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Kinetics of electron transfer between cytochrome *c* and iron hexacyanides. Evidence for two electron-transfer sites

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The electron-transfer reaction between ferrocytochrome *c* and ferricyanide has been studied by the method of photoexcitation. The observed transfer rate shows saturation behaviour at high ferricyanide concentration. Data analysis indicates that there are two binding sites of vastly different affinities at which electron transfer occurs. The binding constant for the strong binding site decreases from 1600 M^{-1} to 80 M^{-1} as the ionic strength increases from 15 mM to 140 mM. At 20 °C, the intramolecular electron-transfer rate for this site is $4.65 \cdot 10^4$ s^{-1}, which gives an electron-transfer distance of approx. 9.7 Å according to Hopfield's model.

Introduction

The electron-transfer reactions between iron hexacyanides and cytochrome *c* have been extensively studied as a model for elucidating the mechanism of electron transfer in biological molecules [1–18]. Based on results of nuclear magnetic resonance studies, Stellwagen and Shulman [3] were the first to propose that electron transfer took place within an encounter complex of hexacyanide ($Fe(CN)_6^{3-}$/$Fe(CN_6^{4-})$ and cytochrome *c* (Cyt^{III}/Cyt^{II}) according to the following scheme

$$Fe(CN)_6^{3-} + Cyt^{II} \rightleftharpoons Fe(CN)_6^{3-} \cdot Cyt^{II} \rightleftharpoons Fe(CN)_6^{4-} \cdot Cyt^{III} \rightleftharpoons Fe(CN)_6^{4-} + Cyt^{III}$$

Subsequently, this reaction has been examined by a variety of techniques including stopped-flow [4,5,7,11,12], continuous-flow [9], temperature-jump [10,16], pulse radiolytic [8] and nuclear magnetic resonance methods [3,14,15,17,18] to demonstrate the existence of the precursor complex and to determine the rate constants involved in various steps of the reaction. Although it is generally accepted that an encounter complex is involved in the reaction mechanism, the results obtained from the large number of studies are not in good agreement with one another. The discrepancies arise partly from errors in the data analysis as indicated in Ref. 12, and partly from the fact that the strong ionic dependence for the rate constants [18] has sometimes not been taken into account. The problem of accuracy is further aggravated by that most of the previous experimental data are analyzed by assuming that cytochrome *c* has only a single binding site for electron transfer, which is inconsistent with recent NMR results [14,15,17].

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In this work, we have used the method of photoexcitation [19–21] to study the kinetics of oxidation of Cyt^{II} by $Fe(CN)_6^{3-}$. Hydrated electrons, generated by photoionization of tyrosine molecules [22], serve to reduce Cyt^{III} to Cyt^{II}. In the subsequent study of the electron transfer between $Fe(CN)_6^{3-}$ and Cyt^{II}, we have carried out measurements up to very high $Fe(CN)_6^{3-}$ concentration (approx. 5 mM), a region not accessible by means of stopped-flow techniques. The experiment is similar but much more complete than the previous pulsed radiolytic work of Ilan and Shafferman [8]. The results indicate that electron transfer occurs via at least two sites for $Fe(CN)_6^{3-}$ with very different binding affinities. The important effect of ionic strength is also demonstrated.

Materials and Methods

Horse-heart cytochrome *c* (Type VI, Sigma Chemical Company) was used as received, and $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$, $K_3[Co(CN)_6]$, tyrosine and other chemicals were of reagent grade. All the samples were prepared in 1 mM (pH 7) phosphate buffer. The ionic strength was adjusted by the addition of NaCl. The overall ionic strength I of the sample was computed from

$$I = \tfrac{1}{2}\sum_i C_i Z_i^2$$

where C_i and Z_i are the molarity and the charge of each species, respectively. The concentration of the protein and $Fe(CN)_6^{3-}$ were determined by absorption spectrophotometry taking $\varepsilon_{550}(Cyt^{III}) = 9.1\ mM^{-1}\cdot cm^{-1}$, $\varepsilon_{550}(Cyt^{II}) = 27.6\ mM^{-1}\cdot cm^{-1}$ [23], and $\epsilon_{420}(Fe(CN)_6^{3-}) = 1.02\ mM^{-1}\cdot cm^{-1}$. The experiments were normally conducted in the presence of O_2. For a few measurements, the samples were deaerated by a number of freeze-pump-thaw cycles, and essentially the same electron-transfer rates (between Cyt^{II} and $Fe(CN)_6^{3-}$) were obtained.

The electron-transfer rates were measured using a conventional flash photolysis set-up with the 266 nm output of a Quanta-Ray DCR-2 Nd:YAG laser (pulse-width, approx. 5 ns) as the excitation source [20,21]. Hydrated electrons were first generated by photoionizing tyrosine molecules (approx. 2 mM) in a sample of Cyt^{III} and hexacyanides. With the following reactions occurring sequentially, the electron-transfer rates between Cyt^{II} and $Fe(CN)_6^{3-}$ can then be obtained,

$$e_{aq}^- + Cyt^{III} \rightarrow Cyt^{II}$$

$$Fe(CN)_i^{3-} + Cyt^{II} \rightarrow Fe(CN)_6^{4-} + Cyt^{III}$$

The reactions were followed by monitoring the transient absorbance change of the protein (primarily at 550 nm) after the laser flash. Typically, the laser energy per pulse was 100 mJ/cm^2. The signals were averaged over 10 shots, and the sample was well stirred between shots. The measurements were conducted at room temperature which was about 20 °C.

Results

$Fe(CN)_6^{3-}$ concentration dependence

Fig. 1 shows the electron-transfer rate between $Fe(CN)_6^{3-}$ and Cyt^{II} as a function of $Fe(CN)_6^{3-}$ concentration for ionic strengths $I = 15$ mM, 35 mM, 90 mM and 140 mM. For all the data, the protein concentration was kept to be less than 10% of that of the $Fe(CN)_6^{3-}$ to simplify the data analysis. At low ionic strengths (15 mM and 35 mM), rate saturation behaviour is clearly seen, consistent with the scheme [3] that electron transfer occurs within the precursor complex. An analysis of the data (see discussion below) shows that a minimum of two electron-transfer sites are present. For comparison, we have also conducted experiments under the condition that NaCl was not added to correct for the variation of ionic strengths due to different concentrations of $Fe(CN)_6^{3-}$. The results were qualitatively similar but the rate parameters deduced from data fitting were slightly different. At a high ionic strength of 140 mM, the rate varies almost linearly with $[Fe(CN)_6^{3-}]$, indicating that the binding of $Fe(CN)_6^{3-}$ to Cyt^{II} is now very weak.

$Co(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ competition studies

The rate of oxidation of Cyt^{II} by $Fe(CN)_6^{3-}$ was measured in the presence of various concentration of $Co(CN)_6^{3-}$, a redox inert analog of

$Fe(CN)_6^{3-}$. The concentration of Cyt^{III} was always kept to be less than 10% of that of $Co(CN)_6^{3-}$. As shown in Fig. 2, the observed electron-transfer rates decrease with increasing concentration of $Co(CN)_6^{3-}$. The effect is most apparent at low ionic strength, which is in accord with the above result that $Fe(CN)_6^{3-}$ binds to Cyt^{II} prior to electron-transfer. The addition of $Co(CN)_6^{3-}$ blocks the binding of $Fe(CN)_6^{3-}$, thereby inhibiting the transfer process. Similar results were also observed when $Fe(CN)_6^{4-}$ was used as the competition agent (Fig. 3).

Cyt^{III} concentration dependence

The binding affinity of $Fe(CN)_6^{3-}$ to Cyt^{III} can also be determined by studying the Cyt^{II} to $Fe(CN)_6^{3-}$ electron-transfer rate as a function of Cyt^{III} concentration. At low ionic strength, it is seen (Fig. 4) that the rate decreases monotonically

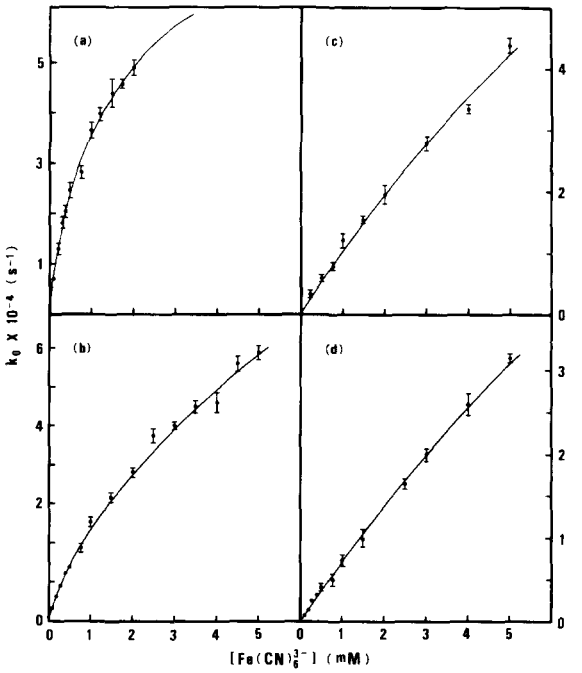

Fig. 1. Plots of the observed electron-transfer rate k_0 vs. the $Fe(CN)_6^{3-}$ concentration for the oxidation of Cyt^{II} by $Fe(CN)_6^{3-}$ at ionic strengths of (a) 15 mM, (b) 35 mM, (c) 90 mM and (d) 140 mM. Solid lines are theoretical curves calculated using Eqn. 7 with the values for K_{32}, k_e and b_{32} listed in Table I.

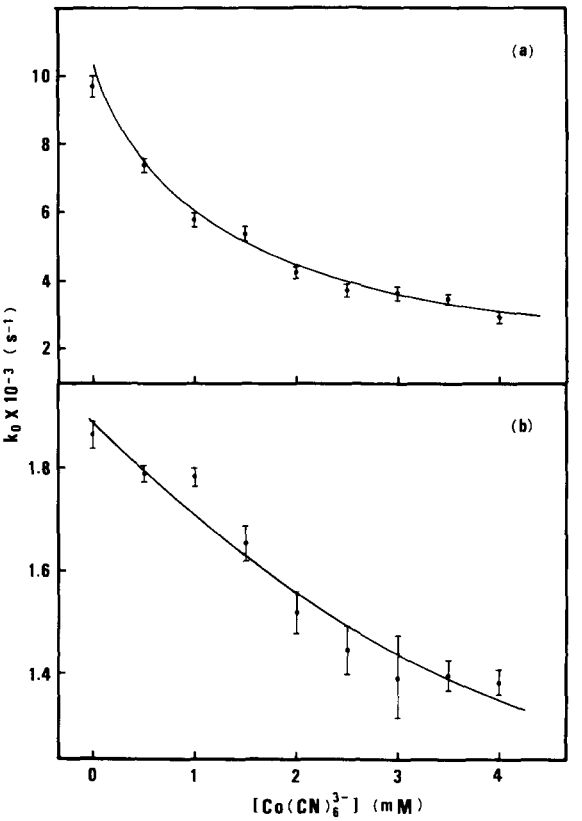

Fig. 2. Plots of k_0 vs. $Co(CN)_6^{3-}$ concentration for the oxidation of Cyt^{II} by $Fe(CN)_6^{3-}$ at ionic strengths of (a) 28.5 mM and (b) 130 mM. The concentration of $Fe(CN)_6^{3-}$ were 250 μM for both (a) and (b). Solid lines are theoretical curves calculated using Eqn. 7a with the values for K_{32}, K'_{32}, k_e and b_{32} listed in Table I.

with the increase in Cyt^{III} concentration. This indicates that the concentration of free $Fe(CN)_6^{3-}$ is much reduced at high [Cyt^{III}], as a result of the formation of $Fe(CN)_6^{3-}$-Cyt^{III} complex. The protein concentration effect is less important at higher ionic strength where the binding affinity is weaker.

Discussion

Our observation that there is a saturation behaviour in the electron-transfer rate between $Fe(CN)_6^{3-}$ and Cyt^{II} supports the scheme proposed by Stellwagen and Shulman [3]. A quantitative analysis of the data is performed by assuming

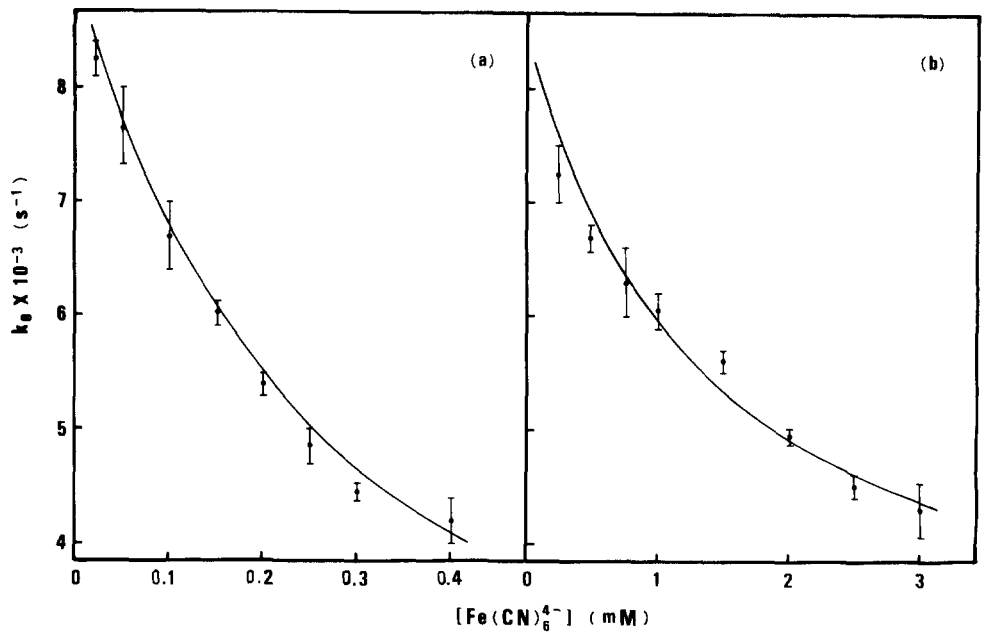

Fig. 3. Plots of k_0 vs. $Fe(CN)_6^{4-}$ concentration for the oxidation of Cyt^{II} by $Fe(CN)_6^{3-}$ at ionic strengths of (a) 15 mM and (b) 40 mM. The total $Fe(CN)_6^{3-}$ and Cyt^{III} concentrations were 109 and 10 μM, respectively, for (a) and 336 and 22 μM, respectively, for (b). Solid lines are theoretical curves calculated using Eqn. 7b with the values for K_{32}, K_{22}, k_e and b_{32} listed in Table I.

that our results can be described by the following reactions:

$$Tyr + h\nu \rightarrow Tyr^+ + e_{aq}^- \quad (1)$$

$$e_{aq}^- + Cyt^{III} \xrightarrow{fast} Cyt^{II} \quad (2a)$$

$$e_{aq}^- + Fe(CN)_6^{3-} \xrightarrow{fast} Fe(CN)_6^{4-} \quad (2b)$$

$$Fe(CN)_6^{3-} + Cyt^{II} \overset{K_{32}}{\rightleftharpoons} Fe(CN)_6^{3-} \cdot Cyt^{II} \xrightarrow{k_e} Fe(CN)_6^{4-} \cdot Cyt^{III} \quad (3a)$$

$$Tyr^+ + Cyt^{II} \rightarrow Tyr + Cyt^{III} \quad (3b)$$

$$Tyr^+ + Fe(CN)_6^{4-} \rightarrow Tyr + Fe(CN)_6^{3-} \quad (3c)$$

$$Fe(CN)_6^{3-} + Cyt^{III} \overset{K_{33}}{\rightleftharpoons} Fe(CN)_6^{3-} \cdot Cyt^{III} \quad (4)$$

The reactions that will contribute to our measured rates are given by Eqns. 3a and 4. Reaction (3b) also provides a pathway for the reoxidation of Cyt^{II}. However, this contribution may be neglected because the amount of Tyr^+ generated is small and there is quenching of Tyr^+ by $Fe(CN)_6^{4-}$ (Eqn. 3c). Note that in Eqn. 3a, we have omitted the backward electron-transfer reaction, the rate of which is negligible compared with the forward rate k_e [18]. Furthermore, we have assumed in Eqns. 3a and 4 that the rates associated with the formation and dissociation of the complex are sufficiently greater than k_e that the complex is always in equilibrium with $Fe(CN)_6^{3-}$ and the protein. As will be shown below, this assumption is valid for essentially all cases, and the observed electron-transfer rate k_0 is given by,

$$k_0 = \frac{K_{32} k_e [Fe(CN)_6^{3-}]_f}{1 + K_{32}[Fe(CN)_6^{3-}]_f} \quad (5)$$

In this equation, $[Fe(CN)_6^{3-}]_f$ is the free $Fe(CN)_6^{3-}$ concentration and is related to the total $Fe(CN)_6^{3-}$ concentration, $[Fe(CN)_6^{3-}]_t$, and the total Cyt^{III} concentration, $[Cyt^{III}]_t$, by

$$[Fe(CN)_6^{3-}]_f = \frac{1}{2K_{33}}\Big(K_{33}\{[Fe(CN)_6^{3-}]_t - [Cyt^{III}]_t\} - 1 + \Big[\big(K_{33}\{[Fe(CN)_6^{3-}]_t - [Cyt^{III}]_t\} - 1\big)^2 + 4K_{33}[Fe(CN)_6^{3-}]_t\Big]^{1/2}\Big) \quad (5a)$$

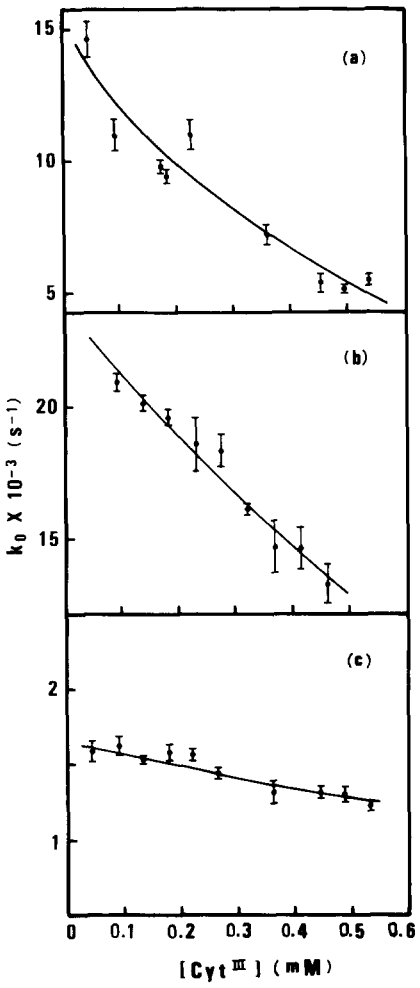

Fig. 4. Plots of k_0 vs. Cyt^{III} concentration for the oxidation of Cyt^{II} by $Fe(CN)_6^{3-}$ at ionic strengths of (a) 15 mM, (b) 15 mM and (c) 140 mM. The $Fe(CN)_6^{3-}$ concentration were 250, 500 and 250 μM for (a), (b) and (c), respectively. Solid lines are theoretical curves calculated using Eqns. 7 and 5a with the values of K_{32}, K_{33}, k_e and b_{32} listed in Table I.

If the binding constant K_{32} is sufficiently small, Eqn. 5 reduces to

$$k_0 = b_{32}[Fe(CN)_6^{3-}]_f \tag{6}$$

where $b_{32} = K_{32}k_e$ is the bimolecular electron-transfer rate constant.

In our initial attempt, the $Fe(CN)_6^{3-}$ concentration dependence data (Fig. 1) were least squared fitted to the single-site model (Eqn. 5), but the result was found to be very poor. The quality of the fit improves tremendously when the following expression for a special two-site model (one strong and one weak site) was used

$$k_0 = \frac{K_{32}k_e[Fe(CN)_6^{3-}]_f}{1 + K_{32}[Fe(CN)_6^{3-}]_f} + b_{32}[Fe(CN)_6^{3-}]_f \tag{7}$$

Note that in this case, $[Fe(CN)_6^{3-}]_f \approx [Fe(CN)_6^{3-}]_t$, since the experiment was conducted under the condition that $[Cyt^{III}]_t \ll [Fe(CN)_6^{3-}]_t$. We have also fitted the data to a general two-site model which involves an additional parameter, namely, the binding constant for the second site. There is very little change in the quality of fit and the parameters obtained. Thus, the data suggest that the electron-transfer between $Fe(CN)_6^{3-}$ and Cyt^{II} occurs via two (or more) sites, one having a large binding constant and the other having a small binding constant (up to 100 M^{-1}).

Table I summarizes the parameters deduced by fitting the data to Eqn. 7 at various ionic strengths. The relative contribution of the two separate sites to the observed rate can be readily obtained from the values of K_{32}, k_e and b_{32}. At $I = 15$ mM, the weak site's contribution is only about 13% as large as that of the strong site at a $Fe(CN)_6^{3-}$ concentration of 100 μM which is, typically, the maximum $Fe(CN)_6^{3-}$ concentration allowed in stopped-flow experiments. This percentage increases to 48% at a higher $Fe(CN)_6^{3-}$ concentration of 2 mM (see Fig. 1), thus enabling contributions from the weak binding site to be identified. The binding constant for the tight binding site decreases monotonically with increasing ionic strength, reaching 80 M^{-1} at $I = 140$ mM at which binding is no longer apparent. From the low ionic strength results (15 mM and 35 mM), the intramolecular electron-transfer rate is $(4.65 \pm 0.2) \cdot 10^4$ s^{-1}, in good agreement with that deduced by Ilan et al. [8] and Eley et al. [18]. Knowing K_{32} and k_e, we can justify as follows the equilibrium assumption on which Eqn. 5 is based. The effective association rate between $Fe(CN)_6^{3-}$ and Cyt^{II} is given by $a_{32}[Fe(CN)_6^{3-}]$, where a_{32} has been estimated from the diffusion-controlled limit to be about $2 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ [20,24]. For the concentration range of $Fe(CN)_6^{3-}$ covered, $a_{32}[Fe(CN)_6^{3-}]$ is therefore larger than $2 \cdot 10^5$ s^{-1}. The dissociation rate constant d_{32} can

TABLE I

VALUES OF RATE AND EQUILIBRIUM CONSTANTS

Typical accuracies for both rate and equilibrium constants are ±10%. The reagents and the protein underlined in the reaction are the ones whose concentration are varied in the experiment.

Reaction	Ionic strength (mM)	K_{32} (M^{-1})	K'_{32} (M^{-1})	K_{33} (M^{-1})	K_{22} (M^{-1})	k_e (10^4 s^{-1})	b_{32} (10^6 $M^{-1}\cdot s^{-1}$)
$\underline{Fe(CN)_6^{3-}} + Cyt^{II}$	15	1600				4.8	8.5
	35	630				4.5	7.5
	90	140				(4.65) [a]	5.0
	140	80				(4.65) [a]	4.0
$Fe(CN)_6^{3-} + Cyt^{II} + \underline{Co(CN)_6^{3-}}$	28.5	1000	1050			(4.65) [a]	4.0
	130	125	166			(4.65) [a]	2.0
$Fe(CN)_6^{3-} + Cyt^{II} + \underline{Fe(CN)_6^{4-}}$	15	1900			4600	(4.65) [a]	7.5
	40	440			860	(4.65) [a]	7.6
$Fe(CN)_6^{3-} + Cyt^{II} + \underline{Cyt^{III}}$	15 [b]	1600		5200		(4.65) [a]	(7.0) [a]
	15 [c]	1550		5500		(4.65) [a]	(7.0) [a]
	140	50		250		(4.65) [a]	(4.0) [a]

[a] The parameters are assumed to have the bracketed values in the least-square fitting.
[b] $Fe(CN)_6^{3-}$ concentration was 250 μM.
[c] $Fe(CN)_6^{3-}$ concentration was 500 μM.

also be estimated from K_{32} and a_{32} to be about $1 \cdot 10^6$ s^{-1}. These rates are sufficiently large compared to k_e for Eqn. 5 to be valid.

The observed electron-transfer rates decrease with the addition of $Co(CN)_6^{3-}$, as a result of the inhibition of the binding of $Fe(CN)_6^{3-}$ to Cyt^{II}. The results of the competition experiment (Fig. 2) were fitted to the following equation

$$k_0 = \frac{K_{32}k_e[Fe(CN)_6^{3-}]_f}{1 + K_{32}[Fe(CN)_6^{3-}]_f + K'_{32}[Co(CN)_6^{3-}]_f} + b_{32}[Fe(CN)_6^{3-}]_f \quad (7a)$$

Note that again $[Fe(CN)_6^{3-}]_f \approx [Fe(CN)_6^{3-}]_t$, since $[Co(CN)_6^{3-}]_t + [Fe(CN)_6^{3-}]_t \gg [Cyt^{III}]$. Fitting was performed by assuming $k_e = 4.65 \cdot 10^4$ s^{-1}, and the close agreement between K_{32} and K'_{32} (Table I) supports the assumption that $Co(CN)_6^{3-}$ behaves simply as a redox inert analog of $Fe(CN)_6^{3-}$. The results obtained using $Fe(CN)_6^{4-}$ as the competition agent were fitted to

$$k_0 = \frac{K_{32}k_e[Fe(CN)_6^{3-}]_t}{1 + K_{32}[Fe(CN)_6^{3-}]_t + K_{22}[Fe(CN)_6^{4-}]_t} + b_{32}[Fe(CN)_6^{3-}]_t \quad (7b)$$

It is seen (Table I) that the binding constant of Cyt^{II} for $Fe(CN)_6^{4-}$, K_{22}, is about a factor of 2 greater than K_{32}. Our result that the more negatively charged $Fe(CN)_6^{4-}$ binds to the site with a stronger affinity is consistent with the fact that $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ bind to a positive patch of the protein surface via electrostatic interaction.

A study of the electron-transfer rate as a function of the Cyt^{III} concentration provides information on the binding affinity between $Fe(CN)_6^{3-}$ and Cyt^{III}, K_{33}. As can be seen from Table I, a fit of the data (Fig. 4) to Eqns. 7 and 5a gives a K_{33} value about 3 times higher than K_{32}. One possible reason is that K_{33} includes contribution from all the binding sites for $Fe(CN)_6^{3-}$ on the protein surface. However, the higher K_{33} may also arise from the difference in the oxidation state of the protein (Cyt^{III} vs. Cyt^{II}). Further work is required to sort out the respective contribution of these two factors.

The electron-transfer reactions between $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ and Cyt^{II}/Cyt^{III} have recently been examined by several groups using NMR spectroscopy [14,15,17,18]. Their results suggest that there are two or more non-equivalent binding sites for hexacyanides on cytochrome *c*,

two of which are located near the heme crevice [14,15]. The amino acid residues responsible for the binding have also been identified. According to Eley et al. [15], lysine residues 25, 27 and 79 are possible binding groups for one site whereas lysine residues 8, 13, 72, 86 and 87 may be involved in the binding for the other site. A similar conclusion is reached by Butler et al. [25] in the study of the kinetic of oxidation of CDNP modified cytochrome *c* by $Fe(CN)_6^{3-}$. More specifically, Hopfield and Urgurbil [14] have assigned from NMR studies the binding groups for the two sites to be (1) lysine residues [25, 27] and (2) 72, 73, with site (1) having a stronger binding affinity. From X-ray crystallography data, the edges of site (1) and (2) have been estimated to be at distances of 9.5 Å and 11 Å, respectively, from the heme edge [14]. In the following, we estimate the electron-transfer distance involved from the intramolecular electron rate k_e. According to Hopfield's electron-transfer model [26].

$$k_e = \frac{2\pi}{\hbar} \frac{1}{4\lambda kT} |T_{ab}|^2 \, e^{-(\Delta E - \lambda)^2/4\lambda kT} \tag{8}$$

where T_{ab} is the electron-transfer tunnelling matrix element, ΔE and λ are the overall potential and reorganization energy for the reaction, respectively. Since $E_f^0(Cyt^{III}/Cyt^{II}) = 0.26$ V and $E_f^0(Fe(CN)_6^{3-}/Fe(CN)_6^{4-}) = 0.41$ V [27], $\Delta E =$ 0.15 V. The reorganization energy for the self-exchange reaction of Cyt^{III}/Cyt^{II} is about 1.5 eV [20] and the charge-transfer band for $Fe(CN)_6^{3-}/Fe(CN)_6^{3-}$ is located at about 1 eV [16], thus giving $\lambda \approx 1.25$ eV. For $k_e = 4.65 \cdot 10^4$ s^{-1}, T_{ab} in Eqn. 8 can be deduced to be about $2.2 \cdot 10^{-4}$ eV. T_{ab} (eV) is related to the electron-transfer distance r (Å) approximately by [26]

$$T_{ab} = 2.7(N_a N_b)^{-1/2} \, e^{-0.72r} \tag{9}$$

where N_a and N_b are the number of aromatic groups over which the electron orbitals are delocalized for site a and site b, respectively. In the present case, $N_a \approx 6$, $N_b \approx 20$, giving $r \approx 9.7$ Å. The excellent agreement with the experimental value of 9.5 Å [14] is probably fortuitous in view of the many assumptions involved in the derivation of Eqns. 8 and 9. However, the result does suggest that the electron transfer under investigation can be understood in terms of current models. At present, we are conducting further studies using other analogs of $Fe(CN)_6^{3-}$, such as $Os(CN)_6^{3-}$ and $Ru(CN)_6^{3-}$, so as to examine the relation between k_e and the driving force for the reaction, ΔE. These results should provide a more critical test of the validity of existing electron-transfer theories.

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