Temperature Dependence of Photoinduced Electron Transfer within Self-Assembled Uroporphyrin−Cytochrome *c* Complexes

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In this report the temperature dependence of photoinduced electron transfer between both singlet- and triplet-state uroporphyrin (^{1}URO and ^{3}URO, respectively) and the heme group of ferricytochrome *c* (Cc) has been examined. Analysis of the forward electron-transfer rate originating from ^{1}URO as a function of temperature gives a reorganizational energy (λ) of 1.0 ± 0.02 eV with an associated electronic coupling term (H_{AB}) of (3.7 ± 0.3) × 10^{-4} eV. In addition, the singlet lifetime data exhibit a temperature-dependent Gaussian distribution that narrows as the temperature increases. This suggests a conformational distribution of bound porphyrin with a narrow range of potential wells at lower temperatures. The corresponding temperature dependence of the electron transfer between ^{3}URO/URO$^+$ and the heme group of Cc gives a $\lambda = 0.49 \pm 0.05$ eV/0.19 ± 0.03 eV and $H_{AB} \sim 4 \times 10^{-4}$ eV/1.4 × 10^{-4} eV, respectively. These observations are consistent with orientational dependent conformational states as well as possible gating of electron transfer within the complex.

Introduction

The importance of inter-/intramolecular electron transfer (ET) in proteins has stimulated extensive research into the molecular basis of these reactions.[1−4] Biological ET reactions occur between various redox-active cofactors embedded within a protein matrix as well as between electrostatically stabilized protein−protein complexes. Within the Marcus framework intramolecular ET rates are modulated by donor−acceptor distance, thermodynamic driving force, donor−acceptor orientation, the nature of the intervening medium, and both inner-sphere and outer-sphere reorganization.[5−11] However, intermolecular ET rates associated with proteins in solution require additional mechanistic steps including: (1) formation of the protein−protein complex, (2) ET between the redox centers of each protein within the complex, and (3) dissociation of the protein−protein complex. The extent to which these additional factors (which influence protein−protein recognition and docking) contribute to the *overall* ET rate constant is of key interest.[12−17]

Zhou *et al.*[18] and Zhou and Rodgers[19] have developed a useful model system for probing the intermolecular ET that involves electrostatic complexes between horse heart cytchrome *c* (Cc) and various derivatives of uroporphyrin (URO). Within this system, excitation of photoactive derivatives of URO bound electrostaticaly to Cc results in both singlet- and triplet-state quenching of the excited porphyrin. In the case of triplet state quenching, reduction of the heme iron of Cc was observed with a forward rate constant = back rate constant ∼2 × 10^6 s^{-1}. Interestingly, the variation in ET rate (originating from triplet state quenching) with reaction free energy is consistent with semiclassical Marcus theory for the back ET reaction while the forward reaction displayed no inverted region within the same range of driving force. The lack of an inverted region was attributed to contributions from the coordinate solvent mode or to conformational gating.

In a previous study, we extended this work by examining photoinduced ET within self-assembled complexes between Cc and both free-base URO and free-base tetrakis(4-carboxyphenyl)porphyrin (4CP) using transient absorption and time-resolved fluorescence spectroscopies.[20] The effects of orientational differences on photoinduced electron transfer between the bound porphyrins and the heme group of Cc were demonstrated in the steady-state and time-resolved fluorescence and triplet−triplet transient absorption data obtained for the complexes. The singlet state of URO was found to be only moderately quenched by complexation to the protein. Fluorescence lifetime data was best fit to two components consisting of a discrete component at 15.7 ns (free URO) and a Gaussian distribution of lifetimes centered at 3.2 ns. However, URO exhibits significant triplet-state quenching, resulting in intracomplex electron transfer in which the observed forward and reverse rates are similar ((1.8 ± 0.2) × 10^6 s^{-1} and (1.6 ± 0.4) × 10^6 s^{-1}, respectively). In contrast, 4CP displayed only singlet-state quenching when complexed with Cc and the singlet state was best fit to a discrete lifetime, $\tau = 1.3$ ns. The difference in ET mechanism within the URO−Cc complex (i.e., singlet versus triplet) was rationalized in terms of distinct dipole orientations of the bound porphyrin relative to the heme group of the protein. We further speculated that the orientational differences between bound URO arise due to the flexibility of the URO side chains.

To better understand the molecular basis for the differences in ET rate within the URO−Cc complex (i.e., ET originating from ^{1}URO/^{3}URO quenching by the heme of Cc within the complex) we have examined the reorganizational energy (λ) and electronic coupling factors (H_{AB}) for intercomplex ET within the URO−Cc complex using the temperature dependence of the ET rate constants. The data provide evidence for distinct binding sites/orientations (giving rise to singlet-/triplet-state

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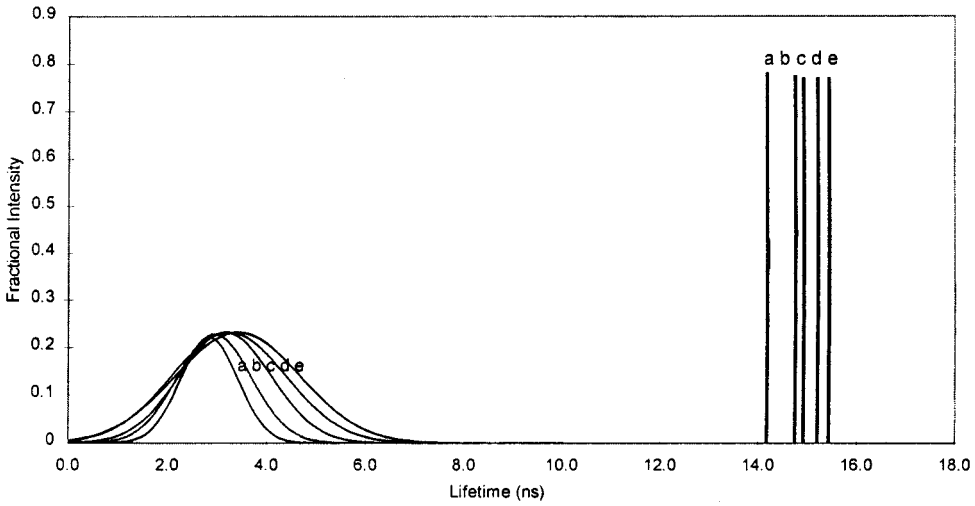

Figure 1. Fluorescence lifetime distributions for URO in the presence of Cc at various temperatures. The shorter lifetime has been best fit to a Gaussian distribution and assigned to intercomplex ET quenching of ^{1}URO, while the longer lifetime (free URO) is fit to a discrete lifetime. Temperatures are (a) 322.8, (b) 313.4, (c) 304.3, (d) 293.7, and (e) 284.5 K.

quenching) as well as possible gating of the thermal back ET (originating from triplet-state quenching).

Material and Methods

Bovine heart cytochrome *c* (Sigma) and free base uroporphyrin (Porphyrin Products) were used without further purification. Cytochrome stock solutions were prepared in 5 mM potassium phosphate bufffer, pH 7.0. Uroporphyrin stock solutions (~1 mM) were prepared in 0.1 N NaOH. The concentrations of the stock solutions were determined by using $\epsilon_{550nm} = 19\ mM^{-1}\ cm^{-1}$ (cytochrome *c* reduced minus oxidized) and $\epsilon_{552nm} = 18.3\ mM^{-1}\ cm^{-1}$ (URO dilute in 0.1 N HCL).[21,22]

Time-resolved fluorescence measurements were performed on an ISSK2 multifrequency and phase modulation spectrofluorimeter equipped with an Ar ion laser (SpectraPhysics model 2045) as the excition source. The sample chamber was temperature controlled via a constant temperature water bath (±0.1 °C). Samples were excited by the 514-nm Ar line, and the emission at $\lambda > 570$ nm was recorded through a Schott RG083 filter (see ref 23 for a description of phase and modulation time-resolved fluorescence methods). Sample concentrations were 15 μM; the sample was prepared to have a 30:70 ratio of complex to free porphyrin, to perform the temperature-dependent study. Fluorescein in 0.1 N NaOH was used as a lifetime reference ($\tau = 4.05$ ns). Lifetime data were analyzed by using software provided by either ISS or Globals Unlimited, using previously described fitting routines.[24,25]

Triplet-state kinetics were examined by using nanosecond transient absorption instrumentation described in detail elsewhere.[20] Briefly, URO−Cc samples were excited at 532 nm with a pulsed Nd:YAG laser (Continuum SureLite I, 7-ns fwhm and 7 mJ/pulse). Changes in absorption were monitored with a Xe arc lamp (Oriel). The Xe probe was overlapped with the pump laser in the sample cuvette and subsequently imaged onto the entrance slit of a Spex 1580 1/4M double monochromator. The temperature of the sample cuvette was digitally controlled (±0.1 °C). The signal from the detector, a thermoelectrically cooled R928 (Hamamatsu) photomultiplier tube, was amplified and then digitized with a Tektronix RTD710A 200-MHz transient digitizer and transferred to a 486-based microcomputer for further processing. Data fits were obtained with Enzfitter software.

TABLE 1: Lifetime Distribution Data as a Function of Temperature[a]

temp, K	τ1,[b] ns	width, ns	fraction	τ2, ns	fraction
284.5	3.42	1.25	0.23	15.43	0.77
293.7	3.24	1.15	0.23	15.20	0.77
304.3	3.17	0.93	0.23	14.92	0.77
313.4	2.95	0.74	0.23	14.74	0.77
322.8	2.84	0.58	0.22	14.17	0.78

[a] All components were allowed to vary for the analysis. [b] Center of a Gaussian distribution.

Results

Temperature Dependence of Singlet-State ET. Previous studies have demonstrated that complexation of URO to Cc results in quenching of both the singlet and triplet states of the bound prophyrin. Singlet-state quenching results have been fit to a Gaussian distribution of lifetimes with a width of 1.2 ns centered at 3.2 ns at 25 °C with a reduced χ^2 of 1.1; the standard errors of the phase angle and modulation ratio used in the reduced χ^2 calculation were 0.2° and 0.004, respectively (Figure 1, trace a) (a discrete component at 15.3 ns corresponding to unbound URO is also observed). The temperature dependence of both the distribution center and width are displayed in Figure 1, traces b−e, and summarized in Table 1. As expected, the lifetime decreases as the temperature increases, indicating an increase in ET rate constant with increasing temperature. Interestingly, the width of the distribution narrows from 1.3 to 0.6 ns over a temperature range of roughly 40 K, suggesting interconversion between conformational distributions (see Discussion). The temperature dependence of the rate constants for ET between the ^{1}URO and the heme group of Cc ($k = 1/\tau_q - 1/\tau_o$) can be fit to the semiclassical Marcus equation to extract the reorganizational energy (λ) and the electronic coupling factor (H_{AB}):

$$k_{ET} = (2\pi/h)H_{AB}/(4\pi\lambda k_B T)^{1/2} \exp[-(-\Delta G^\circ - \lambda)^2/4\lambda k_B T] \quad (1)$$

where k_B is Boltzmann's constant, T is temperature, and ΔG° is the reaction free energy.[26] The reaction free energy (ΔG°) for the photoinitiated forward reaction was calculated by using the Rehm−Weller equation:

$$\Delta G^\circ = e[E^\circ_{URO} - E^\circ_{Cc}] - \Delta E^* + w \quad (2)$$

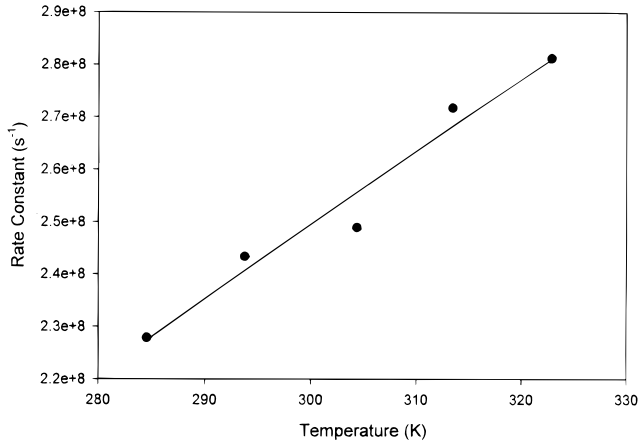

Figure 2. Plot of k_{obs} vs T for the center of the singlet ET Gaussian distribution. Solid line represents the best fit to eq 1.

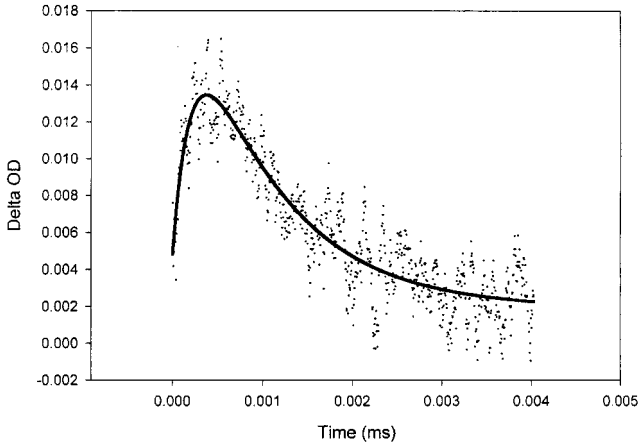

Figure 3. Representative transient absorption trace of the triplet ET within the URO−Cc complex in 5 mM phosphate buffer, pH 7.0, monitored at 550 nm. The sample contained 20 μM Cc and 40 μM URO. The trace is the average of 50 laser pulses (λ_{exc} = 532 nm, 7 mJ/pulse).

where $E°_{Cc}$ is the reduction potential for Cc, $E°_{URO}$ is the reduction potential of the URO π-cation radical, ΔE^* is the energy of the singlet excited state, e is unit electrical charge, and w is a parameter describing the work required to bring two charged molecules to within a reactive distance.[27] For URO, $E°_{URO} = 0.80$ V vs NHE, $E°_{Cc} = 0.26$ V vs NHE, and ΔE^* is 194 kJ/mol.[29] The work term is negligible since there exists an electrostatic complex prior to the electron transfer and therefore it is not necessary to consider the work to bring the donor and acceptor within a distance to facilitate electron transfer. Fitting the rate constants to eq 1 gives $\lambda = 1.0 \pm 0.2$ eV and $H_{AB} = (3.7 \pm 0.3) \times 10^{-4}$ eV (see Figure 2).

Temperature Dependence of Triplet ET. Figure 3 displays a representative transient absorption trace of the URO−Cc complex obtained at 550 nm (absorption maximum for Fe(II)-Cc). The data can be fit to a biexponential process (solid line in Figure 3) with rate constants of $(7.9 \pm 0.8) \times 10^6$ s^{-1} and $(7.3 \pm 0.7) \times 10^5$ s^{-1} for the fast and slow phases, respectively (20 °C). It should be pointed out that these values differ somewhat from those reported in our previous work. This may be due to the fact that the data used in the current study was of significantly better S/N than those of the previous study, yielding more accurate values of the rate constants. The rate constant corresponding to the fast phase data is for the reduction of the heme iron of Cc by ^{3}URO (since this rate constant matches that of the fast phase of the triplet-state decay (data not shown)),

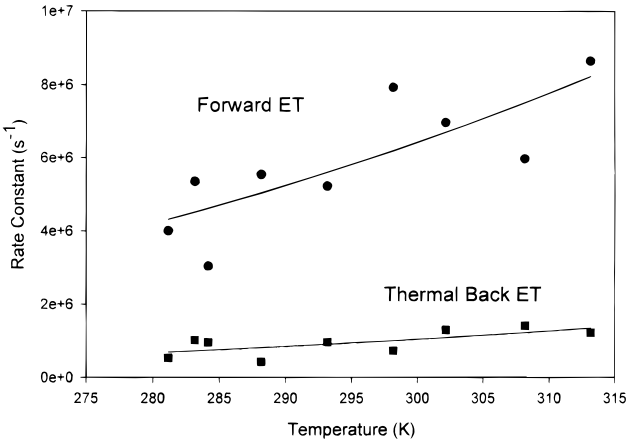

Figure 4. Plot of k_{obs} vs T for the intercomplex triplet ET. Forward (circles) and back (squares) ET are represented. Solid lines represent best fits to eq 1.

while the rate constant for the slower process corresponds to the subsequent thermal back ET from the reduced heme to the URO π-cation radical. These rate constants also exhibit significant temperature dependence. Fitting the rate constants as a function of temperature to eq 1 (Figure 4) yields λ's of 0.49 ± 0.05 eV/0.19 ± 0.03 eV and $H_{AB} \sim 4 \times 10^{-4}$ eV/1.4×10^{-4} eV, for the fast and slow phases, respectively.

Discussion

Reorganizational Energies of ET in URO−Cc Complexes. Earlier work by Clark-Ferris and Fisher[30] and Zhou et al.[18] demonstrated that anionic porphyrins including 4CP and URO bind to a region of Cc comprised of Lys 13, 27, 72, and 86. This region is utilized as a complexation site by a wide range of proteins, including cytochrome c peroxidase (CcP), cytochrome c oxidase (CcO), and cytochrome c reductase (CcR).[31] Our previous work has further demonstrated that excitation of URO bound to this docking region on Cc results in both singlet and triplet quenching due to ET between the excited porphyrin and the heme group of the protein. The observation of both singlet and triplet quenching (reflecting quite different rates of ET) is, at first sight, not suprising since the driving force associated with ET from ^{1}URO to Cc is distinct from that of ET from ^{3}URO to Cc ($\Delta G° = -141.9$ kJ/mol for ^{1}URO to Cc and $\Delta G° = -103.9$ kJ/mol for ^{3}URO to Cc). The ratio of two rate constants associated with ET reactions differing only in reaction driving force can be derived from Semiclassical Marcus theory:

$$(k_S/k_T) = [(H^S_{AB})^2/(H^T_{AB})^2] \exp(-\{2\lambda(\Delta G°_S - \Delta G°_T) + (\Delta G°_S)^2 - (\Delta G°_T)^2)\}/2\lambda kT) \quad (3)$$

where k_S/k_T is the ratio of the singlet-state ET rate constant to the triplet-state ET rate constant, H_{AB} is the electronic coupling term for the singlet-/triplet-state reaction, λ is the total reorganizational energy, k is Boltzmann's constant, T is temperature, and $\Delta G°$ is the free energy for the reaction origination from singlet-/triplet-state quenching.[32] Assuming a reasonable value for λ (1 eV) and assuming H_{AB} is the same for both singlet and triplet quenching the ratio k_S/k_T would be ~0.014. However, the experimentally determined value is 31. Thus, the difference in rates cannot be attributed simply to the difference in reaction driving force.

The temperature dependence of the singlet/triplet ET rate constants reveals distinct differences between these two reac-

tions. The reorganizational energy for the singlet-state quenching ET (1.0 eV) is consistent with previous studies involving Cc and is equivalent to λ for the Cc self-exchange reaction (~1.0 eV).[2] However, the value of λ for the ET reaction between the triplet-state URO and the heme group of Cc is significantly lower than that of the Cc self-exchange reaction (0.49 eV). This is significant since this value should represent a lower limit for ET reactions involving Cc. This value arises predominantly from solvent reorganization after reduction of the heme group of the protein.[2] There are two possible reasons for this low value. First, it has been suggested that anomalous values of λ or H_{AB} obtained from fits of ET rate constant versus temperature to the semiclassical Marcus equation are indicative of gating.[33] Gating implies a conformational change before the ET reaction that is rate limiting. Thus, measurement of the observed rate constant versus temperature actually provides the activation energy for the conformational change and not for the true ET reaction (ΔH^+ for the triplet ET reaction would then be 25.3 kJ/mol). The value of H_{AB} is also somewhat anomalous relative to that of other ET reactions involving Cc (3.4 cm^{-1} for ^{3}URO to Cc versus 0.3 cm^{-1} for Ru−Cc complexes[2]). Although the value of H_{AB} is larger than "typical" values for Cc, it is well below the limit for nonadiabatic ET reactions (i.e., <80 cm^{-1}).[33]

An alternative explanation for the anomalous value of λ for the ET reaction originating from ^{3}URO ET reaction involves the fact that the bound porphyrin alters the overall charge distribution of the protein−porphyrin complex. In fact, previous results by Koppenol and Margoliash[34] have demonstrated significant changes in the overall diopole moment of Cc upon modification of single lysine residues on the surface of the protein. If nothing else, the overall charge of the complex is reduced, relative to free Cc, due to the eight carboxylic acid groups of the bound porphyrin. The positioning of these eight carboxylic acid groups may also alter the overall dipole moment of the protein, thus affecting the solvent reorganizational energy. The fact that the reorganizational energy for the ET reaction between ^{1}URO and the heme group of Cc is closer to the self-exchange value suggests the overall dipole moment of the protein−porphyrin complex is not as perturbed in this conformation.

To better determine the nature of the triplet-state ET reaction the temperature dependence of the rate constants were also fit to

$$k_{et} = k_o \exp(-\beta(r - r_o)) \exp(-(\Delta G^\circ + \lambda)^2/4\lambda RT) \quad (4)$$

where k_o is the nuclear frequency term (10^{13} s^{-1}), r_o is the van der Waals contact distance (3.6 Å), ΔG° is the reaction free energy, λ is the reorganizational energy, and r is the distance between the closest macrocycle atoms in the two redox centers.[2] If the rate constants arise strictly from the ET reaction and no gating is present, then fitting k_{obs} versus T should give reasonable values for λ and r. In fact, the values of λ should match those obtained from eq 1. For the singlet ET reaction fitting the data to eq 4 gives λ of 1.0 eV and r of 12 Å. The distance between the heme iron and the bound porphyrin has previously been reported to be on the order of 12 Å (this value was obtained from molecular modeling studies).[18] For the corresponding triplet-state ET the values of λ and r are found to be 0.42 eV and 10 Å. Although the value of λ is nearly the same as the value determined from eq 4 and the value of r is reasonable, the anomalously low value of λ indicates the forward reaction is gated as has been previously suggested.[18,19] The singlet data give reasonable values for λ and a reasonable distance between the redox sites. These data suggest that the porphyrin binds to Cc with two distinct orientations. One orientation favors fast intermoelcular ET giving rise to singlet-state quenching, while the other is dominated by conformational gating.

The reorganizational energy of the thermal back ET is also quite distinct relative to that of the ET reaction originating from either the singlet or triplet state of URO (roughly 0.2 eV). As discussed in our previous work, the observed thermal back ET arises from recombination of the URO π-cation radical produced from *triplet* ET. Fitting the back ET rate constants as a function of temperature to eq 4 gives λ of 0.18 eV and r of 11.0 Å. In this case the distance term is still fairly reasonable and the value of λ matches that obtained from the fits to eq 1. The low value of λ is inconsistent with previous studies of the URO−Cc system, which show that the back ET reaction follows Marcus behavior (i.e., clear inverted region).[18,19] These studies, however, give a value of λ of 0.7 eV, which is lower than the Cc self-exchange value (1.0 eV) but significantly higher than that provided by the temperature-dependent data (0.2 eV). The discrepancy in the magnitude of the λ values between the current study and the previous work may be due, in part, to the methods used to obtain λ. In the work by Zhou et al.[18] and Zhou and Rodgers[19] various metal substituted derivatives of both URO and Cc were used to vary the reaction driving force. This method is reliable for systems with constant λ (i.e., only ΔG° varies). Differences in the structures of the various porphyrins used can give anomalous values for λ since both λ_{out} and λ_{in} (outer- and inner-sphere reorganization) may be quite distinct between the different porphyrins. For example, the values of λ between the Fe(III)URO/ZnCc and ZnURO/Mn(III)Cc (where ZnCc and Mn(III)Cc are Zn(II) and Mn(III) substituted cytochrome *c*, respectively) are likely to be distinct since oxidation of the Zn porphyrin in Cc results in formation of a porphyrin π-cation radical, while reduction of the Mn(III) to Mn(II) is metal centered. Thus, the protein response to the ET event (contributing to the outer-sphere reorganization, λ_{out}) may not be the same for these two systems. In addition, the vibrational frequencies of the porphyrins involved are not likely to make equivalent contributions to the inner-sphere reorganization (λ_{in}). In the case of the temperature-dependent studies λ is constant since the nature of the system is not varied. It is also noted that the validity of fitting rate constants as a function of temperature to eq 1 implies that E° does not vary as a function of temperature. In the case of Cc reduction, E° should vary by roughly 20 mV (from the 261 mV value used to calculate ΔG°). This variation does not significantly alter the values obtained from the fit. We do point out that the variation in E° for the overall reaction (URO−Cc) as a function of temperature is not known. It is quite possible that the values of λ reported for the back reaction are anomalously low due to temperature variations in the reaction ΔG°. Thus, overall evidence suggests that the back ET reaction is not gated, although additional data will be required to verify this point.

Singlet Lifetime Distribution. We have previously shown that singlet-state lifetimes associated with the URO−Cc complex can be fit to a Gaussian distribution. Data fit to a Gaussian function significantly better than those fit to a discrete or Lorentz model. The choice of Gaussian is not meant to indicate that this distribution is the only one or the best but to demonstrate that the decay is not optimally represented by several discrete exponentials. We further speculated that this distribution arises from the flexibility of the URO side chains resulting in small fluctuations in the intermolecular ET distance. Analysis of the temperature dependence of the width provides a characterization

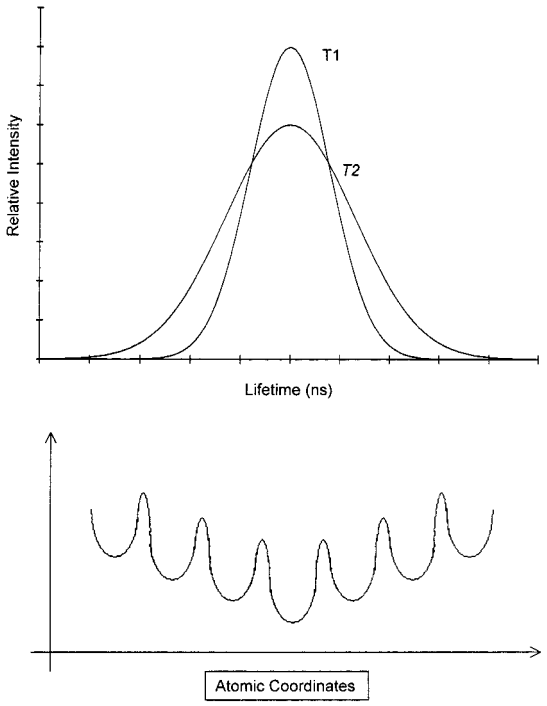

Figure 5. Schematic diagram of the potential surface representing multiple shallow potential wells (T1 > T2).

of the potential surface of the interconversion among these different conformations. The observed width of the shorter lifetime component, representing the intracomplex ET, increases as the temperature is decreased (Table 1). The data are consistent with a model in which a potential surface for the URO−Cc is made up of many shallow potential wells within an overall global minimum (Figure 5). As the temperature increases, the activation barrier of the shallow wells is overcome, resulting in rapid interconversion between conformations. In contrast, lowering the temperature results in reduced interconversion rates giving a wider distribution of lifetimes. If the temperature is lowered sufficiently far, the distribution could be resolved into several discrete lifetimes as the populations within the wells would not have sufficient energy to interconvert.

Conclusion

In this study we have determined the temperature dependence of the rates of singlet and triplet ET within the URO−Cc electrostatic complex. These results suggest that URO binding to Cc gives a distribution of orientations/conformations and that these distinctive conformations give rise to significantly different rate constants for intermolecular ET (in part due to gating). In addition, within the conformation giving rise to singlet-state quenching, considerable flexibility is observed, suggesting distinctive conformational substates varying in distance and/or orientation relative to the heme group of Cc.

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