Reduction of Cobalticytochrome c by Dithionite*

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The kinetics of reduction of cobalticytochrome c (CoCyt c') by dithionite has been measured spectrophotometrically using an excess of the reducing agent. The reaction is biphasic. The observed first order rate constant of the rapid phase is temperature-dependent and is attributed to reduction by the monomeric SO$_2^-$ whereas the slower process is that of the dimeric S$_2$O$_4^{2-}$. From the temperature dependent dissociation constants and concentration of dithionite, the second order rate constants for CoCyt c' + SO$_2^-$ ($k_4$) and for CoCyt c' + S$_2$O$_4^{2-}$ ($k_5$) were obtained. The effect of ionic strength ($\mu = 0.0725$ to 2.01 m) on $k$ has been determined; the dependence gives an estimate for the effective active site charge of +1.7 for CoCyt c'. Measurements made from pH 6 to 11 showed both $k_4$ and $k_5$ have maximum values at pH 9.3 ± 0.1; the dissociation equilibrium of dithionite is pH-independent from 4 to 12. This pH effect is believed to be related to the reported pH = 9.3 transition for ferricytochrome c (FeCyt c'). Reactions were studied from 20° to 35°C. The observed $k_4$ has an activation energy of 16.6 ± 0.5 kcal·mol$^{-1}$. After the contribution from the activation energy for the dissociation of dithionite is subtracted, the activation energy for $k_5$ is about 6 kcal·mol$^{-1}$. These results and those for the native cytochrome c, FeCyt c' + SO$_2^-$ ($k_2$) and FeCyt c' + S$_2$O$_4^{2-}$ ($k_3$) were compared with the theory of vibronically coupled electron tunneling using the same two vibronic coupling parameters previously applied to some twenty other electron transfer reactions involving biological molecules in solution. Good agreements were found between theory and experiment except for $k_2$. The experimental rate constants all in 10$^7$ s$^{-1}$ (25°C, pH 8 to 9.3, $\mu = 0.2$) are: $k_2 = 5.6 \times 10^7$, $k_3 = 2.6$, $k_4 = 3.9 \times 10^7$, $k_5 = 1.5 \times 10^7$; the theoretical values are $k_2 = 2.0 \times 10^7$, $k_3 = 6.6$, $k_4' = 4.4 \times 10^7$, and $k_5' = 3.2 \times 10^7$. For the SO$_2^-$ reduction of FeCyt c', the experimental activation parameters are $\Delta H^\ddagger = 6$ kcal·mol$^{-1}$, $\Delta S^\ddagger = -22$ e.u. to be compared with theoretical values of $\Delta H^\ddagger = 5.7$ kcal·mol$^{-1}$ and $\Delta S^\ddagger = -24$ e.u.

A theory of electron transfer via vibronically coupled tunneling has been formulated by Hopfield (1) and Jortner (2) to interpret the results of light-induced oxidation of cytochrome in the photosynthetic bacterium Chromatium (5, 6). Detailed studies (5, 6) showed that the rate of electron transfer from 4.5 to 100 K is constant at 3 \times 10^7 s$^{-1}$; above that temperature the rate constant increases to 3 \times 10^8 s$^{-1}$ at 300 K with an apparent activation energy of about 3.3 kcal·mol$^{-1}$. These results were quantitatively reproduced by theories with some judicious choice of parameters to be detailed below. We have been interested in extending the theories to electron transfer of biological molecules in solution. The rate constants for the FeCyt c' oxidation of Cyt c (7), the oxidations of CoCyt c' by oxygen and by Fe(EDTA)$^-$, and the reduction of methemoglobin by CoCyt c catalyzed by mediators (8) have been determined. The observed rate constants and enthalpies and entropies of activation agree well with theoretical values using known midpoint oxidation-reduction potentials, distances of closest approach from x-ray structures or molecular models, and a reasonable and consistent set of vibronic parameters. Similar agreements were also found (9) for the following reactions: self-exchange of FeCyt c (10-12), forward and reverse electron transfer between FeCyt c and Pseudomonas Cyt Cm (13, 14), reduction of FeCyt c' by Fe(EDTA)$^-$ (15), oxidation of FeCyt c by Co(phen)$_3^{3+}$ (16) and by Fe(CN)$_6^{3-}$ (17, 18), self-exchange of CoCyt c (7), the oxidations of CoCyt c' by oxygen and by Fe(EDTA)$^-$ (19), oxidation of spinach ferredoxin by Fe(EDTA)$^-$ (20), oxidation of bovine cardiac cytochrome c, by Fe(CN)$_6^{3-}$ and its reversible electron transfer with FeCyt c (21, 22), reduction of oxidized cytochrome oxidase by its natural substrate FeCyt c' (23-25) and the reaction of reduced cytochrome oxidase with oxygen (26, 27). The purpose of this work is to add to this extensive list of comparison between theory and experiment the reduction of CoCyt c' by dithionite.

Sodium dithionite is the most versatile, powerful, and widely used reducing agent available to biochemists. It is almost invariably employed in the preparation of the reduced state of purified enzymes, electron transfer proteins, hemoglobins, and cofactors. It is largely through the work of Palmer and co-workers (26, 29) that the mode of action of dithionite was elucidated. However, their experiments covered rather limited ranges of pH and temperature and did not include the effect of ionic strength. One purpose of our work is to remedy this situation. Finally, the rate constants for the reduction of FeCyt c' by dithionite have been reported (30). A comparison of the dithionite reduction of FeCyt c' and of CoCyt c' from both experimental and theoretical standpoints is yet another objective of our present work.

EXPERIMENTAL PROCEDURES

Materials

Horse heart ferrocytochrome c, type VI, was from Sigma Chemical Co. Sodium dithionite from Fisher was divided into small portions in a nitrogen glove bag and each vial was sealed anaerobically. Each vial

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The abbreviations used are: FeCyt c, ferricytochrome c; CoCyt c', ferricytochrome c; CoCyt c, cobalticytochrome c; FeCyt c', cobalticytochrome c.
was used only in the preparation of one solution discarding the remainder. Sodium dithionite kept in this manner remains in a free flowing crystalline state and does not have an odor of SO₂. All other chemicals were of the highest purity commercially available and used without further purification. All solutions were prepared in glass-distilled water further deionized by a Milli Q system.

**Methods**

**Preparation of Cobalticytochrome c—Iron-free porphyrin cytochrome c** was prepared by reacting ⁵⁷⁷⁷Cyt c' with liquid anhydrous HF. After purification, cobalt ions were inserted and the resulting ⁵⁷⁷⁷Cyt c' was fractionated on Amberlite CG-50. The detailed procedures have been previously described (30-32).

**Preparation of Anaerobic Solutions—**All solutions were prepared in nitrogen filled glove bags and contained in crown top glass bottles (Ace Glass) crimp-capped with a metal cap with a self-sealing neoprene liner. The metal cap has two holes for the insertion of syringe needles. The solutions were magnetically stirred and flushed with argon which has been freed of oxygen with a chromous ion solution. The following buffers were used: pH 6, 0.1 M citrate phosphate (Na⁺); pH 7, 0.1 M phosphate (Na⁺); pH 8, 0.1 M phosphate (Na⁺); pH 9, 0.1 M borax borate (Na⁺); pH 10, 0.1 M borax borate (Na⁺); pH 11, 0.01 M NaOH plus 0.1 M KCl.

When ionic strength was varied, the solutions were 0.02 M phosphate with 0.05, 0.1, 0.5, 1, and 2 M NaCl.

All experiments were carried out with 5 μM ⁵⁷⁷⁷Cyt c' and 2.1 or 4.05 mM dithionite.

**Kinetic Measurements—**The kinetics of reaction was followed by the decrease of absorbance of the Soret maximum of ⁵⁷⁷⁷Cyt c' at 426 nm (εmax = 106.1 mm⁻¹) (32). A Cary 14 spectrophotometer was employed in this work. The reaction cell, equipped with a tiny magnetic stirring bar and capped with a rubber septum, was flushed with argon. Buffer, ⁵⁷⁷⁷Cyt c' solution, and dithionite solution were introduced in that order. Measurement commenced upon the addition of the reducing agent.

**Analysis of Kinetic Data—**The reaction of ⁵⁷⁷⁷Cyt c' with dithionite as it was for ⁵⁷⁷⁷Cyt c' (30) is clearly biphasic as illustrated by Fig. 1. This is due to the equilibrium (29, 33)

\[ \text{S}_2\text{O}_4^{2-} + \text{Cyt c}^{+} \rightleftharpoons \text{SO}_2 \text{Cyt c} + \text{SO}_4^{2-} \]

Both S₂O₄²⁻ and SO₄²⁻ act as reducing agents, the monomer being the stronger of the two (see below).

\[ \text{SO}_2 \text{Cyt c}^{+} \rightarrow \text{SO}_2 + \text{Cyt c} \]  \hspace{1cm} (2)
\[ \text{S}_2\text{O}_4^{2-} + \text{Cyt c}^{+} \rightarrow \text{S}_2\text{O}_2 \text{Cyt c}^{+} \]  \hspace{1cm} (3)
\[ \text{S}_2\text{O}_4^{2-} + \text{Cyt c}^{+} \rightarrow 2\text{SO}_2 + 4\text{Cyt c} \]  \hspace{1cm} (4)

The concentration of dithionite is 40- to 80-fold excess of Cyt c⁺. The observed rate constants for Reactions 2 and 3 are both first order. The resolution of the kinetic data is usually clean (Fig. 2). The observed pseudo-first order rate constants are related to the true second order rate constants by

\[ k_2 = k_2^{obs} \text{[S}_2\text{O}_4^{2-}]_0 \]  \hspace{1cm} (5)

and

\[ k_3 = k_3^{obs}/[\text{S}_2\text{O}_4^{2-}]_0 \]  \hspace{1cm} (6)

**Measurement of SO₂⁻ Concentration by EPR—Dithionite solutions were transferred anaerobically into an aqueous “flat” cell and closed with standard taper glass stoppers. Care was taken to position the cell in the microwave cavity reproducibly to obtain identical detector current readings. For high ionic strength solutions there is a lowering of cavity Q. A correction for the EPR signal intensity was made by comparing with the signals of 4-amino-2,2,6,6-tetramethyl-piperidinox free radical (Aldrich 16,394-5) solutions of same ionic strengths.**

**RESULTS**

**Reduction of Cobalticytochrome c with Dithionite—**Kinetics of dithionite reduction of ⁵⁷⁷⁷Cyt c’ were determined from 20°-35°C. At the higher temperatures, the two phases of reduction were well resolved; at 20°C and 22.5°C, the slower phase was not sufficiently resolved. The observed first order rate constants are given in columns 2 and 3 of Table I. The kinetic results for reactions from pH 6 to 11 are summarized in Table II; the observed rate constants being contained in columns 2 and 3. The effect of ionic strength on the rate of reduction of ⁵⁷⁷⁷Cyt c’ by dithionite is summarized in Table III. In these reactions, the rapid phase is only a small fraction of the total reaction and is not well resolved. As it will be shown below, increase of ionic strength lowers the equilibrium constant for the dissociation of S₂O₄²⁻ and the monomer concentration.

**Dissociation Equilibria of Dithionite—**Burlamacchi et al.
Reduction of Cyt c+ by Dithionite

TABLE III
Effect of ionic strength on the reduction of "Cyt c+ with dithionite

| ρ (M) | √ρ | k " × 10^-3 | k w | k c |
|-------|----|-------------|-------|
| 0.0725 | 0.269 | 8.90 | 4.24 |
| 0.135 | 0.510 | 5.40 | 2.57 |
| 0.510 | 0.714 | 5.03 | 2.40 |
| 1.01 | 1.00 | 3.98 | 1.90 |
| 2.01 | 1.42 | 3.30 | 1.57 |

TABLE IV
Effect of ionic strength on the dissociation of dithionite

| ρ (M) | √ρ | EPR intensity | [SO₂⁻] x 10^6 | K x 10^9 |
|-------|----|---------------|----------------|---------|
| 2.26 | 1.50 | 68.7 | 2.01 | 5.39 |
| 1.26 | 1.12 | 79.2 | 2.32 | 7.18 |
| 0.76 | 0.872 | 83.4 | 2.44 | 7.94 |
| 0.36 | 0.60 | 96.9 | 2.84 | 10.8 |
| 0.31 | 0.56 | 106.8 | 3.13 | 13.1 |

TABLE V
Variation of EPR intensity of SO₂ with pH

| pH | EPR intensity |
|----|---------------|
| 2.2 | 97 |
| 4.1 | 97.5 |
| 7.7 | 91.5 |
| 8.9 | 98 |
| 10.0 | 99 |
| 10.9 | 96 |
| 12.5 | 96.5 |

DISCUSSION

Biphasic Kinetics—The kinetics of reduction of Cyt c⁺ by dithionite is biphasic. This may be due to the presence of either two protein species or two reducing entities. The former is highly unlikely because other oxidation-reduction reactions of cobalt cytochrome c are monophasic, i.e. oxidation of Cyt c by FeCyt c⁺ (7), by methemoglobin mediated by phenazine methosulfate (8), by oxygen and by Fe(EDTA)⁺, unless at neutral pH Cyt c⁺ exists in two conformation and Cyt c has two conformational states. On the other hand, that SO₂⁻ is in equilibrium with SO₂⁺ is well documented (29, 33-36).

The observed dependences of rates of reduction upon pH and ρ can be readily accounted for by their effects on the equilibria. In the above mentioned oxidation reactions of Cyt c⁺, the rates are either unaffected by or slightly dependent on ionic strength. As it will be shown below, the relative rates of the fast and the slow phase are exactly to be expected from the different reduction potentials of SO₂⁻ and SO₂⁺. Finally, the biphasic reduction of Cyt c by dithionite has also been attributed to the presence of two reducing species (29).

Dissociation Equilibria of Dithionite—Equilibrium Reaction 1 does not involve proton, and should not be affected by ρH as observed over a wide ρH range. Dithionite appears to be stable under careful anaerobic conditions even down to pH 4, but decomposes rapidly at pH ≈ 2. It seems that the often mentioned instability of dithionite at acidic pH may be prevented at least down to pH 4 under certain conditions. The significant dependence of [SO₂⁻] and K on ionic strength, Figs. 3 and 4, respectively, can be readily understood. The dissociation reaction of SO₂⁻ should not be significantly affected by ρ whereas the reverse combination of SO₂⁺ would be. If we designate the rate constants and equilibrium constants at ρ and ρ ionic strength with the appropriate subscript, then

In K₁ = ln k₁ ~/ ln K₁

But according to the Debye-Hückel treatment (37)

In k₁ = ln k₁ / 1 + 2Z²α/ρ - 1 + kR

In Equation 8, Z is the charge (−1 for SO₂⁻), the particular values of parameters for water solvent at 25°C from standard Debye-Hückel treatment are α = 1.17, and κ = 0.329 Ǻ⁻¹. The R, the radius of SO₂⁺, is estimated to be 2.53 Ǻ from molecular model, and R for the dimer transition state may be approximated as 2R. The dissociation constant at ρ ionic strength is then

ln K₁ = ln k₁ ~/ ln k₁

In Figure 3, O²⁻ is decomposed to colloidal products.
Reduction of "Cyt c+ by Dithionite

The tangent of the curve of ln K versus \( \sqrt{\mu} \) (Fig. 4) has the slope given by the quantity in the parenthesis of Equation 10. If \( \sqrt{\mu} = 0.5 \), the tangent gives \( Z = -1.05 \pm 0.1 \) which is the correct value. The charges obtained at higher ionic strengths are less satisfactory; the values for \( Z \) are \(-1.2 \pm 0.1 \) and \(-1.3 \pm 0.1 \) at \( \sqrt{\mu} \) of 1.0 and 1.5, respectively. This is expected since the Debye-Hückel treatment is inappropriate for high ionic strength solutions.

Oxidation-Reduction Potentials—The oxidation-reduction potential of \( \text{S}_2\text{O}_8^{2-} \) was given to be \(-1.12 \) V (38) for

\[
2 \text{SO}_2^{2-} + 2 \text{H}_2\text{O} + 2e^- \rightarrow 4 \text{OH}^- + \text{S}_2\text{O}_8^{2-}
\]

(11)

To obtain an estimate for the oxidation-reduction potential of \( \text{S}_2\text{O}_8^{2-} \) of the process

\[
2 \text{SO}_2^{2-} + 2 \text{H}_2\text{O} + 2e^- \rightarrow 4 \text{OH}^- + 2\text{SO}_4^{2-}
\]

(12)

we note that

\[
K_o = K_1 \cdot K_2
\]

(13)

Taking the value of \( 5.12 \times 10^{-10} \) M for \( K_1 \) at the highest measured ionic strength, one obtains \( \log K_o = -47.26 \) and a value of \(-1.39 \) V for the oxidation-reduction potential of Equation 12.

Kinetics of Dithionite Reduction of "Cyt c+—The rate constants for the rapid phase are temperature-dependent whereas those for the slow phase are not (Table I). Fig. 5 is the Arrhenius plot of the first order rate constant \( k_2 \) for the slope gives an apparent activation energy of \( 16.6 \pm 0.5 \) kcal \cdot mol\(^{-1}\). Since the rapid phase is the reduction of "Cyt c+ by the monomeric \( \text{SO}_2^{2-} \), then according to Equation 5, \( \Delta E_{\text{obs}}^{\text{obs}} = \Delta E_1 = \Delta H_1 \), where \( \Delta H_1 \) is the enthalpy change for the dissociation of dithionite and \( \Delta E_2 \) is the activation energy for Reaction 2. The values of \( \Delta H_1 \) is found to be (33) \( 21.3 \pm 0.4 \) kcal \cdot mol\(^{-1}\). Therefore, \( \Delta E_2 \) is about 6 kcal \cdot mol\(^{-1}\).

The values of rate constants \( k_1 \) were calculated with Equation 5 given in column 5 of Table I. Column 6 of the table lists values of \( k_2 \), calculated with Equation 6. The Arrhenius plot for \( k_2 \) (Fig. 6) gives an activation energy \( E_2 \) of 6 \pm 1 kcal \cdot mol\(^{-1}\), in agreement with the above estimate.

The results of experiments in Table II show the effect of pH on the rate of dithionite reduction of "Cyt c+ were obtained with various buffers given in the experimental section. These buffers have different ionic strengths. To calculate \( k_2 \) from the observed rate constants of the rapid phase, the value of \( K_1 \) for a particular ionic strength was obtained from the data in Fig. 4. The variation of \( k_2 \) and \( k_3 \) with pH are shown in Fig. 7. Both reactions have maximum rates at about pH 9.3 \pm 0.1 which are about 2 to 3 times faster than the rates at neutral pH. The self-exchange reaction rate of native cytochrome c is fastest at about pH 9.8 which is about 4 times greater than the rates at neutral pH (13). In this case, the pH effect was attributed to the isoelectric point for "Cyt c which is at about pH 10. It is said that the protein molecules are electrically neutral, whereas at other pH values the like charged molecules repel each other. This interpretation is inapplicable to the dithionite reduction of "Cyt c+ where the reacting molecules are oppositely charged. Also, reactions involving the reduced state of cobalt cytochrome c have maximum rates at about pH 7.0. This includes the autoxidation of "Cyt c+ and the mediated reduction of methemoglobin by "Cyt c (8). It seems, therefore, the pH effect on \( k_2 \) and \( k_3 \) may be related to some ionizable group in the oxidized molecule.

Optical spectroscopic titrations of "Cyt c+ (39), particularly in the 695 nm region, have shown a heme-linked protonic ionization with a pK of about 9.3. There are also changes in the thermodynamic properties at this pH (40, 41). On the other hand, NMR experiments have shown that the contact-shifted proton resonance of the methyl group of Met-80 disappears with a pK of about 9 (42) or as the azide "Cyt c+ complex is formed (43). Finally, the EPR g values of "Cyt c+ change with increasing pH, again with a pK of about 9, and the EPR spectrum of the major species at alkaline pH is consistent with methionine having been replaced by an \(-\text{NH}_2 \) function (44). This ligand change does not alter the spin multiplicity of "Cyt c+ according to EPR and optical criteria.
It has been postulated (28, 42) that the major species of \( \text{FeCyt c}^+ \) formed in the pH 9.3 transition is coordinated in the ε-amine group of Lys-79.

In contrast of \( \text{FeCyt c}^+ \), the stable species of both \( \text{FeCyt c} \) and \( \text{CoCyt c} \) has Met-90 as the sixth ligand in the pH range of 4 to 12. Neither the optical absorption spectrum (39, 45) nor the contact-shifted methyl proton resonance of Met-90 (42) is sensitive to pH values less than 12. In the case of \( \text{CoCyt c} \), EPR spectra (32, 46) showed that between pH 4 and 11, \( \text{CoCyt c} \) has Met-90 and His-16 as the axial ligands. Above pH 11, \( \text{CoCyt c} \) is five-coordinated having only His-16 as the axial ligand; below pH 4, \( \text{CoCyt c} \) is also five-coordinated but has Met-80 as the axial ligand.

Lambeth et al. (28) proposed that below pH 9, reduction of \( \text{FeCyt c}^+ \) occurs by

\[
\text{Lys-79} \quad \text{Met-80} \quad \text{Lys-79} \quad \text{Met-80} \quad \text{Fe}^{3+} + e^- \Rightarrow \text{Fe}^{2+}
\]

Above pH 9.3, it is the Lys-79 ligated species which is reduced,

\[
\text{Lys-79} \quad \text{Met-80} \quad \text{Lys-79} \quad \text{Met-80} \quad \text{Fe}^{2+} + e^- \Rightarrow \text{Fe}^{3+}
\]

This species cannot be reduced by either ascorbate (47, 48) or ferrocyanide (49). Once reduced, further substitution occurs with Met-80 replacing Lys-79. If this interpretation is accepted for the reduction of \( \text{CoCyt c} \), then our results show that the species with coordinated Lys-79 is more easily reduced than that having coordinated Met-90.

The fact that increase of pH from 6 to 9.3 increases \( k_2 \) and \( k_3 \) by only 2- to 3-fold was somewhat surprising. According to Equations 11 and 12, the midpoint potential at pH 9.3 should be 0.39 V more negative than that at pH 6. The main driving force in the theory of vibronically coupled electron tunneling is the potential difference between the electron donor and acceptor molecules (see below). It is estimated that dithionite reduction of \( \text{CoCyt c}^+ \) should be faster at pH 9.2 by some 30-fold over that at pH 6.0 based on the difference in the oxidation-reduction potential of dithionite at these pH values.

The oxidation-reduction potential of native cytochrome c is insensitive to pH (50) and the same may be assumed for cobalt cytochrome c. A possible explanation is that the rate-determining step is Reactions 2 and 3, and that the subsequent reactions of \( \text{SO}_2^- \) and \( \text{SO}_4^{2-} \) with \( \text{OH}^- \) are rapid.

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The ionic strength on the rate of Reaction 3 is shown as a plot of \( k_2 \) versus the square root of pH (Fig. 8). The plot is markedly curved. To calculate the effective active site charge for \( \text{CoCyt c}^+ \), Equation 8 has to be rewritten to take into account that the reacting molecules are not the same. The expression is

\[
\ln k_2^* - \ln k_2 = \left[ \frac{Z_i^2}{1 + \kappa R_i} + \frac{Z_j^2}{1 + \kappa R_j} - \frac{(Z_i + Z_j)^2}{1 + \kappa R_i} \right] \alpha \sqrt{\mu}
\]

where subscript 1 (2) refers to dithionite (\( \text{CoCyt c}^+ \)). If one takes the slope at \( \sqrt{\mu} = 0.3 \text{ M}^{1/2} \) of Fig. 8, i.e. low ionic strength at which Equation 16 is more apt to be valid, and using \( Z_i = -2 \), \( R_i = 5 \) Å, and \( R_j = 17 \) Å (51), a value of \( Z_j = +1.7 \) is obtained for the protein molecule.

The effective active site charge of a protein molecule is a small fraction of its full charge which in the case of \( \text{CoCyt c}^+ \) would be +7.5 from the sequence data (51) is often noted.

**Fig. 8.** Effect of \( \mu \) on the rate constant of \( \text{CoCyt c}^+ \) reduction by \( \text{S}_4\text{O}_6^{2-} \); data from Table III.
Assuming the $k$ values to be the same for the wave functions with and without the electron, and using the classical probability distribution, Hopfield derived the rate of electron transfer between two fixed sites as

$$k_{ab} = (2\pi/h^2) |T_{ab}(r)|^2 \left(2\pi e^0 \right)^{1/2} \exp \left(-\left(\Delta E_s - E_s - \Delta E_b \right)/k_BT\right)$$  \hspace{1cm} (20)

where

$$\sigma^2 = (h_0 a_s^2/2) k_BT_a \coth (T_a/2T) + (h_0 a_b^2/2) k_BT_b \coth (T_b/2T)$$  \hspace{1cm} (21)

and

$$\Delta = k_0 a_s^2/2 + h_0 a_b^2/2$$  \hspace{1cm} (22)

In equation 21, $k_0$ is the Boltzmann's constant, $T$ is temperature in degree Kelvin, $k_BT_a$ ($k_BT_b$) is equal to $homega_a$ ($homega_b$), the energy separation between the nuclear harmonic oscillator states for site $a$ ($b$), and $\Delta$ is the vibronic coupling parameter. At the limit of high temperature, Equation 20 becomes

$$k_{ab} = (2\pi/h^2) |T_{ab}(r)|^2 \left(4\pi k_BT\Delta\right)^{-1/2} \exp \left[-(E_a - E_s - \Delta E_b)/k_BT\right]$$  \hspace{1cm} (23)

Jortner (2) formulated a complete quantum mechanical treatment of electron transfer incorporating the effect of both low frequency medium modes (10 to 100 cm$^{-1}$) and high frequency molecular modes (300 to 3000 cm$^{-1}$). The nonadiabatic multiphonon electron transfer process occurs between the vibronic levels

$$\sigma > = \psi_a X_a \omega (Q) \text{ and } \sigma \omega > = \psi_b X_b \omega (Q)$$

where $\psi$ is the electronic wave function and $X$ is the nuclear wave function. The transition probability is given by the perturbation theory to be

$$k_{ab} = h^{-1}|T_{ab}(r)|^2 \exp \left[-\sum_i \left(\delta_i^2/2\right)(2\bar{v}_i + 1)\right]$$  \hspace{1cm} (24)

$$\times \int_{-\infty}^{\infty} \exp(-i\Delta \omega t) \sum_i \left\{ \exp(i\delta_i \omega t) + 1 \right\} \delta_i \exp(-i\delta_i \omega t) dt$$

$$\bar{v}_i = [\exp(homega/k_BT) - 1]^{-1}$$  \hspace{1cm} (25)

and

$$\delta_i = (\mu_i \omega_i/k_BT)$$  \hspace{1cm} (26)

Physically, $\bar{v}_i$ denotes the mean thermal population of the $i$th mode, and $\delta_i$ is the dimensionless nuclear displacement. The effective electron-phonon coupling strength is defined as $S = \sum_i (\delta_i/2)$. Equation 24 assumes simplified forms depending upon the magnitudes of $k_0 T_a$ as compared to the energy of the solvent and the molecular phonon modes. For our purpose only the high temperature limiting form is needed. Under these conditions, Equation 6 becomes

$$k_{ab} = (2\pi/h^2) |T_{ab}(r)|^2 \left(2\pi Sh < \omega >/k_BT\right)^{-1/2} \exp \left[-(E_a - E_s - \Delta E_b)/4Sh < \omega >\right]$$  \hspace{1cm} (27)

Therefore, the theories of Hopfield and Jortner are equivalent at the high temperature limit with $\Delta = Sh < \omega >$ aside from a difference of a factor of $1/\sqrt{2}$.

Hopfield approximated the tunneling matrix element as

$$T_{ab}(r) \approx \frac{2.7}{(N_a N_b)^{1/4}} \exp(-0.72 r)$$  \hspace{1cm} (28)

The characteristic temperatures $T_a$ and $T_b$ were assumed to be the same (350 K) which is about twice the temperature marking the transition from the temperature-independent to thermally activated electron transfers.

The unimolecular expressions 25 and 27 may be extended to describe bimolecular electron transfers. When the relative location of the donor and acceptor varies with time, the rate of transfer can be calculated by suitably averaging the rate as a function of distance over the probability distribution of geometries. If one neglects naively a particular geometry for electron transfer, then the bimolecular rate can be expressed as

$$k_{ab} = 6.023 \times 10^{-4} kL (2\pi k^2 R/R_p)$$  \hspace{1cm} (29)

where $\lambda$ is the characteristic decay constant defined as $\lambda = (1/0.72) = 0.7 \, \lambda$, and for hemeproteins, $r$ is the distance of closest approach between the carbon atoms on the exposed edge of the hemes of the two protein molecules. The activation parameters can be shown to be

$$\Delta H^\ddagger = k_BT_a \left(2\pi \lambda^2 R/R_p\right)$$  \hspace{1cm} (30)

$$\Delta S^\ddagger = R \ln \left(\frac{2.38 \times 10^{-2}}{k_BT} \frac{(2\pi \lambda^2 R/R_p)}{R_p} \right)$$  \hspace{1cm} (31)

The physical significance of some of the parameters in Equations 19 to 31 is seen with the aid of Fig. 9. Electron transfer occurs from one vibronic state of $a$ to another vibronic state of $b$ indicated by the arrow. The form of the vibrations is assumed to be simply that of a harmonic oscillator. Therefore, $\frac{1}{2} h_0 q^2$ in Equation 22 is just the vibrational potential energy with $h$ and $q$ being the force constant and displacement from equilibrium nuclear position, respectively. The vibronic parameter $\Delta$ is then the sum of the vibrational potential energies of states $a$ and $b$.

Although $\Delta$ appears to be a curve-fitting parameter as its value was first estimated by Hopfield (1) and Jortner (2), it can actually be determined experimentally. At the high temperature limit, $\Delta$ can be obtained directly from the enthalpy of activation with Equation 30. Furthermore, theory predicts (55) a charge transfer absorption with energy

$$h\nu = E_a - E_b - \Delta$$  \hspace{1cm} (32)

This absorption was found at the expected frequency by excitation modulation technique for the cytochrome $c$-$Fe(CN)_6$ complex (55).
The results of calculation using Equations 29 to 31 are summarized in Table VI. In row 2, the vibronic parameters are the same as used in previous applications of the theory (1, 7-9, 55), which is 1 eV for reactions of iron hemoproteins and 1.5 eV for reactions involving cobalt containing molecules. The component distance of closest approach is 2 Å for the cytochrome c since its heme edge is situated by this distance from the surface of the molecule (51); \( r_b \) is from molecular models. The potentials in row 4 of the table has been corrected to pH 8 and for one-electron processes. \( N_s \) is the number of \( \pi \)-electrons of the porphyrin system and \( N_0 \) are those non-bonded \( \sigma \) hybrid electrons of the reductants. The calculated and experimental rate constants for \( SO_2^- \) reduction (rows 7 and 8, columns 3 and 5) are in excellent agreement. The same can be said for the activation enthalpies and entropies for the \( SO_2^- \) reduction of \( \text{FeCyt c}^- \). No experimental activation parameters have been reported for the corresponding reaction for \( \text{Cyt c}^- \). The agreement between theory and experiment for the reactions involving \( \text{FeCyt c}^- \) is within a factor of 2.6. It is only for the \( SO_2^- \) reduction of \( \text{FeCyt c}^- \), that theory and experimental rate constants are in poor agreement differing by some 20-fold.

The vibronic coupling parameters for oxidation-reduction reactions involving cobalt ions are all significantly greater than those of iron complexes and enzymes. According to the model given above, cobalt complexes probably have larger force constants than iron complexes and enzymes. The infrared spectra band assignments for metal complexes are underway.

In conclusion, the vibronic or electron tunneling theory seems to constitute a good model for the description of electron transfers involving biological molecules in solution. Further studies of electron transfer reactions of proteins are continued in our laboratories.

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Table VI

| Reaction | \( \text{FeCyt c}^-/\text{SO}_2^- \) | \( \text{Cyt c}^-/\text{SO}_2^- \) | \( \text{FeCyt c}^-/\text{SO}_4^{2-} \) | \( \text{Cyt c}^-/\text{SO}_4^{2-} \) |
|----------|-----------------|-----------------|-----------------|-----------------|
| \( \Delta, \text{eV} \) | 1.0 | 1.5 | 1.5 | 1.5 |
| \( r_b(r_b) \), Å | 2 (5.00) | 2 (2.53) | 2 (5.00) | 2 (2.53) |
| \( E_b(E_b) \), | +0.26 (-0.206) | +0.26 (-0.341) | -0.14 (-0.206) | -0.14 (-0.341) |
| \( N_s(N_s) \) | 20 (28) | 20 (15) | 20 (28) | 20 (15) |
| \( |T_{ab}|, \text{eV} \) | 5.7 x 10^{-5} | 5.7 x 10^{-5} | 5.7 x 10^{-5} | 5.7 x 10^{-5} |
| \( k \) (calcd), kcal mol^{-1} | 0.8 | 0.8 | 0.8 | 0.8 |
| \( k \) (exp), kcal mol^{-1} | 20.9 | 20.9 | 20.9 | 20.9 |
| \( \Delta S^\circ \) (calcd), kcal mol^{-1} | -23 | -23 | -23 | -23 |
| \( \Delta S^\circ \) (exp), kcal mol^{-1} | n.d. | n.d. | n.d. | n.d. |

* Not determined.
Reduction of $\text{Cyt c}^+$ by Dithionite

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