of the reactions in eqs 3 and 5. The rest of our discussion is devoted to these important subjects.

Dependence on Solution Viscosity of the Intracomplex Rate Constant in the Electrostatic Complexes ^{3}Zn(cyt)/pc(II) and ^{3}Sn(cyt)/pc(II). Table III shows that the rate constant k_1 for the intracomplex reaction in eq 3 markedly decreases as the concentration of glycerol increases. Although this decrease correlates smoothly with an increase in solvent viscosity, as Figure 2 shows, a decrease in solvent polarity (because glycerol is less polar than water) has to be ruled out as a possible cause of the observed decrease in k_1. Control experiments gave the practically equal k_1 values of 2.5×10^5, 2.3×10^5, and 2.4×10^5 s^{-1}, respectively, in the pure aqueous, 30% methanol, and 25% *p*-dioxane solutions, all of them at the ionic strength of 2.5 mM. Because the dielectric constants of these three (pure) solvents are 80, 65, and 59, k_1 evidently does not depend on the solvent polarity. (Its independence of ionic strength was discussed above.) Dielectric constants of 32% ethylene glycol (65) and 52% glycerol (60) are similar, and yet the respective rate constants are different: 2.0×10^5 and 1.4×10^5 s^{-1} (Table V). These experiments militate against solvent polarity as the cause of the variation in k_1. As Table VIII shows, methanol and *p*-dioxane

(78) Meyer, T. E.; Rivera, M.; Walker, F. A.; Mauk, M. R.; Mauk, A. G.; Cusanovich, M. A.; Tollin, G. *Biochemistry* **1993**, *32*, 622.

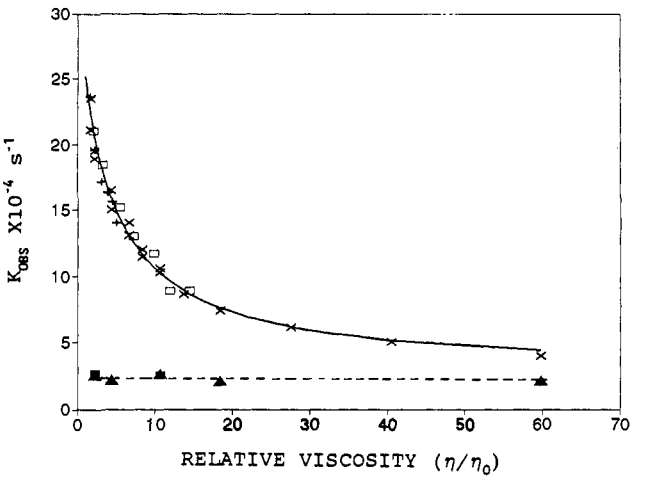


Figure 3. Rate of the unimolecular electron-transfer reaction within the electrostatic (curved points) and covalent (horizontal points) complexes ^{3}Zn(cyt)/pc(II) in a phosphate buffer at ionic strength of 2.5 mM and pH 7.0 whose viscosity was adjusted with glycerol (×), ethylene glycol (+), or D-glucose (□) for the electrostatic complex and with glycerol for the covalent complex. Concentrations are 10 μM zinc cytochrome *c* and 20 μM cupriplastocyanin for the electrostatic complex and 10 μM *N*-acylurea derivatives, chromatographic fractions 1 (▲) and 5 (■), for the covalent complex. The error bars are smaller than the symbols. The solid line is the best fit to eqs 11 and 12, and the dashed line is the best linear fit.

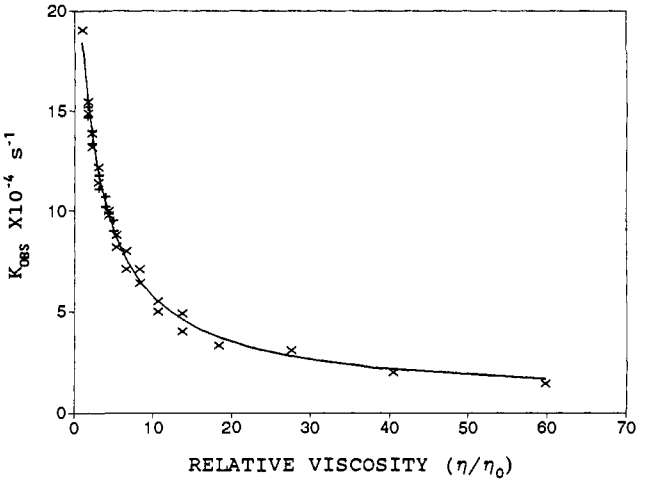
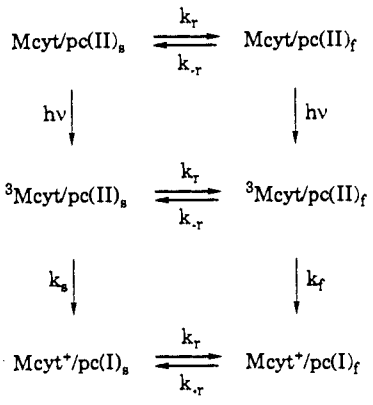


Figure 4. Rate of the unimolecular electron-transfer reaction within the electrostatic complex ^{3}Sn(cyt)/pc(II) in a phosphate buffer at ionic strength 2.5 mM and pH 7.0 whose viscosity was adjusted with glycerol. Concentrations are 20 μM tin cytochrome *c* and 20 μM cupriplastocyanin. The error bars are smaller than the symbols. The solid line is the best fit to eqs 11 and 12.

do not appreciably change the viscosity of the aqueous solution, whereas glycerol changes it drastically. The rate constants in Table V apparently do not correlate with the osmosity of the solutions. For these reasons, we focused on viscosity.

The results in Table V are plotted against viscosity in Figure 3. In solutions adjusted to the same viscosity with three different solutes—glycerol, ethylene glycol, and D-glucose—the k_1 values are equal. All 27 values of k_1 for the reaction in eq 3, determined in buffered mixtures of water with these three different solutes, fall on the same smooth curve when plotted versus the solvent viscosity. All 20 values of k_1 for the analogous reaction in eq 5, determined in buffered mixtures containing two different solutes (glycerol and ethylene glycol), likewise fall on the same smooth plot versus viscosity, shown in Figure 4. Evidently, the decrease in k_1 is caused not by specific protein–solvent interactions but by

Scheme I



M is Zn(II) or Sn(IV)

the increase in viscosity. This decrease is intrinsic to the electrostatic complexes Zn(cyt)/pc(II) and Sn(cyt)/pc(II).

Redox Quenching of the Triplet State in the Covalent Complex ^{3}Zn(cyt)/pc(II). Because the eight chromatographic derivatives of the covalent complex Zn(cyt)/pc(II) have identical properties except for the number and location of the *N*-acylurea groups on the surface, we chose for the kinetic study only two of them, derivatives number 1 and 5. The faster quenching process is the unimolecular intracomplex reaction in eq 3, whereas the slower quenching process is the bimolecular intercomplex reaction in eq 9. Only the intracomplex reaction is relevant to this study, and

$$^3\text{Zn(cyt)/pc(I)} + \text{Zn(cyt)/pc(II)} \rightarrow \text{Zn(cyt)}^+\text{/pc(I)} + \text{Zn(cyt)/pc(I)} \quad (9)$$

only it was examined further.

Independence on Solution Viscosity of the Intracomplex Rate Constant in the Covalent Complex ^{3}Zn(cyt)/pc(II). As Figure 3 shows, for both *N*-acylurea derivatives of this covalent complex $k_1 = (2.4 \pm 0.2) \times 10^4\ s^{-1}$ regardless of the solvent composition. This independence is further evidence that the intracomplex reaction per se is not affected by solvent properties. Absence of any viscosity effects when the proteins are rigidly cross-linked strongly indicates that marked effects upon the electrostatic complexes, which are flexible, are caused by structural rearrangement. The k_1 values for the electrostatic complex converge to those for the covalent complex as viscosity increases; the two extrapolated lines in Figure 3 merge at the relative viscosity of ca. 150, which could not be reached experimentally. This convergence is further evidence that covalent cross-linking reinforces the natural electrostatic complex between the two protein molecules. In other words, cross-linking captures the initial orientation of the electrostatic complex, the same one that is "trapped" by viscous solvents and thus prevented from rearranging.

Kinetics of Rearrangement of the Electrostatic Complexes. The dependence on viscosity of the intracomplex rate constants in ^{3}Zn(cyt)/pc(II) and ^{3}Sn(cyt)/pc(II) can be explained in terms of Scheme I. According to this simple mechanism, the precursor complex ^{3}M(cyt)/pc(II) can rearrange from the initial configuration that is less reactive (hence the subscript s, for "slow" electron transfer) to a different configuration that is more reactive (hence f, for "fast"). The latter complex is such a minor component of the equilibrium mixture that the intracomplex reaction is monophasic under our experimental conditions. The designations s and f of the corresponding successor complexes M(cyt)$^+$/pc(I) are mere labels because their possible structural difference is inconsequential for kinetics. The reversible rearrangement process is marked with subscripts r and –r. The laser flash shifts the equilibrium in favor of the "fast" form because the electron-transfer reaction occurs predominantly along the k_f "branch". The observed intracomplex rate constant k_1 depends on the microscopic rate constants according to eq 10. There is a direct

$$k_1 = k_s + \frac{k_r k_f}{k_{-r} + k_f} \quad (10)$$

link between viscous molecular friction and macroscopic viscosity.[58] When the solute molecules are larger than the solvent molecules, as in our case, the rate of solute reorientation is inversely proportional to viscosity. Only the rate constants k_r and k_{-r} are thus expected to depend on solvent viscosity.

We will consider two special cases. At very high viscosity, when $k_r \ll k_s$, the rate constant k_1 should approach the value of k_s. Indeed, Figure 3 shows that the data points for the flexible electrostatic complex approach the constant value for the rigid covalent complex.

In the viscosity range covered by our experiments, when $k_f \gg k_{-r}$, eq 10 yields eq 11. Fitting to eqs 11 and 12 the data for

$$k_1 = k_s + k_r \quad (11)$$

$$1/k_r = A\eta/\eta_0 + B \quad (12)$$

Zn(cyt)/pc(II) in Table V and Figure 3 yields $k_s = 2.7 \times 10^4\ s^{-1}$ and $1/k_r = (0.94\ \eta/\eta_0 + 3.56)\ \mu s$. The fidelity of this fitting is evident in Figure 3. The fitted value of k_s is very close to the observed rate constant of $(2.4 \pm 0.2) \times 10^4\ s^{-1}$ for the electron-transfer reaction within the covalent complex. This agreement again shows that cross-links capture the initial, "slow", configuration of the electrostatic complex.

In pure buffer, when $\eta/\eta_0 = 1.00$, the rate constant for the rearrangement process is $k_r = 2.2 \times 10^5\ s^{-1}$. The rate constants for the reaction in eq 3 are $(2.5 \pm 0.4) \times 10^5\ s^{-1}$ in the preformed complex, at low ionic strength, and $(2.8 \pm 0.6) \times 10^5\ s^{-1}$ in the encounter complex, at high ionic strength.[77] These two values are equal, and the present study shows that this reaction is gated in both cases.

Fitting to eqs 11 and 12 the data for Sn(cyt)/pc(II) in Table V and Figure 4 yields $k_s = 6.7 \times 10^3\ s^{-1}$ and $1/k_r = (1.56\ \eta/\eta_0 + 4.07)\ \mu s$. In pure buffer, $k_r = 1.8 \times 10^5\ s^{-1}$. The rate constant k_1 for the reaction in eq 5 in the preformed complex, at low ionic strength, is $(1.9 \pm 0.1) \times 10^5\ s^{-1}$. Again, we conclude that the overall electron-transfer reaction is gated by the rearrangement process.

The analogous photoinduced reactions in eqs 3 and 5 have driving forces of 1.2 and 0.8 eV and fitted rate constants k_s of 2.7×10^4 and $6.7 \times 10^3\ s^{-1}$, respectively. The thermal reaction in eq 1 within the covalent complex has a driving force of 0.14 eV and an observed rate constant k_s of less than $0.2\ s^{-1}$.[44,45] If, as is often done, the photoinduced and thermal reactions are treated together by Marcus theory, these differences in k_s can be attributed to the difference in the driving force, provided the reorganization energy is ca. 1.2 eV. This estimate agrees with values reported for other similar electron-transfer reactions. Three points, however, do not define a parabola, and further discussion along these lines is unwarranted.

The Gating Process and the Viscosity Effects. Two facts show that the rearrangement process shown in Scheme I is not simply dissociation and reformation of the electrostatic complex. First, our previous study[77] of the electrostatic complex Zn(cyt)/pc(II) showed that k_{off} is less than $1 \times 10^4\ s^{-1}$. Clearly, dissociation is too slow to interfere with the rearrangement. Second, the rate constant k_{off} depends on ionic strength,[77] whereas the observed rate constant k_1 in this study does not.

Covalent cross-linking lowers 10-fold the rate constant k_1 for the photoinduced reaction in eq 3 but does not abolish the reaction. The rearrangement that is coupled with electron transfer probably

does not involve a major motion, such as migration of zinc cytochrome *c* all the way from the acidic patch to the hydrophobic patch on plastocyanin. Minor motion, such as conformational fluctuation, is more likely.[32,79]

External effects of viscosity (η) on the rate constant (k) can be analyzed in terms of eq 13, in which the standard symbols have their usual meanings. The parameter δ can vary between

$$k = A\eta^{-\delta} \exp(-E_a/k_B T) \qquad (13)$$

0 and 1; when $\delta = 1$, eq 13 becomes the familiar Kramers's equation for the overdamped limit.[62] The data in Table V obey eq 13 and give linear plots of log k versus log η. The slope δ is 0.66 for ^{3}Zn(cyt)/pc(II) and 0.49 for ^{3}Sn(cyt)/pc(II). The deviation of these values from unity may be due to different causes because there are various interactions by which the solvent exchanges energy and momentum with the exposed parts of the protein surface and various interactions between these exposed parts and the regions of the protein molecules that are involved in the dynamic processes. The viscosity effects are dampened as they are transmitted by the proteins from the surface to the dynamic interface.[62] A full analysis of viscosity effects would require molecular dynamics simulations and detailed cryokinetic studies.

The photoinduced reactions in eqs 3 and 5, investigated in this study, can be compared with the thermal reaction in eq 1, investigated previously.[44,45,49] Because the native ferrocytochrome *c* and its derivatives reconstituted with zinc(II) and tin(IV) do not significantly differ in conformation, surface topography, and distribution of the surface charge, the dynamic process in the complexes that they form with cupriplastocyanin can be characterized by the same rate constant, k_r. The fitted values of 2.2×10^5 and 1.8×10^5 s^{-1} for the zinc(II) and tin(IV) derivatives are practically equal, probably because the surface charges, which are important for the protein interactions with each other and with the solvent, are unaffected by the metal replacement. The net charges of the two derivatives differ by two units, but these charges are relatively unimportant for the interactions. The reaction in eq 1, which has the driving force of 0.10 eV, is not gated because the observed process ($k_1 = 1300 \pm 200$ s^{-1})[44,45] is slower than the rearrangement. The increase in the driving force to 0.8 eV, for the reaction in eq 5, enhances the electron-transfer step beyond the point at which it becomes faster than the rearrangement, and rearrangement becomes rate limiting. Consequently, the reaction in eq 5 is gated. A further increase in the driving force to 1.2 eV, for the reaction in eq 3, probably enhances the electron-transfer step even more. But since the overall reaction remains gated by the same rearrangement process, no significant kinetic difference is detected in our experiments, on the microsecond scale.

The possibility that apparently similar electron-transfer reactions (such as those in eqs 1, 3, and 5) may be gated or not gated depending on the driving force should be kept in mind when independence of the observed rate constant on driving force is taken as evidence for gating. A goal of future work in this laboratory will be to determine the threshold driving force, presumably between 0.10 and 0.80 eV, at which the reaction just becomes gated.

Acknowledgment. This research was supported by the NSF Grant MCB-9222741. We thank Dr. Ling Qin for his contribution to the analysis of viscosity effects.

(79) Northrup, S. H.; Erickson, H. P. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3338.