Driving Force Dependence of Rate Constants of Electron Transfer within Cytochrome c and Uroporphyrin Complexes

Jian S. Zhou*,† and Michael A. J. Rodgers*

Contribution from the Center for Photochemical Sciences, Bowling Green State University, Bowling Green, Ohio 43403. *Received January 14, 1991. Revised Manuscript Received June 10, 1991*

Abstract: Long-range electron-transfer reactions within preformed electrostatically associated complexes between metallocytochrome *c* (Mn(III)cytc and Zncytc) and metallouroporphyrins (Up, ZnUp, Mn(III)Up, and Fe(III)Up$(CN)_2$) have been studied by time-resolved absorption spectroscopy. Rate constants for photoinduced forward and thermal backward electron-transfer reactions are as follows: 8.2×10^5 s^{-1} (^{3}ZnUp*/Mn(III)cytc); 9.1×10^5 s^{-1} (^{3}Up*/Mn(III)cytc); 1.5×10^4 s^{-1} (^{3}Zncytc*/Mn(III)Up); 2.1×10^6 s^{-1} (ZnUp$^{\bullet+}$/Mn(II)cytc); 1.9×10^6 s^{-1} (Up$^{\bullet+}$/Mn(II)cytc); 5.5×10^4 s^{-1} (Zncytc$^{\bullet+}$/Mn(II)Up); 9.1×10^4 s^{-1} (^{3}Zncytc*/Fe(III)Up$(CN)_2$); and 6.0×10^3 s^{-1} (Zncytc$^{\bullet+}$/Fe(II)Up$(CN)_2$). These data, together with those reported previously from this laboratory, provide seven unimolecular electron-transfer reactions for each type of reaction spanning an 0.8-V driving force range. The variation of rate constants with driving force for the thermal reactions confirms the predictions of semiclassical theory of the electron-transfer reaction; i.e., a maximum rate constant and an *inverted region* are clearly observed. Fitting the data yields a reorganization energy (λ) of 0.70 eV. However, no inverted region was observed for the photoinduced electron-transfer reactions over the same range of driving force (0.30–1.29 V). The lack of the inverted region for photoinduced electron-transfer reactions is possibly explained by a contribution of the coordinate solvent mode to the total outer-sphere reorganization energy or by a two-step mechanism.

Introduction

Electron-transfer reaction theories[1,2] predict that, in a series of homogeneous electron-transfer reactions, the rate constants increase with the driving force ($-\Delta G$) to a maximum at $-\Delta G = \lambda$, and then decrease with increasing $-\Delta G$ in the inverted region ($-\Delta G > \lambda$); λ is termed the reorganization energy. A number of experiments have been carried out to verify this driving force dependence of the electron-transfer rate constant. To date, some success has been achieved in observing the inverted region for intramolecular electron transfers in organic radical anions,[3,4] for fast geminate charge recombination in photoinduced bimolecular electron transfers,[5,6] for photoinduced intramolecular electron transfers in covalently linked porphyrin–quinone systems,[7,8] and for photoinduced intramolecular electron transfers in transition-metal complexes.[9] Although interesting findings of unimolecular electron transfers in protein-based systems have been made in recent years,[10-16] the experimental confirmation of the inverted region has been found to be difficult. This partly arises from the lack of the systematic variation of the driving force over a wide range (e.g., from 0 to 1.5 eV), and partly from complications such as conformation changes involved in the electron transfer.[17-19] The driving force effect on the rate constant of electron transfers in porphyrin–protein complexes is the subject of this paper.

We previously reported the transfer of electrons between metallouroporphyrin and metallocytochrome *c*,[13] and determined directly the rate constants for photoinduced forward unimolecular electron transfer and the consequent thermal backward unimolecular electron transfer within preformed electrostatically bound porphyrin–protein complexes.[13] This is reminiscent of protein–protein complexes in natural systems. Using such preformed complexes eliminates the diffusion step which can mask electron transfer to an extent that the most interesting features of electron transfer never show up. However, the diffusional situation can be completely restored and studied by using high ionic strengths where complexation is minimized. An advantage of this system is that it is relatively facile to change the driving force by metal substitution in the heme and/or in the uroporphyrin moiety. In our previous work,[13] the variation of rate constants with driving force for six unimolecular electron-transfer reactions was found to apparently follow an inverted parabolic relationship consistent with the semiclassical theory of electron-transfer reactions. In the present work, we have studied unimolecular photoinduced and thermal electron transfer between Mn-substituted cytochrome *c* and a pair of metallouroporphyrins (uroporphyrin and zinc uroporphyrin), as well as between Zn-substituted cytochrome *c* and a pair of metallouroporphyrins (manganese(III) uroporphyrin chloride and iron(III) uroporphyrin dicyanide), in order to extend

† Present address: Department of Chemistry, Iowa State University, Ames, IA 50011.

(1) Marcus, R. A. *Annu. Rev. Phys. Chem.* **1964**, *15*, 155–196.
(2) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322.
(3) (a) Beitz, J. V.; Miller, J. R. *J. Chem. Phys.* **1979**, *71*, 4579–458. (b) Miller, J. R.; Beitz, J. V. *Ibid.* **1981**, *74*, 6476–648. (c) Miller, J. R.; Beitz, J. V. *J. Am. Chem. Soc.* **1984**, *106*, 5057–506.
(4) (a) Closs, G. L.; Calcaterra, L. T.; Green, N. J.; Penfield, K. W.; Miller, J. R. *J. Phys. Chem.* **1986**, *90*, 3673–3683. (b) Closs, G. L.; Miller, J. R. *Science* **1988**, *240*, 440–447.
(5) (a) Ohno, T.; Yoshimura, A.; Mataga, N.; Tazuke, S.; Kawanishi, Y.; Kitamura, N. *J. Phys. Chem.* **1989**, *93*, 3546–3551. (b) Ohno, T.; Yohimura, A.; Shioyama, H.; Mataga, N. *Ibid.* **1987**, *91*, 4365–4370. (c) Mataga, N.; Asahi, T.; Kanda, Y.; Okada, T.; Kakitani, T. *Chem. Phys.* **1988**, *127*, 249–261.
(6) (a) Gould, I. R.; Ege, D.; Moser, J. E.; Farid, S. *J. Am. Chem. Soc.* **1990**, 4290–4301. (b) Gould, I. R.; Ege, D.; Mattes, S. L.; Farid, S. *J. Am. Chem. Soc.* **1987**, *109*, 3794–3796. (c) Gould, I. R.; Moser, J. E.; Armitage, B.; Farid, S. *Ibid.* **1989**, *111*, 1917–192. (d) Gould, I. R.; Moody, R.; Farid, S. *Ibid.* **1988**, *110*, 7242–724.
(7) Wasielewski, M. R.; Niewczyk, M. P.; Svec, W. A.; Pewitt, E. B. *J. Am. Chem. Soc.* **1985**, *107*, 1080–101.
(8) (a) Irvine, M. P.; Harrison, R. J.; Beddard, G. S.; Leighton, P.; Sanders, J. K. M. *Chem. Phys.* **1986**, *104*, 315–31. (b) Archer, M. P.; Gadzekpo, V. P. Y.; Bolton, J. R.; Schmidt, J. A.; Weedon, A. C. *J. Chem. Soc., Faraday Trans. 2* **1986**, *82*, 2305.
(9) Chen, P.; Duesing, R.; Tapolsky, G.; Meyer, T. J. *J. Am. Chem. Soc.* **1989**, *111*, 8305–8306.
(10) (a) McLendon, G.; Miller, J. R. *J. Am. Chem. Soc.* **1985**, *107*, 7811–7816. (b) Conklin, K. C.; McLendon, G. *J. Am. Chem. Soc.* **1988**, *110*, 3345.
(11) (a) Axup, A. W.; Albin, M.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435–439. (b) Karas, J. L.; Lieber, C. M.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 599–600.
(12) (a) Bechtold, R.; Kuehn, C.; Lepre, C.; Isied, S. S. *Nature* **1986**, *322*, 286–288. (b) Bechtold, R.; Gardineer, M. B.; Kazmi, A.; van Hemelryck, B.; Isied, S. S. *J. Phys. Chem.* **1986**, *90*, 3800–3804.
(13) (a) Zhou, J. S.; Granada, E. S. V.; Leontis, N. B.; Rodgers, M. A. J. *J. Am. Chem. Soc.* **1990**, *112*, 5074–5080.
(14) (a) Liang, N.; Mauk, A. G.; Pielak, G. L.; Johnson, J. A.; Smith, M.; Hoffman, B. M. *Science* **1988**, *240*, 311–313. (b) Natan, M. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1989**, *111*, 6468–6470.
(15) (a) Hazzard, J. T.; McLendon, G. L.; Cusanovich, M. A.; Tollin, G. *Biochem. Biophys. Res. Commun.* **1988**, *151*, 429–434. (b) Hazzard, J. T.; Poulos, T. L.; Tollin, G. *Biochemistry* **1987**, *26*, 2836–2848.
(16) (a) Elias, H.; Chou, M. H.; Winkler, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 429–434. (b) Meade, T. J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 4353–4356.
(17) Hoffman, B. M.; Ratner, M. A. *J. Am. Chem. Soc.* **1987**, *109*, 6237–6243.
(18) Betchtold, R.; Kuehn, C.; Lepre, C.; Isied, S. *Nature (London)* **1986**, *322*, 286–288.
(19) Brunschwig, B. S.; Sutin, N. *J. Am. Chem. Soc.* **1989**, *111*, 7454–7465.

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and to refine the previous study. The composite data set shows that the rate constant of the thermal back electron transfer adheres to the Marcus theory, but that there seems to be some gating effects for the photoinduced forward electron transfer.

Experimental Section

Materials. Horse heart cytochrome *c*, type VI, was purchased from Sigma Chemical Co. Manganese cytochrome *c*, Mn(III)cytc, was prepared from horse heart cytochrome *c* according to the procedure of Dickinson and Chien.[20] The manipulation of the free base porphyrin cytochrome *c* was performed with the exclusion of light. The purity of Mn(III)cytc was checked by the absorption spectroscopy, which is consistent with that in the literature.[20] Zn-substituted cytochrome *c*, Zncytc, was prepared from horse heart cytochrome *c* according to the procedure reported in the literature.[21]

Uroporphyrin I dihydrochloride (Up) was supplied by Porphyrin Products Inc. and used without further purification. Zinc, manganese, and iron uroporphyrins were prepared as described previously.[13]

All aqueous solutions were prepared with distilled water purified by a Barnstead NANOpure II water purification system. The sodium phosphate buffer (pH 7.26, 4 mM ionic strength) was prepared from molecular biological reagent-grade monobasic and dibasic sodium phosphate.

Methods. Electron-transfer kinetics of the triplet excited state were performed by laser flash photolysis[22,13] under anaerobic conditions. Samples were held in 1 × 1 cm fluorescence cuvettes and excited with a Q-switched Quantel YG 571-C10 Nd:YAG laser (532 nm, 10-ns pulse). Kinetic absorption spectrophotometric measurements were obtained with a computer-linked system supplied by Kinetic Instruments. The reaction rates were measured as a function of quencher concentration, up to 30 μM.

Results

1. Zinc Uroporphyrin and Manganese(III) Cytochrome *c* Redox Couple. The photoinduced electron-transfer reactions between ZnUp and Mn(III)cytc were followed by monitoring the decay of the triplet excited state of ZnUp (^{3}ZnUp*) at 448 nm, which is the isosbestic point for the Mn(III)cytc/Mn(II)cytc absorbance. Figure 1a shows the decay curve of the ^{3}ZnUp* state in the presence of Mn(III)cytc at pH 7.26 and μ = 4 mM. The ^{3}ZnUp* state had a lifetime of 3.5 ms in the absence of the protein but was quenched dramatically by Mn(III)cytc. The decay curve could not be fitted by a single exponential but could be fitted successfully by a two-exponential function. The rate constant of the slower component was found to increase with Mn(III)cytc concentration. The decay rate of the faster component had a mean value of (8.2 ± 1.4) × 10^5 s^{-1}, independent of the Mn(III)cytc concentration up to 30 μM. The fractional contribution of the faster component to the total decay increased with Mn(III)cytc concentration.

The thermal backward electron transfer was determined by monitoring the Mn(II)cytc absorption at 417 nm (Figure 1b), the isosbestic point for the ^{3}ZnUp*/ZnUp absorbance. A rapid buildup of Mn(II)cytc absorption was observed, and this species decayed subsequently with recovery of the starting material Mn(III)cytc. Using a double exponential function to analyze this kinetic profile, we obtained a mean growth rate constant of (2.1 ± 0.4) × 10^6 s^{-1}, and a mean decay rate constant of (7.2 ± 0.4) × 10^5 s^{-1}, very close to that of the faster component of the ^{3}ZnUp* state. These values were independent of the Mn(III)cytc concentration.

We have evaluated the yield of electron-transfer products formed from the ^{3}ZnUp* quenching in the fast process according to the following formula:

$$Y_{ET} = \frac{[\text{Mn(II)cytc}]}{[^3\text{ZnUp*}]} = \frac{\Delta A_{417}}{\Delta A_{448}} \frac{\Delta \epsilon_{448}}{\Delta \epsilon_{417}}$$

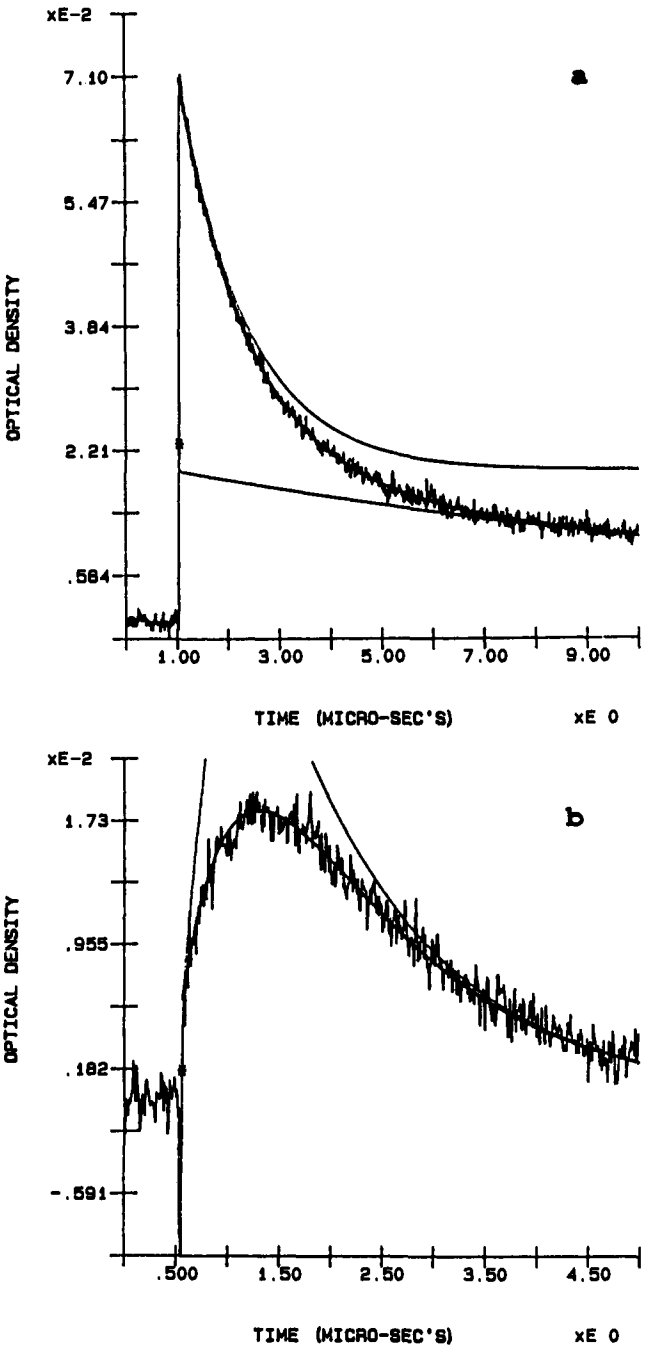


Figure 1. Transient absorbance changes for ZnUp/Mn(III)cytc systems: (a) triplet decay of ZnUp at 448 nm, (b) formation and decay of Mn(II)cytc monitored at 417 nm. Solid lines are fitting curves using a double exponential function. Conditions: [ZnUp] = [Mn(III)cytc] = 10 μM, at 25 °C, pH 7.26, μ = 4 mM.

where ΔA_{448} is the absorbance of ^{3}ZnUp*, obtained from ΔA_{448} at time zero less that amount of the slower component; ΔA_{417} is the absorbance of Mn(II)cytc, derived from fitting curves when the computer provides the total extent of Mn(II)cytc as if no decay occurred; $\Delta\epsilon$ is the extinction coefficient change. A mean ratio ($\Delta A_{417}/\Delta A_{448}$) of 0.77 ± 0.08 has been found for this series of experiments. Together with the estimated $\Delta\epsilon$ values of (5.9 ± 0.7) × 10^4 M^{-1} cm^{-1} for Mn(III)cytc to Mn(II)cytc[20] and (7.2 ± 0.5) × 10^4 M^{-1} cm^{-1} for ^{3}ZnUp*/ZnUp,[23] we find that the yield of electron-transfer products from the ^{3}ZnUp* quenching is 0.94 ± 0.1, which indicates a unit efficiency of electron transfer in the Mn(III)cytc-induced quenching of ^{3}ZnUp*.

(20) Dickinson, L. C.; Chien, J. C. W. *J. Biol. Chem.* **1977**, *252*, 6156–6162.

(21) (a) Vanderkooi, J. M.; Erecinska, M. *Eur. J. Biochem.* **1975**, *60*, 199–207. (b) Vanderkooi, J. M.; Adar, F.; Erecinska, M. *Eur. J. Biochem.* **1976**, *64*, 381–387.

(22) Rodgers, M. A. J. In *Primary Photo-Process in Biology and Medicine*; Bensasson, R. V., Jori, G., Land, E. J., Truscott, T. G., Eds.; Plenum: New York, 1985; pp 1–23.

(23) Carapelluci, P. A.; Mauzerall, D. *Ann. N.Y. Acad. Sci.* **1975**, *244*, 214–238.

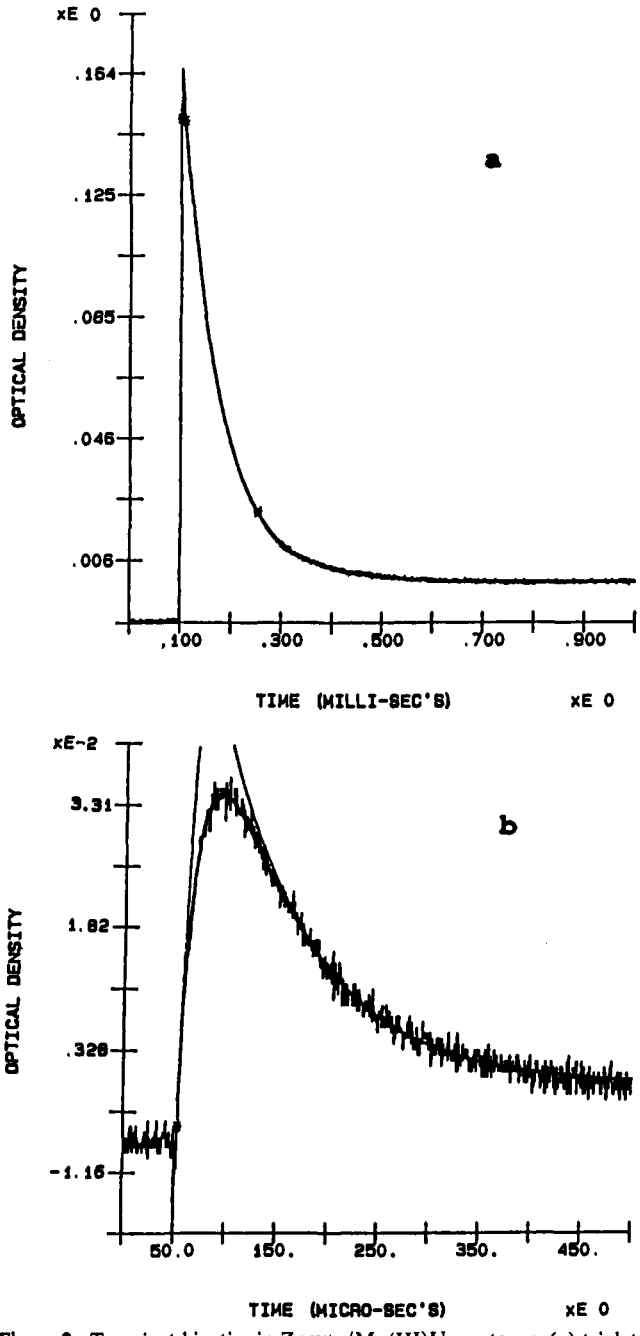


Figure 2. Transient kinetics in Zncytc/Mn(III)Up systems: (a) triplet decay of Zncytc at 440 nm, (b) formation and decay of Mn(II)Up at 428 nm. Solid lines are fitting curves. Conditions: [Zncytc] = 10 μM, [Mn(III)Up] = 20 μM, at 25 °C, pH 7.26, μ = 4 mM.

2. Uroporphyrin and Manganese(III) Cytochrome *c* Redox Couple. In a related series of experiments, ZnUp was replaced by free base Up. The transient kinetic behavior was extremely similar to that of the ZnUp/Mn(III)cytc couple. The decay of the triplet excited state of Up(^{3}Up*) at 448 nm was nonexponential but could be successfully fitted with a double exponential routine. The faster component had a rate constant of (9.1 ± 1.0) × 10^5 s^{-1}, which was independent of Mn(III)cytc concentration up to 30 μM. The slower component had a rate constant that increased with Mn(III)cytc concentration. At 416 nm, the isosbestic point for ^{3}Up*/Up, the Mn(II)cytc absorbance rose rapidly with a rate constant of (1.9 ± 0.2) × 10^6 s^{-1}, and decayed subsequently with a first-order rate constant ((8.0 ± 0.9) × 10^5 s^{-1}) close to that of the faster triplet component measured at 448 nm. Again there was no dependence on Mn(III)cytc concentration.

3. Zn-Substituted Cytochrome *c* and Manganese(III) Uroporphyrin Chloride Redox Couple. Following excitation, the triplet excited state of Zncytc at 440 nm decayed slowly to ground state

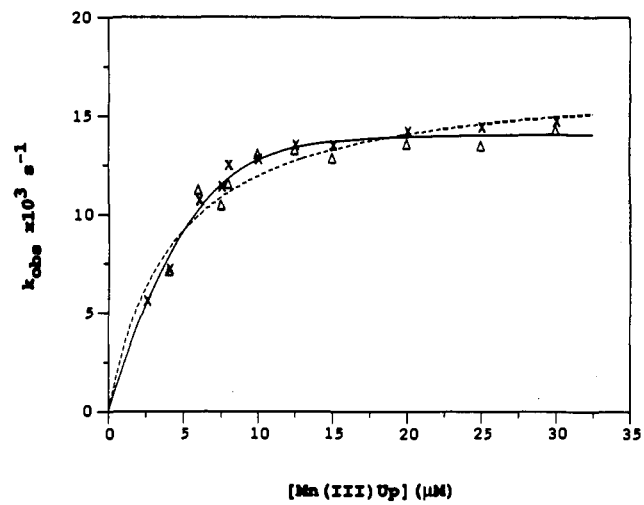


Figure 3. Rate constants as a function of Mn(III)Up concentration over the range from 2.5 to 30 μM: decay rate constants of ^{3}Zncytc* at 440 nm (X) and Mn(II)Up at 428 nm (Δ). The solid line is a theoretical curve of eq 1 with the following parameters: k_F = 1.5 × 10^4 s^{-1} and K_A = 7.2 × 10^5 M^{-1}. The broken line is the theoretical fitting curve with K_A = 2.3 × 10^5 M^{-1} and k_F = 1.7 × 10^4 s^{-1} based on the preequilibrium mechanism. Conditions: [Zncytc] = 10 μM, at 25 °C, pH 7.26, μ = 4 mM.

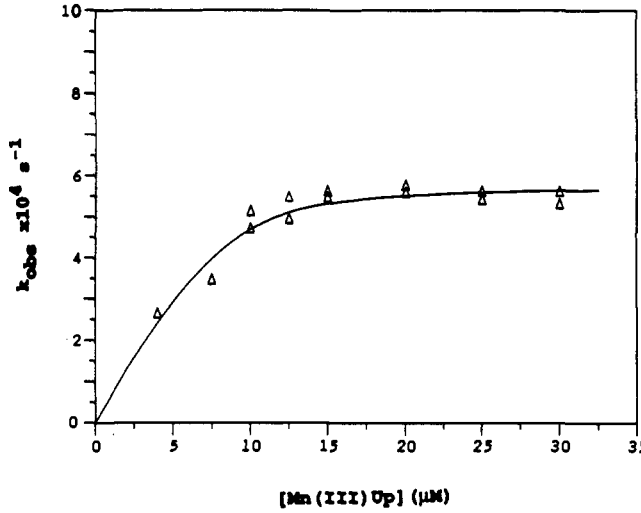
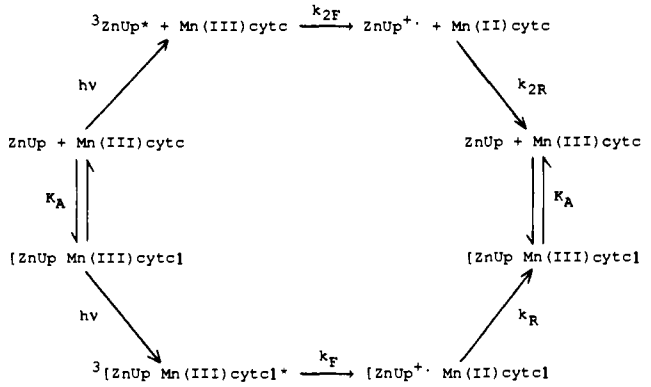


Figure 4. Rate constant for the rising portion of Mn(II)Up at 428 nm as a function of Mn(III)Up concentration over the range from 4 to 30 μM. The solid line is the theoretical curve with parameters K_A = 1.2 × 10^6 M^{-1}, k_R = 5.5 × 10^4 s^{-1}, and b = 0.7 (see text for details). Conditions: [Zncytc] = 10 μM, at 25 °C, pH 7.26, μ = 4 mM.

with a lifetime of 10 ms in argon-saturated solution at pH 7.26 and μ = 4 mM. In the presence of Mn(III)Up, the decay of the triplet state of Zncytc was markedly enhanced (Figure 2a). In Figure 2a, the triplet decay follows a single exponential decay to a nonzero base line. This latter was always present in flash excitation of Zncytc in the absence or presence of quencher and probably corresponds to the formation of a weakly absorbing permanent product. This phenomenon was observed previously by others.[16a] It has no significance in the T_1 decay. The decay rate of the triplet increased with Mn(III)Up concentration before reaching a plateau (Figure 3). Meanwhile, at 428 nm, which is the isosbestic point for ^{3}Zncytc*/Zncytc, we observed a rapid but noninstantaneous rise and subsequent decay of the transient absorbance, identified as Mn(II)Up (Figure 2b). The residual absorbance in Figure 2b decayed slowly in a longer time scale with a recovery of the ground state. This residual absorbance decreased with the increase of the Mn(III)Up concentration. Analysis by a two-exponential function showed the decaying portion to have a rate constant that is identical with that of triplet decay at all Mn(III)Up concentrations used (Figure 3). The rate constant

Scheme I



for the growing portion depended on the Mn(III)Up concentration (Figure 4) in an analogous manner, i.e., being a function of the quencher concentration up to ca. 15 μM where a plateau was reached.

4. Zn-Substituted Cytochrome *c* and Iron(III) Uroporphyrin Dicyanide Redox Couple. In a related set of experiments, Fe(III)Up$(CN)_2$ was prepared and used to replace Mn(III)UpCl. As with ZnUp/Mn(III)cytc and Up/Mn(III)cytc systems, in the presence of an increasing concentration of Fe(III)Up$(CN)_2$, the triplet of Zncytc decayed in a complex manner that was fitted by a two-exponential function. The decay rate constant of the fast component was $(9.1 \pm 1.0) \times 10^4$ s^{-1} independent of Fe(III)Up$(CN)_2$ concentration up to 30 μM and concomitant with the formation rate constant of a 428-nm absorption arising from Fe(II)Up$(CN)_2$. This later species decayed with a mean rate constant of $(6.0 \pm 1.0) \times 10^3$ s^{-1}, which is independent of Fe(III)Up$(CN)_2$ concentration.

Discussion

Transient Kinetics. 1. ZnUp and Mn(III)cytc. First it is necessary to convince ourselves that a preformed complex between ZnUp and Mn(III)cytc is involved in the transient kinetics. Using difference spectroscopy, we previously established that a strong electrostatically associated 1:1 complex is formed between ground-state Up and cytc(III) at low ionic strength.[13] We assume that such a complex exists in the ZnUp/Mn(III)cytc couple also since the lysine groups on the protein surface and the anionic groups on the periphery of Up are unlikely to be affected by the insertion/replacement of new metal ions. This, our basic assumption, is supported by the appearance of the kinetic traces as the protein concentration was changed, similar to what had been observed in our earlier work.[13] The irradiation of ZnUp/Mn(III)cytc solution gives rise to two populations of the triplet excited state, each of which exhibits different exponential decay behavior (Scheme I, Figure 1a). The slower component at 10 μM Mn(III)cytc results from the triplet state of residual uncomplexed ZnUp. It exhibits the typical characteristics of a bimolecular process, being dependent on the quencher concentration. The faster component most probably arises from the triplet excited state of the complex, ^{3}[ZnUp Mn(III)cytc]*, which decays with a first-order rate constant of 8.2×10^5 s^{-1} *independent* of Mn(III)cytc concentration. Furthermore, the yield of Mn(II)cytc formed from the quenching of ^{3}ZnUp* is ca. 100% for the faster component, indicating that the electron transfer is uniquely responsible for the intramolecular quenching process. In view of this, the rate constant of the faster component of excited-state decay will be taken as the electron-transfer rate constant k_F in Scheme I.

The kinetic behavior of Mn(II)cytc is consistent with a series first-order reaction of the type A → B → C in which the rate constant for the B → C step exceeds that for the A → B reaction.[24] In our case, A would correspond to the triplet state of the complex, B to the ion pair, and C to the ground-state complex. Analysis of the buildup and decay of the Mn(II)cytc absorbance at 417 nm allows the evaluation of k_R as the rapidly rising part in Figure 1b, and k_F as the decaying part in the same figure. This phenomenon has been observed previously by us and others[13,16a,25] for electron transfer in protein-based systems.

2. Up and Mn(III)cytc. The kinetic behavior of the Up/Mn(III)cytc couple is similarly consistent with forward and backward electron transfers within the porphyrin–protein complex. The k_R is again higher than k_F, like the couples Up/cytc(III) and ZnUp/Mn(III)cytc. The k_F was obtained from the faster decay component of the triplet excited state at 448 nm or from the slow decay of the Mn(II)cytc absorbance at 416 nm, the k_R, from the growth part of the Mn(II)cytc absorbance at 416 nm (Table I).

3. Zncytc and Mn(III)UpCl. Every other couple of the type protein and metallouroporphyrin that we have studied both here and in ref 13 has shown double exponential decay of the T_1 state, or a rapid concentration-independent part and a slower concen-

Table I. Rate Constants for the Unimolecular Electron-Transfer Reactions in Uroporphyrin–Cytochrome *c* Complexes at 25 °C

reaction	ΔE (V)[c]	k_R (s^{-1})	ref
ZnUp$^{+\cdot}$ + cytc(II) → ZnUp + cytc(III)	0.47	8.5×10^5	*a*
Up$^{+\cdot}$ + cytc(II) → Up + cytc(III)	0.60	3.7×10^6	*a*
ZnUp$^{+\cdot}$ + Mn(II)cytc → ZnUp + Mn(III)cytc	0.77	2.1×10^6	*b*
Up$^{+\cdot}$ + Mn(II)cytc → Up + Mn(III)cytc	0.80	1.9×10^6	*b*
Zncytc$^{+\cdot}$ + Fe(II)Up → Zncytc + Fe(III)Up	1.23	1.4×10^4	*a*
Zncytc$^{+\cdot}$ + Mn(II)Up → Zncytc + Mn(III)Up	1.26	5.5×10^4	*b*
Zncytc$^{+\cdot}$ + Fe(II)Up$(CN)_2$ → Zncytc + Fe(III)Up$(CN)_2$	1.38	6.0×10^3	*b*
reaction	**ΔE (V)[c]**	**k_F (s^{-1})**	**ref**
^{3}Zncytc* + Fe(III)Up$(CN)_2$ → Zncytc$^{+\cdot}$ + Fe(II)Up$(CN)_2$	0.30	9.1×10^4	*b*
^{3}Zncytc* + Mn(III)Up → Zncytc$^{+\cdot}$ + Mn(II)Up	0.42	1.5×10^4	*b*
^{3}Zncytc* + Fe(III)Up → Zncytc$^{+\cdot}$ + Fe(II)Up	0.45	5.5×10^5	*a*
^{3}Up* + Mn(III)cytc → Up$^{+\cdot}$ + Mn(II)cytc	0.86	9.1×10^5	*b*
^{3}ZnUp* + Mn(III)cytc → ZnUp$^{+\cdot}$ + Mn(II)cytc	0.99	8.2×10^5	*b*
^{3}Up* + cytc(III) → Up$^{+\cdot}$ + cytc(II)	1.06	1.7×10^6	*a*
^{3}ZnUp* + cytc(III) → ZnUp$^{+\cdot}$ + cytc(II)	1.29	2.0×10^6	*a*

[a] Reference 13. [b] This work. [c] Driving forces are derived from the following half-cell potentials (vs NHE, 25 °C): ZnUP$^{+\cdot}$/^{3}ZnUp*, −1.03 V;[26] ZnUp$^{+\cdot}$/ZnUp, 0.73 V;[26] Up$^{+\cdot}$/^{3}Up*, −0.80 V;[28] Up$^{+\cdot}$/Up, 0.86 V;[28] cytc(III)/cytc(II), 0.26 V;[30] Zncytc$^{+\cdot}$/^{3}Zncytc*, −0.88 V;[31] Zncytc$^{+\cdot}$/Zncyt, 0.80 V;[31] Mn(III)cytc/Mn(II)cytc, 0.06 V;[14] Mn(III)Up/Mn(II)Up, −0.46 V;[32] Fe(III)Up/Fe(II)Up, −0.43;[13] Fe(III)Up$(CN)_2$/Fe(II)Up$(CN)_2$, −0.58 V.[33]

(24) Moore, J. W.; Pearson, R. G. *Kinetics and Mechanism*, 3rd ed.; Wiley: New York, 1981; p 290.

(25) (a) Liang, N.; Kang, C. H.; Ho, P. S.; Margoliash, E.; Hoffman, B. M. *J. Am. Chem. Soc.* **1986**, *108*, 4665–4666. (b) Liang, N.; Pielak, G. L.; Mauk, A. G.; Smith, M.; Hoffman, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1249–1252.

(26) Estimated from the redox potentials of zinc octaethylporphyrin (ref 27).

(27) (a) Kalyanasundaram, K.; Shelnutt, J. A.; Gratzel, M. *Inorg. Chem.* **1988**, *27*, 2820–2825. (b) Fuhrhop, J. H.; Mauzerall, D. *J. Am. Chem. Soc.* **1969**, *91*, 4174–4181.

(28) Estimated from the redox potentials of octaethylporphyrin (ref 29).

(29) Felton, R. H. In *The Porphyrins*; Dolphin, D., Ed.; Academic: New York, 1978; Vol. V, Part c.

(30) Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. Q. *Rev. Biophys.* **1986**, *18*, 261–322.

(31) Magner, E.; McLendon, G. *J. Phys. Chem.* **1989**, *20*, 7130–7134.

(32) E° = −0.46 V vs NHE for Mn(III)Up/Mn(II)Up is determined from the cyclic voltammetric measurement.

(33) The redox potential of iron uroporphyrin dicyanide is estimated to be E° for iron uroporphyrin chloride[13] plus a value of −0.15 V. This value (−0.15 V) is obtained by averaging the difference values of redox potentials between cyanide and chloride iron porphyrins.[29,34,35]

(34) Phillips, J. N. *Compr. Biochem.* **1963**, *9*, 34–73.

(35) Kadish, K. M.; Davis, D. G. *Ann. N.Y. Acad. Sci.* **1973**, *206*, 495–503.

tration-dependent part, the fractional contribution of the former increasing with quencher concentration. This behavior has been interpreted as arising from a mixture of intracomplex and intermolecular quenching, occurring on well-separated time scales. For the case of ^{3}Zncytc* quenching by Mn(III)Up, however, we find a single exponential decay only, which is dependent on quencher concentration in a nonlinear way, reaching a concentration-independent region above 15 μM of Mn(III)Up (Figure 3). This apparently anomalous behavior can be explained on the above kinetic scheme if the concentration-dependent part and the concentration-independent part of the quenching are occurring fortuitously in the same time regime, i.e., if the magnitudes of the two first-order constants are similar. In such a situation, the measured first-order rate constant (k_{obs}) is a composite of k_F and k_{2F}, which are the rate constants of the unimolecular and bimolecular parts, respectively (see Scheme I); i.e.

$$k_{obs} = fk_F + (1 - f)k_{2F} \quad (1)$$

where f is the fraction of Zncytc that is complexed with the porphyrin prior to excitation. The fitting curve of the data with eq 1 is superimposed in Figure 3. Values of $k_F = 1.5 \times 10^4$ s^{-1} and $K_A = 7.2 \times 10^5$ M^{-1} are derived from the fitting. This association constant for the Zncytc/Mn(III)Up couple is comparable with the value of 9.5×10^5 M^{-1} determined from the difference spectroscopy for the Up/cytc couple.[13] It should be pointed out that this three-parameter fitting could be arbitrary. However, the value of k_F, which is the only parameter of interest in this study, is equal to the plateau value in Figure 3 independent of other parameters. Thus, this value obtained from fitting is certainly reliable.

The kinetic behavior of Mn(II)Up at 428 nm provides further support for the involvement of the preformed complex in the electron transfer. The growth rate constant of Mn(II)Up absorbance at 428 nm is greater than the decay rate constant of the ^{3}Zncytc*, indicating that the preformed complex is involved in the electron transfer in the Mn(III)Up/Zncytc couple. The Mn(II)Up species monitored at 428 nm were produced from the uni- and bimolecular quenchings of ^{3}Zncytc*. The kinetics of electron transfer within the preformed complex are similar to that for the two couples discussed earlier; i.e., the thermal back electron transfer is faster than the photoinduced electron transfer. The observed formation of the Mn(II)Up in the bimolecular process is mainly derived from the step of the separation of the ion pair. This step yields a relatively long lifetime of the separated ions, which corresponds to the long-lived residual absorbance. The rate constant for the formation of Mn(II)Up should have a component equal to that of the triplet decay of Zncytc in the bimolecular process. As for the triplet decay, the rate constant for the rising part of Mn(II)Up absorbance, therefore, is a composite given by $fk_R + b(1 - f)k_{2F}$, where b is a factor to account for the efficiency of the ion pair separation and is not important for determining the value of k_R since k_R is equal to the plateau value. A value of 0.7, which is obtained by averaging the values of the efficiency of the ion pair separation reported by Ohno et al.[5a] for ruthenium complex systems, was used for the fitting. The fitting of the data yields $k_R = 5.5 \times 10^4$ s^{-1} and $K_A = 1.2 \times 10^6$ M^{-1} (Figure 4). The association constant again is comparable with that for the Up/cytc(III) couple in support of our above argument. The decay rate constant of Mn(II)Up absorption at 428 nm is equal to that for the triplet decay of Zncytc.

4. Zncytc and Fe(III)Up(CN)$_2$. As with ZnUp/Mn(III)cytc and Up/Mn(III)cytc systems, the transient kinetic behavior for the couple Zncytc/Fe(III)Up(CN)$_2$ is consistent with the photoinduced forward and thermal back electron transfer within the preformed porphyrin–protein complex. But, the k_F is faster than k_R. The k_F was determined from the faster decay of the triplet at 450 nm or from the growth part of the Fe(II)Up(CN)$_2$ absorbance at 428 nm, the k_R, from the decay of the Fe(II)Up(CN)$_2$ absorbance at 428 nm (Table I).

Driving Force Dependence of Rate Constants. So far, we have studied two distinct types of electron-transfer reactions within the preformed electrostatically porphyrin–protein complexes: seven photoinduced forward reactions and seven thermal backward reactions. All values of k_F and k_R are tabulated along with driving force values (ΔE) in Table I. The rate constants vary from 6.0×10^3 to 3.7×10^6 s^{-1} over a range of driving force from 0.30 to 1.38 V. The differences in driving force for the reactions do not unexpectedly give rise to these variations of rate constants.[1,2,36-37] Semiclassical theory[1,2] has provided the following expressions for the rate constant (k_{ET}) of nonadiabatic electron transfer between two centers held at fixed distance and orientation:

$$k_{ET} = \nu_N k_E \exp[-(\Delta G + \lambda)^2/4\lambda RT] \quad (2)$$

where ν_N is the frequency of motion along the reaction coordinate and equal to 10^{13} s^{-1}; k_E is the term describing the electronic coupling between redox partners, which is a function of the separation and orientation of the redox partners; ΔG is the driving force for the electron transfer (equal to $-\Delta E$); λ is the nuclear reorganization energy derived from the reacting molecules (λ_{in}) and surrounding medium (λ_{out}), which accompanies the electron transfer. When the series of electron-transfer reactions under study are homogeneous (i.e., constant λ and constant k_E), the semiclassical theory predicts that a parabolic relationship should be observed between the logarithm of rate constants and the driving force (eq 2).

We need to examine the question of whether our reactions, i.e., thermal backward electron transfer or photoinduced electron transfer, are in fact a homogeneous set. It is established that the effects of the solvation changes on the electron transfer are constant or identical in the same solvent for metalloporphyrins over a wide range of central metals and free base porphyrin.[35] Therefore, in our case, fluctuations in λ_{out} should be insignificant since the solvent medium is not changed. The substitution of different metals in the heme and uroporphyrin moieties is used to vary only the driving force; consequent fluctuations in λ_{in} should therefore be relatively minor since no changes have been made in the gross structure of the protein and porphyrin. It can be argued that a much higher value of λ_{in} could be expected for Fe(III)UpCl and Mn(III)UpCl since the coordination number of the central metal is changed and an anion axial ligand is necessitated to be removed on reduction.[38] This would lead to a much lower rate constant of electron transfer for these two uroporphyrins (as observed). However, Brown and Lantzke[39] have demonstrated elegantly that, in solvents such as DMSO and water, the iron(III) porphyrin is largely dissociated from its anion ligand Cl$^-$ and that two solvent molecules are coordinated to Fe(III) as an axial ligand. This dissociation should result in an enhancement of the rate of electron transfer since there is no necessity for the chloride ion removal on reduction, as proven by Kadish and Davis.[35] We argue therefore that λ_{in} for Fe(III)UpCl in water cannot be high because of the dissociation of the axial ligand Cl$^-$ (since it is always dissociated) and it should be similar to the value for Up and ZnUp. This argument is further supported by experiments with the couple Zncytc/Fe(III)Up(CN)$_2$.[40] Although the coordination number change of the iron on reduction is prevented by binding a high-field, strong ligand (CN$^-$),[41] the rate constants of electron transfer are not enhanced but even slower than those for the Zncytc/Fe(III)UpCl couple. This decrease in rate constant is likely attributed to the change in driving force (Table I). It is expected that Mn(III)UpCl behaves in a like

(36) (a) Levich, V. O. *Adv. Electrochem. Electrochem. Eng.* **1966**, *4*, 249. (b) Kestner, N. R.; Logan, J.; Jortner, J. *J. Phys. Chem.* **1974**, *78*, 2148–2166. (c) Ulstrup, J.; Jortner, J. *J. Chem. Phys.* **1975**, *63*, 4358–4368.

(37) (a) Redi, M.; Hopfield, J. *J. Chem. Phys.* **1980**, *72*, 6651. (b) DeVault, D. Q. *Rev. Biophys.* **1980**, *13*, 387. (c) Brunschwig, B. S.; Logan, J.; Newton, M. D.; Sutin, N. *J. Am. Chem. Soc.* **1980**, *102*, 5798.

(38) A reviewer has properly pointed out this possibility.

(39) Brown, S. B.; Lantzke, I. R. *Biochem. J.* **1969**, *115*, 279–285.

(40) The data for the Zncytc/Fe(III)Up(CN)$_2$ couple were obtained in order to respond to a suggestion by a reviewer.

(41) The stability constant of 8.3×10^9 M^{-1} for Fe(II)P(CN)$_2$ (ref 34) implies a CN$^-$ dissociation rate constant of ca. 1 s^{-1}. Thus, the reduced complex is stable to CN$^-$ dissociation over the time scale required for the back electron transfer to occur.

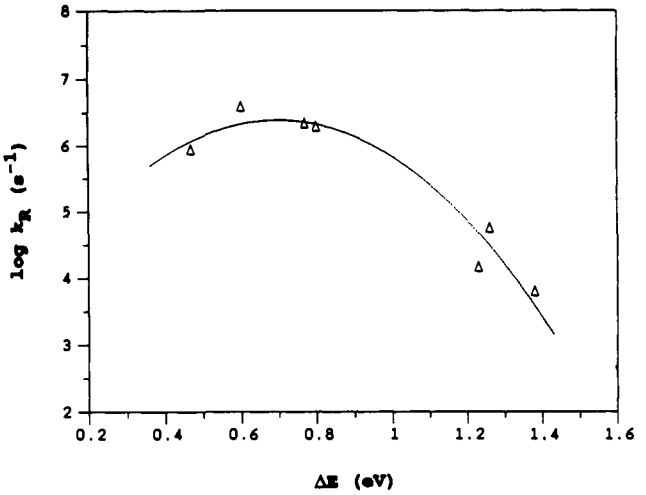


Figure 5. Log k_R as a function of ΔE. The solid line is a theoretically fitted curve of eq 2 with λ = 0.70 eV and $\nu_N k_E = 2.4 \times 10^6$ s^{-1}.

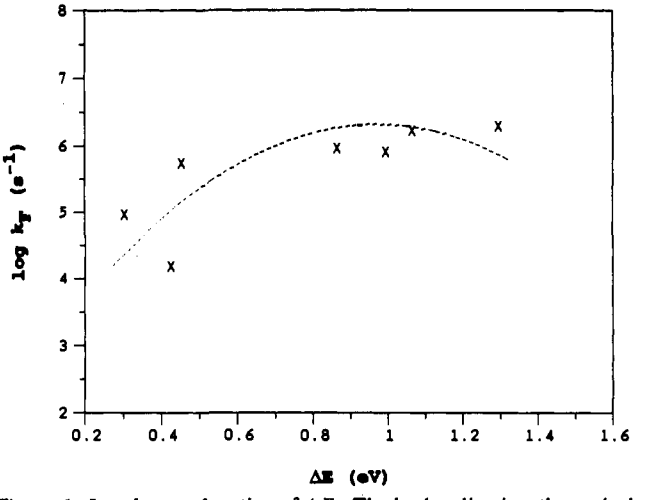


Figure 6. Log k_F as a function of ΔE. The broken line is a theoretical curve of eq 2 with λ = 0.98 eV and $\nu_N k_E = 2.1 \times 10^6$ s^{-1}.

manner to iron(III) porphyrin.[34,42-44] The next most important consideration is whether the donor–acceptor distance and orientation (and therefore k_E) are constant throughout all seven intracomplex electron-transfer reactions we studied. In the preformed electrostatic complex, the uroporphyrin, planar molecule (ca. 1.6 nm in diameter), binds probably to the domain near the exposed heme edge on cytochrome *c*, where the cationic lysine residues are concentrated. This view of the docking gains some support from computer modeling[13] and from the findings of Clark-Ferris and Fisher.[45] They have found that the 1:1 porphyrin/cytochrome *c* complex is established by the electrostatic interactions between the positively charged lysine residues around the exposed heme edge on cytochrome *c* and the negatively charged carboxylate residues on the porphyrin periphery. Previously reported studies[46] demonstrated strong similarities in conformation and binding properties among metallocytochromes. We intend to examine the binding site of metallouroporphyrins on cytochrome *c* by topographical mimic, energy-minimized modeling, and two-dimensional NMR experiments. However, for the moment we rely only on the surmise that, as with Up, metallouroporphyrins bind electrostatically to the same site on cytc. In this assumption, it is not unreasonable to infer that the differences in electron density at the metal center of the tetrapyrrole ring are not significant in governing the donor–acceptor distance and orientation in the complex.

Consider first the thermal back electron-transfer reaction. Figure 5 shows the variation of rate constants as a function of the driving force. This set of data are consistent with the predictions of semiclassical theory of electron-transfer reactions. The rate constant increases with the driving force to a maximum and then decreases with further increase of the driving force in the *inverted region*. The best fitting of data to eq 2 yields λ = 0.70 eV and $\nu_N k_E = 2.4 \times 10^6$ s^{-1}. It is unlikely that the rate constant changes arise from the differences in λ among the various metalloporphyrins and from the conformational differences among the various metallocytochromes. The more than 2 orders of magnitude of difference in k_R over the driving force range of 0.8 V and the regular dependence of k_R on ΔE seem an unlikely result of those changes.

In contrast to the thermal backward electron-transfer reactions, the rate constants for photoinduced forward electron-transfer reactions increased from 9.1×10^4 to 2.0×10^6 s^{-1} when the driving force increased from 0.30 to 1.29 V, and no inverted region was observed to 1.29 V (Figure 6). In this vein, it is important to note that the k_F values for ^{3}Zncytc* quenching by Mn(III)Up and by Fe(III)Up are a factor of 50 apart whereas the driving force difference is only 30 mV. Thus, five of the seven values show only a very shallow increase in log k_F with increase in ΔE. The differences in inner-sphere reorganization contribution for these two types of reactions should be relatively minor,[16b] at most 0.2 eV. Therefore, it may be that the lack of the inverted region for photoinduced forward electron-transfer reactions is due to the contribution of λ_{out} or a conformation change.[17-19] Kakitani and Mataga[47] have discussed the contribution of the coordinated-solvent mode (C mode) to the total outer-sphere reorganization energy in three types of reactions: charge separation, charge recombination, and charge shift. They found that, in charge separation reactions, the C mode works to strongly annihilate the inverted region when at least one reactant is uncharged, whereas in charge recombination the C mode is not significant to annihilate the inverted region. Our results for these two types of reactions are not out of line with these arguments. On the other hand, the two-step mechanism described by Brunschwig and Sutin[19] could also be operable. The reduction in rate constants with increasing driving force in the inverted region can be masked by a two-step mechanism, surface diffusion and subsequent electron transfer, for photoinduced forward electron-transfer reactions. The first step is needed to reach an optimal conformation for photoinduced electron transfer and probably is a control step, while this step could be unnecessary for the thermal back electron transfer since the reached conformation in the photoinduced electron transfer is likely to be optimal also. However, for an extensive and detailed discussion about the origin of the lack of inverted region for photoinduced forward electron-transfer reactions, we clearly need

Table II. Reorganization Energy (λ) for Cytochrome *c* Electron-Transfer Reactions

redox couple	λ (eV)	ref
Ru/cytc	1.2	16a, 48, 49
cytc/CCP	1.5, 1.3	10b
cytc/cytc	0.7	50
cytc/cyt b_5	0.7	10a
cytc/porphyrin	0.70	this work

(42) (a) Fleischer, E. B.; Palmer, J. M.; Srivastava, T. S.; Chatterjee, A. *J. Am. Chem. Soc.* **1971**, *93*, 3160–3167. (b) Fleischer, E. B.; Jacobs, S.; Mestichelli, L. *J. Am. Chem. Soc.* **1968**, *90*, 2527–2531.
(43) Boucher, L. J. *Ann. N.Y. Acad. Sci.* **1973**, *206*, 409–419.
(44) Kolski, G. B.; Plane, R. A. *Ann. N.Y. Acad. Sci.* **1973**, *206*, 604–613.
(45) Clark-Ferris, K. K.; Fisher, J. *J. Am. Chem. Soc.* **1985**, *107*, 5007–5008.
(46) (a) Moore, G.; Williams, R. J. P.; Chien, J. C. W.; Dickinson, L. C. *J. Inorg. Biochem.* **1980**, *13*, 1–15. (b) Vanderkooi, J. M.; Adar, F.; Erecinska, M. *Eur. J. Biochem.* **1976**, *64*, 381–387.
(47) Kakitani, T.; Mataga, N. *J. Phys. Chem.* **1987**, *91*, 6277–6285.
(48) Nocera, D. G.; Winkler, J. R.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145.
(49) Isied, S. S.; Kuehn, C.; Worosila, G. *J. Am. Chem. Soc.* **1984**, *106*, 1722.
(50) Dixon, D. W.; Hong, X.; Woehler, S. E.; Mauk, A. G.; Sishta, B. P. *J. Am. Chem. Soc.* **1990**, *112*, 1082–1088.

data over a wider range of driving force (e.g., 2 eV).

It is interesting to compare values of the reorganization energy λ for the electron transfers in cytochrome *c* systems. It is seen from Table II that $\lambda = 0.70$ eV from our data for thermal back reactions is comparable with the values estimated by others for protein-protein systems. It is perhaps surprising that our value is consistent with those for the systems with two hemes (cytc/cytc and cytc/cyt b_5). Although these systems are close to identical from the view of reaction centers, the environment of the heme porphyrin is different from that of the Up moiety. The inner-sphere contribution λ_{in} to λ for these systems should be very similar. But the outer-sphere contribution (λ_{out}) of the solvent reorganization should be higher for Up/cytc systems since Up is more exposed to bulk solvent. The similarity in λ between cytc/cyt b_5 and Up/cytc probably arises from the compensation of the contribution of the reorganization of the protein matrix.[51]

We have calculated the solvent reorganization using the dielectric continuum model proposed by Brunschwig et al.[52] A value of 0.37 eV was obtained based on a model of a single sphere of 3.1-nm diameter, where the center is supposed to be that of the heme as is revealed by computer modeling for the Up/cytc complex. Taking account of the contribution of the reorganization of the protein matrix ($\lambda = 0.15$ eV[51]), $\lambda_{out} = 0.52$ eV is the predominant component in the nuclear reorganization energy for thermal backward electron-transfer reactions in Up/cytc systems.

In conclusion, our data indicate that the existence of an inverted region in electron-transfer reactions depends on the type of reaction under study. As predicted by electron-transfer theory, the rate constant for thermal electron-transfer reactions between charged species increase with the driving force and then undergo a decrease with further increase in driving force. The reorganization energy λ is 0.70 eV. In the case of photoinduced electron-transfer reactions, the variation of rate constants does not follow Marcus theory predictions. There is an apparent gating effect that can be conceptually explained by the contribution of the coordinated-solvent or by the two-step mechanism. Further work along this line is in progress in this laboratory.

Acknowledgment. This work is supported in part by NIH Grant GM31603 and by the Center for Photochemical Sciences at Bowling Green State University. We are grateful to Dr. G. McLendon for useful discussions.

(51) Churg, A. K.; Weiss, R. M.; Warshel, A.; Takano, T. *J. Phys. Chem.* **1983**, *87*, 1683-1694.
(52) Brunschwig, B. S.; Ehrenson, S.; Sutin, N. *J. Phys. Chem.* **1986**, *90*, 3657-3668.

Mechanism of Action of Vitamin K†

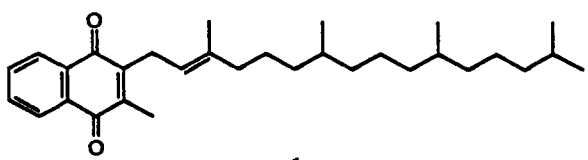
Paul Dowd,* Seung Wook Ham, and Steven J. Geib

Contribution from the Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received January 22, 1991*

Abstract: Vitamin K is the blood clotting vitamin; it is important as an obligatory cofactor for the enzyme which carboxylates selected glutamate residues in the proteins of the blood coagulation cascade, including prothrombin (factor II), factors VII, IX, and X and proteins C, S, Z, and M. Molecular oxygen is required for the carboxylation. A new model, based on 2,4-dimethyl-1-naphthol demonstrates that spontaneous oxidation of the corresponding 2,4-dimethyl-1-naphthoxide anion **1** leads to the tertiary alkoxide **9**. The latter is a sufficiently strong base to effect the Dieckmann condensation of diethyl adipate (**2**) to ethyl cyclopentanonecarboxylate (**4**), a model for the carboxylation. In thermochemical terms this *base strength amplification* is driven by the oxidation to the extent of approximately −50 kcal/mol. It is proposed that the model oxidation proceeds through a dioxetane intermediate **8**, which demands that the epoxide be cis to the alcohol in the product **3**. This has been demonstrated by X-ray crystallography. Likewise, treatment of 2,3,4-trimethyl-1-naphthol (**16**) with oxygen in chloroform yielded the crystalline hydroperoxide **15**. The latter rearranged to the epoxy alcohol **18** upon treatment with potassium hydride. The structure of **17** was established by X-ray crystallography. Thermochemical analysis shows that the oxidation of vitamin K hydroquinone to vitamin K oxide is exothermic to the extent of −62 kcal/mol. The new mechanism suggests that a second atom of oxygen-18 might be incorporated in an $^{18}O_2$ labeling experiment. Analysis of mass spectral data in the literature indicates that this probably is the case, lending support to the new mechanistic proposal.

Introduction

Vitamin K (**1**) is an obligatory cofactor in the complex enzymic sequence known as the blood cotting cascade and is, therefore, a vital component of the chemistry governing blood coagulation.[1-6]



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The importance of vitamin K in blood coagulation was widely appreciated, but its specific function was not understood until γ-carboxyglutamic acid (Gla) was discovered independently by Stenflo,[7] Nelsestuen,[8] and Magnusson in 1974.[9,10] The critical observation that γ-carboxyglutamic acid was absent from bovine prothrombin circulating in the blood of animals administered the

† Dedicated with warmest best wishes to Professor Ronald Breslow on the occasion of his 60th birthday.

(1) Review of early work: Wagner, A. F.; Folkers, K. *Vitamins and Coenzymes*; Interscience: New York, 1964; pp 407-434.
(2) Dam, H. *Biochem. Z.* **1929**, *215*, 475. Dam, H. *Biochem. Z.* **1930**, *220*, 158. Dam, H. *Nature* **1934**, *133*, 909.
(3) Dam, H. *Nature* **1935**, *135*, 652. Dam, H. *Biochem. J.* **1935**, *29*, 1273.
(4) McFarlane, W. D.; Graham, W. R., Jr.; Hall, G. E. *J. Nutr.* **1931**, *4*, 331. McFarlane, W. D.; Graham, W. R., Jr.; Richardson, F. *Biochem. J.* **1931**, *25*, 358. Almquist, H. J. *J. Biol. Chem.* **1936**, *114*, 241. Almquist, H. J. *J. Biol. Chem.* **1936**, *115*, 589. Dam, H.; Schonheyder, F. *Biochem. J.* **1936**, *30*, 897. Thayer, S. A.; MacCorquodale, D. W.; Binkley, S. B.; Doisy, E. A. *Science* **1938**, *88*, 243. McKee, R. W.; Binkley, S. B.; MacCorquodale, D. W.; Thayer, S. A.; Doisy, E. A. *J. Am. Chem. Soc.* **1939**, *61*, 1295. Fieser, L. F. *J. Am. Chem. Soc.* **1939**, *61*, 2561. Dam, H.; Geiger, A.; Glavind, J.; Karrer, P.; Karrer, W.; Rothschild, E.; Salomon, H. *Helv. Chim. Acta* **1939**, *22*, 310. Binkley, S. B.; MacCorquodale, D. W.; Thayer, S. A.; Doisy, E. A. *J. Biol. Chem.* **1939**, *130*, 219. Karrer, P.; Geiger, A.; Legler, R.; Ruegger, A.; Salomon, H. *Helv. Chim. Acta* **1939**, *22*, 1464. McKee, R. W.; Binkley, S. B.; Thayer, S. A.; MacCorquodale, D. W.; Doisy, E. A. *J. Biol. Chem.* **1939**, *131*, 327.

0002-7863/91/1513-7734$02.50/0