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The Kinetics of Oxidation of Reduced Cytochrome c by Ferricyanide Derivatives[†]

James C. Cassatt* and Claudia P. Marini

ABSTRACT: The kinetics of oxidation of reduced horse heart cytochrome c by ferricyanide derivatives, $Fe(CN)_5X^{n-}$, where X = PPh$_3$, SCN$^-$, CN$^-$, NH$_3$, and N$_3^-$, were measured by stopped-flow techniques. The reactions were found to be first order in the concentration of both reduced cytochrome c and oxidizing agent. The rate constant for the oxidation increased as the reduction potential increased. The values were 3.0×10^7 M^{-1} sec^{-1} for $Fe(CN)_5PPh_3^{2-}$, 1.0×10^7 M^{-1} sec^{-1} for $Fe(CN)_5CNS^{3-}$, 8.0×10^6 M^{-1} sec^{-1} for $Fe(CN)_6^{3-}$, 2.5×10^6 M^{-1} sec^{-1} for $Fe(CN)_5NH_3^{2-}$, and 9.0×10^5 M^{-1} sec^{-1} for $Fe(CN)_5N_3^{3-}$. The rate of oxidation decreased markedly as the ionic strength increased and also decreased somewhat in 10% 1-propanol. The entropy of activation of about -20 eu was found to be nearly independent of the oxidizing agent but the energy of activation was found to decrease with increasing rate constant from a value of 3.5 kcal/mol for $Fe(CN)_5N_3^{3-}$ to an unusually low value of about 1 kcal/mol. The abnormally low value for the energy of activation was explained in terms of two mechanisms: one in which the oxidizing agent was first bound to the cytochrome c followed by slow electron transfer, and the other in which the cytochrome c exists in a labile equilibrium with another more easily oxidizable conformation.

Numerous investigations of the kinetics of oxidation of reduced cytochrome c by various oxidizing agents have previously been reported. Among these are several studies on the rate of oxidation of reduced cytochrome c by potassium ferricyanide over a wide range of pH, ferricyanide concentration, temperature, and ionic strength (Brandt *et al.*, 1966; Havsteen, 1965; Sutin and Christman, 1961; Morton *et al.*, 1970). There are, however, no data available on how the redox potential of the oxidizing agent affects the rate of oxidation. To study this effect of redox potential we have selected a series of ferricyanide derivatives, $Fe(CN)_5X^{n-}$, where X = azide, cyanide, triphenylphosphine, ammonia, and thiocyanate. Also included in the study are the oxidizing agents $Fe(dipy)(CN)_4^-$ and $Fe(dipy)_2(CN)_2^+$.[1] This

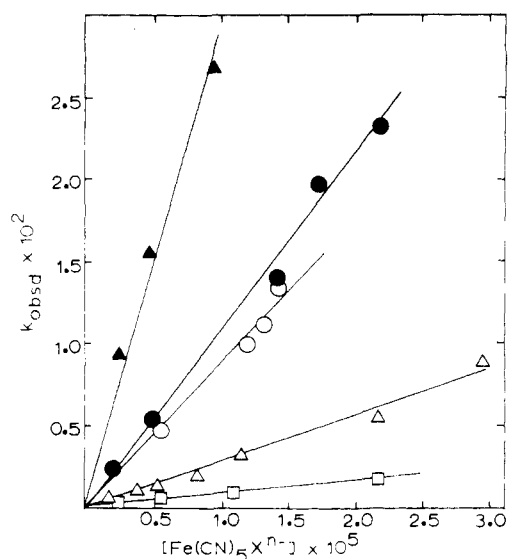[1] Abbreviations used are: dipy, α,α'-dipyridyl; PPh$_3$, triphenylphosphine.

FIGURE 1: The observed rate constant (sec^{-1}) as a function of the concentration of oxidizing agent (M) for the oxidation of reduced cytochrome c by $Fe(CN)_5PPh_3^{2-}$ (▲), $Fe(CN)_5CNS^{3-}$ (●), $Fe(CN)_6^{3-}$ (O), $Fe(CN)_5NH_3^{2-}$ (△), and $Fe(CN)_5N_3^{2-}$ (□), at 25°.

FIGURE 2: Log of the rate constant ($M^{-1} sec^{-1}$) for the oxidation of the reduced cytochrome c by $Fe(CN)_5PPh_3^{2-}$ (▲), $Fe(CN)_5CNS^{3-}$ (●), $Fe(CN)_6^{3-}$ (O), $Fe(CN)_5NH_3^{2-}$ (△), and $Fe(CN)_5N_3^{3-}$ (□), as a function of $1/T$.

series of oxidizing agents had previously been used in a study of the effect of redox potential on the rate of oxidation of ferrocyanide (Stasiw and Wilkins, 1969). With the exception of the azide and thiocyanate derivatives the reactions were shown to be outer sphere. For these reasons the ferricyanide series seemed ideal for this investigation. In view of the rather surprising observation by Morton *et al.* (1970) that the rate of reaction of reduced cytochrome c with ferricyanide is independent of temperature, we have determined the activation parameters for each of the reactions.

Experimental Section

Materials. Horse heart cytochrome c, type III, was purchased from Sigma. Reduced cytochrome c was prepared by reduction with potassium ascorbate (Wharton and Tzagoloff, 1967). Ascorbate was removed on a Sephadex G-10 column. $H[Fe(dipy)(CN)_4]$ and $[Fe(dipy)_2(CN)_2]NO_3$ were prepared by reaction of $Fe(dipy)_3^{2-}$ with cyanide followed by oxidation (Shilt, 1960). $Na_2[Fe(CN)_5NH_3]$ and $Na_2[Fe(CN)_5PPh]_3$ were prepared by reaction of sodium nitroprusside with ammonia (Brauer, 1963) or triphenylphosphine (purchased from Aldrich) (Nast and Kreiger, 1965) followed by oxidation. $Fe(CN)_5N_3^{3-}$ and $Fe(CN)_5CNS^{3-}$ were prepared *in situ* by dissolving $Na_2[Fe(CN)_5NH_3]$ in 0.01 M NaN_3 or 0.01 M $NaCNS$ and allowing these solutions to stand at least 2 hr (Jaselskis, 1961). All compounds were characterized spectrally and the spectra agreed well with literature values. Extinction coefficients used to standardize the solutions for the kinetic runs were 1450 M^{-1} cm^{-1} at 379 nm for $H[Fe(dipy)(CN)_4]$, 200 M^{-1} cm^{-1} at 544 nm for $[Fe(dipy)_2(CN)_2]NO_3$ (Shilt, 1960), 1500 M^{-1} cm^{-1} at 400 nm for $Na_2[Fe(CN)_5NH_3]$, 3700 M^{-1} cm^{-1} at 560 nm for $Fe(CN)_5N_3^{3-}$, and 2680 M^{-1} cm^{-1} at 590 nm for $Fe(CN)_5CNS^{3-}$ (Jaselskis, 1961). The spectrum of the triphenylphosphine derivative was not available; so it was standardized by reducing it with an excess of ferrocyanide and determining spectrophotometrically the concentration of ferricyanide produced using an extinction coefficient of 1000 M^{-1} cm^{-1} at 420 nm. As a further check, the rate

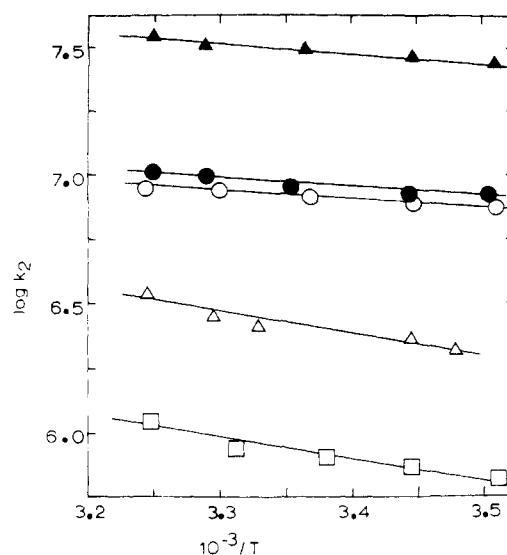constant for the oxidation of potassium ferrocyanide by the triphenylphosphine derivative was measured in excess oxidizing agent. The results were in excellent agreement with those of Stasiw and Wilkins (1969) reported earlier. All other chemicals were reagent grade or better.

Methods. The rates of all reactions were measured by following the loss of the Soret peak of reduced cytochrome c at 420 nm on an Aminco-Morrow stopped-flow apparatus. The temperature was controlled to ±0.1° with a circulating water bath. Due to the sandwich design of the mixing-observation chamber, we had difficulty avoiding leaks at reduced temperature. To circumvent this difficulty the quartz windows provided were replaced with plexiglass.

A pH of 7 was maintained with 0.01 M potassium phosphate buffer. The ionic strength was maintained at 0.1 M with added KCl. All reactions were carried out under anaerobic conditions to prevent autoxidation of reduced cytochrome c which occurred slowly at the low protein concentrations used here.

Results

All reactions, except where noted, were carried out under pseudo-first-order conditions with the oxidizing agent in at least fivefold excess. Plots of log (OD − OD_∞) as a function of time yielded good straight lines, from which the observed rate constants, k_{obsd}, were determined. The collected results are shown in Table I. Plots of k_{obsd} as a function of the concentration of oxidizing agent are shown in Figure 1 for the various oxidizing agents used in this study. The second-order rate constants for the oxidation, k_2, calculated from the slopes of the lines in Figure 1, are tabulated in Table II. The rate constants quoted are accurate to ±10%. The rates of oxidation of reduced cytochrome c by $H[Fe(dipy)(CN)_4]$ and $[Fe(dipy)_2(CN)_2]NO_3$ were so large that second-order conditions using equimolar concentrations of reactants of 1.0 × 10^{-6} M were employed. Even under these conditions the rates were barely measurable; hence the rate constants quoted in Table II should only be taken as estimates. Our value for the rate constant for the oxidation of reduced cytochrome c by $K_3Fe(CN)_6$ agrees well with those measured previously. The rate constants for all of the

TABLE I: Collected Kinetic Data.

Oxidant	Concn of Oxidant (M)	Concn of Reductant (M)	Temp (°C)	k_{obsd} (sec^{-1})
$Fe(CN)_6{}^{3-}$	5.85×10^{-6}	1.2×10^{-6}	25	47
	1.17×10^{-5}	1.2×10^{-6}	25	99
	1.30×10^{-5}	1.6×10^{-6}	25	107
	1.40×10^{-6}	5.0×10^{-7}	25	133
	8.50×10^{-6}	1.2×10^{-6}	11	62.4
	8.50×10^{-6}	1.2×10^{-6}	17	64.3
	8.50×10^{-6}	1.2×10^{-6}	24	68.3
	8.50×10^{-6}	1.2×10^{-6}	30	73.5
	8.50×10^{-6}	1.2×10^{-6}	35	74.3
$Fe(CN)_5NH_3{}^{2-}$	2.95×10^{-5}	9.0×10^{-7}	25	86
	2.20×10^{-5}	9.0×10^{-7}	25	54
	1.15×10^{-5}	9.0×10^{-7}	25	33
	7.90×10^{-6}	9.0×10^{-7}	25	19
	5.40×10^{-6}	9.0×10^{-7}	25	13
	3.80×10^{-6}	9.0×10^{-7}	25	9.9
	2.06×10^{-6}	7.0×10^{-7}	25	5.0
	6.00×10^{-6}	7.5×10^{-7}	14	12.5
	6.00×10^{-6}	7.5×10^{-7}	18	13.3
	6.00×10^{-6}	7.5×10^{-7}	27	15.7
	6.00×10^{-6}	7.5×10^{-7}	31	16.8
	6.00×10^{-6}	7.5×10^{-7}	36	20.4
$Fe(CN)_5PPh_3{}^{2-}$	9.50×10^{-6}	1.0×10^{-6}	25	267
	4.75×10^{-6}	1.0×10^{-6}	25	154
	2.40×10^{-6}	5.0×10^{-7}	25	92
	3.50×10^{-6}	7.0×10^{-7}	11	95.6
	3.50×10^{-6}	7.0×10^{-7}	17	102
	3.50×10^{-6}	7.0×10^{-7}	24	110
	3.50×10^{-6}	7.0×10^{-7}	31	112
	3.50×10^{-6}	7.0×10^{-7}	35	125
$Fe(CN)_5N_3{}^{3-}$	2.16×10^{-5}	1.0×10^{-6}	25	18.2
	1.08×10^{-5}	1.0×10^{-6}	25	9.25
	5.40×10^{-6}	1.0×10^{-6}	25	5.33
	2.16×10^{-6}	7.0×10^{-7}	25	1.77
	1.05×10^{-5}	4.7×10^{-7}	12	6.8
	1.05×10^{-5}	4.7×10^{-7}	17.5	7.7
	1.05×10^{-5}	4.7×10^{-7}	23	8.4
	1.05×10^{-5}	4.7×10^{-7}	29	9.03
	1.05×10^{-5}	4.7×10^{-7}	35	11.5
$Fe(CN)_5CNS^{3-}$	2.20×10^{-5}	4.0×10^{-7}	25	231
	1.70×10^{-5}	1.7×10^{-6}	25	198
	1.40×10^{-5}	1.7×10^{-6}	25	138
	5.00×10^{-6}	1.7×10^{-6}	25	55.6
	2.20×10^{-6}	4.0×10^{-7}	25	24.8
	5.20×10^{-6}	7.0×10^{-7}	12	43
	5.20×10^{-6}	7.0×10^{-7}	17.5	44
	5.20×10^{-6}	7.0×10^{-7}	24.5	47
	5.20×10^{-6}	7.0×10^{-7}	30.5	52
	5.20×10^{-6}	7.0×10^{-7}	35	54

reactions are observed to increase as the $E°$ values increase.

Activation energies (E_a) were determined from plots of log k_2 as a function of T^{-1} (Figure 2). The entropies of activation (ΔS^*) were determined from the equation

$$k_2 = \frac{kT}{h} e^{\Delta S^*/R - \Delta H^*/RT}$$

where k is Boltzmann's constant, h, Planck's constant, R, the gas constant, and T, the absolute temperature. These values are also shown in Table II. The activation energies are accurate to no better than ± 1 kcal/mol. In the cases of the lowest values of E_a the rate constant at 35° is only about 20% higher than that at 10°. This difference is, nevertheless, outside the error limits for the individual rate constants and represents a real difference. Furthermore, even though this difference is small, it is consistent. The fact that the variation of the rate constant with temperature is so small probably explains a previous report that the rate

TABLE II: Rate Constants and Activation Parameters for the Oxidation of Reduced Cytochrome c.

Oxidizing Agent	E^{0a}	K^b	k_2 (M^{-1} sec^{-1})^c	k_r (M^{-1} sec^{-1})^d	k_2 (Calcd)^e	E_a (kcal/mol)	ΔS^* (eu)
Fe(dipy)$_2$(CN$_2$)$^-$			1.9×10^5				
Fe(dipy)(CN$_4$)$^-$	0.55	8.13×10^4	1.6×10^5	1.9×10^3	4.3×10^8		
Fe(CN)$_5$PPh$_3$$^{2-}$	0.54	5.56×10^4	3.0×10^7	5.4×10^2	1.7×10^7	1.8	-21
Fe(CN)$_5$CNS^{3-}			1.0×10^7			1.8	-23
Fe(CN)$_6$$^{3-}$	0.40	235	8.0×10^6	3.4×10^4	7×10^5	1.7	-24
Fe(CN)$_5$NH$_3$$^{2-}$	0.33	15.4	2.5×10^6	1.6×10^5	3×10^6	3.0	-22
Fe(CN)$_5$N$_3$$^{3-}$	0.24	0.458	9.0×10^5	1.96×10^6	9×10^3	3.5	-22

^{a} Reduction potentials taken from Stasiw and Wilkins (1969). ^{b} Calculated from the $E°$ values using a value of 0.26 for the reduction potential of cytochrome c (Margalit anf Schejter, 1973). ^{c} At 25°. ^{d} The rate constant for the reduction of cytochrome c calculated from the rate constant for the oxidation and equilibrium constant. ^{e} Calculated from Marcus-Hush theory (see Discussion).

constant for the oxidation of reduced cytochrome c by K$_3$Fe(CN)$_6$ was independent of temperature (Morton et al., 1970). Because of low values of the activation energies, the values obtained for oxidation of reduced cytochrome c by the various oxidizing agents cannot be distinguished from one another. The entropy of activation within an experimental error of about 6 eu is the same (-22 eu) for all the reactions studied. Because of the difficulties in obtaining accurate rate constants, the activation parameters for the oxidation of reduced cytochrome c by H[Fe(dipy)(CN)$_4$] and [Fe(dipy)$_2$(CN)$_2$]NO$_3$ were not determined.

Kaminsky et al. (1969,1971,1972) have reported that high concentrations (5–10 mol % of methanol, ethanol, and 1-propanol) altered the conformation of reduced cytochrome c and enhanced the binding of carbon monoxide and the rate of oxidation by molecular oxygen. With these results in mind, we measured the rate of oxidation of reduced cytochrome c by Fe(CN)$_5$NH$_3$$^{2-}$ as a function of 1-propanol concentration at two pH's. The results are shown in Figure 3. The results of Kaminsky et al. (1971) are included for comparison. (Note the change in the ordinate.) Over the range of alcohol concentration where the rate of

oxidation of reduced cytochrome c by molecular oxygen increases by over 1000-fold, the rate of oxidation by Fe(CN)$_5$NH$_3$$^{2-}$ decreases about twofold, an effect probably due simply to a change in solvent composition.

The effect of ionic strength on the rate of oxidation of reduced cytochrome c by Fe(CN)$_6$$^{3-}$ and Fe(CN)$_5$NH$_3$$^{2-}$ was measured by varying the concentration of added KCl (0.02–0.1 M) at constant phosphate concentration (0.001 M) and pH (7.0). Plots of k_2 as a function of $\sqrt{I}/(1 + \sqrt{I})$ (where I is equal to the ionic strength) are shown in Figure 4. Our results agree well with the earlier results of Morton et al. (1970) which are also included. The rate constant decreases with increasing ionic strength as would be expected for the reaction between the highly positively charged reduced cytochrome c (the isoelectric point is around 10 (Barlow and Margoliash, 1966)) and the negatively charged oxidizing agent. The line shows considerable curvature, but at the higher ionic strengths, greater than 0.05 M, it is nearly linear. As expected, the more highly charged Fe(CN)$_6$$^{3-}$ ion gives a somewhat steeper slope, especially in the linear region, than the Fe(CN)$_5$NH$_3$$^{2-}$ ion.

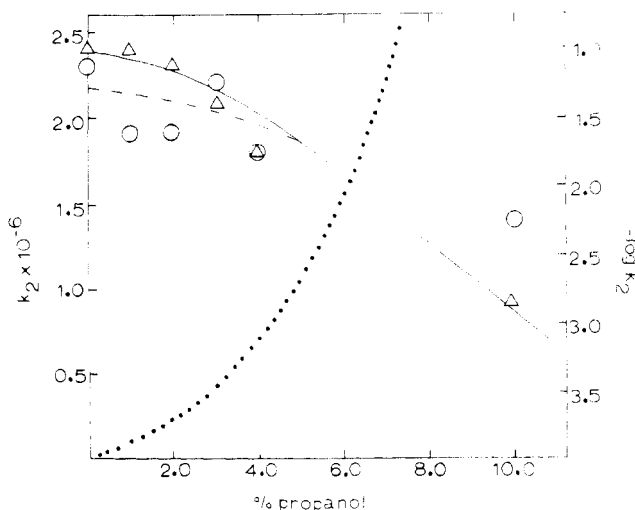Discussion

According to Marcus-Hush theory (Reynolds and

FIGURE 3: The rate constant (M^{-1} sec^{-1}) for the oxidation of reduced cytochrome c by Fe(CN)$_5$NH$_3$$^{2-}$ (left-hand ordinate) at pH 7 (O) and 5 ($\triangle$) compared to $-$log rate constant for the oxidation by O$_2$ (right-hand ordinate from Kaminsky et al. (1971)) as a function of 1-propanol concentration (mole per cent) (dotted line).
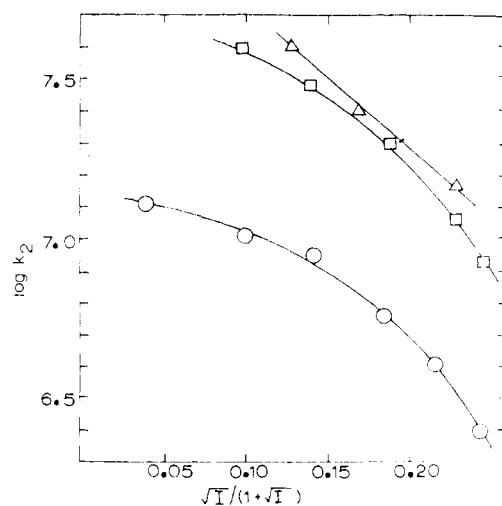

FIGURE 4: Log of the rate constant (M^{-1} sec^{-1}) for the oxidation of reduced cytochrome c by Fe(CN)$_5$NH$_3$$^{2-}$ (O) and Fe(CN)$_6$$^{3-}$ ($\square$) as a function of $I^{1/2}$ (1 + $I^{1/2}$). Also shown for comparison are the results of Morton et al. (1970) for the oxidation by Fe(CN)$_6$$^{3-}$ ($\triangle$).

Lumry, 1966), the rate constant for an outer-sphere redox reaction can be calculated from

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

where

$$\log f = (\log K_{12})^2/4 \log (k_{11}k_{22}/Z^2)$$

where k_{12} is equal to the rate constant for a redox reaction involving two reactants whose electron exchange rate constants are denoted by k_{11} and k_{22}, K_{12} is equal to the equilibrium constant for the redox reaction, and Z is a frequency factor usually taken as 10^{11}. Using the above equation, Sutin (1972) has calculated k_{12} for the reaction of ferricyanide with reduced cytochrome c. The calculated value of 1×10^6 M^{-1} sec^{-1} agrees reasonably well with the observed value of 8×10^6 M^{-1} sec^{-1}.

Using the electron exchange rate constants and E° values calculated by Stasiw and Wilkins (1969) we have calculated values of k_{12} for the oxidants used here. These values are shown in Table II. Comparison with the observed values shows that for the oxidants $Fe(dipy)(CN)_4^-$, $Fe(CN)_5PPh_3^{2-}$, and $Fe(CN)_6NH_3^{2-}$ agreement between observed and calculated values is remarkably close. For $Fe(CN)_6^{3-}$, the agreement is reasonable. However, from the calculated values one would predict that $Fe(CN)_6NH_3^{2-}$ would oxidize reduced cytochrome c faster than $Fe(CN)_6^{3-}$ due to the larger values of the electron exchange constant even though the potential is less positive. The opposite, however, is observed.
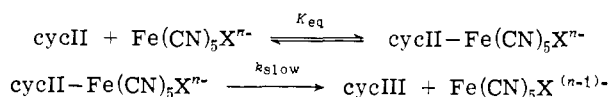
For $Fe(CN)_5N_3^{3-}$, the disparity between observed and calculated values is two orders of magnitude. With both $Fe(CN)_6^{3-}$ and $Fe(CN)_5N_3^{3-}$ the reaction occurs faster than predicted. This may be a reflection of an interaction between the negatively charged oxidant and positively charged reduced cytochrome c although some of the charge effects may be reduced by ion pair formation between K^+ and oxidant (Eaton et al., 1967).

The rate constants calculated for the reduction of cytochrome c by the appropriate ferrocyanide derivatives are given in Table II. As expected, as the reduction reaction becomes more favorable, the rate of reduction increases.

Activation Parameters. All the reactions studied here are characterized by entropies of activation of around -20 eu. This value compares well with that observed by Stasiw and Wilkins (1969) for the oxidation of ferrocyanide by the same series of compounds and is similar to that usually observed for outer-sphere redox reactions.

Because of the relatively large error inherent in measuring low activation energies meaningful comparison is impossible. Nevertheless, the observed activation energies are much less than expected for a simple bimolecular reaction. Even for diffusion controlled reactions, an activation energy of about 3.5 kcal/mol is expected (North, 1966); the rate constants for these reactions are at least two orders of magnitude less than that expected for a diffusion controlled reaction.

One possible explanation for the abnormally low activation energies is that the oxidizing agent may first form a complex with the reduced cytochrome c via a labile equilibrium and followed by slow electron transfer.

$$cycII + Fe(CN)_5X^{n-} \underset{}{\overset{K_{eq}}{\rightleftharpoons}} cycII-Fe(CN)_5X^{n-}$$
$$cycII-Fe(CN)_5X^{n-} \xrightarrow{k_{slow}} cycIII + Fe(CN)_5X^{(n-1)-}$$

This mechanism is consistent with the observed E_a values and second-order kinetics if the first step is characterized by a negative ΔH° to offset the positive E_a of the second step and the equilibrium lies far to the left. At sufficiently high concentrations of oxidizing agent, the rate is predicted to become independent of the concentration of oxidizing agent. This has never been observed even at concentrations of $Fe(CN)_6^{3-}$ as high as 8×10^{-3} M used in temperature-jump experiments (Brandt et al., 1966). If such a complex is formed, a formal inner-sphere mechanism, in which the oxidizing agent is bound via a CN^- or other bridging group to the heme iron with the attendant displacement of one of the bound protein ligands, is inconsistent with the observed decrease of the rate constant in alcohol since Kaminsky et al. (1971) have shown that the rate of the inner-sphere oxidation of reduced cytochrome c by molecular oxygen was greatly increased in the presence of alcohol. Some sort of outer-sphere complex where $Fe(CN)_5X^{n-}$ is bound in some fashion to the polypeptide chain—perhaps through a charge interaction with a positively charged region—cannot be ruled out.

Another possible explanation for the abnormally low activation energy is that prior to electron transfer the protein undergoes a conformational change,

$$cycII \underset{}{\overset{K_{eq}}{\rightleftharpoons}} cyc^*II$$
$$cyc^*II + Fe(CN)_5X^{n-} \longrightarrow cycIII + Fe(CN)_5X^{(n-1)-}$$
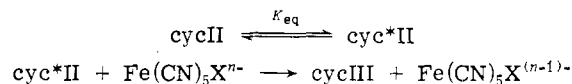
where cyc^*II represents a conformation of reduced cytochrome c more easily oxidizable by the oxidizing agent. This mechanism is consistent with the observed data if the equilibrium is a labile equilibrium which lies far to the left and is characterized by a negative ΔH°. It has recently been shown by X-ray crystallography (Takano et al., 1973) that the conformations of oxidized and reduced cytochrome c differ significantly in the neighborhood of the heme. In the oxidized form, the heme is more exposed to solvent than in the reduced form where it is essentially buried. Hence, the conformational change proposed in the above scheme may involve formation of a species which resembles the oxidized conformation where the heme would presumably be more accessible to the oxidizing agent. A similar mechanism has been proposed for the reduction of oxidized cytochrome c (Yandell et al., 1973; Salemme et al., 1973).

Regardless of the mechanism, it is evident that the oxidation of reduced cytochrome c has been greatly facilitated by an abnormal lowering of the activation energy. Because of the changes in protein structure which could be involved in this process, this facilitation of electron transport may play a significant role in the transfer of electrons through the electron transport chain.

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Hydrogen–Tritium Exchange of Partially and Fully Reconstituted Zinc and Cobalt Alkaline Phosphatase of *Escherichia coli*[†]

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ABSTRACT: The influence of metals upon the rate of hydrogen–tritium exchange of *Escherichia coli* alkaline phosphatase has been studied by the gel filtration technique. At pH 8.0, 20°, nearly 90% of the 1200 potentially exchangeable hydrogens of the metal-free apoenzyme exchange within a few minutes; an additional 8% exchange more slowly over the ensuing 12 hr. In contrast, at all times of exchange enzymes *fully* reconstituted with 4 g-atoms/mol of Co or Zn retain 60 or 70 more hydrogens than the apoenzyme indicating that the metal atoms markedly stabilize the dynamic protein structure. The greater conformational stability of the metalloenzymes is also apparent from examination of the temperature dependence (4–35°) of the exchange reaction. The rates of exchange of phosphatases only partially reconstituted with metals, *i.e.,* 2 g-atoms/mol of either Zn or Co, differ both from apo- and holometalloenzymes and from each other. Thus, addition of only two Zn atoms appears to stabilize a discrete set of equilibrium conformers which differ from those generated by two Co atoms, suggesting differing modes of metal binding; both, in turn, are less stable than the equilibrium structures of their corresponding holometalloenzymes. However, in the presence of phosphate, the proton exchange rates of the partially reconstituted metallophosphatases coincide with those observed for their corresponding holometalloenzymes. Hence, phosphate appears to act in concert with metals to promote conformational stabilization. Phosphate also protects the partially reconstituted Zn enzyme against a slow loss of catalytic activity evident under these experimental conditions.

The metal atoms in metalloenzymes may function in two important ways: metals may participate in the catalytic process directly as components of active enzymatic sites; alternatively, they may affect protein tertiary or quaternary structure and may thereby influence catalysis indirectly. In some enzymes, different atoms of the same metal or atoms of different metals appear to have catalytic and structural roles, respectively (Vallee and Wacker, 1970). Delineation of the precise manner in which metals participate in enzyme action has provided important approaches to understanding the mechanisms of action of metalloenzymes. As would be anticipated, investigations of polymeric systems have proven considerably more complex than have comparable studies of monomeric proteins (Ulmer and Vallee, 1971).

Escherichia coli alkaline phosphatase, a dimeric metalloenzyme, mol wt 89,000, exemplifies these problems since its zinc atoms appear to have *both* functional and structural roles (Simpson and Vallee, 1968). Preparations of the enzyme isolated in our laboratory consistently contain 3.8–4.2 g-atoms of zinc/mol of enzyme (Simpson *et al.,* 1968) which may be removed and replaced completely with corresponding loss and restoration of catalytic activity. Moreover, enzymatic function can be restored also by substitution of cobalt for zinc (Plocke and Vallee, 1962; Simpson and Vallee, 1968). On the basis of kinetic and physicochemical studies employing both zinc and cobalt, the metal atoms in alkaline phosphatase appear to fall into at least