Electron Transfer from the Heme of Cytochrome *c* to Two Equidistant Redox-Modified Sites, Histidine 33 and Methionine 65: The Importance of Electronic Effects and Peptide Networks

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Electron transfer (ET) proteins, covalently modified with transition metal redox reagents at different locations on the surface of the protein, have been used to study the distance dependence of intramolecular electron transfer rates and the role of the protein network in providing a pathway for the ET reaction.[1-7]

We report here new, unexpected ET results from a comparative study of two horse heart (hH) cytochrome *c* (cyt *c*) donor–acceptor complexes: one modified at Met 65[8] with $[Fe(CN)_5(H_2O)]^{3-}$[9] (Fe(Met 65)) and one modified at His 33 with t-$[Ru(NH_3)_4isn(H_2O)]^{2+}$ (isn = isonicotinamide) (Ru(His 33)). The two amino acid modification sites Met(65) and His(33) are located at *similar distances* from the heme iron,[10] but in different segments of the protein (Figure 1). In each case, the direction of the ET reaction studied is from the reduced heme of cyt *c* to the transition metal acceptor complex; and both metal acceptor complexes have comparable self-exchange rates and redox potentials.[11,12]

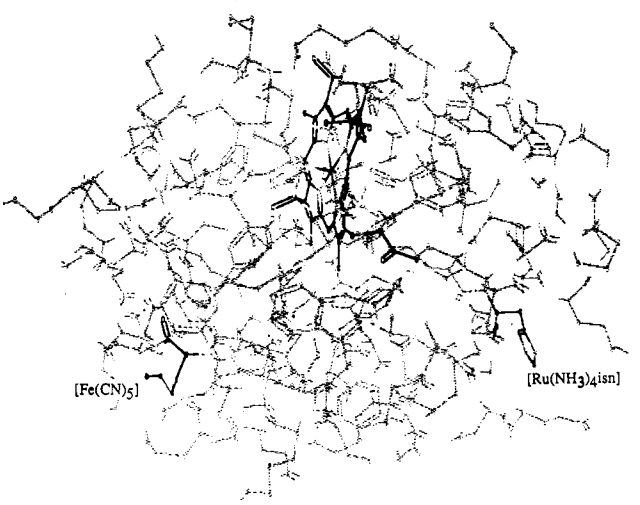

Figure 1. Two equidistant sites in horse heart cytochrome *c*: Met 65 and His 33. The Fe(heme) to S and to N_1 (imz) distances are 15.3 Å (Met 65) and 16.0 Å (His 33) (ref 10). Horse heart cyt *c* was modified with $[Fe^{II}(CN)_5]$ at Met 65 and $[Ru^{II}(NH_3)_4(isn)]$ at His 33 in two separate experiments.

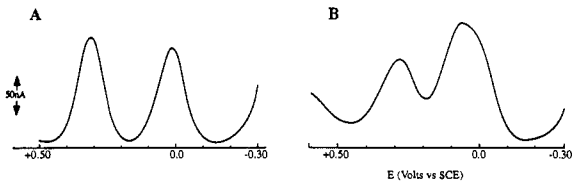

Figure 2. Electrochemistry of hH cyt c^{II}-$[Fe^{III}(CN)_5$(Met 65)] (A) and hH cyt c^{II}-$[Fe^{III}(CN)_5$(Met 65)][$Fe^{III}(CN)_5$(His 33)] (B).

In spite of the similar distances and driving force for the reaction, the observed rates of intramolecular ET are profoundly different. In the [Ru(His 33)-cyt *c*(hH)], intramolecular ET occurs rapidly from the heme to the $[Ru^{III}(His\ 33)]^{3+}$ group, $k_{et} = 440 \pm 30\ s^{-1}$. In contrast, in the [Fe(Met 65)-cyt *c*(hH)] under identical conditions, only *intermolecular* ET is observed ($k_b = 7.1 \pm 0.5 \times 10^5\ M^{-1}\ s^{-1}$) and the *intramolecular* ET reaction from the heme to the $[Fe^{III}$(Met 65)] group is estimated to be $k_{et} \leq 0.6 \pm 0.3\ s^{-1}$. In this communication we describe the experiments and our interpretation of these unusual results.

The preparation of [Ru(His 33)-cyt *c*(hH)] has been reported,[13,14a] and more details are being published separately.[14b]

The reaction of $[Fe(CN)_5(NH_3)]^{3-}$ (20 mM) with hH cyt *c* (6 mM) proceeds rapidly (~1 h). Two hH cyt *c* species were obtained by ion exchange chromatography, with one or two $[Fe^{II}(CN)_5]$ groups per heme, respectively.

Electrochemistry of the singly substituted derivative shows two redox sites, that of the [Fe(Met 65)] (0.56 V vs NHE) (similar to that for the amino acid complex $[(CN)_5Fe\text{-}Met]^{3-}$ (0.575))[11] and that of the heme of cyt c (0.26 V vs NHE) (Figure 2). The doubly substituted derivative (which was not used in the ET studies) shows an additional wave, which is assigned to the [Fe(His 33)] (~0.33 V) and which overlaps that of the heme of cyt *c* (0.26 V) (Figure 2). Slow scan cyclic voltammetry (CV) (50 mV/s) of the singly substituted derivative [Fe(Met 65)-cyt *c*] did not show any detectable loss of $[Fe^{III}(CN)_5]$ from the modified cyt *c* on the CV time scale.

Tryptic digestion of the [Fe(Met 65)-cyt *c*] shows that the $[Fe^{II}(CN)_5]$ is present with Met 65 on the T11 peptide fragment, with its spectrum similar to that for the amino acid complex, $[(CN)_5Fe\text{-}Met]^{3-}$.[11a,b]

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(2) (a) For a recent review of the work in this area, see: Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369 and references therein. (b) In order to study ET distance dependence, cytochromes (from horse heart, yeast mutants, and semisynthetic forms) were modified with histidine residues at different locations and covalently bound to ruthenium complexes. The observed kinetics can be interpreted best using a distance function involving an effective number of covalent bonds, or a tunneling pathway length. See, for example: Wuttke, D. S.; Bjerrum, M. J.; Winkler, J. R.; Gray, H. B. *Science* (*Washington, D.C.*) **1992**, *256*, 1007.

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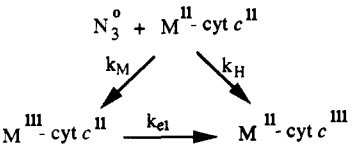
Scheme 1[a]



[a] k_M and k_H are the rates for the direct oxidation of the metal center or the heme by the $N_3^\bullet$ radical; k_{et} is the rate of intramolecular ET.

Table 1. Intra- and Intermolecular Electron Transfer Rates and Driving Forces for Modified Protein Complexes

complex	k_{et}, s^{-1} (25 °C)	k_b, M^{-1} s^{-1} (25 °C)	$\Delta G°$, eV
hH cyt c^{II}-[$Ru^{III}(NH_3)_4$isn(His 33)][a] 16–4 μM	440 ± 30	$2.8 \pm 0.2 \times 10^4$ [b]	0.18
hH cyt c^{II}-[$Fe^{III}(CN)_5$(Met 65)][c] 10.3–1.7 μM	≤0.6 ± 0.3	$7.1 \pm 0.5 \times 10^5$	0.30
tuna cyt c^{II}-[$Fe^{III}(CN)_5$(Met 65)][c] 19.6–1.4 μM	≤0.2 ± 0.1	$5.2 \pm 0.2 \times 10^5$	0.30

[a] 0.05 M phosphate buffer, pH 7.0, 10^{-3} M NaN_3. [b] k_b is for $[Ru(NH_3)_5isn]^{3+}$ + hH cyt c^{II} (ref 16), which is an upper limit for the intermolecular ET reaction between two molecules of hH cyt c^{II}-[Ru^{III}(His 33)]. [c] 0.10 M phosphate buffer, pH 7.4, 10^{-3} M NaN_3.

The spectrum of the [Fe^{II}(Met 65)-cyt c^{III}(hH)], (oxidized with $[Fe(CN)_6]^{3-}$) shows the expected 695-nm band (ϵ = 850 M^{-1} cm^{-1}) for cyt c^{III} (hH), indicating that the Met ligation to the ferric heme is intact.[15]

Intramolecular ET from the heme to the [Fe^{III}(Met 65)] or to the [Ru^{III}(His 33)] complex was studied according to Scheme 1. The driving force for these two reactions, calculated from electrochemical data, is 0.300 and 0.180 eV, respectively.

The ET intermediates (Scheme 1), which were produced by the reaction of azide radicals (generated by pulse radiolysis techniques) with an excess of [Ru^{II}(His 33)-cyt c^{II}] or [Fe^{II}(Met 65)-cyt c^{II}], were monitored at λ = 550, 504, and 432 nm ([Ru^{III}(His 33)-cyt c^{II}]) and at λ = 550 nm ([Fe^{III}(Met 65)-cyt c^{II}]).

The [Ru^{III}(His 33)-cyt c^{II}] intermediate undergoes rapid *intramolecular* ET from the reduced heme to the Ru^{III}(His 33) site, k_{et} = 440 ± 30 s^{-1} (Table 1). The corresponding *intermolecular* ET rate between $[Ru(NH_3)_5isn]^{3+}$ and hH cyt c^{II} was determined to be 2.8×10^4 M^{-1} s^{-1} in a separate experiment.[16]

For the [Fe^{III}(Met 65)-cyt c^{II}] intermediate, kinetic studies with 10–1.7 μM cyt *c* show that the ET reaction is concentration dependent (Table 1) and the intramolecular ET rate from the reduced heme to the Fe^{III}(Met 65) site (inferred from the intercept at zero concentration in a plot of rate versus concentration) is estimated to be $k_{et} \leq 0.6$ s^{-1}. The *intermolecular* rate between two [Fe^{III}(Met 65)-cyt c^{II}] molecules (where an electron is transferred from the heme of one modified cyt *c* to the $[Fe(CN)_5]$ of another cyt *c*) was determined (from the slope of a rate vs concentration plot) to be $k_b = 7.1 \pm 0.5 \times 10^5$ M^{-1} s^{-1}.[17]

To further confirm the [Fe^{II}(Met 65)-cyt c^{III}(hH)] derivative, $[Fe^{II}(CN)_5]$ was used to modify tuna cyt *c* using procedures similar to those used for the hH species. Because tuna cyt *c* has a Met 65 residue, but *no* His 33 residue (rather a Trp 33), only a singly modified tuna cytochrome species with the $[Fe(CN)_5]$ group bound to the Met 65 residue was isolated, as expected. The tuna [Fe(Met 65)-cyt *c*] exhibited electrochemistry and ET rates ($k_{et} \leq 0.2$ s^{-1} and $k_b = 5.2 \pm 0.2 \times 10^5$ M^{-1} s^{-1}) similar to those of the corresponding hH modified species. Most significantly, both the tuna and hH modified cyt *c* species show unobservably slow ET rates from the reduced heme to the $[Fe^{III}(CN)_5]$ at Met 65, $k_{et} \leq 0.2$ s^{-1} (tuna) and $k_{et} \leq 0.6$ s^{-1} (hH).

If the same electronic coupling mechanism is assumed between the heme of hH cyt *c* and His 33 or Met 65, and the free energy and reorganizational energy differences between $[Fe^{II/III}(CN)_5]$ and $[Ru^{II/III}(NH_3)_4isn]$ are corrected for, a rough estimate of the expected rate of intramolecular ET from the heme(II) to $[Fe^{III}(CN)_5]$ is calculated to be 10^4–10^5 s^{-1}, i.e., 1–2 orders of magnitude faster than that observed for the [Ru^{III}(His 33)-cyt *c*] derivative, instead of the <0.6 s^{-1} as observed here.[18]

For the two hH cyt *c* derivatives modified in different regions of the protein, [Fe(Met 65)] and [Ru(His 33)], the large difference in the experimental rates of ET (>730) (Table 1) from the heme to two sites located *at similar distances* has rarely been observed. This observation demonstrates that *the through-space distance between the donor and acceptor is not the primary determinant of the rate of ET in this protein,* even when the reorganization energy and $\Delta G°$ are corrected for.

In principle, the nuclear and/or the electronic effects[19,20] can account for the differences in the observed rates; however, the observation of rapid *intermolecular* ET rates for both modified protein complexes argues against inhibition of the overall ET processes by the nuclear effects of the donor and acceptor.[21] Other factors such as the electronic effects of the peptide network[22] and the nature of the electronic interaction between the transition metal acceptors and the protein (i.e., the connectivity of the metal complex to the protein) must play an important role in determining the ET rates.

Application of the electronic pathway analysis[6] leads to the predicted rate ratio ~800 in favor of the His 33 site (compared with the observed rate ratio >730). Recent theoretical analyses[7,23] also reinforce the effects of the peptide medium on the rate of these reactions. In contrast to this work, activationless intraprotein ET rates were interpreted using a single edge to edge distance decay factor of 1.4 $Å^{-1}$, indicating that, for limited sets of ET reactions, average medium descriptions can serve as a useful first approximation for calculating ET rates.[24]

In the results reported here, electronic effects arising from the peptide network cannot be differentiated from the electronic interaction of the metal complexes with the proteins. The effect of the electronic interactions can be further explored in future experiments with complexes such as $[Ru(CN)_5(OH_2)]^{3-}$. This analogue[25] is expected to result in kinetically stable cytochrome derivatives at both the His 33 and Met 65 sites, so that ET experiments with the same acceptor complex and the same distance can be compared.

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