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Cytochrome *c* Peroxidase Catalyzed Oxidations of Substitution Inert Iron(II) Complexes

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Abstract: The kinetics of the reduction of compound II of cytochrome *c* peroxidase as a function of temperature have been determined for a variety of iron(II) reductants of varying redox potential. A linear free-energy relationship has been obtained between free-energy changes for the reactions and the activation free energies consistent with the Marcus theory for outer-sphere electron transfer. Application of the Marcus theory to these reactions has permitted a calculation of the previously unknown redox potential for the compound II, native enzyme couple, and the homonuclear electron self-exchange rate between native enzyme and compound II. The values so obtained are 1.087 V and 7.5×10^{-5} M^{-1} s^{-1}, respectively, at pH 5.26 and 25 °C. The activation free-energy barrier for this self-exchange process is 23.2 ± 0.2 kcal mol^{-1}. The kinetics of the peroxidase-catalyzed oxidation of ferrocytochrome *c* can be accommodated by the Marcus theory as well if a more stable precursor complex is assumed for the cytochrome *c*–cytochrome *c* peroxidase reaction than for the reaction between the small inorganic complexes and the enzyme.

Cytochrome *c* peroxidase[1] is an enzyme present in yeast mitochondria which catalyzes the oxidation of ferrocytochrome *c* to ferricytochrome *c* by hydrogen peroxide according to the reaction sequence

$$\text{CcP} + \text{H}_2\text{O}_2 \xrightarrow{k_1^{app}} \text{CcP-I}$$

$$\text{CcP-I} + \text{cyt } c'' \xrightarrow{k_2^{app}} \text{CcP-II} + \text{cyt } c''' \qquad (1)$$

$$\text{CcP-II} + \text{cyt } c'' \xrightarrow{k_3^{app}} \text{CcP} + \text{cyt } c'''$$

Cytochrome *c* peroxidase, which has been the subject of a

recent review,[2] has a molecular weight of approximately 34 100 daltons and contains an iron(III) protoporphyrin IX moiety. Compound I of CcP (CcP-I in eq 1) is oxidized 2 equiv above the native state and is considered to be in an iron(IV) state with the second oxidizing equivalent residing in radical form on the protein. Compound II (CcP-II in eq 1) is formed by the one-electron reduction of CcP-I and is also considered to be primarily an iron(IV) specie.[2]

In order to investigate the mechanism of reaction 1 in more detail, this laboratory has been concentrating initially on the reactions of CcP with various inorganic substrates. Studies have previously appeared on the ferrocyanide[3,4] and dicyanobis(1,10-phenanthroline)iron(II)[5] reductions of CcP-I and CcP-II. This report extends the studies with these reductants to other temperatures and also investigates the reactions of CcP-catalyzed oxidations of other iron(II) mono-, bis-, and tris(1,10-phenanthroline) and 2,2′-bipyridine complexes.

Reductants of the type used here are commonly employed in studies on the applicability of the relative Marcus theory[6] to outer sphere electron-transfer reactions.[7] We have examined the utility of Marcus' theory to the enzymatic oxidation of the iron(II) complexes with extremely encouraging results.

Experimental Section

CcP was isolated from baker's yeast as described previously.[3] Sodium acetate buffer was employed for pH control at 5.26. Buffer concentration was 0.01 M. Ionic strength was maintained at 0.10 with potassium nitrate. Stock solutions of potassium nitrate were standardized by charging aliquots onto a column of Dowex 50W-X8 cation-exchange resin and titrating the liberated acid with standard sodium hydroxide.

$Fe(phen)_2(CN)_2 \cdot 2H_2O$, $Fe(TMphen)_3SO_4$, $Fe(DMbipy)_3(ClO_4)_2$, and 0.025 M aqueous solutions of $Fe(bipy)_3SO_4$, $Fe(phen)_3SO_4$, and $Fe(5\text{-}NO_2\text{-}phen)_3SO_4$ were purchased from the G. F. Smith Chemical Co. and used without further purification. Potassium ferrocyanide trihydrate was purchased from Matheson Coleman and Bell.

$K_2[Fe(phen)(CN)_4] \cdot 3H_2O$ was prepared from $Fe(phen)_2(CN)_2 \cdot 2H_2O$ and KCN (Matheson Coleman and Bell) according to the method of Schilt.[8]

Hydrogen peroxide was 30% Superoxol from J. T. Baker Chemical Co.

Enzyme concentrations were determined spectrophotometrically using a molar extinction coefficient of 93 mM^{-1} cm^{-1} at 408 nm.[9] Standard solutions of solid iron(II) complexes were prepared by weight. Concentrations of iron(II) complexes purchased as solutions were determined spectrophotometrically using published molar extinction coefficients.[10]

Kinetic experiments were performed under pseudo-first-order conditions using a large excess of reductant. Reaction progress was followed on either a Cary 15 recording spectrophotometer or a Durrum-Gibson stopped-flow spectrophotometer. Studies were carried out at approximately 5, 10, 15, 20, and 25 °C.

Enzyme compound I was formed just prior to kinetic runs by adding a slightly less than stoichiometric amount of hydrogen peroxide. Enzyme concentrations of 4–5 μM were employed for studies on the Cary 15 and concentrations of 0.4–0.5 μM were employed for stopped-flow studies. Progress of the reactions were followed at either 414.5 or 424 nm where disappearance of CcP-I and CcP-II was observed.

Three of the reductants employed in these studies, $Fe(phen)_2(CN)_2$, $Fe(TMphen)_3^{2+}$, and $Fe(DMbipy)_3^{2+}$, are relatively insoluble in the buffered solutions. Hence, these complexes were prepared as concentrated methanol solutions and diluted just prior to kinetic runs with buffer. The effects of methanol concentration on the rate constants for the latter two reductants were determined as previously reported for $Fe(phen)_2(CN)_2$,[5] and concentrations of methanol were maintained below the level of significant interference with the measured rates (i.e., less than a 10% reduction in the rate). For the three systems mentioned, the maximum methanol concentrations allowed were 5, 4, and 2% respectively.

Equation 2 was analyzed by utilizing a nonlinear least-squares curve-fitting program, KINET, obtained from Dr. J. L. Dye, Michigan State University.[11] The error limits reported are $\pm 1\sigma$.

Theory

Two broad classes of electron-transfer reactions have been recognized, so-called "inner-sphere" and "outer-sphere" electron transfers. Outer-sphere electron transfers, i.e., reactions in which no formal bond is formed bridging the oxidant and reductant, have proved amenable to theoretical treatment. The theory of Marcus[7] has enjoyed considerable success in predicting the rate parameters of many electron-transfer reactions of the outer-sphere type. Marcus has formulated a theoretical treatment capable of calculating rate constants from basic nonkinetic parameters, the "absolute Marcus theory".[12] However, a simpler approach to the calculations is possible in some cases by use of the "relative Marcus theory".[12]

The basic equation of the relative Marcus theory is the cross relation[7,12,13] (eq 2)

$$k_{12} = (k_{11}k_{22}K_{12}f)^{1/2} \tag{2}$$

where k_{12} is the rate constant for the electron-transfer reaction of interest, k_{11} and k_{22} are the rate constants for the homonuclear electron self-exchange processes of the reductant and oxidant, K_{12} is the equilibrium constant for the redox reaction, and f is given by eq 3

$$\ln f = \frac{(\ln K_{12})^2}{4 \ln (k_{11}k_{22}/Z^2)} \tag{3}$$

where Z is the collision frequency of two molecules in solution ($\sim 10^{11}$ M^{-1} s^{-1}). Equation 2 can also be expressed in terms of activation free energies (eq 4)

$$\Delta G_{12}^{\ddagger} = 0.5(\Delta G_{11}^{\ddagger} + \Delta G_{22}^{\ddagger} + \Delta G_{12}^{\circ} - RT \ln f) \tag{4}$$

where again the "12" subscripts refer to the redox reaction of interest and "11" and "22" subscripts correspond to the homonuclear self-exchange processes. The conditions implicit in the utilization of eq 2 and 4 have been summarized recently.[13]

Consider now the reactions of a common oxidant with a homonuclear self-exchange rate of k_{22} with a series of reductants with similar homonuclear self-exchange rates, k_{11}. If f in eq 2 is approximately 1, and electrostatic interactions are negligible, a plot of $\ln k_{12}$ vs. $\ln K_{12}$ (or equivalently, ΔE°) should be linear, and the intercept will be proportional to $\ln (k_{11}k_{22})$. If $\ln f$ is not negligible, a slight curvature will result. Similarly, from eq 4 a plot of $\Delta G_{12}^{\ddagger} + 0.5(RT \ln f)$ *vs.* ΔG_{12}° should be linear with a slope of 0.5 and an intercept of $0.5(\Delta G_{11}^{\ddagger} + \Delta G_{22}^{\ddagger})$.

Results

It has been established that electrostatic interactions between CcP-I (and CcP-II) and reductants can influence the rate of electron transfer considerably.[3] Also it has been demonstrated that the reductant is influenced by the net charge on the enzyme.[3,5] Therefore it is possible to minimize electrostatic interactions in the present study by working at the isoelectric point of CcP, i.e., a pH of 5.26.[14]

The form of the rate law for the electron-transfer processes with the various reductants was established by varying the reductant concentrations over a wide range at 25 °C while maintaining a large excess of reductant over enzyme. No reaction was observed between $Fe(5\text{-}NO_2\text{-}phen)_3^{2+}$ and the oxidized enzyme. $Fe(5\text{-}NO_2\text{-}phen)_3^{2+}$, representing the weakest reducing agent employed in these studies. $Fe(phen)_3^{2+}$ showed apparent reversible kinetic behavior, but due to the complexity and uncertainty of the nature of the oxidized forms of this reductant under conditions of low acidity,[15] analysis of the kinetic data for this system was not attempted.

The remaining reductants follow the rate law

Table I. Experimental Rate Constants and Reductant Redox Potentials

Reductant	k_3^{app}, M^{-1} s^{-1} [a]	$E°$, V (temp, °C; medium; ref)
$Fe(5\text{-}NO_2\text{-}phen)_3^{2+}$	No reaction	1.25 (25; 1 N H_2SO_4; 16)
$Fe(phen)_3^{2+}$	Reversible	1.099 (25.5; I = 0.01, pH 3.25; 17)
$Fe(bipy)_3^{2+}$	360 ± 30	1.066 (25; 0.1 M K_2SO_4, pH 5.7; 18)
$Fe(DMbipy)_3^{2+}$	4250 ± 320[b]	0.89 (20; 0.5 M KCl; 19)
$Fe(TMphen)_3^{2+}$	$(1.2 \pm 0.1) \times 10^4$	0.81 (25; 1 N H_2SO_4; 20)
$Fe(phen)_2(CN)_2$	3.4×10^4 [c]	0.806 (25; 2 M H_2SO_4; 21)
$Fe(phen)(CN)_4^{2-}$	$(8.0 \pm 0.4) \times 10^5$ [b]	0.61 (25; 1 M KCl, pH 5.2; 22)
$Fe(CN)_6^{4-}$	$(8.1 \pm 0.5) \times 10^4$ [d]	0.4103 (25; I = 0.104, pH 4.75; 23)

[a] 25 °C, I. = 0.10, pH 5.26 (0.01 M acetate). [b] 24.5 °C. [c] From ref 5. [d] From ref 3.

Table II. Experimental Activation Parameters for CcP-II Oxidations

Reductant	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, eu	$\Delta G^{\ddagger}$, kcal mol^{-1} (25 °C)
$Fe(bipy)_3^{2+}$	4.7 ± 2.5	−32 ± 9	14.1 ± 0.1
$Fe(DMbipy)_3^{2+}$	4.3 ± 0.4	−28 ± 2	12.50 ± 0.02
$Fe(TMphen)_3^{2+}$	6.6 ± 1.0	−18 ± 3	11.86 ± 0.05
$Fe(phen)_2(CN)_2$	1.8 ± 1.4	−32 ± 5	11.38 ± 0.07
$Fe(phen)(CN)_4^{2-}$	6.5 ± 1.5	−10 ± 5	9.48 ± 0.08
$Fe(CN)_6^{4-}$	3.6 ± 0.3	−24 ± 1	10.78 ± 0.02

$$-d[\text{CcP-II}]/dt = k_3^{app}[\text{CcP-II}][\text{Fe(II) complex}] \quad (5)$$

This rate law had been verified previously for two of the systems under consideration, the $Fe(phen)_2(CN)_2$[5] and the $Fe(CN)_6^{4-}$ [3,4] system. It should be noted that the reaction between the oxidized enzyme and these reductants is biphasic, showing an initial fast reaction corresponding to CcP-I reduction with a rate constant k_2^{app}, and a slower reaction corresponding to CcP-II reduction with rate constant k_3^{app}. The difficulty in obtaining good values for k_2^{app} has been alluded to before.[3] In this study, values of k_2^{app} could not be consistently obtained at all temperatures and, hence, have not been included in the results reported herein. However, in those cases where k_2^{app} was determined, $k_2^{app} \cong 3k_3^{app}$ as reported in earlier works.[3-5]

Equation 5 was verified for $Fe(bipy)_3^{2+}$ in the range 54–250 μM iron(II). For $Fe(DMbipy)_3^{2+}$, eq 5 was followed from 10 to 100 μM iron(II). For the $Fe(TMphen)_3^{2+}$ and $Fe(phen)(CN)_4^{2-}$ complexes, the corresponding concentration ranges for iron(II) were 10–50 μM and 5–30 μM, respectively. The experimental rate constants are summarized in Table I.

In addition to the electron-transfer reaction, which causes a decrease in the absorbance of the enzyme at 424 nm, all systems investigated exhibited a reaction which increased the absorbance at 424 nm. In most cases, this reaction was either too slow or too fast to interfere with observation of the redox reaction. In those few cases where this reaction interfered with the observation of the electron-transfer process, a change of wavelength to 414.5 nm resulted in the masking of this reaction. The exact nature of this reaction has not been ascertained at this time; however, it appears to correspond to a complexation between the iron(II) reductant and native CcP. Studies with $Fe(bipy)_3^{2+}$ and native CcP show a similar absorbance increase on the approximate time scale of the effect observed in the redox kinetic studies. Previous investigations indicate ferrocyanide binds to the enzyme with an association equilibrium constant of 5.3×10^2 M^{-1} at pH 6.3.[3]

In order to apply the relative Marcus theory in the form of eq 2, it is necessary to know the redox potentials ($E°'$) of the reductants employed under our experimental conditions, i.e., a pH of 5.26 and an ionic strength of 0.1. The majority of $E°'$ determinations for this class of complexes has been obtained in concentrated acid. However, we have been able to find $E°'$ values corresponding roughly to our experimental conditions for many of the systems of interest (see Table I). A few of the redox potentials referenced in Table I have been determined at a variety of pH values. In such cases, little variation in $E°'$ is found over the intermediate pH range.[22,23]

With the rate constants and $E°'$ values given in Table I, eq 2 reverts to an equation with two unknowns, $k_{11}k_{22}$ and $E°'$, for the CcP-II, CcP couple. Utilizing eq 2 in the form

$$\ln k_{12} = 0.5\left[\ln (k_{11}k_{22}) + \frac{2.303n\Delta E°'}{0.05916} + \frac{\left(\frac{2.303n\Delta E°'}{0.05916}\right)^2}{4[\ln (k_{11}k_{22}) - 2\ln Z]}\right] \quad (6)$$

the values for $\ln (k_{11}k_{22})$ and $E°'$ were obtained using the nonlinear least-squares program KINET (see Experimental Section). The values obtained from such an analysis are $\ln (k_{11}k_{22}) = 10.12$ and $E°'$ (CcP-II,CcP) = 1.087 V. Figure 1 shows a plot of the experimental $\ln k_3^{app}$ ($\ln k_{12}$ in eq 6) vs. $E°'$ for the reductant compared to those calculated for $\ln k_3^{app}$ using the values given above. Note that the $\ln k_3^{app}$ for the ferrocyanide reaction was not included in these calculations since its homonuclear exchange rate is slower than that of the other reductants.[24]

The activation parameters for the various systems under study were determined from temperature studies between 5 and 25 °C. A few studies were performed at 30 °C, but the rates obtained at this temperature were consistently low when compared with the data obtained at lower temperatures. The lower values may be due to partial enzyme denaturation at 30 °C. The experimental values for the rate constants did increase somewhat when incubation times for the enzyme at 30 °C prior to a kinetic run were reduced. Considering these difficulties, data points at 30 °C were omitted from our calculations.

The activation parameters obtained from Eyring plots of the experimental data are given in Table II. Figure 2 shows a plot of $\Delta G_{12}^{\ddagger} + 0.5(RT \ln f)$ vs. $\Delta G_{12}°$. The slope of this plot is 0.50 ± 0.03, in excellent agreement with theory, and the intercept which is equal to $(\Delta G_{11}^{\ddagger} + \Delta G_{22}^{\ddagger})/2$ is 14.5 ± 0.2 kcal mol^{-1} (see Theory).

Discussion

One of the most difficult tasks in applying the relative Marcus theory is the determination of the homonuclear exchange rates for the two reactant complexes. No direct measurements of the exchange rate have been made for any of the reductants appearing in Figure 1 in aqueous solution. However, the good correlation shown in Figure 1 suggests that the ex-

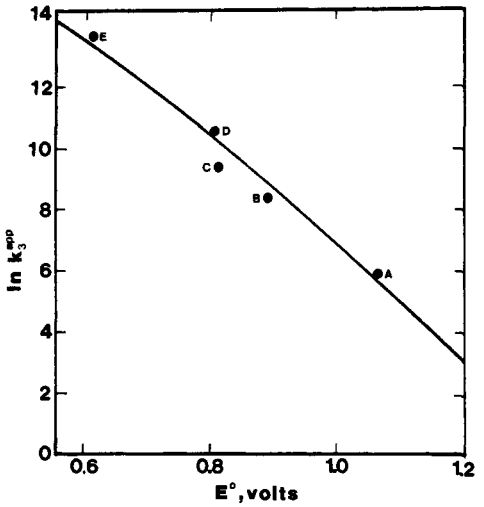

Figure 1. Graph of logarithms of second-order rate constants for electron transfer vs. the redox potentials of the iron(II) reductants compared to the calculated curve when ln $(k_{11}k_{22})$ = 10.12 and $E^{\circ\prime}$ for CcP, CcP-II = 1.087 V. A, $Fe(bipy)_3^{2+}$; B, $Fe(DMbipy)_3^{2+}$; C, $Fe(TMphen)_3^{2+}$; D, $Fe(phen)_2(CN)_2$; E, $Fe(phen)(CN)_4^{2-}$.

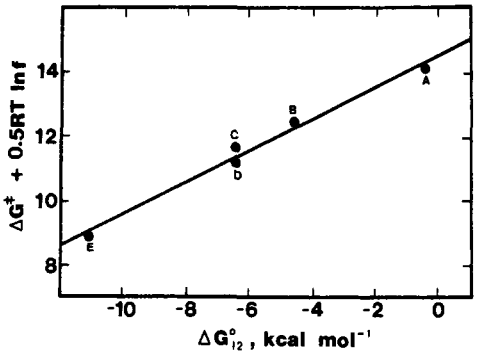

Figure 2. Graph of $\Delta G^{\ddagger} + 0.5RT \ln f$ vs. the free-energy change for the redox reaction compared to a least-squares line. A, $Fe(bipy)_3^{2+}$; B, $Fe(DMbipy)_3^{2+}$; C, $Fe(TMphen)_3^{2+}$; D, $Fe(phen)_2(CN)_2$; E, $Fe(phen)(CN)_4^{2-}$.

change rates are essentially the same for all the reductants. In addition, it is expected that the exchange rates would have values similar to that of the closely related complex $Fe(phen)_3^{2+}$. The homonuclear exchange rate of $Fe(phen)_3^{2+/3+}$ has been determined at 25 °C in saturated Na_2SO_4 with a reported value of $(3.3 \pm 1.4) \times 10^8$ M^{-1} s^{-1}.[25] Utilizing this value for k_{11} and the value of 10.12 for ln $(k_{11}k_{22})$ determined from the Marcus theory correlation, Figure 1, a value for k_{22} (the homonuclear exchange rate for the CcP, CcP-II couple) of 7.5×10^{-5} M^{-1} s^{-1} at 25 °C and pH 5.26 is obtained.

An independent check on the values of k_{22} and $E^{\circ\prime}$ for the CcP, CcP-II couple, determined by using the Marcus theory correlation on the reaction rates with the phen and bipy iron complexes, can be made using the ferrocyanide data. Utilizing the value of k_{22} and $E^{\circ\prime}$ for the CcP, CcP-II couple determined above, $E^{\circ\prime}$ for the $Fe(CN)_6^{4-/3-}$ couple (see Table I), and k_{11} for the ferro-ferricyanide exchange at 25 °C (calculated from ref 24 as 7×10^4 M^{-1} s^{-1}), a value of 2×10^5 M^{-1} s^{-1} can be calculated from eq 6 for k_3^{app} for the reduction of CcP-II by $Fe(CN)_6^{4-}$. This value is within a factor of 3 of the experimentally determined value, 8.1×10^4 M^{-1} s^{-1}.

Similarly, the intrinsic free-energy barrier to electron exchange between CcP-II and CcP may be obtained from the intercept of Figure 2 by again utilizing the data of Ruff[25] for $Fe(phen)_3^{2+/3+}$. The value quoted above for the homonuclear exchange rate of the tris-phen system yields a value of 5.82 kcal mol^{-1} for $\Delta G_{11}^{\ddagger}$. Therefore, from the intercept of Figure 2, a value of 23.2 ± 0.2 kcal mol^{-1} is indicated for $\Delta G_{22}^{\ddagger}$, the intrinsic free-energy barrier for CcP-II, CcP self-exchange.

Again we may perform an independent check of the validity of this calculated value using eq 4 and the data of Shporer et al. for the $Fe(CN)_6^{4-}$ system. Reference 24 quotes a value of 10.9 kcal mol^{-1} for $\Delta G_{11}^{\ddagger}$. Therefore, eq 4 yields a value of 10.3 kcal mol^{-1} for $\Delta G_{12}^{\ddagger}$ in comparison to the experimental value of 10.8 kcal mol^{-1} (Table I).

Some comment on the extremely slow homonuclear self-exchange rate for CcP-II, CcP of 7.5×10^{-5} M^{-1} s^{-1} seems appropriate. Since no direct measurements of self-exchange rates for Fe(IV,III) porphyrin systems have been reported, we cannot say if this type of behavior is typical. However, there are data in the literature sufficient to make a Marcus theory calculation for the horseradish peroxidase system (HRP). The isoelectric point of HRP is 8.7.[26] From the kinetic data of Dunford et al.[27,28] in the vicinity of the isoelectric point, an average value of 8.2×10^3 M^{-1} s^{-1} for the HRP-II + $Fe(CN)_6^{4-}$ rate constant is obtained.[29] The E° for the HRP-II, HRP couple has been reported as ~1.0 V.[30] $E^{\circ\prime}$ for the $Fe(CN)_6^{4-/3-}$ couple at pH 9, 25 °C, and I = 0.1 is 0.4075 V.[23] Therefore, from eq 6 a value of 9×10^{-7} M^{-1} s^{-1} is calculated for the homonuclear self-exchange rate between HRP-II and HRP.

The only study of iron(III,IV) electron exchange to appear in the literature is that of Palazzotto and Pignolet on the tris(dithiocarbamato)iron(III,IV) complexes.[31] In this study electron exchange between the low-spin iron(IV) state and an equilibrium mixture of high- and low-spin iron(III) was very rapid with k on the order of 10^8 M^{-1} s^{-1}. The authors note that electron exchange between an entirely high-spin iron(III) complex and low-spin iron(IV) is slower by approximately an order of magnitude. The iron in CcP is a mixture of high- and low-spin iron(III) in thermal equilibrium at 25 °C and pH 5.[32] The slow self-exchange rates for the peroxidases imply that an additional contribution to the energy barrier is present with the peroxidases that is absent in the tris(dithiocarbamato)iron systems. Studies with labeled peroxide[33] indicate that an oxygen is incorporated into the coordination sphere of the iron in the oxidized enzyme.[34] The difference in exchange rate between the peroxidases and the dithiocarbamato complexes may be due to modifications in the sixth coordination site of the iron in the enzyme accompanying electron exchange.

A very rapid rate of electron transfer has been reported between cyt c'' and the oxidized enzyme intermediates.[35] At pH 6.0 this rate is $5\text{-}6 \times 10^8$ M^{-1} s^{-1}. Again eq 6 has been applied in order to calculate this rate. Using an $E^{\circ\prime}$ of 0.261 V[36] and a self-exchange rate of 1×10^3 M^{-1} s^{-1} [37] for the ferro-ferricyt c system, a value of 2×10^5 M^{-1} s^{-1} is calculated for k_{12}. The large discrepancy between the calculated and observed values could be due to errors in some of the values employed in this calculation. For instance, the value used for the homonuclear exchange rate for CcP of 7.5×10^{-5} M^{-1} s^{-1} is probably only good to within an order of magnitude. Also a value for the ferro-ferricyt c homonuclear exchange rate of 5×10^4 M^{-1} s^{-1} has been reported by Kowalsky.[38] However, using this value for k_{11} in eq 6 still gives a calculated value for k_{12} lower than the experimental value by more than two orders of magnitude. One explanation for this discrepancy would be that the reaction between cyt c'' and the oxidized intermediates of CcP may proceed by an entirely different and more energetically favorable mechanism than the simple outer-sphere process considered here.

However, the cyt$''$ case can be explained with equal satisfaction if a difference in the stability of the collision complex prior to electron transfer (the precursor complex) is assumed for cyt c with respect to the other reductants. If such a situation

is realized, then according to Marcus and Sutin[13] eq 2 takes the form of eq 7

$$k_{12} = (P_{12}P_{21}k_{11}k_{22}K_{12}f/P_{11}P_{22})^{1/2} \quad (7)$$

where P_{11} and P_{22} are the stability constants of the precursor complexes for the homonuclear reactions, and P_{12} and P_{21} are the stability constants of the precursor complex and successor complex for the heteronuclear electron-transfer reaction, respectively. Due to the excellent correlations obtained without such considerations for the kinetic behavior of the five reductants of Figure 1 and ferrocyanide, these six species must exhibit a relatively constant value for the $P_{12}P_{21}/P_{11}P_{22}$ term. If it can be assumed that the association equilibrium constants of the various reductants with CcP are proportional to $(P_{12}P_{21}/P_{11}P_{22})^{1/2}$, then the cyt c'' data can be correlated. The association equilibrium constant for the binding of ferrocyanide and ferrocytochrome c to CcP has only been determined at pH 6.3[3] and 7,[35] respectively. However, the association equilibrium constants correspond well to the reciprocal of the Michaelis constants determined in the steady-state oxidation of ferrocyanide[4] and ferrocytochrome c.[35,39] The Michaelis constants have been determined over a wider range of pH and are relatively independent of pH (within an order of magnitude). At pH 5.25 the equilibrium association constants for ferrocyanide and ferrocytochrome c binding to CcP are estimated to be about 2×10^2 and 2×10^5 M^{-1}, respectively. Using these values of the equilibrium association constants, the calculated value for the ferrocytochrome c oxidation rate constant is corrected upward to 2×10^8 M^{-1} s^{-1} in comparison to the experimentally determined rate of 5×10^8 M^{-1} s^{-1}. These results suggest that complex formation may be very important in determining the rate of biological electron-transfer reactions.

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References and Notes

(1) Abbreviations appearing in the text are cytochrome c peroxidase = CcP; primary and secondary oxidized forms of CcP = CcP-I and CcP-II, respectively; ferro- and ferricytochrome c = cyt c'' and cyt c''', respectively; 1,10-phenanthroline = phen; 2,2′-bipyridine = bipy; 4,4′-dimethyl = DM; and 3,4,7,8-tetramethyl = TM.
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Catalysis of Internucleotide Bond Formation by Divalent Metal Ions

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Abstract: Adenosine 5′-phosphorimidazolide condensed in aqueous solution in the presence of various divalent metal ions to form short oligoadenylic acids. The effect of divalent metal ions on the synthesis of oligoadenylic acids is in the following order: $Pb^{2+} > Co^{2+} > Zn^{2+} \gtrsim Mn^{2+} > Ni^{2+} > Cd^{2+}$ $(Fe^{2+}) > Ca^{2+} \gtrsim Mg^{2+} \approx$ none $\approx Cu^{2+} \gtrsim Hg^{2+}$. When Pb^{2+} was used, the total yield of oligoadenylic acids was as high as 57%. The internucleotide linkage in the resulting pApA and pApApA were mainly 2′–5′.

An efficient procedure for the synthesis of polynucleotides from activated nucleotides in aqueous solution is very important both in prebiotic chemistry and biochemistry. Nucleoside 5′-polyphosphates are used as activated monomers in biological systems. ATP or ADP forms high molecular weight polynucleoides in aqueous solution in the presence of some enzymes. Without such enzymes, ATP and ADP hydrolyze but no polynucleotide can be obtained.

Adenosine 5′-phosphorimidazolide (ImpA) is an activated derivative of adenylic acid (pA) and hydrolyzes to pA and imidazole in the absence of a template or other catalysts.[1] In a previous communication,[2] we reported that Zn^{2+} ion cata-