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Free Energy Effects on Biological Electron Transfer: Reactions of Iron(IV) Cytochrome *c* Peroxidase (ES) with Metallocytochromes *c*

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Abstract: Reaction rates are reported for the oxidation by Fe(IV) cytochrome *c* peroxidase (ccp; ES) of horse heart cytochrome *c* (cytc) derivatives containing Fe(II), Zn(II), or H_2 porphyrin at the cytc heme site. For these reactions, k_{obsd} and ΔG are 10^3 s^{-1} and −0.9 eV for Fe(II), ~0.2 s^{-1} and −0.35 eV for Zn(II), and ~0.01 s^{-1} and ca. −0.05 eV for H_2 porphyrin. These results can be well predicted by Marcus theory, assuming a reorganization energy of $\lambda \approx 1.5$ eV. These results are briefly compared to other studies of intraprotein redox reactions.

Since protein electron transfer reactions provide the pathways for channeling biological energy, these reactions remain a subject of intense interest.[1] Although much progress has been made in understanding small molecule electron transfer reactions,[2] only recently have experiments begun to test the applicability in protein systems of fundamental theories[4] of electron transfer. For example, Marcus' theory[4b] suggests $k_{et} = A \exp[-(\Delta G + \lambda)^2/4\lambda kT]$. A depends on donor–acceptor electronic coupling, and thus on distance: $A \propto \exp(-\alpha R)$, where R is the edge–edge distance. The parameter α depends on electron binding energy, $\alpha \approx 1.2$ Å^{-1}. ΔG is the reaction free energy, and λ is the "reorganization energy" required for all the nuclear displacements that accompany electron transfer. In protein redox reactions, the dependence of rate on distance, reaction free energy, and protein structure remains poorly understood. One attractive system for detailed studies of how rate is controlled in protein electron transfer reactions is provided by the cytochrome *c* (cytc)/cytochrome *c* peroxidase (ccp) couple.[5]

The ccp/cytc system is particularly noteworthy in that a wide range of physiochemical data are already available for this system, including detailed steady state kinetic measurements,[5] independent protein–complex equilibrium[5,6] studies, and oxidation–reduction potential measurements.[7] Most important, high-resolution crystal structures are available for ccp and cytc, as well as a detailed model for the structure of the cytc/ccp complex (Figure 1).[8] A key feature of this model is that the cytc/ccp complex is stabilized by specific salt bridges. These links define a unique conformation in which the cytc heme and ccp heme are held in parallel planes

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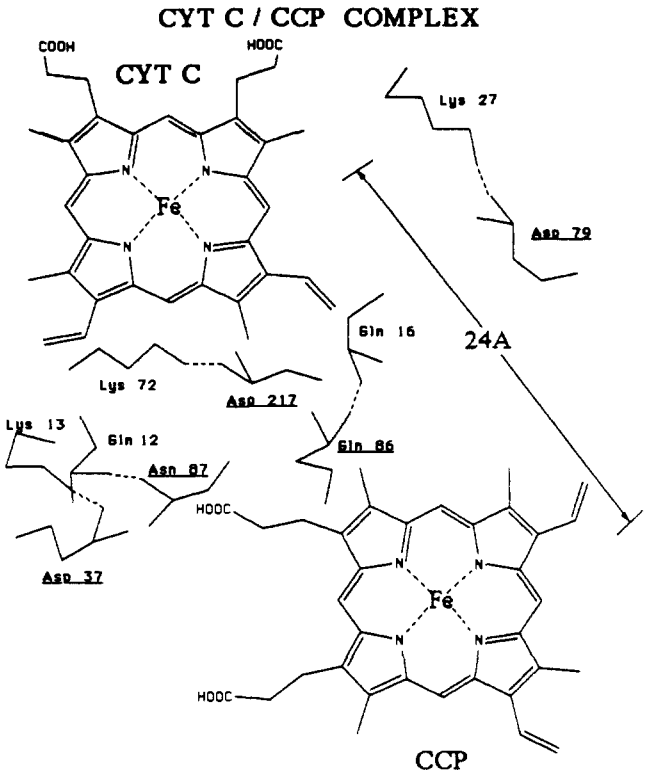

Figure 1. Model of the cytc/ccp complex focusing on the heme–heme interface. Cytochrome *c* peroxidase amino acids are underlined. The dotted line represent H bonds between the two proteins. The Fe–Fe distance is about 24 Å.

with a Fe–Fe distance of about 24 Å. Such model structures are supported by a host of independent chemical data.[9] Finally, as noted elsewhere,[3g] cytc and ccp readily yield a range of metal derivatives of widely varying redox potentials and reactivity, which can be used advantageously in mechanistic studies. A key question for this system is how does rate depend on ΔG; what is λ for this protein/protein couple? Previous investigations of cytochrome *c* reactions have led to widely varying estimates of λ, ranging from $\lambda \sim 0.8$ eV in the $cytb_5$/cytc couple[3g] to $\lambda \leq 0.2$ eV for electron transfer in the His 33 $Ru(NH_3)_5$–cytochrome *c* derivative.[3j] In order to better understand these observations, we now report simple ground-state reactions for metal-substituted cytochrome *c* derivatives (Mcytc) with the peroxide oxidation product of ccp (compound ES): ES + 2(M cytc) → Fe^{III} ccp + 2(M cytc)$^{\bullet+}$. The results of these studies suggest that $\lambda \simeq 1.5$ eV for electron transfer in the cytc/ccp complex. These results are briefly compared with analogous systems, and their implications for understanding physiological protein electron transfer reactions are assessed.

Experimental Section

Cytochrome *c* peroxidase was isolated by the method of Nelson et al.[13] with the following modifications: cross-linked DEAE agarose was substituted for DEAE cellulose; cytochrome *c* peroxidase was extracted in 0.05 M sodium acetate, pH 5.0, thereby allowing the enzyme to be loaded directly onto the DEAE column; and the enzyme was concentrated by ultrafiltration, using an Amicon cell and a 10000 molecular weight cutoff filter rather than by using a second DEAE column. The enzyme was then crystallized by dialysis against cold, twice distilled water and stored at −196 °C. The enzyme prepared in this manner had a purity index of 1.2, as determined by the ratio of absorbance at 407 to 280 nm.

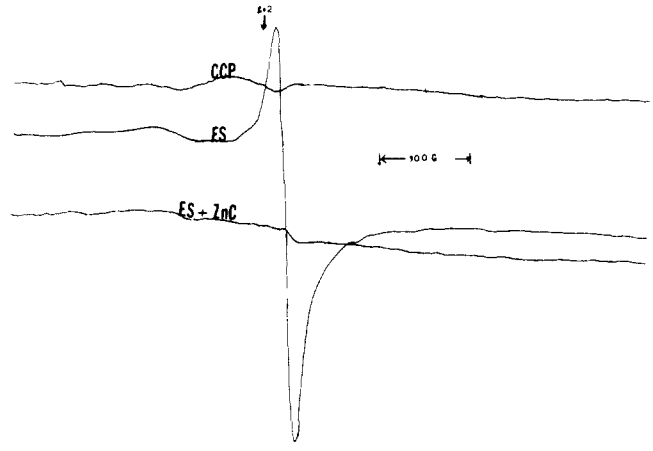

Figure 2. (A, top) EPR spectrum of Fe^{III}ccp (200 μM) measured at 77 K (10 mM P_i pH 7 at room temperature), microwave frequency 9.283 GHz, gain 2×10^4, 5 G field modulation, 20 mW microwave power, 1000 G scan. (B, middle) EPR spectrum of the H_2O_2 oxidation product of ccp (compound ES 15 μM) measured from 3000.0 to 4000.0 G with a microwave frequency of 9.5000 to 9.53440 GHz. (C, bottom) EPR spectrum of ES + Zn cytc (200 μM each) measured as above. Note the disappearance of the sharp $g = 2$ signal characteristic of ES.

Porphyrin cytochrome *c* was prepared from Sigma Type VI cytochrome *c* by the method of Vanderkooi et al.[14] with the following modifications: the protein was dissolved in 100 mM potassium phosphate buffer pH 7.1 and then the solution was adjusted to pH 5.0 prior to concentration; the protein solution was concentrated by ultrafiltration, using a 5000 molecular weight cutoff filter and redissolved in cold, twice distilled water rather than dialysis against phosphate buffer; and the protein was purified on a Sephadex G-25 (Sigma) column (1.5 cm × 25 cm) that was pre-equilibrated with twice distilled water rather than an Amberlite CGH-50 column. The purity of the porphyrin cytochrome *c* was checked by visible absorption spectroscopy. The spectrum of purified porphyrin cytochrome *c* (Figure 3B) showed no residual iron cytochrome *c* peaks. All sample handling was done in the dark to prevent photodegradation of the porphyrin cytochrome *c* in the presence of O_2.

Zinc cytochrome *c* (Zn cytc) was prepared by the addition of 100 mM zinc acetate to an acidic, aqueous solution of porphyrin cytochrome *c*. The mixture was then heated to 40 °C and maintained at that temperature during the entire time course of the reaction. The reaction was followed by periodically removing aliquots of the solution and monitoring the visible absorption spectrum. The reaction was complete when all porphyrin cytochrome *c* bands had totally disappeared and the spectrum contained only the characteristic zinc cytochrome *c* bands (Figure 3C). The reaction was complete in 1 h. Excess zinc acetate was then removed by ultrafiltration with a 5000 molecular weight cutoff filter. The protein was then redissolved in twice distilled water and applied to a Sephadex G-25 (Sigma) column (1.5 cm × 25 cm) for final purification. Again the purity was checked by visible absorption spectroscopy and the spectrum showed only bands due to zinc cytochrome *c* with no residual porphyrin cytochrome *c* bands. Again, all sample handling was done in the dark to prevent photodegradation of the zinc cytochrome *c* in the presence of O_2.

All potassium phosphate buffers were prepared with Baker Analyzed reagent grade monobasic and dibasic potassium phosphate and twice distilled water. The correct ionic strength and pH were obtained by mixing appropriate concentrations of monobasic and dibasic stock solutions.

Samples for difference spectra and kinetic data were prepared by dissolving various concentrations (5 μm–30 μM) of ccp and zinc or

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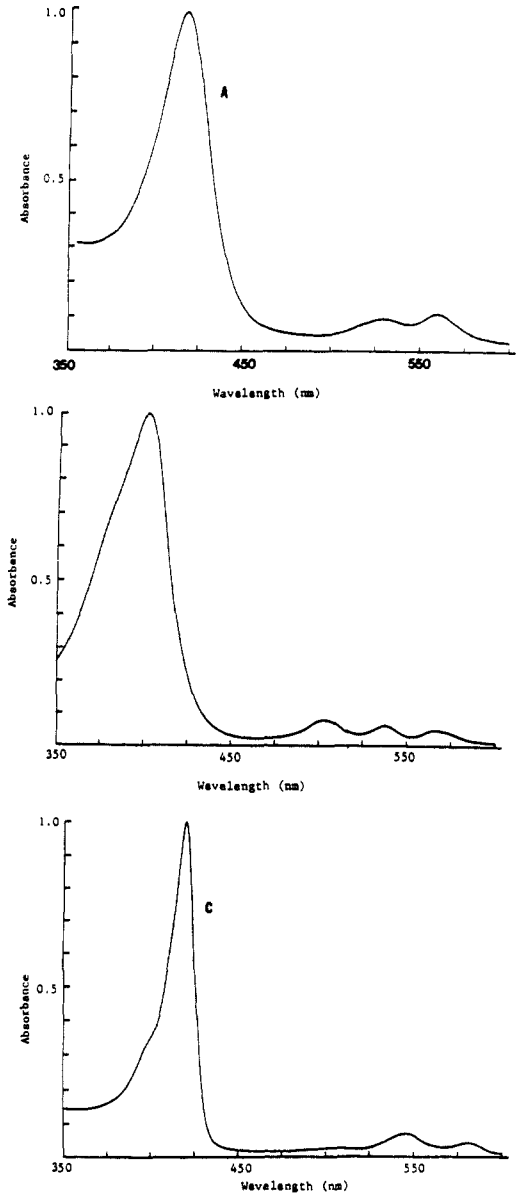

Figure 3. (A) Visible absorption spectrum of ES in 10 mM potassium phosphate, pH 7.0 buffer, 25 °C, measured from 350 to 600 nm. (B) Visible absorption spectrum of porph cytc in 10 mM potassium phosphate, pH 7.0 buffer, 25 °C, measured from 350 to 600 nm. (C) Visible absorption spectrum of Zn cytc in 10 mM potassium phosphate, pH 7.0 buffer, 25 °C, measured from 350 to 600 nm.

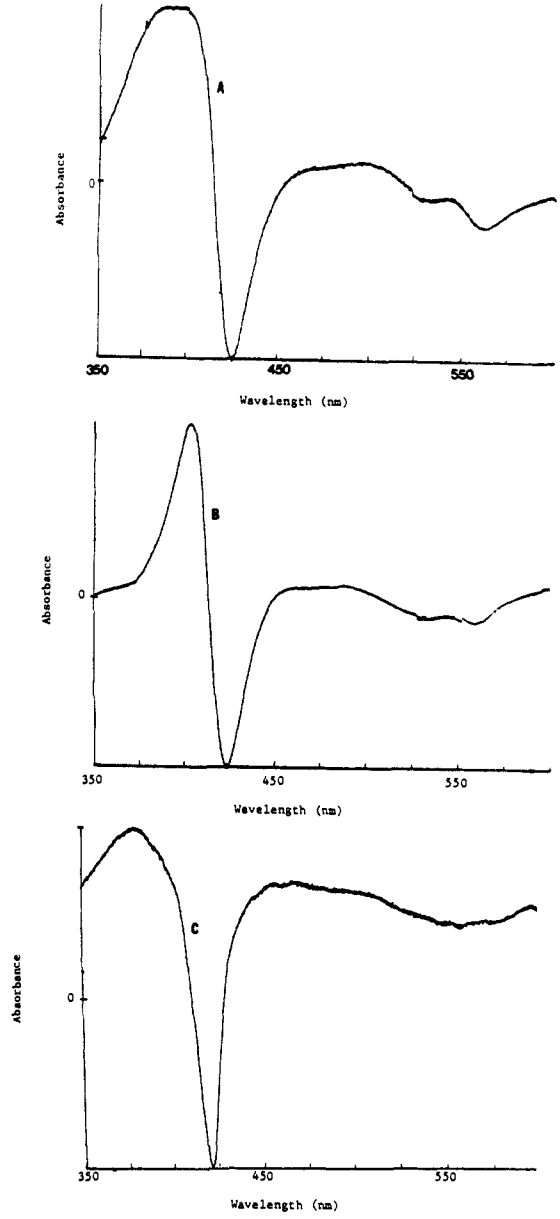

Figure 4. Room temperature visible absorption difference spectra in the region 350 to 600 nm of (A) (ccp spectrum) − (ES spectrum); (B) (porph cytc + ES mixed in tandem cells) − (porph cytc + ES unmixed in tandem cells); and (C) (Zn cytc + ES mixed in tandem cells) − (Zn cytc + ES unmixed in tandem cells). All samples were 10 μM protein in 10 mM phosphate pH 7.0 buffer. Difference spectra were obtained in 50, 100, 200, 500 mM phosphate pH 6.0, pH 8.0 buffers and were identical with those shown.

porphyrin cytochrome *c* in appropriate buffers. Buffers ranged in ionic strength from 10 to 500 mM and pH 6.0 to 8.0. ES was then prepared by the dropwise addition of 180 μM H_2O_2 (diluted from 3% Mallinkrodt stock into appropriate buffer) and checked by visible absorption spectroscopy. After H_2O_2 addition the Soret band shifted from 407 to 420 nm indicative of the ES complex. The ES solutions prepared in this manner were stable for ≥1 h. The one-electron oxidant abbreviated Fe^{IV}ccp, was prepared by careful addition of H_2O_2 to Fe^{II}ccp after the method[10] of Hoffman, but using a lumiflavin/EDTA photoreduction system.

Difference spectra and kinetics data were obtained on a Perkin-Elmer λ3 ultraviolet–visible spectrophotometer interfaced to a DEC LSI 11-03 computer. Difference spectra were obtained with use of tandem mixing cells (Hellma Cells, Inc.). The first spectrum was of ES or Fe^{IV}ccp (10 μM) and zinc or porphyrin cytochrome *c* (10 μM) unmixed in the tandem cells. The second spectrum was of ES or Fe^{IV}ccp (10 μM) and zinc or porphyrin cytochrome *c* (10 μM) mixed in the tandem cells. Spectra were obtained in the region 350 to 600 nm. The "unmixed spectrum" was then subtracted from the "mixed spectrum" and the resulting spectrum was essentially identical with that obtained by subtracting a standard ES spectrum (10 μM) from a standard ccp spectrum (10 μM) (Figure 4A). Difference spectra were obtained at all ionic strengths (10, 50, 100, 200, and 500 mM) and were identical.

Kinetic experiments were carried out at 420 nm (the Fe^{IV} ccp and/or ES maximum) and monitored for 5 min. For determination of activation parameters, the temperature was controlled to ±0.2 °C with a thermostated cell holder which was refrigerated with use of a Haake FK2 constant temperature circulator. Temperature readings were obtained with a Cu versus Cu–Ni thermocouple. The reported kinetic constants were obtained with samples mixed 1:1, 2:1, 3:1, 1:2, and 3:2 (ES: porphyrin cytochrome *c*) to ensure that the reaction was strictly first order. The reported kinetic constants are averages of multiple runs with different preparations of ccp and porphyrin cytochrome *c*.

Electron paramagnetic resonance (EPR) experiments were carried out at −196 °C on a Bruker ER 200D EPR spectrometer interfaced to a Z-80 computer. The sweepwidth was 3000.0–4000.0 G and the microwave frequency was 9.50000–9.53440 GHz. Three samples were prepared for each experiment. The first sample contained 500 μL of ccp (200 μM) (Figure 2A); the second sample contained 500 μL of ES (200 μM) (Figure 2B); and the third sample contained 250 μL of ES + 250 μL of zinc or porphyrin cytochrome *c* (200 μM). All samples were flash frozen in liquid nitrogen immediately after mixing. The experiment was repeated three times with different preparations of ccp and zinc or por-

Table I. Parameters for the Intracomplex Reaction Sequence[a]

$$\text{M cytc} + \text{ccp (ES)} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} [\text{M cytc/ES}] \xrightarrow{k_{obsd}} [\text{M cytc}^+/\text{ccp(III)}]$$

M	ΔG (eV)	k_{obsd} (s^{-1})	k_f (M^{-1} s^{-1})[c]	k_r (s^{-1})[c]
Fe^{II}	−0.9	800[b]	10^9	10^3
Zn^{II}	−0.35	0.2	*d*	*d*
H_2 porphyrin	−0.05	0.01	*d*	*d*

[a] Conditions: pH 7.0, 10 mM Pi, 25 °C. [b] Reference 18. [c] Values of k_f, k_r from ref 5. [d] Values of k_f, k_r for M cytc assumed similar to Fe^{II} cytc, since the binding constants are similar.

phyrin cytochrome *c* to ensure reproducibility of data.

Results

Porph cytc/ES Reaction. When highly purified, the hydrogen peroxide oxidation product of ccp (compound ES) is stable for more than 1 h at room temperature in dilute phosphate buffer (pH ≤7.0), as judged by its characteristic EPR (Figure 2B) and visible (Figure 3A) spectra.

A visible difference spectrum of a mixture of porph cytc and ES shows the loss of features associated with the ferryl [$Fe^{IV}O$] chromorphore in ES, and the corresponding growth of ferric [Fe^{III}] ccp features (Figure 4B). By monitoring the absorbance change at 420 nm, the rate of reduction of Fe^{IV}ccp by porphyrin cytochrome *c* can be accurately monitored. The ES compound contains two oxidizing equivalents: one at the oxo ferryl porphyrin center and one protein based organic radical cation. These two reaction centers introduce possible kinetic complexity in the oxidation kinetics. In order to test for such complexity, we independently prepared the *single* site oxidant Fe^{IV}ccp, which is the sole product of the reaction of Fe^{II}ccp + H_2O_2 → Fe^{IV}ccp.[10a] Importantly, there was *no* significant difference in the observed reaction rates for the reactions

$$Fe^{IV}\text{ccp} + \text{porph} \xrightarrow{k = 0.011\ s^{-1}} \text{products}$$

$$\text{ES} + \text{porph cytc} \xrightarrow{k = 0.01\ s^{-1}} \text{products}$$

In independent studies, Hazzard et al.[18] have shown that the reaction rates are also quite similar for the reactions

$$\text{ES} + Fe^{II}\text{cytc} \xrightarrow{k = 800\ s^{-1}} Fe^{III}\text{ccp} + Fe^{III}\text{cytc}$$

$$\text{vs } Fe^{IV}\text{ccp} + Fe^{II}\text{cytc} \xrightarrow{k = 800\ s^{-1}} Fe^{III}\text{ccp} + Fe^{III}\text{cytc}$$

Thus the *observed* (intracomplex) rate processes measured here obviously are *not* complicated by multiple reaction pathways. The observed kinetics thus correspond to a simple one-electron transfer

$$Fe^{IV}\text{ccp/Mcytc} \xrightarrow{k} Fe^{III}\text{ccp/(Mcytc)}^+$$

In this way, we measure a rate constant of 0.01 ± 0.002 s^{-1} (10 mM phosphate buffer, pH 7.0, 25.0 °C) for the oxidation of the porphyrin cytochrome *c* by compound "ES" of cytochrome *c* peroxidase.

On addition of equimolar horse heart porphyrin cytochrome *c* to ES the characteristic EPR spectrum of compound ES also is rapidly altered. Within 5 min, the characteristic sharp EPR signal at $g = 2$ due to the ES radical is completely lost. (This signal has been alternately assigned to a sulfur based or tryptophan radical).[5] Measurements of the decrease in the $g = 2$ signal, while less precise, are in qualitative agreement with the spectral data. We cannot directly measure the reaction between the protein radical site (R^+) and the cytochrome *c*. This inability is not surprising since the rate of equilibration between the Fe(IV) and radical site as measured by Ho[10] is much faster than the net reaction rate observed here. Ho et al. have measured the rate for the reaction $Fe^{III} + R^+ \rightarrow R + Fe^{IV}$ to be ≃6 s^{-1}. Therefore, the reactions we studied proceed according to the sequence below, with the rate constants summarized in Table I.

1. ES + M cytc $\underset{k_r}{\overset{k_f}{\rightleftharpoons}}$ ES/M cytc (or Fe^{IV}ccp)
2. Fe^{IV}ccp/M cytc $\xrightarrow{k_{obsd}}$ Fe^{III}ccp/(M cytc)$^+$
3. For ES, reaction 2 is itself a multistep process which likely proceeds via the sequence

$$Fe^{IV}OR^+ \text{ ccp/(M cytc)} \xrightarrow{k'} Fe^{III}R^+ \text{ ccp} + \text{(M cytc)}^{\bullet+} \quad \text{(a)}$$

$$Fe^{III}R^+ \text{ ccp} \xrightarrow{k} Fe^{IV}O \text{ ccp} \quad \text{(b)}$$

$$Fe^{IV}O \text{ ccp/(M cytc)} \xrightarrow{k''} Fe^{III} \text{ ccp} + \text{(M cytc)}^{\bullet+} \quad \text{(c)}$$

Under our reaction conditions, both of the oxidizing equivalents of ES "funnel" through a common $Fe^{IV}O$ intermediate and a single rate constant is observed which is essentially identical with that found for the singly oxidizing Fe^{IV}ccp species (i.e., in scheme 3b, $k \approx k'$). With the rate constants noted in Table I, the single rate constant, k_{obsd}, is due to reaction of the Fe^{IV}ccp site by M cytc, which is clearly rate determining for the H_2 porph and Zn reactions reported here ($k' > k \approx k'$).[10] Since both ES and Fe^{IV}ccp behave as kinetically equivalent oxidants, we will hereafter refer to ES, for the sake of simplicity. Direct detection of the porphyrin and zinc cation radical intermediates is not possible since such radicals decay quite rapidly in this and related systems ($\tau < 10$ ms),[3k,l] relative to the rate of radical formation ($\tau \sim$ 5–100 s). Thus, no significant concentration of the intermediate builds up. There is some net loss of the porphyrin spectrum in porph cytc upon oxidation by ES, consistent with oxidation of the unstable radical. To rule out any role of adventitious reductants, control experiments were performed with native Fe^{III} cytc, as well as Fe^{III} cytc prepared by reconstitution of H_2 porph cytc. Neither case, on addition of Fe^{III} cytc to ES, exhibited loss of the ES spectrum over a period >20 min.

Several explanations are possible for the 10^5-fold rate difference between the Fe^{II}cytc/ES and porph cytc/ES reactions. A simple explanation is that the conformations of porph cytc and Fe^{II}cytc differ dramatically, and porph cytc is therefore "inactive" in long distance electron transfer. However, a number of lines of evidence argue against this explanation. For example, binding measurements of porph cytc to ccp give a binding constant for porph cytc which is quite similar to that of Fe^{III}cytc.[16] It should be noted that under the experimental conditions used, ≥95% of all ccp is bound to porphyrin cytc. Further evidence is available from energy transfer measurements[3k] and NMR studies of metal-substituted cytochromes.[17] These arguments are summarized in detail by McLendon and Miller.[3g] An attractive alternative, which is consistent with limited observations for other protein/protein couples (e.g., cytc/cyt b_5) is that this large rate difference is due to the large free energy difference between the reactions. For Fe^{II}cytc/ES $\Delta G \approx -0.90 \pm 0.15$ eV, while for porph cytc/ES $\Delta G \approx -0.05 \pm 0.1$ eV. These calculated ΔG values are based on a cytc III/II potential of 0.24 eV,[7a] on Erman's estimate of the ES/Fe^{III}ccp potential of 1.1 eV,[7f] and on a (porph cytc)$^{\bullet+}$/porph cytc potential of 1.0 eV, as measured in our lab by Magner.[7c] The quoted number for the ES couple was obtained by measuring redox equilibria between ES and a series of graded Fe(phenanthroline)$_3^{2+}$ homologues. Since reduction of ES involved proton uptake, this estimate should be valid only near neutral pH (as in the present studies).[7d] Estimates for the oxidation potentials of H_2 porph cytc ($E° = 1.0 \pm 0.2$ eV), Zn cytc ($E° = 0.8 \pm 0.2$ eV), and cytc ($E° = 0.24$ eV) have been described in detail elsewhere.[7] For present purposes, we note that these values are close to the well-known redox potentials of simple Zn porphyrins (for Zn meso porphyrin $E° \simeq 0.7$ eV) and H_2 porphyrins ($E° = 0.90$ eV).[7e] Assuming these free energy values, a 10^5 rate difference would occur between Fe^{II}cytc and H_2 porph cytc if the total protein reorganization energy $\lambda = 1.5$ eV. This explanation leads to two experimentally testable predictions. First, a cytc derivative of intermediate oxidation potential, like Zn cytc, should react more rapidly with ES than does porph cytc, but less rapidly than Fe^{II}cytc. Second, the porph cytc/ES reaction should show a relatively high activation

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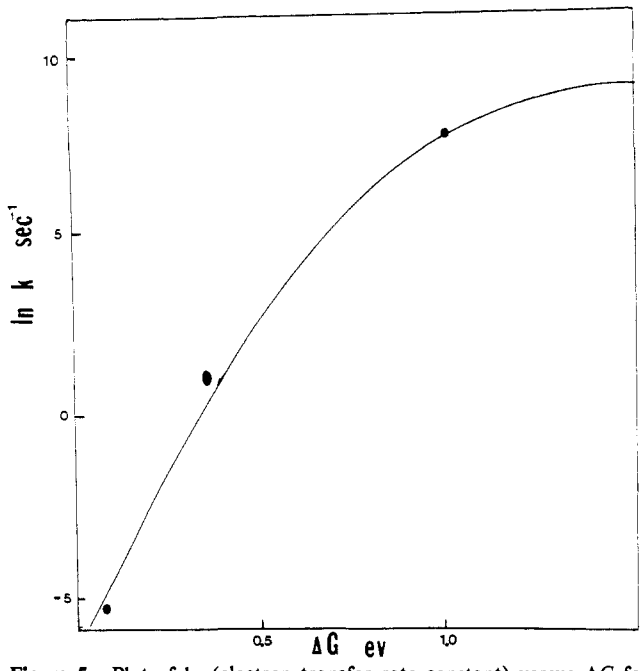

Figure 5. Plot of ln (electron transfer rate constant) versus ΔG for ES/porph cytc, ES/Zn cytc, ES/Fe^{II}cytc systems. The solid line is a theoretical prediction of the rate constant using classical Marcus theory assuming $\lambda = 1.5$ eV and $k = A \exp(-(\Delta G + \lambda)^2/4\lambda kT)$.

energy, ~7 kcal/mol. Tests of these predictions are presented in the following sections.

Reactions of Zn cytc and ES. The reaction of ES with Zn cytc completely parallels the behavior observed for porph cytc. On mixing equimolar Zn cytc and ES, the characteristic $g = 2$ EPR signal of ES is lost, and the visible difference spectrum shows ES is fully converted to Fe^{III}ccp (Figures 2 and 4C). By monitoring the change at 420 nm in the visible absorption spectra, the rate constant obtained for ES + Zn cytc, k_{zn}[Fe^{III}ccp + (Zn cytc)$^{++}$], is $k_{zn} \sim 0.2 \pm 0.1$ s^{-1} (Table I). The consistency of the kinetic data with Marcus theory for Fe^{II}, Zn^{II} cytc, and H_2 porph cytc oxidations by ES can now be tested. A plot of the observed and theoretically calculated rate constants versus ΔG is shown in Figure 5, assuming $\lambda = 1.5$ eV. (A best error estimate is 1.5 ± 0.5: more points would be needed to unambiguously establish a turnover in rate as ΔG approaches λ.) The theoretical fit of the data is probably fortuitous, but the general consistency between theory and experiment is clear. With so few points, of course, a *linear* relationship might seem equally valid (although it has no theoretical basis). The Marcus plot shown *does*, however, predict a specific activation energy, which can be tested as outlined below.

Temperature Dependence of k_{porph}. In principle, a particularly stringent test of the assumed value $\lambda = 1.5$ eV can be obtained by measuring the temperature dependence of k_{porph}. In practice, rate–temperature studies in proteins are fraught with potential complications. The most serious complications arise from thermal population of new protein conformations. Such conformations could have different local environments, which affect λ or ΔG, or even different donor–acceptor distances. Fortunately, for the cytc/ccp couple, such problems are likely to be small. Both cytc and ccp are quite thermally stable toward denaturation. Furthermore, it is known that the equilibrium constant for binding cytc and ccp depends only very weakly on temperature.[6]

A final problem is that, in general, ΔH and ΔS values are unknown for protein redox reactions. Since the value calculated by Marcus theory is an activation *free energy*, if $\Delta S°$ were large, the activation enthalpy and activation free energy could differ significantly. With these caveats in mind, we decided nonetheless to measure the temperature dependence of the ES/porph cytc reaction for comparison with theory. The results of these experiments are shown in Figure 6. The activation parameters so obtained are $A \simeq 10^2$ and $E_a \sim 5.5$ kcal/mol (0.25 eV). For $\Delta G = -0.05$ eV and $E_a = 0.25$ eV, Marcus theory[4b] predicts a

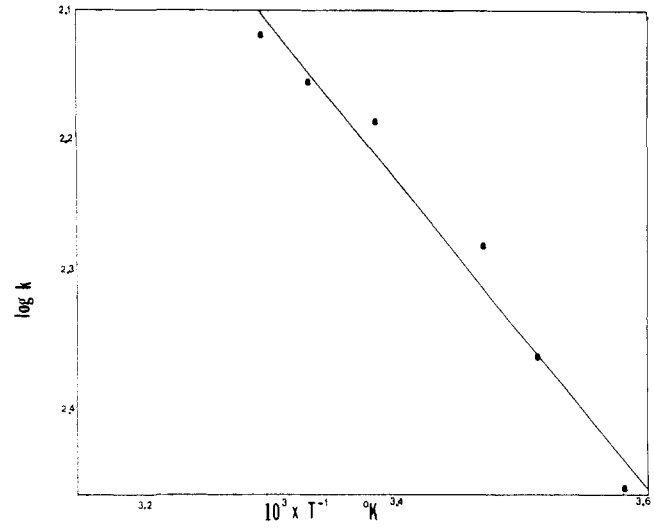

Figure 6. Temperature dependence of the electron transfer rate constant for the ES/porph cytc reaction. Results were obtained with use of 10 mM phosphate buffer, pH 7.0.

total reorganization energy of $\lambda \sim 1.3$ eV in good agreement with the dependence of k_{obsd} on ΔG. The actual λ value could be higher than suggested from E_a. For example, the λ value inferred from the dependence of rate on ΔG may include high-frequency modes, which are not thermally populated. Thus, the dependence of rate on ΔG and of k_{porph} on temperature both suggest a value of $\lambda \geq 1.3$ eV for the ES/cytc reaction.

The very small prefactor of $A \sim 10^2$ is qualitatively consistent with a nonadiabatic electron-transfer reaction. For long-distance transfer, we assume $A_{obsd} \simeq A° \exp(-1.2R)$ where $A°$ is the frequency factor for an adiabatic reaction, and R is the distance between donor and acceptor.[2] Using the best available estimates of $A° \sim 10^{13}$ s^{-1} and $R = 16$ Å, we obtain an estimate of $A_{obsd} \approx 10^3$ s^{-1}. Considering the uncertainty in these parameters, the agreement between the calculated and observed values is considered surprisingly good. Since the rate of the ccp/cytc reaction essentially can be explained by using parameters from simple model systems, we tentatively suggest that the ES/cytc reaction need not necessarily involve unusual mediation via aromatic residues ("π ways") as previously proposed.[8] We note, however, that the critical Phe 82 residue which has been proven important in the elegant experiments of Hoffman et al.[11] is unchanged in our experiments.

Discussion

Rapid progress is starting to emerge in the area of biological electron transfer. One important question that a number of experiments are designed to answer is the following: How does the rate of electron transfer vary with free energy, i.e., what is the reorganization energy associated with protein electron transfer reactions?

To this end, a variety of different protein electron transfer experiments have been undertaken.[3] In general, the experiments can be classified in two broad categories: transfer between physiological partners such as cytc/ccp, cytc/cyt b_5, cyt b_5/Hb and transfer between two redox groups within the same protein. The latter studies range from work on photoactive site heme derivatives in metal hybrid hemoglobins done by Hoffman et al.[3c,h] to studies using Ru substituted proteins (cytc, azurin, or Mb) by Gray[3a,i,j] and Iseid.[3d,e]

The available data on Ru modified proteins suggested originally that reorganization energies in such modified proteins are small. These results are consistent with classical continuum theory calculations on model systems, which predict that $\lambda < 0.3$ eV. This is supported by the work of Gray and co-workers in the $(NH_3)_5Ru^{III}$His33–Fe^{II} cytc system.[3a,j] They found that the rate of intramolecular electron transfer from Ru to Fe was slow, $k_{et} \sim 50$ s^{-1}, and exhibited little temperature dependence, indicating $\Delta H^{\ddagger} \sim 1.5$ kcal/mol (0.068 eV) and $\lambda \leq 8$ kcal/mol (0.36 eV).

However, more recent data suggest that λ may be significantly larger in these systems: $\lambda \sim 2$ eV.[19]

In those cases so far characterized, the reorganization energy associated with electron transfer in protein/protein complexes is large (0.8 eV $< \lambda <$ 2.0 eV). For example, Hoffman and coworkers[3h] have reported detailed temperature dependence studies of photoinduced electron transfer in Zn^{II}/Fe^{III} hybrid Hb. These results are consistent with $\lambda \sim 2$ eV for this system. Recent work by McLendon and Miller[3g] employed metal substitution and pulse radiolysis techniques to the physiological cytc/cyt b_5 couple. By varying ΔG, a value of $\lambda \simeq 0.8$ eV was obtained, and this value was further supported by studies of the temperature dependence of the rate. Finally, the present results for the cytc/ccp system, $\lambda = 1.5$ eV, suggest that reorganization energy in this protein/protein complex is rather large.

What is the source of the large reorganization energy found in protein/protein systems? Recent work by Rackovsky and Goldstein[15] suggests that a large contribution to λ may come from a redox dependent reorientation of the interface between proteins. In physiological partners such as cytc/ccp and cytc/cyt b_5 detailed crystallographic and modelling studies have shown that there is an electrostatic binding region on the complex in which several lysine residues on cytc align with appropriate acidic amino acids on the redox partner to form strong "salt links". Similarly, the interface between the α and β subunits of Hb is also comprised of "salt bridges". The movement of these salt links at the interface may contribute to the large observed total electron transfer reorganization energies.

Using a three-point superposition model, Rackovsky and Goldstein were able to demonstrate that there are definite conformational changes which occur at the cytc/redox partner interface when cytc is oxidized. In particular in cytc, Lys-27 undergoes a large, observable motion upon oxidation which leads to a change in the binding affinity of partners. This motion then provides a dissociation signal to the complex. In addition, there is a closing motion of the protein shell upon the heme on oxidation and a "double hinge" action of the nonbinding region which guides the motion of the two binding edges. Furthermore, upon oxidation, the heme undergoes charge redistribution and conformational changes which cause the heme to tilt within the protein envelope.

Although no direct evidence is available for the Hb subunits, it is tempting to speculate that the salt bridges between subunits undergo conformational changes similar to the cytc system. This would help explain the large λ observed in the Zn/Fe hybrid system.

An obvious test of such models involves direct modification of the binding iterface via (site deirected) mutagenesis. Such studies are in progress in our labs, and elsewhere. It is noteworthy that the data to date indeed demonstrate that significant rate differences (>2×) can be observed by mutation of those lysines (e.g., Lys 13, Lys 27) of cytochrome *c* which are involved in binding to ccp. These data also suggest that binding of cytc to ccp is a highly dynamic process, as might be required for large λ values.[20]

Summary

1. The intramolecular electron transfer rate was measured for the oxidation by Fe^{IV}ccp (ES) of Zn^{II} and H_2 porph cytc. For the ES/porph cytc reaction $k_{porph} \simeq 0.01 \pm 0.02$ s^{-1} ($\Delta G \simeq -0.05$ eV), and for the ES/Zn cytc reaction $k_{zn} \simeq 0.2$s $\pm$ 0.1 s^{-1} ($\Delta G \simeq -0.35$ eV). These results are consistent with Marcus theory which predicts an exponential dependence of rate on reaction free energy: $k_{et} = A \exp[-(\Delta G + \lambda)^2/4\lambda kT]$, with $\lambda \simeq 1.5$ eV.

2. The activation parameters for the ES/porph cytc system determined by a temperature dependence study are $\Delta H^{\ddagger} = 0.21$ eV, $\Delta S^{\ddagger} = -53$ eu, $E_{act} = 0.24$ eV, and the prefactor, $A \simeq 10^2$. The dependence of k_{et} on ΔG and of k_{porph} on temperature both suggest a value of $\lambda \simeq 1.5$ eV for the ES/porph reaction.

3. The results for the cytc/ccp system ($\lambda \simeq 1.5$ eV) are consistent with results obtained on other protein/protein complexes such as cytc/cyt b_5 and Zn/Fe hybrid Hb (0.8 eV $< \lambda <$ 2.0 eV). These results suggest that proteins may not be optimized for maximum rate, but rather for other properties like reactant specificity.

4. It is clear that proteins can undergo efficient electron transfer over relatively long distances. This apparent dependence of electron transfer rate on distance is generally consistent with data based on small molecule reactions.

(19) Gray, H. B., personal communication.

(20) McLendon, G.; Short Rogalskyj, J.; Magner, E.; Taylor Conklin, K. In *Oxidases and Related Redox Systems*; Mason, H., Ed.; A. Liss: New York, 1988. This work was supported by NIH (GM33881) and NSF.