# Photoinduced Electron Transfer from the Triplet State of Zinc Cytochrome *c* to Ferricytochrome $b_5$ Is Gated by Configurational Fluctuations of the Diprotein Complex†

Ling Qin and Nenad M. Kostić*

*Department of Chemistry, Iowa State University, Ames, Iowa 50011*

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ABSTRACT: The intracomplex electron-transfer reaction $^3$Zncyt/$b_5$(III) → Zncyt$^+$/$b_5$(II) within electrostatic and covalent complexes of zinc(II) cytochrome *c* and ferricytochrome $b_5$ is studied by laser flash photolysis. Kinetic effects of protein cross-linking and of solution viscosity are interpreted in terms of dynamic mobility of the associated proteins with respect to each other. The rate constant for the monoexponential reaction in the electrostatic complex is $3.5 \times 10^5$ s$^{-1}$ in aqueous solution; this value is independent of protein concentration and ionic strength, but it decreases markedly as viscosity is raised by addition of glycerol or sucrose. The multiexponential reaction in the covalent complex was analyzed also in terms of the stretched exponential, $\exp[-(kt)^n]$. The best fit requires $k = 6.8 \times 10^4$ s$^{-1}$ and $n = 0.56$ in aqueous solution; this rate constant is independent of protein concentration and ionic strength, but it decreases slightly as viscosity is raised. Fitting of the viscosity dependence to a simple two-state kinetic model yields a rate constant of $3.0 \times 10^5$ s$^{-1}$ for rearrangement of the electrostatic complex $^3$Zncyt/$b_5$(III) from the initial docking configuration to a different, more reactive, configuration. The corresponding rate constant for rearrangement of the electrostatic complex $^3$Zncyt/pc(II) containing plastocyanin, determined previously, is $2.5 \times 10^5$ s$^{-1}$. Evidently, the intracomplex reaction in both electrostatic complexes is gated by a rearrangement process. Fitting of the viscosity dependences to two types of modified Krammers's equation shows that cross-links raise internal friction between the associated protein molecules from 3 cP in both electrostatic complexes Zncyt/$b_5$(III) and Zncyt/pc(II) to 15 cP in the covalent complex Zncyt/$b_5$(III); the rate constant for intracomplex electron transfer in the covalent complex $^3$Zncyt/pc(II) is independent of viscosity, so that in this case internal friction is undefined. This study shows that electrostatic complexes which zinc(II) cytochrome *c* forms with plastocyanin and with cytochrome $b_5$ have similar dynamic properties even though plastocyanin has two distinct reactive patches on its surface, whereas cytochrome $b_5$ has only one such patch. The rearrangement process responsible for gating of the intracomplex electron-transfer reaction probably amounts to configurational fluctuations during which the exposed heme edge in zinc(II) cytochrome *c* remains within or near the broad acidic patch in plastocyanin or cytochrome $b_5$. Additional conformational fluctuation should be considered, especially in the Zncyt/$b_5$(III) system, because covalent cross-linking of these two proteins does not completely abolish viscosity dependence of the intracomplex electron-transfer reaction. Configurational mobility of the associated proteins with respect to each other and conformational mobility of individual proteins should be distinguished in future experimental and theoretical studies of electron-transfer reactions between metalloproteins.

*Electron-Transfer Reactions of Metalloproteins.* Because metalloproteins are involved in various biological oxidoreduction processes, the kinetics and mechanism of metalloprotein electron-transfer reactions are being intensely studied (Hoffman et al., 1991; Kostić, 1991; Mauk, 1991; McLendon, 1991a,b; McLendon & Hake, 1992; Therien et al., 1991; Winkler & Gray, 1992). We are interested in these reactions within diprotein complexes, especially in protein–protein orientation and its modulation by dynamic processes (Cheng et al., 1994; Peerey & Kostić, 1989; Peerey et al., 1991; Qin & Kostić, 1992, 1993; Zhou et al., 1992; Zhou & Kostić, 1991a,b, 1992a,b, 1993a,b). A pair of metalloproteins can form various complexes in solution, and an orientation that is optimal for recognition and binding need not be optimal for electron transfer. In gated reactions the rate of electron transfer is limited by the rate of rearrangement among different configurations of the protein–protein complex. Gating may be common in proteins; this phenomenon is not limited to electron-transfer reactions.

*Previous Studies of Cytochromes and Plastocyanin.* The blue copper protein plastocyanin is notable because it has on its surface two distinct areas through which it can exchange electrons with redox partners (Sykes, 1991a,b). The broad, negatively charged acidic patch is remote from the copper site, whereas the electroneutral hydrophobic patch is proximate to this site. Both of these patches are well coupled electronically to the copper site (Betts et al., 1992), although the electron-tunneling paths connecting the two patches to the copper site may differ in "conductivity" (Christensen et al., 1990, 1991; Lowery et al., 1993). Plastocyanin uses its acidic patch for docking with the positively charged (basic) patch in cytochrome *c* and also in cytochrome *f* (Bagby et al., 1990; He et al., 1991; King et al., 1985; Martinez et al., 1994; Modi et al., 1992b; Morand et al., 1989; Roberts et al., 1991). The latter protein is a physiological partner of plastocyanin.

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Detailed studies of the reactions in eqs 1–6, involving these

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

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

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

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

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

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

heme proteins and their isostructural derivatives containing zinc(II) and tin(IV) in the place of iron, in our laboratory (see the references above) and in other laboratories (Meyer et al., 1993a,b; Modi et al., 1992a,b) showed that the initial diprotein complexes must rearrange for the electron-transfer step.

The rates of the reactions in eqs 3 and 5 within the electrostatic complexes, which are structurally flexible, decrease smoothly as the solution viscosity is raised. An analysis of this dependence yielded the rate constant of $2.5 \times 10^5\ s^{-1}$ for the rearrangement of the initial (docking) complex that is less reactive in electron transfer into a different complex that is more reactive (Zhou & Kostić, 1992a, 1993b). The rates of these same reactions (in eqs 3 and 5) within the covalent complexes, in which the initial (docking) configuration is noninvasively reinforced by rigid cross-links (Zhou et al., 1992), do not depend on solution viscosity (Zhou & Kostić, 1993b).

*The Present Study.* We investigate here the nature of this configurational rearrangement. Two mechanisms are kinetically indistinguishable and consistent with the results of the viscosity experiments. In one mechanism, the cytochrome molecule migrates outside of the acidic patch, presumably toward, or into, the hydrophobic patch and reduces the copper atom from the new site. In the other mechanism, the cytochrome molecule fluctuates at, or near, the initial docking site while remaining within the acidic patch. In either mechanism, the associated protein molecules may also undergo conformational changes. We consider the configuration of the protein complex (the protein–protein orientation) and the conformation (secondary and tertiary structure) of the associated proteins. We distinguish conceptually between the corresponding configurational and conformational changes, and we offer some indirect evidence on this intricate question.

To distinguish experimentally between the two general mechanisms for configurational rearrangement, we study here the effects of covalent cross-linking and of solution viscosity on the photoinduced reaction in eq 7 between zinc cytochrome

$$^3\text{Zncyt/b}_5\text{(III)} \rightarrow \text{Zncyt}^+\text{/b}_5\text{(II)} \quad (7)$$

*c* and ferricytochrome $b_5$, designated simply $b_5$(III). There are several reasons for using this latter protein as the electron acceptor. It is well characterized, and its three-dimensional structure is known in detail (Mathews & Czerwinski, 1986; Dixon, 1988). Most important, cytochrome $b_5$ differs from plastocyanin in having on its surface only one area through which it exchanges electrons with its redox partners. This area is a negatively charged (acidic) patch surrounding the exposed heme edge. Comparison between cupriplastocyanin and ferricytochrome $b_5$ as electron acceptors in the reactions with the same electron donor, $^3$Zncyt,[1] will throw light on the question whether the dynamic effects observed with the former acceptor were related to the presence of two distinct reactive patches on the surface.

## MATERIALS AND METHODS

*Chemicals.* All chemicals were obtained from Sigma Chemical Co. and were of reagent grade. Distilled water was further demineralized to a resistance greater than 17 MΩ cm. Iron was removed from horse-heart cytochrome *c*, the free-base protein was purified, zinc(II) was inserted, and the reconstituted protein was purified on a CM-52 column, with an 85 mM sodium phosphate buffer at pH 7.0 as an eluent (Vanderkooi & Erecińska, 1975; Vanderkooi et al., 1976). Reconstituted cytochrome *c* was always handled in the dark. The tryptic fragment of cytochrome $b_5$ was purified by a published procedure (Reid & Mauk, 1982) until its absorbance quotient $A_{412}/A_{280}$ became 5.8, thus indicating a purity greater than 95%. Ultrafiltration was done in Amicon cells, with YM-3 membranes, at 4 °C, under pressure of purified nitrogen.

*Covalent Complex Zncyt/$b_5$.* A solution in a 1 mM sodium phosphate buffer at pH 7.0 that was 200 μM in zinc cytochrome *c*, 200 μM in cytochrome $b_5$, and 10 mM in the carbodiimide designated EDC was incubated at room temperature, in the dark, for 4 h. The reaction mixture was chromatographed, in the dark, on a G-75 superfine gel filtration column sized 1 × 18 cm; the eluent was a 10 mM sodium phosphate buffer at pH 7.2. The diprotein complex was eluted in the first band, and the constituent proteins were more strongly retained by the column. Purity and molecular mass of the diprotein complex denatured by sodium dodecyl sulfate (SDS) were verified by electrophoresis, with a Protean II apparatus by Bio-Rad, Inc. A 12% polyacrylamide separating gel was overlaid by a 4% stacking gel, and a potential difference of 100 V was applied for 2 h.

*Redox Potentials.* They were determined by spectrophotometric titrations (Kawai et al., 1963; Velick et al., 1956), with a Hewlett-Packard 8452A diode-array spectrophotometer. Buffers were deaerated with argon; solutions in these buffers were deaerated by slow bubbling, to avoid frothing. A solution in a 1 mM sodium phosphate buffer at pH 7.0 that was 10 μM in free ferricytochrome $b_5$ or in the covalent complex Zncyt/$b_5$(III) and 10 μM in methylene blue, in a rubber-stoppered spectrophotometric cuvette, was titrated with a 1.50 mM solution of sodium dithionite in the same buffer, delivered with a syringe. The reduction of ferricytochrome *c* and of methylene blue was followed at 422 and 664 nm, respectively. The redox potentials were calculated with the Nernst equation, assuming that the reduction potential of methylene blue is 5 mV vs NHE.

*Kinetics.* Laser kinetic spectroscopy (so-called laser flash photolysis) at microsecond resolution was done with a standard apparatus, containing a Phase-R DL1100 laser with Rhodamine 590 dye (Zhou & Kostić, 1993a). The ionic strength of a 1 mM solution of sodium phosphate at pH 7.0 was adjusted with NaCl. Protein solutions were deaerated by

[1] Abbreviations: cyt, cytochrome *c* or cytochrome *f*; cyt(II), ferrocytochrome *c* or ferrocytochrome *f*; cyt(III), ferricytochrome *c* or ferricytochrome *f*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; NHE, normal hydrogen electrode; pc, plastocyanin; pc(I), cuproplastocyanin; pc(II), cupriplastocyanin; Zncyt, zinc(II) cytochrome *c*; $^3$Zncyt, triplet state of zinc(II) cytochrome *c*; Zncyt$^+$, zinc(II) cytochrome *c* cation radical; Sncyt, tin(IV) cytochrome *c*; $^3$Sncyt, triplet state of tin(IV) cytochrome *c*; Sncyt$^+$, tin(IV) cytochrome *c* cation radical; $b_5$, cytochrome $b_5$; $b_5$(II), ferrocytochrome $b_5$; $b_5$(III), ferricytochrome $b_5$; Tml, trimethyllysine.

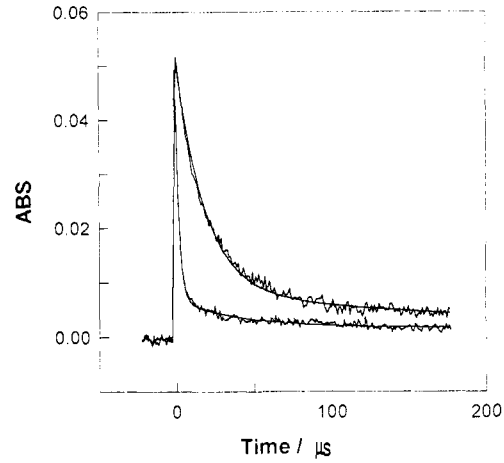



FIGURE 1: Redox quenching of the triplet state $^3$Zncyt, monitored at 460 nm. Lower trace: the solution contained 10 $\mu$M zinc cytochrome *c* and 15 $\mu$M ferricytochrome $b_5$ in a 1 mM sodium phosphate buffer at pH 7.0. Upper trace: the solution contained also 76% by weight glycerol. The time scale is chosen so as to highlight the effect of glycerol. The solid lines are biexponential fits. The relative amplitude of the fast (unimolecular) and the slow (bimolecular) components of the reaction and the unimolecular rate constant are as follows: lower trace, 85%, 15%, and $3.4 \times 10^5$ s$^{-1}$; upper trace, 83%, 17%, and $5.3 \times 10^4$ s$^{-1}$.

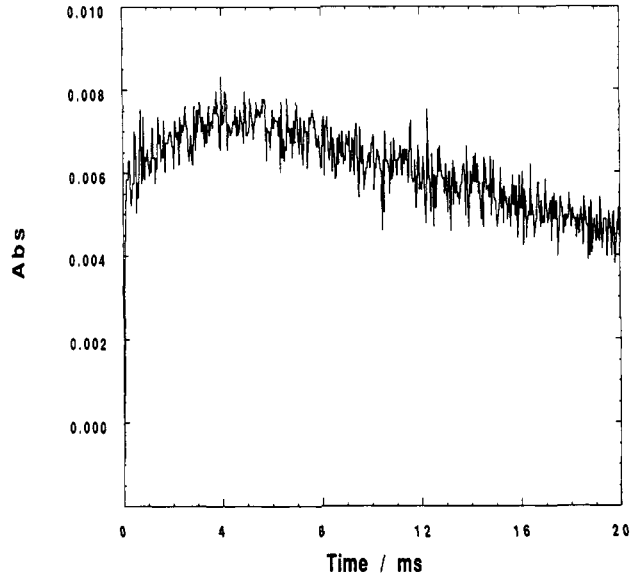



FIGURE 2: Transient absorbance of the cation radical Zncyt$^+$, monitored at 675 nm. The solution contained 10 $\mu$M zinc cytochrome *c*, 15 $\mu$M ferricytochrome $b_5$, and 1.00 M NaCl in a 1 mM sodium phosphate buffer at pH 7.0.

gentle bubbling of pure argon. Typically, the solutions were 10 $\mu$M in zinc cytochrome *c* and 15 $\mu$M in ferricytochrome $b_5$; other solutions were 10–40 $\mu$M in the covalent complex Zncyt/$b_5$(III). The reactions were initiated with a 0.4-$\mu$s laser pulse. Transient absorbance changes of the triplet state $^3$Zncyt and of the cation radical Zncyt$^+$ were monitored at 460 and 675 nm, respectively. The absorbance–time curves were analyzed with a program by OLIS, Inc., and with KaleidaGraph programs by Synergy Software PCS, Inc. The rate constants were average values from at least four experiments, and individual values deviated from the mean by 15% or less.

## RESULTS

*Ultraviolet–Visible Spectra of the Proteins.* The characteristic absorption bands of zinc cytochrome *c* and of cytochrome $b_5$ in a sodium phosphate buffer at pH 7.0 are unaffected by addition of glycerol (up to 80% by weight) and sucrose (up to 60% by weight). Addition of ethylene glycol (up to ca. 95% by weight) causes the Soret band of free cytochrome $b_5$ to grow in intensity by as much as 20% and to shift by as much as 4 nm toward shorter wavelengths, depending on the ethylene glycol concentration. Addition of ethylene glycol does not affect the Soret band of free zinc cytochrome *c*. The covalent complex Zncyt/$b_5$ behaves exactly like the free constituent proteins.

*Reduction Potential.* This property of free ferricytochrome $b_5$ in a 50 mM sodium phosphate buffer at pH 7.0 changes from 6 to −11 mV vs NHE when the aqueous solution is made 80% by weight in glycerol. The reduction potential of the covalent complex Zncyt/$b_5$(III) in the pure buffer is 33 mV.

*Quenching of the Triplet State in the Electrostatic Complex $^3$Zncyt/$b_5$(III) and Solvent Effects on Kinetics.* The triplet excited state of zinc cytochrome *c* in deoxygenated phosphate buffer at pH 7.0 and in the absence of quenchers decays with the rate constant of $100 \pm 10$ s$^{-1}$ at 25 °C. This rate constant remains unchanged when the protein solution is made up to 60% by weight in sucrose and up to 80% by weight in glycerol.

The triplet state is quenched by ferricytochrome $b_5$; typical results are shown in Figures 1 and 2. Decay of the triplet state could be fitted very well with two exponential functions, according to eq 8. The symbols $A$ and $k$ stand for amplitude

$$F(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) \qquad (8)$$

and first-order rate constant. The rate constant for the fast or early component of the decay ($k_1 = 3.5 \times 10^5$ s$^{-1}$) was independent of the zinc cytochrome *c* concentration (5.0–30 $\mu$M) and of ionic strength (1.0–500 mM). The rate constant $k_2$ for the slow or late component decreased as the protein concentration was lowered and as ionic strength was raised, in separate experiments. The relative amplitude of the fast component, $A_1/(A_1 + A_2)$, increased as the protein concentrations were raised and as ionic strength was lowered. For example, in the presence of 10 $\mu$M zinc cytochrome *c* this relative amplitude increased from 70% to 85% as the concentration of ferricytochrome $b_5$ was raised from 3.0 to 10 $\mu$M in a 1 mM phosphate buffer at pH 7.0. The relative amplitudes were unaffected by the concentration of sucrose and of glycerol in solution. The fast component of the reaction was detectable at ionic strengths lower than 20 mM. Most interesting, $k_1$ decreased as the concentration of added glycerol or sucrose in the buffer was raised. The time scale in Figure 1 was chosen so as to highlight this decrease. At longer times, the fast and the slow components of the reaction are separable. The results are given in Table 1 and Figure 3.

*Quenching of the Triplet State in the Covalent Complex $^3$Zncyt/$b_5$(III) and Solvent Effects on Kinetics.* Decay of the triplet state $^3$Zncyt in the covalent complex with ferricytochrome $b_5$ in solution without viscous solutes could be fitted well with more than two exponential functions or with a stretched exponential function in eq 9. The best fitting in the

$$A = A_0 \exp[-(kt)^n] \qquad (9)$$

absence of viscous solutes was obtained with $n = 0.56 \pm 0.06$ and $k = 6.8 \times 10^4$ s$^{-1}$. This apparent rate constant was independent of the diprotein complex concentration (5.0–30 $\mu$M) and, as Figure 4 shows, of ionic strength (1.0–500 mM). The dependence of the rate constant on viscosity is shown in Table 2 and Figure 3.

Table 1: Rate Constant, $k_1$, for the Fast Component of the Decay of $^3$Zncyt (10 $\mu$M) in the Presence of Ferricytochrome $b_5$ (15 $\mu$M) in a 1.0 mM Sodium Phosphate Buffer at pH 7.0, at Different Concentrations (Percentages by Weight) of Added Organic Solutes

| | | $10^{-4}k_1$ ($s^{-1}$)[a] | |
|---|---|---|---|
| wt % | rel viscosity | glycerol | sucrose |
| 0 | 1.0 | 34 | 34 |
| 20 | 1.7 | 29 | |
| 20 | 1.9 | | 31 |
| 28 | 2.3 | 23 | |
| 28 | 2.8 | | 25 |
| 44 | 4.4 | 18 | |
| 36 | 4.6 | | 18 |
| 52 | 6.7 | 13 | |
| 56 | 8.3 | 12 | |
| 44 | 8.6 | | 13 |
| 60 | 11 | 11 | |
| 64 | 14 | 9.1 | |
| 68 | 18 | 7.9 | |
| 52 | 19 | | 8.4 |
| 72 | 28 | 6.5 | |
| 56 | 32 | | 6.6 |
| 76 | 40 | 5.3 | |
| 60 | 58 | | 3.5 |
| 80 | 60 | 3.2 | |

[a] Relative error ±10%.

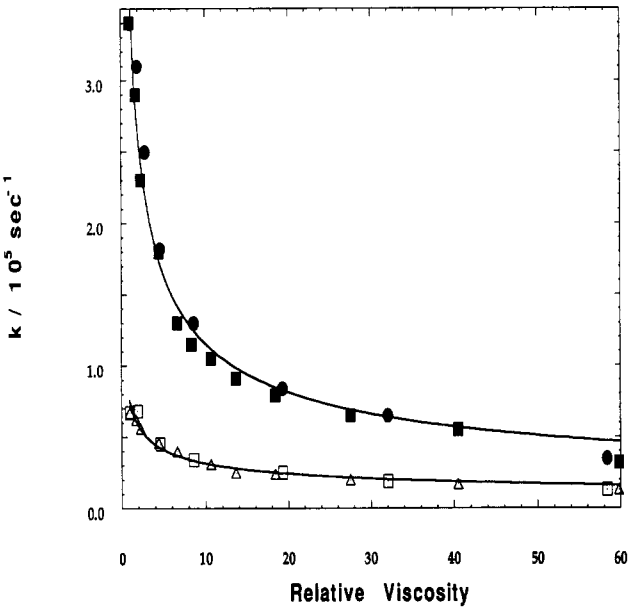



FIGURE 3: Observed rate constant for the unimolecular electron-transfer reaction within the electrostatic complex $^3$Zncyt/$b_5$(III) (upper curve) and the average rate constant for this reaction within the covalent complex $^3$Zncyt/$b_5$(III) (lower curve) in a sodium phosphate buffer at ionic strength 1.0 mM and pH 7.0, the viscosity of which was adjusted with glycerol (■) or sucrose (●) for the electrostatic complex and with glycerol (△) or sucrose (□) for the covalent complex. Concentrations are 10 $\mu$M zinc cytochrome $c$ and 15 $\mu$M ferricytochrome $b_5$ for the electrostatic complex and 10 $\mu$M for the covalent complex. The solid lines are best fits to eq 12.

## DISCUSSION

*Previous Studies of the Complexes between Cytochrome c and Cytochrome $b_5$.* Association between these prototypical redox metalloproteins has been much studied because their structures are known in detail. In a pioneering application of computer graphics to analysis of protein–protein interactions, four particular lysine residues in cytochrome $c$ were matched with three particular acidic residues and one particular heme propionate in cytochrome $b_5$ in a way that maximizes electrostatic attraction and minimizes steric crowding (Salemme, 1976). A fifth specific salt bridge was

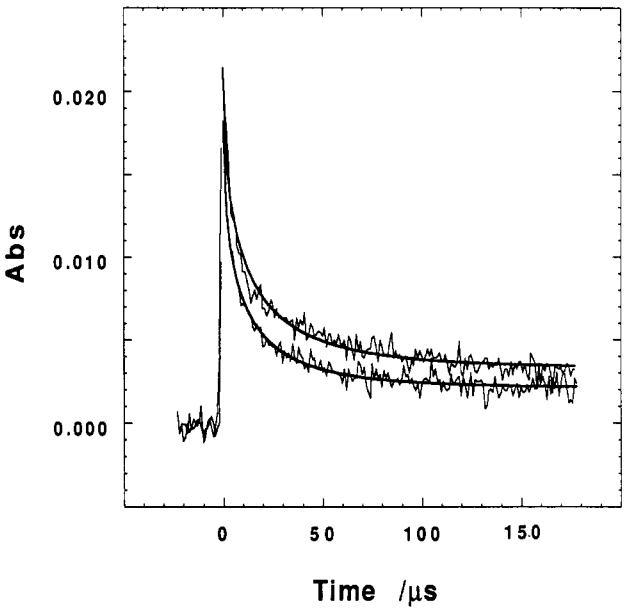



FIGURE 4: Redox quenching of the triplet state $^3$Zncyt, monitored at 460 nm, in a 10 $\mu$M solution of the covalent complex Zncyt/$b_5$(III) in a 1 mM sodium phosphate buffer at pH 7.0 (lower trace) and in the same solution that is also 0.500 M in NaCl (upper trace). The solid lines are fits to stretched exponentials (eq 9).

Table 2: Average Rate Constant, $k_{av}$,[a] for Triplet Decay in $^3$Zncyt/$b_5$(III), in a 10 $\mu$M Solution of the Covalent Complex Zncyt/$b_5$(III) in a 1.0 mM Sodium Phosphate Buffer at pH 7.0, at Different Concentrations (Percentages by Weight) of Added Organic Solutes.

| | | $10^{-4}k_{av}$ ($s^{-1}$)[b] | |
|---|---|---|---|
| wt % | rel viscosity | glycerol | sucrose |
| 0 | 1.0 | 6.8 | 6.8 |
| 20 | 1.7 | 6.2 | |
| 20 | 1.9 | | 7.1 |
| 28 | 2.3 | 5.8 | |
| 44 | 4.4 | 4.6 | |
| 36 | 4.6 | | 4.5 |
| 52 | 6.7 | 4.1 | |
| 44 | 8.6 | | 3.5 |
| 60 | 11 | 3.4 | |
| 64 | 14 | 2.5 | |
| 68 | 18 | 2.4 | |
| 52 | 19 | | 2.5 |
| 72 | 28 | 2.1 | |
| 56 | 32 | | 1.8 |
| 76 | 40 | 1.7 | |
| 60 | 58 | | 1.2 |
| 80 | 60 | 1.2 | |

[a] $k_{av} = nk$ (eq 9). In all fittings $n = 0.56 \pm 0.06$. [b] Relative error ±10%.

proposed later (Stonehuerner et al., 1979). There is much experimental evidence for the existence of the cyt/$b_5$ complex and for the primacy of electrostatic interactions in it (Mauk et al., 1982, 1986, 1991; McLendon et al., 1985; Eley & Moore, 1983; Hartshorn et al., 1987; Kornblatt et al., 1988; Holloway & Mantsch, 1988).

Previous experimental studies (Mauk et al., 1986; Rodgers et al., 1988) gave evidence for coexistence of two electrostatic complexes in solution. Molecular dynamics simulations (Wendoloski et al., 1987) revealed a multitude of complexes, some of which seem to be more favorable for electron transfer than the single complex in the original static model. Highly resolved NMR spectra indicated that at least six lysine residues in cytochrome $c$ are involved in binding; two or more structurally similar complexes probably coexist in solution, and their interconversion may resemble two balls rolling against

each other's surface (Burch et al., 1990). Electrochemical studies of the complexes that cytochrome $b_5$ forms with mutants of yeast cytochrome *c* were interpreted in terms of multiple overlapping binding and recognitition domains on the surface of cytochrome *c* (Burrows et al., 1991). The two proteins were covalently attached to each other by a carbodiimide, but the cross-linked residues could not be identified (Mauk & Mauk, 1989). The cross-links likely reinforce the electrostatic complex (Mauk & Mauk, 1989; Zhou et al., 1992), in which the oppositely charged patches around the exposed heme edges in each protein abut each other.

Because replacement of iron(II) by zinc(II) does not noticeably perturb the conformation of cytochrome *c* and its association with other proteins (Vanderkooi & Erecińska, 1975; Vanderkooi et al., 1976; Moore et al., 1980), findings from the aforementioned investigations apply also to zinc cytochrome *c* and cytochrome $b_5$, the subject of this study. Indeed, some of the previous studies of the cyt/$b_5$ system were done with zinc cytochrome *c* (McLendon et al., 1985).

*Previous Studies of the Electron-Transfer Reactions between Cytochrome c and Cytochrome $b_5$.* In these studies, as in those discussed above, native cytochrome *c* and its zinc derivative were used interchangeably. In the early investigations (McLendon & Miller, 1985), kinetic results were analyzed by Marcus theory. This kinetic work has been questioned (Willie et al., 1992). The more recent investigations concerned not only kinetic but also dynamic aspects of the electron-transfer reactions. A rearrangement of the electrostatic diprotein complex before external reduction by flavin semiquinones was considered as a possibility but not investigated (Eltis et al., 1988). Effects of protein dynamics and of electronic fluctuations were analyzed in a theoretical study of the electron-transfer reaction (Kuznetsov & Ulstrup, 1989). Although the bimolecular reaction in eq 10 and the self-

$$b_5(II) + cyt(III) \rightarrow b_5(III) + cyt(II) \qquad (10)$$

exchange reactions of cytochrome *c* and of cytochrome $b_5$ are not the subject of our study, theoretical treatments of these three reactions by methods of Brownian dynamics and by van Leeuwen theory of electrostatic interactions are relevant here because electrostatic properties of the proteins were analyzed, because electron transfer was presumed to occur via the partially exposed heme edge in each protein, and because different configurations of the homodiprotein complexes were considered (Eltis et al., 1991; Andrew et al., 1993; Dixon et al., 1989). The intracomplex electron transfer from the heme group in a $[Ru(bpy)_3]^{2+}$ derivative of ferrocytochrome $b_5$ to the heme group in ferricytochrome *c* is biphasic ($1 \times 10^5$ and $2 \times 10^4$ $s^{-1}$) at very low ionic strength and monophasic at higher, but still relatively low, ionic strength (Willie et al., 1992, 1993). The two phases (rate constants) were reasonably attributed to two or more structures of the diprotein complex with different electron-transfer properties, but it is impossible to determine the number of conformations by mere fitting of the kinetic results. The interprotein electron-transfer reaction in this study was initiated by photoexcitation of a $[Ru(bpy)_3]^{2+}$ derivative attached to residue 65 in cytochrome $b_5$. Although this tag seems not to affect the overall equilibrium constant for binding to cytochrome *c*, it has to be proven that this bulky cationic complex does not affect the conformation of cytochrome $b_5$ and the configuration and dynamics of its complex with cytochrome *c*. Only then can it be accepted that the biphasic reaction is an intrinsic property of this diprotein system.

Kinetics of bimolecular (external) reduction of the cyt(III)/$b_5$(III) electrostatic complex by a flavin semiquinone and by ferrocytochrome $b_5$ allow indirect analysis of the intracomplex electron transfer in the cyt(III)/$b_5$(II) electrostatic complex. Such a study gave indications of structural heterogeneity and flexibility of the diprotein complex (Meyer et al., 1993a,b). A very recent simulation by Brownian dynamics of diffusional docking and of electron transfer from ferrocytochrome $b_5$ to the wild-type form and mutants of yeast ferricytochrome *c* indicated that at least two classes of cyt/$b_5$ complexes, having different electrostatic energies, coexist in solution. In each class four salt bridges were proposed: Arg13–Glu48, Lys87–Glu56, Lys86–Asp60, and Tml72–heme in the more stable complexes; Lys27–Glu44, Arg13–Glu48, Tml72–Asp60, and Lys79–heme in the less stable complexes (Northrup et al., 1993).

A preliminary study indicated that the rate of the thermal reaction in eq 10 depends on solution viscosity, but a detailed investigation was not possible for several reasons (Qin et al., 1991). This previous study was hampered by the use of external reducing agents; under these conditions both the protein–protein reaction and the bimolecular reactions by which this reaction of interest was induced depended on viscosity. Moreover, the kinetic traces were noisy.

The present study of the photoinduced reaction in eq 7 is free of these difficulties. The reaction is initiated by a laser pulse, and there are no external redox agents. The electron-transfer step occurs within the preformed complex, under unimolecular conditions. In this case kinetic effects of solution viscosity can be attributed to the protein–protein electron-transfer reactions within the electrostatic and covalent diprotein complexes.

*Properties of the Covalent Complex Zncyt/$b_5$ and of Its Constituent Proteins.* Cross-links do not appreciably perturb redox potentials and UV–visible spectra of the two proteins. A change of 0.02 V is negligible in comparison with the driving force of 1.2 eV for the reaction in eq 7. Glycerol and sucrose added to buffered solutions proved to be noninvasive solutes, which raised the solution viscosity without appreciably perturbing the individual proteins and their covalent complex.

*Quenching of the Triplet State in the Electrostatic Complex $^3$Zncyt/$b_5$(III).* The results in Figures 1 and 2 show that the triplet state is oxidatively quenched; the quencher is ferricytochrome $b_5$. The fast component of the quenching, which predominates at low ionic strength, is due to the unimolecular electron-transfer reaction between the associated proteins (eq 7). The slow component, which predominates at higher ionic strength, is due to the analogous bimolecular reaction between the unassociated proteins. Since this latter reaction is less interesting from the viewpoint of protein–protein dynamics and since the bimolecular reaction in eq 10 has been studied before (Northrup et al., 1993), the bimolecular quenching process will not be discussed further.

The intracomplex reaction is monophasic at the microsecond time resolution. Its rate constant, $3.5 \times 10^5$ $s^{-1}$, is independent of concentration and ionic strength and is virtually identical to the value of $2.5 \times 10^5$ $s^{-1}$ for the reaction in eq 3 (Zhou & Kostić, 1991a) even though ferricytochrome $b_5$ is a weaker oxidant than cupriplastocyanin; the respective driving forces for the reactions in eqs 7 and 3 are 0.80 and 1.2 eV.

*Quenching of the Triplet State in the Covalent Complex $^3$Zncyt/$b_5$(III).* The apparent rate constant is independent of ionic strength and the complex concentration, as expected for the intracomplex reaction. The multiple kinetic components may be due to multiple configurations of the electrostatic

complex, reinforced by cross-links that impede their rapid (submicrosecond) interconversion. A stretched exponential appropriately represents such a distribution of rate constants and has often been used in similar studies (Siebrand & Wildman, 1986; Ansari et al., 1992; Nocek et al., 1991). This empirical fitting function is consistent with cooperativity or, more likely, with the aforementioned multiple configurations of the diprotein complex having different intrinsic electron-transfer rates (Lindsey & Patterson, 1980; Siebrand & Wildman, 1986).

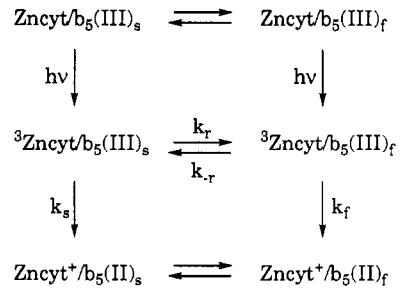
In the case of $^3$Zncyt/pc(II), the intracomplex reaction was monophasic in both the electrostatic and the covalent diprotein complex (Zhou & Kostić, 1991a,b, 1992a, 1993b). In the present case of $^3$Zncyt/$b_5$(III), however, the intracomplex reaction is monophasic in the electrostatic complex but multiphasic in the covalent complex. This contrast may indicate functional homogeneity of the complex Zncyt/pc and functional heterogeneity of the complex Zncyt/$b_5$. More evidence for this conclusion will be presented below.

*Viscosity Effect on the Electron-Transfer Reaction within the Electrostatic Complex $^3$Zncyt/$b_5$(III).* The dependence in Table 1 is not caused by specific protein–solute interactions because glycerol and sucrose have similar effects on the rate constant. Solutions of sucrose and glycerol at the same viscosity can differ much in water content. For example, respective solutions with the relative viscosity of 59 contain 51% and 24% by weight of water. The dielectric constants of pure sucrose (42) and glycerol (3.3) also differ much. Of various properties of the solution that change with addition of these organic solutes to the buffered proteins, only the increase in viscosity correlates with the observed decrease in the rate constant for the intracomplex electron-transfer reaction. All 19 values of $k_1$ for the reaction in eq 7 fall on the same smooth curve when plotted versus the solution viscosity (Figure 3). When this viscosity is adjusted to the same value by glycerol and by sucrose, the rate constants are identical within the error bounds of the experiments. Therefore, the effect in Table 1 is most likely caused by the hydrodynamic friction, which impedes protein motion, and not by dielectric friction, by which the solvent responds to changes in the charge or charge distribution of the solute (Flemming & Wolynes, 1990). Dampening of conformational fluctuations with increasing viscosity has been observed also in other proteins (McCammon & Wolynes, 1977).

A previous spectroscopic study showed that the cyt/$b_5$ binding constant is independent of viscosity (Qin et al., 1991). Our kinetic study consistently shows that the relative amplitude, $A_1/(A_1 + A_2)$, of the intracomplex electron-transfer reaction is independent of glycerol or sucrose concentration at a fixed ionic strength. We conclude that changes in viscosity do not affect the protein association equilibrium. Changes in viscosity effect the rate of electron transfer, as explained above.

*Viscosity Effect on the Electron-Transfer Reaction within the Covalent Complex $^3$Zncyt/$b_5$(III).* As Table 2 and Figure 3 show, the reaction within the covalent complex depends slightly on solution viscosity. This dependence is smaller than the marked dependence found for the electrostatic complex, but it indicates that the covalent complex is not completely rigid. The plots in Figure 3 for both electrostatic and covalent complexes at very high viscosity converge to the same rate constant of $1 \times 10^4$ s$^{-1}$. This convergence indicates that hydrodynamic friction (in viscous solution) and cross-links (in the covalent complex) stabilize the same configuration of the diprotein complex or different configurations with the same intrinsic electron-transfer reactivity.

Scheme 1




Our findings concerning redox proteins agree with the well-known findings concerning the oxygen carrier myoglobin. The multiexponential conformational changes of this protein at room temperature were fitted to a stretched exponential (eq 9) with $n = 0.6$ (Ansari et al., 1992). Myoglobin is conformationally heterogeneous (Breece et al., 1980; Fraunfelder et al., 1988, 1991), but at higher temperatures the interconversion of conformational substates becomes fast, and first-order kinetics (i.e., $n = 1.0$) is observed. The effect of cross-linking in our present study may be similar to the effect of cooling in these previous studies. We will test this hypothesis in future studies of temperature effects on the electron-transfer reactions within the electrostatic and covalent diprotein complexes.

*Analysis of Viscosity Effects and Comparison between $^3$Zncyt/pc(II) and $^3$Zncyt/$b_5$(III) Systems.* The dependence on viscosity of the intracomplex rate constants in Tables 1 and 2 and Figure 3 can be explained in terms of Scheme 1. According to this simple mechanism, the precursor complex $^3$Zncyt/$b_5$(III) can adopt two interconverting configurations: a less reactive one, designated s (for "slow" electron transfer), and a more reactive one, designated f (for "fast"). The latter complex is such a minor component of the equilibrium mixture that the reaction within the electrostatic complex is monophasic under our experimental conditions. The reversible rearrangement process is marked with subscripts r and –r. The laser flash (designated $h\nu$) triggers the electron-transfer reaction, which occurs predominantly along the $k_f$ "branch". Kinetic analysis, like the previous analysis in this laboratory (Zhou & Kostić, 1992a, 1993b), involves the steady-state assumption for $^3$Zncyt/$b_5$(III)$_f$. It nicely reproduces the convergence of the two curves at very high viscoosity (Figure 3) and yields the fitted value $k_r = 3.0 \times 10^5$ s$^{-1}$ in aqueous buffer in the absence of viscous solutes. This rate constant is practically equal to the corresponding rate constant of $2.5 \times 10^5$ s$^{-1}$, obtained for the $^3$Zncyt/pc(II) complex (Zhou & Kostić, 1992a, 1993b).

Although both of these diprotein systems conform to the two-state model in Scheme 1, their dynamic properties may be different. The dynamic process may involve configurational fluctuations of protein molecules relative to each other or conformational fluctuation of individual proteins or both. Cytochrome *c* is a tightly folded protein (Moore & Pettigrew, 1990), and our experiments showed that added solutes do not noticeably affect its redox properties. Since the relevant properties of plastocyanin are not affected by the presence of ethylene glycol (Zhou & Kostić, 1993b), whereas those of cytochrome $b_5$ are affected, conformational fluctuations are more likely for Zncyt/$b_5$ than for Zncyt/pc.

Although the two-state model may not be fully applicable to systems involving multiple states (Hoffman & Ratner, 1987, 1988), it allows a useful conceptual analysis. Since we did not observe Zncyt/$b_5$(III)$_f$ or its triplet excited state, we assume it to be a very minor component of the equilibrium mixture

(see above). Alternatively, our kinetic results can be explained in terms of a single protein complex, corresponding to the transition state and therefore undetectable. These two alternatives, the gated and the not gated reaction, cannot be distinguished by our experimental methods.

Very recent Brownian dynamics simulations showed multiple rotational orientations of the cytochrome *c* molecule docked in the region of the exposed heme edge in cytochrome $b_5$ (Northrup et al., 1993). In such cases the kinetic effects of solution viscosity can appropriately be treated by modified Kramers's theory, eq 11, in which $\eta$ is the viscosity and $\sigma$ is

$$k_{obs} = \frac{1}{\eta + \sigma} \exp(-E/RT) \qquad (11)$$

"protein friction" (Ansari et al., 1992). The temperature parameter is implicitly included in the regression coefficient. This theory recognizes the importance of Brownian fluctuations in overcoming the energy barrier for a chemical reaction, and it does not assume only two configurations.

The concept of internal friction describes how motion of neighboring atoms or groups affects the motion of an atom or group and its exchange of energy and momentum with its surroundings in the protein. Fitting of our kinetic results in eq 11 yields "internal frictions" of 3 and 15 cP, respectively, for the electrostatic and the covalent complex Zncyt/$b_5$(III) and 3 cP for the electrostatic complex Zncyt/pc(II). Since the intracomplex reaction in the covalent complex Zncyt/pc(II) is independent of viscosity (Zhou & Kostić, 1992a, 1993b), internal friction is undefined in this case. Equality of the frictions in the two electrostatic complexes is consistent with the aforementioned equality of the rearrangement rate constants, $k_r$. The qualitative difference between the two covalent complexes is consistent with the hypothesis that Zncyt/$b_5$ is more flexible than Zncyt/pc.

Viscosity effects can also be described with eq 12 (Khoshtariya et al., 1991), which reduces to Kramers's equation when $\delta = 1$. As above, temperature is treated only implicitly. Fitting of the kinetic data in Tables 1 and 2 yields $\delta$ values of 0.36 and 0.57, respectively, for the covalent and electrostatic complexes Zncyt/$b_5$(III). Again, the difference between these values indicates that the internal friction is greater in the covalent than in the electrostatic complex.

$$k_{obs} = A\eta^{-\delta} \exp(-E/RT) \qquad (12)$$

## CONCLUSION

This study shows that electrostatic complexes which cytochrome *c* forms with plastocyanin and with cytochrome $b_5$ have similar dynamic properties even though plastocyanin has two distinct patches through which its metal site may exchange electrons with redox partners, whereas cytochrome $b_5$ has only one such patch. Evidently, the rate of the intracomplex electron-transfer reaction may depend on solution viscosity even if the dynamic rearrangement of the diprotein complex does not involve grossly different docking configurations. In initial studies of ground-state (thermal) reactions, we proposed migration of ferrocytochrome *c* on the cupriplastocyanin surface from the initial docking site in the acidic patch to a different reactive site in the hydrophobic patch (Peerey & Kostić, 1989; Peerey et al., 1991). In the more recent studies of excited-state (photoinduced) reactions, we favored fluctuations within one general docking configuration, in which the exposed heme edge of zinc cytochrome *c* remains within or near the acidic patch on the plastocyanin surface (Zhou & Kostić, 1992a, 1993a,b; Qin & Kostić, 1993). Since, however, the basic patch in cytochrome *c* (and its zinc derivative) and the acidic patches in plastocyanin and cytochrome $b_5$ are extensive and cover appreciable fractions of the respective protein surfaces, these two concepts—migration and fluctuation—are not mutually exclusive. These two kinds of dynamic processes differ in degree more than in kind, as the present study shows.

The very recent determination of the surprising three-dimensional structure of cytochrome *f* (Martinez et al., 1994) lends credence to our conclusion, based on kinetic studies, that the physiological partners ferrocytochrome *f* and cupriplastocyanin do not undergo electron transfer in the same orientation in which they dock (Qin & Kostić, 1992, 1993). The docking site on the cytochrome *f* surface lies more than 20 Å away from the heme. Questions concerning dynamical aspects of electron-transfer reactions should be amenable to calculations by a modified Brownian dynamics method, in which even the interacting proteins, and not only their complexes, are treated as dynamic and flexible objects.

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