Effects of Viscosity and Temperature on the Kinetics of the Electron-Transfer Reaction between the Triplet State of Zinc Cytochrome *c* and Cupriplastocyanin†

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ABSTRACT: This is a study of the effects of viscosity (in the range of 0.8−790 cP), of temperature (in the range of 260.7−307.7 K), and of ionic strength (in the range of 2.5−20.0 mM) on the kinetics of photoinduced electron-transfer reaction ^{3}Zncyt/pc(II) → Zncyt$^+$/pc(I) within the electrostatic complex of zinc cytochrome *c* and cupriplastocyanin at pH 7.0. The unimolecular rate constant is k_F. The apparent activation parameters $\Delta H^‡$, $\Delta S^‡$, and $\Delta G^‡$ for this reaction were obtained in experiments with aqueous glycerol solutions having a constant composition. The interpolation of k_F values obtained at the constant composition into the dependence of k_F on temperature at constant viscosity gave the proper activation parameters, which agree with those obtained in experiments with solutions having a constant viscosity. This agreement validates the latter method, which is more efficient than the former, for determining activation parameters of processes that are modulated by viscosity. The smooth change in k_F is governed by the change in viscosity, not in other properties of the solvent, and it does not depend on the choice of the viscosigen. Donor/acceptor electronic coupling (H_{AB}) and reorganizational energy (λ), obtained by fitting of the temperature dependence of k_F to the Marcus equation, are consistent with true electron transfer and with electron transfer that is coupled to, or gated by, a preceding structural rearrangement of the diprotein complex ^{3}Zncyt/pc(II). The fact that at very high viscosity k_F approaches zero shows that the reaction is probably gated throughout the investigated range of viscosity. Kinetic effects and noneffects of ionic strength, viscosity, and thermodynamic driving force indicate, but do not prove, that the reaction under consideration is gated. The kinetic effect of viscosity is analyzed in terms of two models. Because ln k_F is a nonlinear function of ln η, protein friction has to be considered in the analysis of viscosity effects on kinetics.

Biological electron transfer is a very important process in nature. Because metalloproteins are involved in electron transport in the cell, it is important to understand the mechanism of the reaction between these electron-carrier proteins. Two metalloproteins transfer electrons at the rate that may be determined by different steps. If this step is actually electron transfer, the process can be described by the Marcus equation (Marcus & Sutin, 1985). Among the properties that govern the rate are electronic coupling and reorganizational energy. If the rate-determining step is some nonredox process, that process can be described generally, using activation parameters from Arrhenius or Eyring equations. If the rate-limiting step is a process other than electron transfer, the reaction can be gated or coupled (Davidson, 1996; Brunschwig & Sutin, 1989; Hoffman & Ratner, 1987, 1988; Nocek et al., 1991; Feitelson & McLendon, 1991; Walker & Tollin, 1992; Sullivan et al., 1992; Hoffman et al., 1990).

A pair of metalloproteins can form multiple complexes in solution (Wendoloski et al., 1987; Northrup et al., 1988; Rodgers et al., 1988; Burch et al., 1990; Wallin et al., 1991; McLendon et al., 1993; Nocek et al., 1991; Kostić, 1991; Chen et al., 1992, 1994; Willie et al., 1992; Mauk et al., 1994). The orientation that is most stable thermodynamically may not be optimal for electron transfer (Peerey & Kostić, 1989; Peerey et al., 1991; Kostić, 1991; Zhou & Hoffman, 1994; Ullmann & Kostić, 1995; Ullmann et al., 1997).

Plastocyanin (pc)[1] and cytochrome *c* (cyt) are both well-characterized and much studied (Sykes, 1991a,b; Moore & Pettigrew, 1990; Scott & Mauk, 1996). Replacement of iron(II) in cytochrome *c* by zinc(II) does not perturb the conformation of cytochrome *c* (Moore et al., 1980; Anni et al., 1995; Angiolillo & Vanderkooi, 1995). Zinc cytochrome *c* (Zncyt) associates with other metalloproteins in the same manner as cytochrome *c* itself does (Vanderkooi et al., 1976, 1977; Erecińska & Vanderkooi, 1978; Ye et al., 1997). Much spectroscopic and chemical evidence agrees that both cytochrome *c* and zinc cytochrome *c* bind to plastocyanin with their basic patch surrounding the exposed heme edge. Plastocyanin binds to each of them with its broad acidic patch (King et al., 1985; Bagby et al., 1990; Roberts et al., 1991; Geren et al., 1983; Zhou et al., 1992b).

Studies of the quenching of the triplet state ^{3}Zncyt by cupriplastocyanin (Zhou & Kostić, 1991a,b, 1992a−c; 1993a,b; Qin & Kostić, 1992, 1993, 1994, 1996; Kostić, 1996) show that at low ionic strengths, at which the diprotein complex persists, the unimolecular reaction in eq 1 can be

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[1] Abbreviations: b_5, cytochrome b_5; b_5(II), ferrocytochrome b_5; b_5-(III), ferricytochrome b_5; cyt, cytochrome *c*; MADH, methylamine dehydrogenase; MEDH, methanol dehydrogenase; pc, plastocyanin; pc-(I), cuproplastocyanin; pc(II), cupriplastocyanin; Sncyt, tin(IV) cytochrome *c*; ^{3}Sncyt, triplet (excited) state of tin(IV) cytochrome *c*; Sncyt$^+$, cation radical of tin(IV) cytochrome *c*; Zncyt, zinc cytochrome *c*; ^{3}Zncyt, triplet (exited) state of zinc cytochrome *c*; Zncyt$^+$, cation radical of zinc cytochrome *c*.

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observed as the faster of the two kinetic phases. (The Roman numerals are the oxidation states of copper.) The slower phase, which corresponds to the bimolecular reaction between the separate proteins, is not the subject of this study.

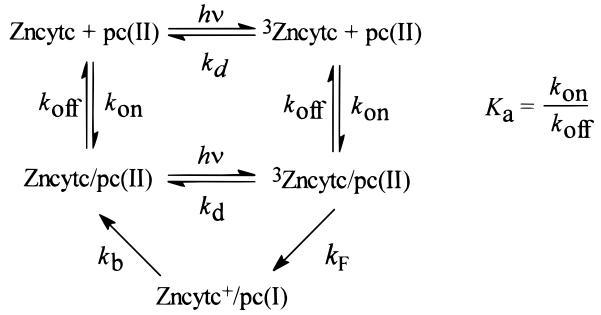
$$^3\text{Zncyt/pc(II)} \xrightarrow{k_F} \text{Zncyt}^+\text{/pc(I)} \qquad (1)$$

The intracomplex reaction in eq 1 has a driving force of 1.2 eV. It has been studied in two types of diprotein complexes of zinc cytochrome *c* and cupriplastocyanin: the electrostatic complex, which is flexible, and the covalent complex, in which the electrostatic protein/protein configuration (orientation) is reinforced by rigid but noninvasive covalent cross-links (Zhou et al., 1992). The respective rate constants are 3.0×10^5 and 2.5×10^4 s^{-1} at 298 K, in aqueous buffers of unit viscosity ($\eta = 1.00$). As the viscosity is raised, the former rate constant decreases smoothly while the latter remains unchanged within the experimental error. A viscous solvent dampens the rearrangement process that occurs in the electrostatic but not in the covalent complex (Zhou & Kostić, 1992a, 1993b). Very similar results were obtained also for the following two pairs of reductants and oxidants: the triplet state of tin(IV) cytochrome *c* and cupriplastocyanin (Zhou & Kostić, 1993b) and the triplet state of zinc cytochrome *c* and ferricytochrome b_5 (Qin & Kostić, 1993). In all these three protein pairs, the rate-limiting process seems to be configurational fluctuation, a process during which the donor and the acceptor remain docked in the same general orientation but wiggle with respect to each other. A theoretical analysis of electron-transfer paths between the heme and blue copper sites in various configurations of the cyt(II)/pc(II) complex confirmed the experimental findings (Ullmann & Kostić, 1995). The configuration that optimizes the surface interactions does not optimize the heme/copper electronic coupling. Motions of the cytochrome *c* molecule, whose basic patch explores the area within or near the acidic patch in plastocyanin, enhance the electronic coupling.

At high viscosity, the apparent rate of reduction in the electrostatic complex approaches the rate of reduction in the covalent complex, in both cases. At low viscosity, the measured unimolecular rate of the quenching of the triplet state of zinc cytochrome *c* is a protein rearrangement that is highly dependent on viscosity. And at very high viscosity, this rate may correspond to the true electron-transfer rate within the thermodynamically most stable complex or to a rearrangement. In this study, we will show which is probably true.

These previous studies from our laboratory (Zhou & Kostić, 1993b) showed that the dependence of k_F on the concentrations of glycerol, ethylene glycol, and glucose is due to changes in viscosity, not to changes in other properties of the solutions containing these viscosigens. Viscosity, however, was varied over a relatively narrow range, at a single temperature. We now vary these conditions more widely in order to understand better the unimolecular reaction k_F in Scheme 1. In the first part of our study, we examined the effects of temperature and ionic strength at constant viscosity, still over a narrow range of viscosity (Ivković-Jensen & Kostić, 1996). These experiments yielded the thermodynamic parameters ΔH and ΔS for the formation of the complex Zncyt/pc(II) and the proper activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the reaction in eq 1 and Scheme 1. We interpreted these parameters in terms of molecular

Scheme 1



surfaces (Connolly, 1983) and confirmed that the rate of the electron-transfer reaction is limited by rearrangement at low viscosities.

In this, the second part of our study, we greatly widen the viscosity range (up to $\eta = 790$ cP) by varying temperature (in the range of 260.7−307.7 K) as well as the glycerol concentration (up to 80% w/w). Temperature influences the kinetics both indirectly, by affecting viscosity, and directly. After having already examined direct effects of temperature (Ivković-Jensen & Kostić, 1996), we can now study the effects of both temperature and viscosity. We are interested in the mechanism of the reaction k_F at very high viscosity, but still in liquid solution. Does the reaction remain gated or conformationally coupled (as it is at low viscosity), or is it a true electron-transfer reaction within the initial configuration of the diprotein complex, entrapped by the viscous medium?

MATERIALS AND METHODS

Chemicals. Distilled water was demineralized to a resistivity of greater than 17 MΩ·cm. Chromatography gels (CM Sephadex C-50, Sephadex G-25 and G-75, and Sephadex DEAE A-25) were purchased from Sigma Chemical Co. Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co. All other chemicals were purchased from Fischer Chemical Co.

Buffers. Sodium dihydrogen phosphate and sodium monohydrogen phosphate were used to make buffers with ionic strengths (μ) of 2.5 and 10.0 mM. All buffers were at pH 7.0. The buffer with an ionic strength of 20.0 mM was prepared from the buffer at 10.0 mM by addition of NaCl. The buffers were always prepared fresh.

Viscosity. The concentration of glycerol in the buffers was 0, 20, 40, 60, and 80% w/w. The absolute viscosities (η) of water and of aqueous solutions of glycerol at different temperatures were taken from tables (*CRC Handbook of Chemistry and Physics, 1986; CRC Handbook of Biochemistry and Molecular Biology*, 1975; Miner & Dalton, 1953). The relative viscosity (η/η_o) of buffers with and without glycerol was measured with a thermostated glass viscometer; the absolute error was ±0.05 cP. Given $\eta_o = 1.002$ cP, the absolute viscosity (η) was calculated. The absolute viscosity of glycerol solutions at experimental temperatures was interpolated and extrapolated from the fitting of the literature values to virial equations; see the Supporting Information (Figure S1). Both calculations and experiments showed the contribution of salts to viscosity to be negligible. The contribution of proteins, at micromolar concentrations, was neglected.

Proteins. Horse heart cytochrome *c* was purchased from Sigma Chemical Co. The iron-free (so-called free base) form

was made, purified, and reconstituted with zinc(II) by a modification (Ye et al., 1997) of the original procedure (Vanderkooi & Erecińska, 1975; Vanderkooi et al., 1976). The product, zinc cytochrome *c*, was handled at 4 °C, in the dark. Two of the criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$. The third was the rate constant for natural decay of the triplet state ($k_d < 110$ s^{-1}); it was checked before each series of kinetic experiments. Plastocyanin was isolated from French bean by a standard procedure (Milne & Wells, 1970) and purified repeatedly by gel-filtration chromatography on Sephadex G-25 and G-75 columns and by an ion exchange chromatography on a Sephadex DEAE A-25 column; the criterion of purity was the absorbance quotient $A_{278}/A_{597} < 1.20$. Both proteins were desalted, transferred into a 2.5 mM phosphate buffer at pH 7.0, and stored in liquid nitrogen. Before each series of kinetic experiments, plastocyanin was treated with a small excess of dissolved $K_3[Fe(CN)_6]$, which was removed with Centricon ultrafiltration cells. Concentrations of the two proteins were determined from their UV−vis spectra, on the basis of the known absorptivities: $\epsilon_{423} = 2.43 \times 10^5$ M^{-1} cm^{-1} for zinc cytochrome *c* (Vanderkooi et al., 1976) and $\epsilon_{597} = 4500$ M^{-1} cm^{-1} (Milne & Wells, 1970) for cupriplastocyanin.

Flash Kinetic Spectrophotometry. So-called laser flash photolysis on the microsecond scale was done with a standard apparatus (Zhou & Kostić, 1991a,b, 1992b, 1993a,b). Argon was passed first through glycerol solutions and then through the buffered solution to be deaerated. Buffers with glycerol were deaerated by vigorous bubbling of argon for 1−3 h (the time depending on the glycerol concentration) at room temperature. The required volume of the buffer was further deaerated in a 10 mm cuvette for 30 min. Next, the cell jacket was connected to a 30 L Forma 2067 circulating bath, which maintained the temperature within ±0.2 °C. The actual temperature in the cell was calibrated with an Omega HH82 digital thermometer and was known with a precision of ±0.1 °C. The temperature range was 260.7−307.7 K. After the temperature of the buffer was adjusted, other solutions were added. The concentrations of zinc cytochrome *c* and cupriplastocyanin were always 10.0 and 30.0 μM, respectively. After each exposure to air, the solution in the cuvette was gently deaerated for 10−15 min. Determinations of k_d in control experiments proved this deareation to be thorough. Formation and decay (natural or by quenching) of the triplet state, ^{3}Zncyt, were monitored at 460 nm. Its concentration depended on the intensity of the laser pulse and was ca. 1.0 μM, much lower than the cupriplastocyanin concentration. Kinetic conditions for the pseudo-first order were thus satisfied. For each set of conditions (temperature, glycerol concentration, and ionic strength), 5−20 laser pulses were delivered. Error bars in Figure 1 enclose all the corresponding experimental values.

Kinetics. All the rate constants were obtained from the change in the absorbance at 460 nm with time. The absorbance decrease corresponds to the disappearance of the ^{3}Zncyt. The traces were analyzed with kinetic software from OLIS, Inc., and with the fitting program SigmaPlot 1.02, from Jandel Scientific Co. The standard errors of fitting, which are given in the tables, are computed by dividing the standard deviation by the square root of the number of measurements.

RESULTS

Natural Decay of the ^{3}Zncyt. The rate constant k_d was obtained from fittings of the traces to the monoexponential eq 2. We purified Zncyt until k_d became less than 110 s^{-1} at 298 K.

$$\Delta A = a \exp(-k_d t) \qquad (2)$$

Its value was independent of Zncyt concentration, ionic strength, and wavelength but dependent on glycerol concentration and temperature [see the Supporting Information (Figure S2)]. In buffered solutions containing 20, 40, 60, and 80% w/w glycerol, k_d is lower than in pure buffer at each temperature. It decreases at each temperature as the concentration of glycerol is raised. The rate constant k_d at an ionic strength of 2.5 mM ranges from 61 ± 2 s^{-1} in 80% w/w glycerol at 265.5 K, to 145 ± 5 s^{-1} in 20% w/w glycerol at 307.7 K, to 191 ± 5 s^{-1} in pure buffer at 307.7 K.

Modes of Quenching of ^{3}Zncyt by Cupriplastocyanin. The quenching of ^{3}Zncyt in the presence of 30 μM cupriplastocyanin is biphasic at all ionic strengths and temperatures used here. The faster phase (amplitude a_1) is the unimolecular reaction within the diprotein complex (eq 1), and the slower phase (amplitude a_2) is the bimolecular reaction between the unassociated protein molecules (Zhou & Kostić, 1991a; Ivković-Jensen & Kostić, 1996). The respective rate constants, the unimolecular k_F and the composite k_{obs}, were obtained by fitting to eq 3. In this work, we are interested

$$\Delta A = a_1 \exp(-k_F t) + a_2 \exp(-k_{obs} t) + b \qquad (3)$$

only in the unimolecular reaction k_F, shown in eq 1 and Scheme 1.

Unimolecular Reaction in Buffers without and with Glycerol. We reported earlier the rate constant for the faster component (k_F) in the phosphate buffer at pH 7.0 and ionic strengths of 2.5, 10, and 20 mM. Their viscosities span a narrow range and slightly depend on temperature, as follows: 1.77−1.82 cP at 273.3 K and 0.81−0.90 cP at 300.8 K (Ivković-Jensen & Kostić, 1996). Now we report this rate constant (k_F) in the phosphate buffers at pH 7.0 containing 20, 40, 60, and 80% w/w glycerol at ionic strengths of 2.5 mM (Figure 1a), 10.0 mM (Figure 1b), and 20 mM (Figure 1c). The temperature range was 270.4−307.7 K for the buffered 20% w/w glycerol and 260.7−307.7 K for the other three buffered glycerol solutions. We measured and interpolated the viscosity of buffered glycerol solutions in the stated temperature range [see the Supporting Information (Figure S1)]. The viscosity varied from 0.8 cP in the pure buffer at the highest temperature to 790 cP in the buffered 80% w/w glycerol at the lowest temperature.

Fractional Contribution of the Unimolecular Reaction. We measured the absolute values of the amplitudes of the two phases, a_1 and a_2 in eq 3, in the phosphate buffers at pH 7.0 and ionic strengths of 2.5, 10.0, and 20 mM containing glycerol at different concentrations. The fractional contributions of the faster phase, at a single cupriplastocyanin concentration but at different ionic strengths, are given in the Supporting Information (Table S1).

DISCUSSION

Unimolecular Reaction. The proteins associate at low ionic strength because the complementary patches on their

surfaces bear opposite charges. This association therefore depends greatly on ionic strength. Only at low ionic strength can both unimolecular and bimolecular reaction be observed. Already at the ionic strength of 20 mM, the unimolecular phase can be observed only if the cupriplastocyanin concentration is higher than 25 μM; in this study, we set it at 30 μM.

Determination of the Activation Parameters. As Figure 1 shows, the unimolecular rate constant k_F increases as the temperature increases and decreases as the viscosity increases. Fittings to Arrhenius (eq 4) and Eyring (eqs 5 and 6) equations yield the apparent activation parameters A, E_a, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$. In our previous study, the viscosity

$$k_F = A \exp \frac{-E_a}{RT} \tag{4}$$

$$k_F = \frac{k_B T}{h} \exp \frac{-\Delta G^{\ddagger}}{RT} \tag{5}$$

$$k_F = \frac{k_B T}{h} \exp \frac{\Delta S^{\ddagger}}{R} \exp \frac{-\Delta H^{\ddagger}}{RT} \tag{6}$$

spanned a narrow range (0.81−16.00) cP and was kept constant by adjustments of the solution composition as the temperature was changed. Therefore, the true activation parameters were obtained at each viscosity (Ivković-Jensen & Kostić, 1996). In this study, in which much wider ranges of viscosity (0.80−790 cP) and temperature (260.7−307.7 K) are examined, such adjustments were deemed impractical. We kept the glycerol concentration constant and allowed the viscosity to vary with temperature. In this way, we obtain apparent activation parameters, for a given constant concentration of glycerol; they are given in the Supporting Information (Table S2). The advantages of this study are the quicker experimental procedure and the greater intervals of viscosities and temperatures. Since k_F strongly depends on viscosity, the activation parameters (determined from the dependence of k_F on temperature) also depend on viscosity whether glycerol concentration [Supporting Information (Table S2)] or viscosity (Ivković-Jensen & Kostić, 1996) is kept constant. In the former case, however, the apparent activation parameters depend on temperature both directly and indirectly, because viscosity depends on temperature. This indirect dependence can be eliminated in two ways: by adjusting the viscosity at each temperature with sucrose (Ivković-Jensen & Kostić, 1996) and by interpolations (Beece et al., 1980). The interpolation method consists of the following. The logarithm of viscosity at different temperatures for different glycerol concentrations is described by a set of virial equations [see the Supporting Information (Figure S1)]. The intersection of the curves that represent these virial equations and straight lines that represent logarithms of viscosities of interest (log 2, log 4, log 8.4, log 16, log 50, and log 100) define temperatures at which the rate constants are calculated using apparent activation parameters in the Supporting Information (Table S2). The rate constants thus obtained were grouped by viscosity. Their fitting to Arrhenius (eq 4) and Eyring (eqs 5 and 6) equations gave proper activation parameters, shown in Table 1. The apparent activation parameters for the raw experimental results are given in the Supporting Information (Table S2), whereas those for the results obtained by interpolation are given in Table 1. The

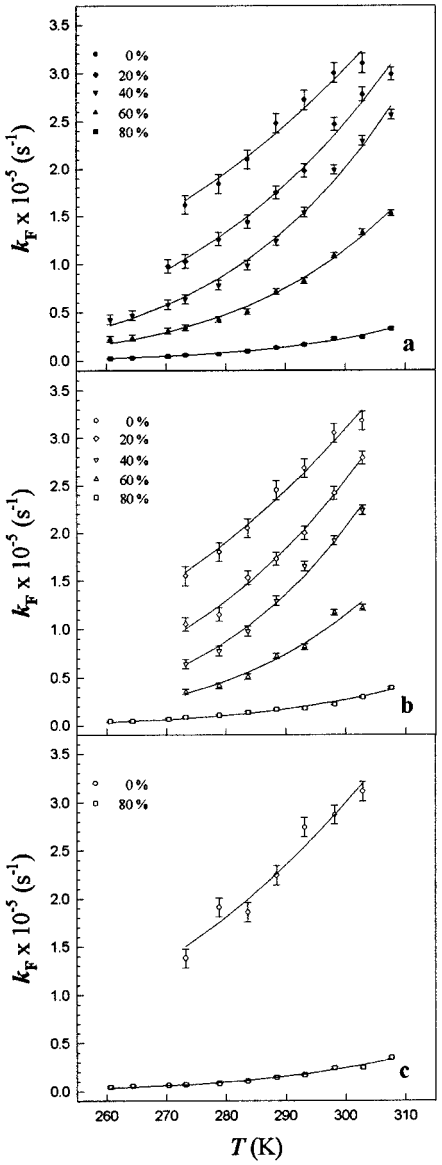


FIGURE 1: Dependence on temperature of the rate constant (k_F) for the unimolecular component of the reaction between ^{3}Zncyt and pc(II), shown in eq 1, in sodium phosphate buffers at pH 7.0 and ionic strengths of (a) 2.5 mM, (b) 10.0 mM, and (c) 20.0 mM. The lines are fittings to eq 6; the same lines are obtained also by fitting to eq 4. The glycerol concentrations, in percentage by weight, increase from top to bottom.

true activation parameters obtained by the latter method are valid, because they are corrected for the dependence of solution viscosity on temperature.

Fittings to nonlinear and linear forms of eqs 4−6 gave the same activation parameters. The linear plots are shown in the Supporting Information (Figure S3). The values of the rate constant k_F at the given temperature and viscosity and also the activation parameters are the same, regardless of the method for adjusting viscosity: with sucrose and direct adjustment or with glycerol and interpolation.

Because the frequency factor (A) does not have an obvious physical meaning, and because it contains temperature-dependent terms that may not cancel, fittings to eq 4 are uninformative. Fittings to eq 5 give the free energy of activation ($\Delta G^{\ddagger}$). Since, however, the experimental data require biexponential treatment, the assumption that $\Delta G^{\ddagger}$ is independent of temperature is unjustified. Fittings to eq 6

Table 1: Dependence on Ionic Strength (μ) and Solution Viscosity (η) of the Activation Parameters for the Unimolecular Reaction Shown in eq 1[a]

		eq 4		eq 5	eq 6	
μ (mM)	η (cP)	$A \times 10^{-6}$	E_a (kJ/mol)	$\Delta G^{\ddagger}$ (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/K mol)
2.5	2.0	19 ± 6	11 ± 2	44 ± 2	8 ± 1	−114 ± 3
	4.0	100 ± 200	16 ± 5	45.1 ± 0.8	13 ± 5	−100 ± 20
	8.4	30 ± 70	14 ± 6	44.0 ± 0.8	12 ± 5	−100 ± 20
	16.0	1 ± 2	6 ± 7	49 ± 2	3 ± 7	−140 ± 30
	50	0.2 ± 0.4	4 ± 5	48 ± 2	2 ± 5	−150 ± 20
	100	0.1 ± 0.1	3 ± 4	47 ± 2	13 ± 4	−160 ± 20
10.0	2.0	700 ± 100	19 ± 1	43.2 ± 0.7	17 ± 1	−84 ± 1
	4.0	15 ± 9	11 ± 2	45 ± 2	9 ± 2	−116 ± 6
	8.4	7 ± 3	10 ± 2	44 ± 2	8 ± 1	−122 ± 4
	16.0	1 ± 1	20 ± 3	49 ± 3	4 ± 3	−150 ± 20
	50	0.1 ± 0.02	19 ± 9	48 ± 2	3 ± 9	−162 ± 4
	100	0.1 ± 0.01	16 ± 5	47 ± 2	6 ± 7	−166 ± 3

[a] The parameters are obtained by fitting of interpolated values of the rate constant k_F to Arrhenius and Eyring equations.

Table 2: Dependence on Temperature and Solvent Viscosity of the Free Energy of Activation and of the Friction Parameters for the Unimolecular Reaction Shown in eq 1 at the Ionic Strength of 2.5 mM[a]

	eq 13		eq 14	eq 7	eq 16		eq 17
T (K)	δ	$\Delta G^{\ddagger}$ (kJ/mol)	δ	$\Delta G_{calc}{}^{\ddagger}$ (kJ/mol)	σ (cP)	$\Delta G^{\ddagger}$ (kJ/mol)	σ (cP)
260.7	0.5 ± 0.2	37.1 ± 0.8	0.39 ± 0.04	38 ± 2	38 ± 3	39.7 ± 0.1	9 ± 2
264.4	0.6 ± 0.1	37.6 ± 0.6	0.42 ± 0.04	39 ± 2	27 ± 3	40.0 ± 0.1	7 ± 2
270.4	0.55 ± 0.05	38.5 ± 0.2	0.41 ± 0.04	39 ± 2	7 ± 3	39.6 ± 0.1	4.8 ± 0.7
273.3	0.60 ± 0.04	38.7 ± 0.1	0.40 ± 0.08	40 ± 2	2 ± 1	39.1 ± 0.3	5 ± 1
278.9	0.58 ± 0.03	39.5 ± 0.1	0.43 ± 0.07	40 ± 2	2.4 ± 0.8	39.8 ± 0.2	3.6 ± 0.8
283.7	0.56 ± 0.03	40.1 ± 0.1	0.45 ± 0.06	41 ± 2	2.4 ± 0.8	40.3 ± 0.2	3.2 ± 0.6
288.5	0.54 ± 0.04	40.6 ± 0.1	0.44 ± 0.07	41 ± 2	2.5 ± 0.9	40.8 ± 0.2	3.5 ± 0.9
293.2	0.51 ± 0.05	41.2 ± 0.2	0.46 ± 0.06	41 ± 2	2.8 ± 0.8	41.4 ± 0.2	3.2 ± 0.6
298.2	0.45 ± 0.07	41.8 ± 0.2	0.43 ± 0.06	42 ± 2	3.6 ± 0.4	41.9 ± 0.1	3.9 ± 0.4
302.9	0.4 ± 0.1	42.4 ± 0.2	0.44 ± 0.08	42 ± 2	4.0 ± 0.6	42.5 ± 0.6	3.6 ± 0.5
307.7	0.5 ± 0.2	42.8 ± 0.3	0.48 ± 0.08	43 ± 2	3.6 ± 0.9	43.0 ± 0.2	2.9 ± 0.6

[a] Equations 13, 14, 16, and 17 are used for the fittings, and eq 7 is used for the calculations.

are the most appropriate because $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are practically independent of temperature in the interval studied. From those two activation parameters, $\Delta G^{\ddagger}$ at any temperature may be calculated using eq 7. Results of these calculations are

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (7)$$

given in Table 2. The calculated values of $\Delta G^{\ddagger}$, obtained from temperature dependence of k_F, can be compared to the fitted values of $\Delta G^{\ddagger}$, obtained from the viscosity dependence of k_F (see below).

Although $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, in principle, depend on the solvent viscosity, which in turn depends on temperature, these viscosity effects are usually neglected in experimental studies. Viscosity of aqueous solutions, especially the dilute ones, changes only slightly with temperature. If the rate constant is independent of viscosity, these secondary effects of temperature are justifiably neglected. But if the solvent is viscous, and if the rate constant depends on viscosity, both the direct and indirect effects of temperature must be considered. In such cases, one or more parameters are added in the Eyring equation to describe viscosity dependence (Debrunner & Frauenfelder, 1982).

Values of the Activation Parameters. The values $\Delta H^{\ddagger} =$ 13 kJ/mol and $\Delta S^{\ddagger} = -97$ J/K mol, shown in the first row of Table S2 in the Supporting Information, were successfully explained in our first study (Ivković-Jensen & Kostić, 1996). The enthalpy change was quantitatively reproduced by calculations concerning the change in the character of the exposed surfaces in the rate-determining rearrangement (configurational fluctuation) of the diprotein complex ^{3}Zncyt/pc(II). The negative entropy change was attributed to the closer approach of the two protein molecules as the complex tightens to allow the fast electron-transfer reaction to occur. The decrease in the rate constant for the rearrangement with the increasing viscosity was attributed to the change in $\Delta H^{\ddagger}$ (Ivković-Jensen & Kostić, 1996). The question whether $\Delta S^{\ddagger}$ depends on viscosity remained. This work shows that $\Delta S^{\ddagger}$ decreases (becomes more negative) as the viscosity increases.

Association Constant K_a. The relative fractional contribution of the unimolecular (faster) phase increases as the association constant (K_a) increases, according to eq 8. The

$$\frac{a_1}{a_1 + a_2} = \frac{1}{2[\text{Zncyt}]_0}\{[\text{Zncyt}]_0 + [\text{pc(II)}]_0 + K_a^{-1} - \sqrt{([\text{Zncyt}]_0 + [\text{pc(II)}]_0 + K_a^{-1})^2 - 4[\text{Zncyt}]_0[\text{pc(II)}]_0}\} \quad (8)$$

total concentration of cupriplastocyanin was kept constant; $[\text{pc(II)}]_0 = 30\ \mu$M. As Table S1 in the Supporting Information shows, K_a depends greatly on ionic strength, but not on temperature (in the range studied) and glycerol concentration. The independence of K_a of viscosity agrees with theory; the equilibrium is not affected by solvent viscosity, whereas the rate of configurational fluctuations is (Imry & Gavish, 1974; Beece et al., 1980). The fact that viscosity is the controlling variable is consistent with the idea that the barrier in this inter-protein reaction originates in protein dynamics, which

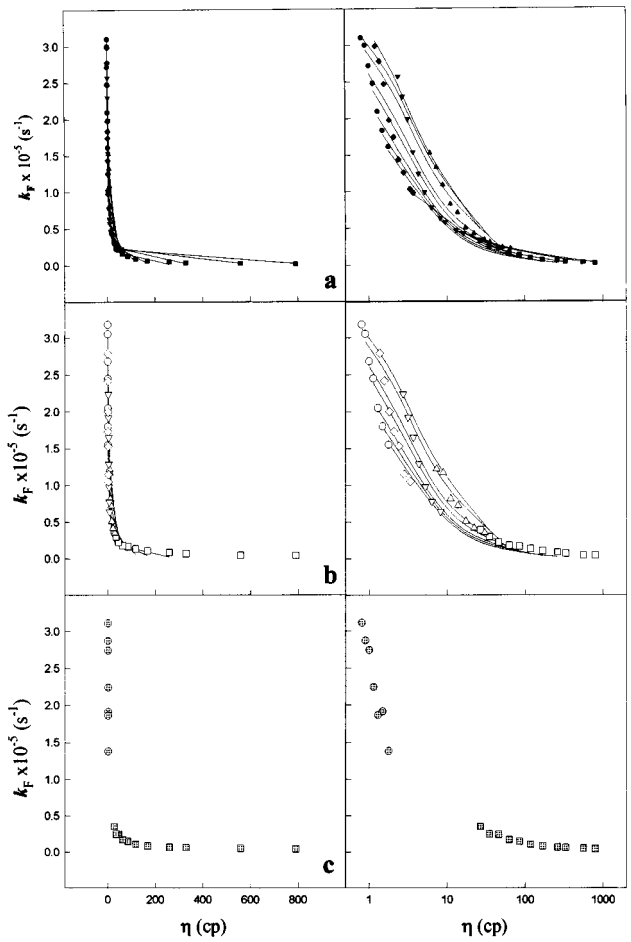


FIGURE 2: Dependence on viscosity of the rate constant (k_F) for the unimolecular component of the reaction between ^{3}Zncyt and pc(II), shown in eq 1, in sodium phosphate buffers at pH 7.0 and ionic strengths of (a) 2.5 mM, (b) 10.0 mM, and (c) 20.0 mM. The symbols are the same as in Figure 1. The lines are fittings to eq 17. Note the linear and logarithmic scales of viscosity at each ionic strength. The logarithmic scale lessens the overlap of data points.

is governed by thermal fluctuations (Cooper, 1976; Beece et al., 1980).

Microscopic Rate Constants. The rate constant k_F for the intracomplex reaction in eq 1 is the sum of microscopic rate constants k_s, for electron transfer in the thermodynamically stable configuration of the complex ^{3}Zncyt/pc(II), and k_r, for the rearrangement into the more reactive configuration, according to eq 9 (Zhou & Kostić, 1993b). Because the

$$k_F = k_r + k_s \tag{9}$$

previous experiments (Zhou & Kostić, 1993b) were done at a single temperature and in the relatively narrow range of viscosity ($1.0 \leq \eta \leq 80$), they could not show whether the reaction is controlled by a rearrangement also at high viscosities. In general, k_s is independent of viscosity, whereas k_r is inversely proportional to viscosity. As Figure 2 shows, k_r decreases as the viscosity increases. As the highly viscous solvent traps the complex ^{3}Zncyt/pc(II) in its initial configuration, k_F approaches k_s. The question is whether k_s is zero, i.e., whether the interprotein electron-transfer reaction can occur at all in the initial docking configuration. The lowest value of k_F in Figure 2 is ca. 2300 s^{-1}, and the curves continue to decrease even at the highest viscosities. We conclude that the rate constant k_F for the unimolecular electron-transfer reaction in eq 1 approaches zero, i.e., that the rate of this reaction is limited by the rearrangement of the diprotein complex throughout the range of temperatures and viscosities examined.

Interplay of Electron Transfer and Protein Rearrangement. An oxidoreduction reaction may consist simply of an electron-transfer step, or that step may be gated by, or coupled with, some nonredox process (Davidson, 1996). Whether the parameters required for fitting of kinetic results to the Marcus equation (Marcus & Sutin, 1985) are reasonable may be used as a criterion whether the rate under consideration is truly that of the electron-transfer step in the overall reaction (Davidson, 1996; Davidson & Jones, 1996; Bishop & Davidson, 1995; Brooks & Davidson, 1994a,b; Harris et al., 1994). Because there are no rules about the expected values of the parameters or about the intervals to which these values belong, their analysis is inevitably somewhat arbitrary. Unusual parameters may be symptoms of gated or coupled electron transfer (Brunschwig & Sutin, 1989; Harris et al., 1994), but such parameters need not rule out electron transfer as the rate-limiting step. In our opinion, these three mechanisms may not be distinguishable simply by analyzing temperature effects on kinetics. In the case of true electron transfer, Marcus parameters are valid. In the case of gated electron transfer (i.e., when the rearrangement is much slower than the subsequent electron-transfer step), Marcus parameters are meaningless; the rate-limiting process is characterized by Eyring parameters. In the case of coupled electron transfer (i.e., when the rearrangement is faster than the subsequent electron-transfer step but the equilibrium for rearrangement is unfavorable), both Marcus and Eyring parameters would have contributions from the rearrangement (Davidson, 1996).

Fittings to Marcus Theory. We take the thermodynamic driving force for the reaction in eq 1 to be the sum of the potentials for oxidation of ^{3}Zncyt to Zncyt$^+$ and for the reduction of pc(II) to pc(I), namely $\Delta G^\circ = -120$ kJ/mol, and fit the dependence of k_F to eqs 10 and 11 (Marcus & Sutin, 1985)

$$k_F = \frac{4\pi^2 {H_{AB}}^2}{h\sqrt{4\pi\lambda RT}} \exp\left[\frac{-(\Delta G^0+\lambda)^2}{4\lambda RT}\right] \tag{10}$$

$$k_F = k_0 \exp[-\beta(r - r_0)] \exp\left[\frac{-(\Delta G^0+\lambda)^2}{4\lambda RT}\right] \tag{11}$$

in which the symbols have their usual meanings: H_{AB} is the electronic coupling between the heme and the copper site, λ is the reorganizational energy, h is the Planck's constant, R is the gas constant, k_0 is the nuclear frequency (10^{13} s^{-1}), r_0 is the contact distance (3 Å), β is the attenuation of electronic coupling (1.4 $Å^{-1}$), and r is the donor−acceptor distance. The units are carefully chosen to cancel each other and yield the net unit of s^{-1}.

The values of λ and H_{AB} obtained from fittings to eq 10 of the kinetic results at the ionic strength of 2.5 mM (Ivković-Jensen & Kostić, 1996) depend somewhat on viscosity: 250 ± 6, 245 ± 8, and 283 ± 3 kJ/mol (or 2.4, 2.4, and 2.7 eV) for λ and 3×10^{-23}, 2×10^{-23}, and 5×10^{-23} J (or 1, 1, and 2.5 cm^{-1}) for H_{AB}, respectively, when the solvent was an unadjusted buffer and when its viscosity was adjusted to 2.00 and 16.0 cP. Similar fittings of the kinetic results

obtained in the present study gave less accurate values of λ and H_{AB}.

The reorganizational energies (λ) for electron-transfer reactions within single metalloproteins labeled with ruthenium complexes are 1.2, 1.3, and 0.9 eV, respectively, for cytochrome *c*, myoglobin, and azurin (Winkler & Gray, 1992). The reorganizational energies for reactions involving pairs of metalloproteins are 0.8, 0.9, and 1.4 eV, respectively, for the following pairs: cytochrome *c* and cytochrome b_5, hemoglobin and cytochrome b_5, and cytochrome *c* and cytochrome *c* peroxidase (McLendon & Hake, 1992; Simmons et al., 1993; Kostić, 1992). Our reorganizational energies are much larger than those mentioned so far but comparable to the values of 2.3, 1.9, and 2.1 eV, respectively, for MADH and amicyanin (Brooks & Davidson, 1994a), MEDH and cytochrome c_{551i} (Harris & Davidson, 1993), and hemoglobins partially reconstituted with zinc(II) ions (Peterson-Kennedy et al., 1984, 1986). Our donor/acceptor couplings fall between the values of 0.071 and 11.6 cm^{-1}, reported for MEDH and cytochrome c_{551} (Harris & Davidson, 1993) and MADH and amicyanin (Brooks & Davidson, 1994a).

The values of λ and *r* obtained from fittings to eq 11 of the kinetic results at the ionic strength of 2.5 mM (Ivković-Jensen & Kostić, 1996) are consistent with the results of the fittings to eq 10 discussed above: 242 ± 6, 239 ± 9, and 276 ± 4 kJ/mol for λ and 10.9, 11.2, and 10.0 Å for *r*, respectively, when the solvent was an unadjusted buffer and when its viscosity was adjusted to 2.00 and 16.0 cP. The values of *r* obtained from fittings to eq 11 with the values of λ from the fittings to eq 10 fall in the interval of 9.6−10.9 Å. Fittings to eq 11 of the kinetic results obtained in the present study gave the values that fall in the intervals of $\lambda = 200 \pm 40$ kJ/mol and $r = 14 \pm 3$ Å at viscosities of 2−100 cP. These fitted parameters span the wider range of viscosity and are less acurate but consistent with those listed above.

Because the definition of the distance (*r*) depends on the details of the electronic structures of the redox sites, we consider all of the common choices of the reference points: the metal atoms, the coordinated atoms, and the outer edge of the ligands. The relevant points in cytochrome *c* are the iron (or zinc) atom, the sulfur atom of Cys 17, and the various atoms at the heme edge. The relevant points in plastocyanin are the copper atom, the nitrogen ligand of His 87, and the sulfur ligand of Cys 84 (Guss & Freeman, 1983; Redinbo et al., 1994; Gross, 1993; Sykes, 1991a,b). The three realistic configurations of the cyt/pc complex (Roberts et al., 1991; Ullmann & Kostić, 1995) are designated as in these previous studies, for the sake of consistency. The configuration max ov, which can reasonably be taken as the model of the initial docking (Ivković-Jensen & Kostić, 1996; Crnogorac et al., 1996), sets Cys 17 and Cys 84 12 Å apart and all the other pairs of reference points more than 14 Å apart. The configuration max ov rot sets all the pairs of reference points more than 14 Å apart. The configuration n/eq sets both Cys 17 and the heme edge 12 Å apart from Cys 84, and it sets the distance between Cys 17 and His 87 at 11 Å.

Mechanism of the Unimolecular Reaction. The low fitted value of H_{AB} may indicate that the quenching in eq 1 is a true electron-transfer reaction. The fitted values of λ, which are relatively large, do not rule out this possibility, but they are consistent also with the mechanism in which the electron-transfer step is gated by, or coupled with, the preceding rearrangement (Davidson, 1996). A slight dependence of λ on viscosity, possibly reflected in the aforementioned results of fitting, can be considered only after more studies of this kind are reported. The fittings to the analogous eqs 10 and 11 yielded the same values of λ. Not surprisingly, the fittings to eq 11 yielded reasonable values of the distance *r*. Interpretation of the fitted parameters alone does not settle the mechanism of the reaction in eq 1. For a more informative analysis of the mechanism, we combine the findings of this and previous studies in our laboratory (Zhou & Kostić, 1992, 1993b). The independence of k_F of ionic strength (Zhou & Kostić, 1993b; Figure 1) is consistent with true electron transfer but possibly inconsistent with coupled electron transfer within the electrostatic diprotein complex because the equilibrium constant for the rearrangement of such a complex is expected to depend on ionic strength (Harris et al., 1994). The great dependence of k_F on viscosity, seen in Figure 2, is inconsistent with true electron transfer because no term in eq 10 is expected to depend markedly on viscosity. This dependence is probably inconsistent also with coupled electron transfer because equilibrium constants generally are independent of viscosity (Imry & Gavish, 1974; Beece et al., 1980).

Indeed, the association constant K_a for the diprotein complex under consideration is independent of viscosity, as Table S1 in the Supporting Information shows. Since the dependence of k_F on viscosity is the same regardless of the viscosigen added to the solution (Zhou & Kostić, 1993b), specific solvation of the protein molecules or the diprotein complex, which in principle might affect the rearrangement equilibrium, is unlikely. The strong dependence of k_F on viscosity, and possibly also the demonstrated independence of ionic strength, are consistent with gated electron transfer. The evidence may be consistent also with coupled electron transfer, if the equilibrium constant for rearrangement is independent of ionic strength. Additional evidence for gating includes the similar values of k_F at the driving force of 0.80 and 1.2 eV (Zhou & Kostić, 1993b). In conclusion, the evidence indicates, but does not prove, that the reaction in eq 1 involves electron transfer gated by a slower rearrangement of the diprotein complex.

Kinetic Effects of Viscosity. Effects of solution viscosity on reactions of proteins have been studied relatively little (Gavish & Werber, 1979; Beece et al., 1980; Khoshtariya et al., 1991; Ansari et al., 1992; Nocek et al., 1991). Molecules in solution affect the motions of one another through viscous friction. We use glycerol because it does not change the spectroscopic and photophysical properties of zinc cytochrome *c* and plastocyanin (Zhou & Kostić, 1993b). In this study, we consider the friction of these two molecules with each other and with the solvent.

Those points in Figure 2 that are obtained at the same temperature define an isotherm. Studying these isotherms, we isolate the effects of viscosity from the effects of temperature. As previous studies (Zhou & Kostić, 1993b; Ivković-Jensen & Kostić, 1996) and Figure S4 in the Supporting Information show, it is viscosity, and not other properties, of the added viscosigens that is responsible for the dependence of the rate constant k_F on the solvent composition. In particular, as Figure S5 in the Supporting Information shows, this dependence cannot be attributed to the change in the dielectric properties of the solvent.

The generally observed linear dependence of log k_F on log η establishes the empirical eq 12 (Khoshtariya et al., 1991), which is based on the Arrhenius equation. Equations 13 and 14 are likewise based on the Eyring equation. The results of the fittings to eqs 13 and 14, on the assumption that the activation parameters are independent of viscosity at constant temperature, are given in Table 2. The activation parameters in eq 14 are set to $\Delta H^{\ddagger} = 13$ kJ/mol and $\Delta S^{\ddagger} = -97$ J/K mol. This is justified by the equality of the $\Delta G^{\ddagger}$ values obtained from eqs 13 and 7 at the same temperature. Values of δ obtained by fittings to eq 14, in which δ is now the only variable, are more accurate than the values obtained by fittings to eq 13, in which $\Delta G^{\ddagger}$ is another variable. Indeed, Table 2 shows the difference in the precision of the two fittings. The parameter δ has no clear physical meaning; it simply shows how much k_F depends on viscosity.

$$k_F = A\eta^{-\delta} \exp\left(\frac{-E_a}{RT}\right) \tag{12}$$

$$k_F = \frac{k_B T}{h}\eta^{-\delta} \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right) \tag{13}$$

$$k_F = \frac{k_B T}{h}\eta^{-\delta} \exp\left(\frac{-\Delta S^{\ddagger}}{R}\right) \exp\left(\frac{-\Delta H^{\ddagger}}{RT}\right) \tag{14}$$

$$k_F = \frac{C}{\eta + \sigma} \exp\left(\frac{-E_a}{RT}\right) \tag{15}$$

$$k_F = \frac{k_B T}{h}\frac{1+\sigma}{\eta+\sigma} \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right) \tag{16}$$

$$k_F = \frac{k_B T}{h}\frac{1+\sigma}{\eta+\sigma} \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \exp\left(\frac{-\Delta H^{\ddagger}}{RT}\right) \tag{17}$$

As Table 2 and Figure 3a show, δ is independent of temperature within the experimental error. This independence is also evident in the overlap of data points at different temperatures [Figure 2 and Supporting Information (Figure S4)]. The applicability of eqs 12−14 to protein reactions is questionable because the dependence of log k_F on log η is not exactly linear. Therefore, the exponential and linear forms of these equations yield different fittings of experimental results; see the Supporting Information (Figure S6).

Another approach to the study of viscosity effects is possible when the solvent molecules are much smaller than the solute (protein) molecules. The contribution of the protein friction to the total friction is designated σ (Ansari et al., 1992). The Kramers equation is combined with the Arrhenius treatment (eq 15) and Eyring treatment (eqs 16 and 17). Because η is absolute viscosity, σ also has units of viscosity. Analysis of any of these three equations shows that protein friction dominates when the solution viscosity is very low, that both kinds of friction are important when the solution viscosity is low, and that solvent friction dominates when the solution viscosity is high. In this last case, the rate constant becomes inversely proportional to the solvent viscosity. This regime is called overdamped Kramers limit (Kramers, 1940), and it was also the limiting case for eqs 12−14 when $\delta = 1$. In our experiments, the viscosity spans all three aforementioned regimes.

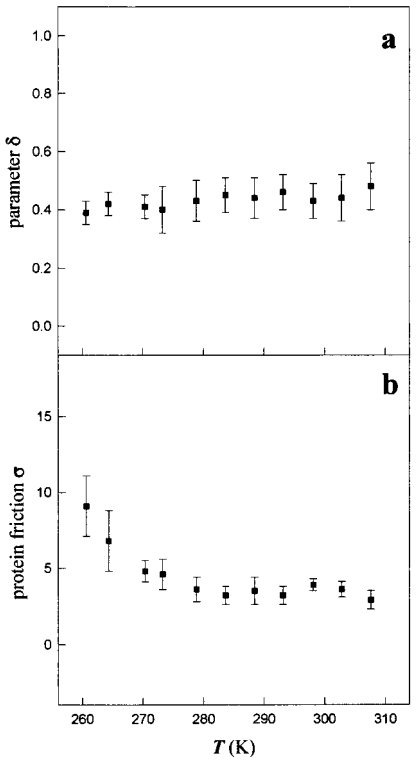


FIGURE 3: Dependence on temperature of (a) the empirical parameter δ from eq 14 and (b) the protein friction parameter σ (in centipoise) from eq 17. The data are taken from Table 2.

The results of fittings to eq 16, with two parameters, are shown in Table 2. As before, $\Delta G^{\ddagger}$ is assumed to be independent of viscosity. Reassuringly, the $\Delta G^{\ddagger}$ values obtained from eqs 16 and 7 are equal. When the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ obtained for solutions in pure aqueous buffers are used in eq 17, kinetic results in glycerol-containing buffers can be fitted with a single parameter, σ. The fitting to eqs 16 and 17 is much better than the fitting to eqs 13 and 14 because, as mentioned before, the dependence of log k_F on log η is not exactly linear. Again, setting the activation parameters in eq 17 to $\Delta H^{\ddagger} = 13$ kJ/mol and $\Delta S^{\ddagger} = -97$ J/K mol is justified by the aforementioned equality of the $\Delta G^{\ddagger}$ values. Again, values of σ obtained by the one-variable fittings to eq 17 are somewhat more accurate then those obtained by the two-variable fittings to eq 16. Because the σ values when $T < 273$ K and when $T = 307.7$ K are the results of fittings of only three or four data points in Figure 1a, these values are less precise than the others, each of which was obtained by fitting to five data points. Despite the considerable error margins in Figure 3b, σ seems to decrease slightly as the temperature increases.

Fitting of the data for the conformational change of myoglobin after photodissociation of the CO ligand yielded a σ of 4.1 ± 1.3 cP (Ansari et al., 1992). This single value, obtained in the intervals of temperature and viscosity that are somewhat narrower than those in our study, does not reveal conceivable differences in the protein behavior at different temperatures. Moreover, the Zncyt/pc system has different contributions to σ, arising from the friction between and within the individual proteins. The temperature dependence of σ in Table 2 and Figure 3b is consistent with the notion that the rate of rearrangement is governed by thermal fluctuations; the higher the temperature, the more flexible the diprotein complex.

CONCLUSION

Both temperature and solvent viscosity can modulate dynamic processes involved in inter-protein electron-transfer reactions. The dependence of the rate constant on these two properties defines a three-dimensional surface shown in the Supporting Information (Figure S7). For a thorough understanding of dynamics, such surfaces should be explored as widely as experimental limitations allow. Because changes in temperature may also cause changes in viscosity, care must be taken to separate the direct and indirect effects of temperature on the rate constant in experiments in which the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are determined. To this end, effects of temperature at constant viscosity can be examined first, as in our previous work (Ivković-Jensen & Kostić, 1996), and the effects of viscosity at constant temperature can be explored next, as in the present work.

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SUPPORTING INFORMATION AVAILABLE

Two tables showing the fractional contribution of the unimolecular reaction and the dependence on glycerol concentration of the apparent activation parameters for the unimolecular reaction and seven figures showing the dependence of viscosity on temperature, fitted to a virial equation; the dependence of k_d on temperature, fitted to a virial equation; the dependence of $\ln(k_F/T)$ on temperature, fitted to the Eyring equation in the linear form; the dependence of k_F on solution viscosity; the dependence of the solvent dielectric constant on temperature; the dependence of k_F on the solvent dielectric constant; three ways of fitting dependence of k_F on solution viscosity; and the interdependence of T, η, and k_F (9 pages). Ordering information is given on any current masthead page.

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