chrome *c* peroxidase and its two oxidized intermediates, I and II. Complex formation between ferrocyanide and the enzyme intermediates does influence the electron-transfer reactions. At low concentrations of ferrocyanide, I oxidizes ferrocyanide with an intrinsic bimolecular rate of $(3.8 \pm 0.8) \times 10^5$ $M^{-1}$ $sec^{-1}$ and II oxidizes ferrocyanide with an intrinsic rate of $(1.4 \pm 0.3) \times 10^5$ $M^{-1}$ $sec^{-1}$. The variation of the apparent rate constants with pH and ionic strength is due to the variation in electrostatic interactions between reactant and buffer ions in solution.

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# Cytochrome *c* Peroxidase Catalyzed Oxidation of Ferrocyanide by Hydrogen Peroxide. Steady-State Kinetics†

Howard C. Jordi and James E. Erman*

ABSTRACT: The steady-state oxidation of ferrocyanide by hydrogen peroxide as catalyzed by cytochrome *c* peroxidase is characterized by saturation kinetics between pH 4 and 8.1 at 0.1 M ionic strength and 25°. The maximum turnover number decreases from a value of $1.2 \times 10^3$ $sec^{-1}$ at pH 4.25 to 2.5 $sec^{-1}$ at pH 8.1. The apparent Michaelis constant varies between 0.9 and 10 mM, the maximum occurring at pH 5.5 in acetate buffer. The steady-state rate parameters are dependent upon specific ion effects, with acetate buffers increasing the oxidation rate over that in phosphate buffers at high ferrocyanide concentrations. Above pH 5.5, the steady-state oxidation of ferrocyanide is faster than predicted by transient-state studies at low ferrocyanide concentrations. The discrepancy is explained on the basis of side reactions at high pH between the enzyme and hydrogen peroxide producing a more reactive oxidant of ferrocyanide than compound II of cytochrome *c* peroxidase.

In order to elucidate the mechanism of electron-transfer reactions mediated by cytochrome *c* peroxidase, we have investigated the oxidation of ferrocyanide, a simple model for the iron atom of the natural enzyme substrate, ferrocytochrome *c*. In an accompanying paper, we have presented the results of a transient state study of ferrocyanide oxidation by compounds I and II of cytochrome *c* peroxidase (Jordi and Erman, 1974). In that study it was found that ferrocyanide binds to the native enzyme. In addition, it was found that the transient state oxidation of ferrocyanide by I and II reached limiting values at high ferrocyanide concentrations, indicating complex formation between ferrocyanide and these two enzyme intermediates. Because of the number of reactions observed at high ferrocyanide concentrations, accurate rate constants were difficult to obtain and the transient state study at high ferrocyanide concentrations was limited to a single pH value near 6. In this report, we present the results of a steady-state investigation of the cytochrome *c* peroxidase catalyzed oxidation of ferrocyanide by hydrogen peroxide.

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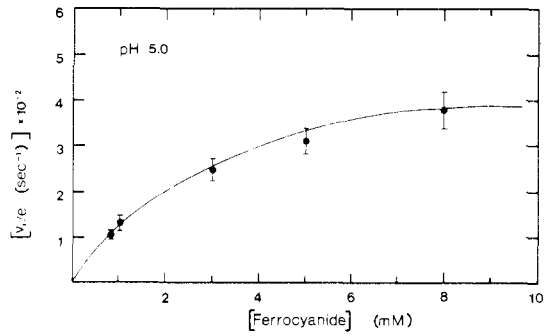


FIGURE 1: Plot of initial velocity as a function of ferrocyanide concentration at pH 5 in 6.7 mM acetate buffer adjusted to 0.1 M ionic strength with $KNO_3$. The solid line was calculated using a value of 540 $sec^{-1}$ for $V_m^{app}/e$ and a value of 3.3 mM for $K_m^{app}$.

## Experimental Procedure

Preparation of enzyme, ferrocyanide, hydrogen peroxide, and buffer solutions was the same as described previously (Jordi and Erman, 1974). Ionic strength was normally 0.1 M adjusted with $KNO_3$. Variation in ionic strength was accomplished by changing either the buffer concentration or the $KNO_3$ concentration as specified in the Results section.

Steady-state kinetic measurements were carried out on a Cary Model 14 recording spectrophotometer with cell compartment thermostated at 25°. All solutions except the stock enzyme were thermally equilibrated at 25° prior to the kinetic runs. The enzyme solution was kept on ice. Appropriate amounts of buffer, ferrocyanide, and enzyme were pipetted into a cuvet and the reaction was initiated by addition of hydrogen peroxide. No change in results was observed when the reaction was initiated by the addition of enzyme.

The initial velocity was determined from the initial linear portion of the spectrophotometer tracing at 420 nm as a function of time. An extinction coefficient of 1.0 $mM^{-1}$ $cm^{-1}$ for ferricyanide was used. The absorption due to ferrocyanide is negligible at this wavelength. The enzymatic rates were corrected for the blank reaction in the absence of enzyme.

## Results

At constant initial hydrogen peroxide concentration and high ferrocyanide concentration, the initial steady-state velocity of ferrocyanide oxidation reaches a saturation level as seen in Figure 1. Phenomenologically, the initial velocity fits eq 1,

$$\frac{v_i}{2e} = \frac{(V_m^{obsd}/e)[S]_0}{K_m^{obsd} + [S]_0} \qquad (1)$$

where $e$ is the total enzyme concentration and $[S]_0$ is the initial ferrocyanide concentration. The factor of two in the denominator on the left-hand side of eq 1 is included since each turnover

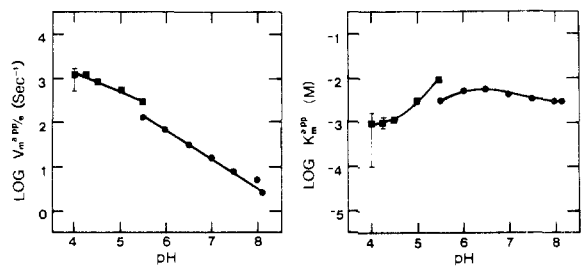


FIGURE 2: Left-hand side: plot of log $V_m^{app}/e$ as a function of pH. Right hand side: plot of log $K_m^{app}$ as a function of pH; (■) acetate; (●) phosphate. The solid lines have no theoretical significance.

of the enzyme oxidizes two molecules of ferrocyanide (Yonetani, 1966).

The most plausible explanation for the observed saturation in the initial velocity is that ferrocyanide forms a complex with each of the enzyme intermediates and that the concentration of the complexes limits the oxidation rate. The complexes which are formed could be either active Michaelis–Menten complexes, Scheme I, where the electron transfer occurs intramolecularly in the complex or inactive complexes in which electron transfer is prevented from occurring, Scheme II. Abbreviations

SCHEME I

$$\mathrm{CcP} + \mathrm{H_2O_2} \xrightarrow{k_1} \mathrm{CcP\text{-}I}$$

$$\mathrm{CcP\text{-}I} + \mathrm{Fe(CN)_6^{4-}} \underset{k_{-22}}{\overset{k_{22}}{\rightleftharpoons}} \mathrm{CcP\text{-}I{\cdot}Fe(CN)_6^{4-}} \xrightarrow{k_{23}} \mathrm{CcP\text{-}II} + \mathrm{Fe(CN)_6^{3-}}$$

$$\mathrm{CcP\text{-}II} + \mathrm{Fe(CN)_6^{4-}} \underset{k_{-24}}{\overset{k_{24}}{\rightleftharpoons}} \mathrm{CcP\text{-}II{\cdot}Fe(CN)_6^{4-}} \xrightarrow{k_{25}} \mathrm{CcP} + \mathrm{Fe(CN)_6^{3-}}$$

SCHEME II

$$\mathrm{CcP} + \mathrm{H_2O_2} \xrightarrow{k_1} \mathrm{CcP\text{-}I}$$

$$\mathrm{CcP\text{-}I} + \mathrm{Fe(CN)_6^{4-}} \xrightarrow{k_{33}} \mathrm{CcP\text{-}II} + \mathrm{Fe(CN)_6^{3-}}$$

$$k_{-32} \downarrow\uparrow k_{32}$$

$$\mathrm{CcP\text{-}I{\cdot}Fe(CN)_6^{4-}}$$

$$\mathrm{CcP\text{-}II} + \mathrm{Fe(CN)_6^{4-}} \xrightarrow{k_{35}} \mathrm{CcP} + \mathrm{Fe(CN)_6^{3-}}$$

$$k_{-34} \downarrow\uparrow k_{34}$$

$$\mathrm{CcP\text{-}II{\cdot}Fe(CN)_6^{4-}}$$

used are: CcP, cytochrome $c$ peroxidase; CcP-I, compound I of cytochrome $c$ peroxidase; CcP-II, compound II of the enzyme.

It has not been possible to obtain conclusive evidence favoring one mechanism over the other. However, it is quite difficult to visualize the structure of the inactive complex postulated by Scheme II. An inactive complex would mean that ferrocyanide binds in such a manner that electron transfer could not occur between it and the oxidized enzyme site. In addition, the binding of ferrocyanide would have to perturb the enzyme in such a manner that the enzyme could not accept electrons from other ferrocyanide ions. These seem to be quite stringent and unlikely conditions. It appears more plausible that the negatively charged ferrocyanide ion binds to positively charged sites on the enzyme. At least one of these sites is probably in such a position that rapid electron transfer occurs between the bound ferrocyanide and the oxidized enzyme site. In the interest of brevity, the results will be discussed only on the basis of Scheme I.

For Scheme I, the reciprocal of the initial steady-state velocity is given by eq 2. Comparison of eq 2 with the reciprocal of

$$\frac{2e}{v_i} = \frac{1}{k_1[H_2O_2]_0} + \frac{1}{k_{23}} + \frac{1}{k_{25}} + \left(\frac{k_{-22} + k_{23}}{k_{22}k_{23}} + \frac{k_{-24} + k_{25}}{k_{24}k_{25}}\right)\frac{1}{[S]_0} \qquad (2)$$

eq 1 confirms that the initial velocities calculated from Scheme I have the same dependence on ferrocyanide concentration.

The maximum velocity, at constant hydrogen peroxide concentration, is given by eq. 3. At the hydrogen peroxide concen-

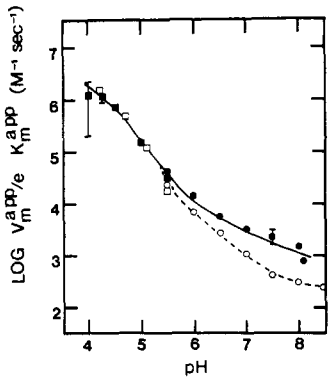


FIGURE 3: Plot of log $V_m^{app}/eK_m^{app}$ as a function of pH. The squares represent data obtained in acetate buffer; the circles represent data obtained in phosphate buffer. The solid symbols are experimental values obtained from steady-state measurements. The open symbols were calculated from eq 9 of the text using data from Jordi and Erman (1974). The lines have no theoretical significance.

$$\frac{e}{V_m^{obsd}} = \frac{1}{k_1[H_2O_2]_0} + \frac{1}{k_{23}} + \frac{1}{k_{25}} \quad (3)$$

tration used in this study, 0.5 mM, the contribution of the $1/k_1[H_2O_2]_0$ term in eq 3 is negligible except at low pH, where the observed electron transfer reactions become fast enough to be influenced to some extent by the rate of formation of I. Since the value of $k_1$ (Yonetani and Ray, 1966; Jordi, 1973) and the initial hydrogen peroxide concentration are known, the contribution of $1/k_1[H_2O_2]_0$ to $e/V_m^{obsd}$ can be calculated. An apparent maximum velocity due only to the electron transfer rate between compounds I and II and ferrocyanide, the quantity of interest, can be evaluated from eq 4. The apparent Mi-

$$\frac{e}{V_m^{app}} = \frac{e}{V_m^{obsd}} - \frac{1}{k_1[H_2O_2]_0} \quad (4)$$

chaelis constant can be evaluated from the observed Michaelis constant by eq 5.

$$K_m^{app} = K_m^{obsd} V_m^{app}/V_m^{obsd} \quad (5)$$

In terms of the rate constants in Scheme I, $V_m^{app}/e$, $K_m^{app}$, and $V_m^{app}/eK_m^{app}$ are given by eq 6, 7, and 8, respectively. $K_m^{I}$

$$V_m^{app}/e = k_{23}k_{25}/(k_{23} + k_{25}) \quad (6)$$

$$K_m^{app} = K_m^{I}\frac{k_{25}}{k_{23} + k_{25}} + K_m^{II}\frac{k_{23}}{k_{23} + k_{25}} \quad (7)$$

$$\frac{V_m^{app}}{eK_m^{app}} = \frac{k_{23}k_{25}}{K_m^{I}k_{25} + K_m^{II}k_{23}} \quad (8)$$

equals $(k_{-22} + k_{23})/k_{22}$ and $K_m^{II}$ equals $(k_{-24} + k_{25})/k_{24}$; these are the apparent Michaelis constants for compounds I and II oxidation of ferrocyanide, respectively.

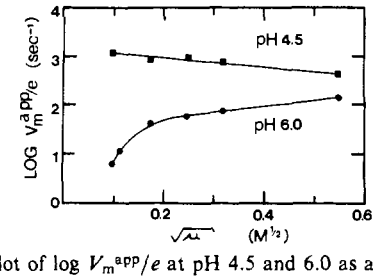


FIGURE 4: Plot of log $V_m^{app}/e$ at pH 4.5 and 6.0 as a function of the ionic strength.

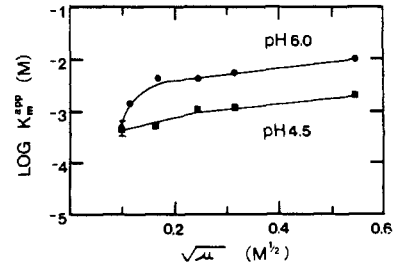


FIGURE 5: Plot of log $K_m^{app}$ at pH 4.5 and 6.0 as a function of the ionic strength.

Plots of log $V_m^{app}/e$ and log $K_m^{app}$ as a function of pH at 0.1 M ionic strength and 25° are shown in Figure 2. In Figure 2 and succeeding figures the standard error is 10%; error bars are included only for those data where the error exceeds the size of the symbols.

The solid symbols in Figure 3 give log $V_m^{app}/eK_m^{app}$ calculated from the two steady-state parameters as a function of pH. A direct comparison between the steady-state results in this report and the transient state oxidation at low ferrocyanide concentrations (Jordi and Erman, 1974) can be made according to eq 9. The rate constants, $k_2^{app}$ and $k_3^{app}$, are the apparent bi-

$$\frac{V_m^{app}}{eK_m^{app}} = \frac{k_2^{app}k_3^{app}}{k_2^{app} + k_3^{app}} \quad (9)$$

molecular rate constants for the oxidation of ferrocyanide by I and II, respectively, at low concentrations. In terms of Scheme I

$$k_2^{app} = \frac{k_{22}k_{23}}{k_{23} + k_{-22}} \quad (10)$$

$$k_3^{app} = \frac{k_{24}k_{25}}{k_{25} + k_{-24}} \quad (11)$$

The open symbols in Figure 3 represent $V_m^{app}/eK_m^{app}$ calculated according to eq 9 from the transient state data. There is good agreement between the steady-state and transient state results below pH 5.5. Above pH 5.5, the steady-state oxidation of ferrocyanide is faster than predicted by the transient state results. This discrepancy complicates the interpretation of the steady-state results above pH 5.5. Reasons for the discrepancy will be explored later.

As an aid in interpreting the steady-state oxidation of ferrocyanide, the kinetics were studied as a function of ionic strength at pH 4.5 and 6.0. The values were chosen to be on either side of the enzymes isoelectric point, pH 5.25 (Yonetani, 1967; Ellfolk and Sievers, 1969). At pH 6.0, adjustment of the ionic strength by either varying the phosphate buffer concentration or by addition of $KNO_3$ gave similar values of the steady-state parameters. At pH 4.5 the enzyme appeared to be unstable when only acetate was used to adjust the ionic strength. However, using $KNO_3$ to adjust the ionic strength of a 6.7 mM acetate buffer gave reproducible data. Figures 4, 5, and 6 are plots of log $V_m^{app}/e$, log $K_m^{app}$, and log $V_m^{app}/eK_m^{app}$, respectively, as functions of ionic strength at both pH 4.5 and 6.0. Again, the steady-state values of $V_m^{app}/eK_m^{app}$ agree with the previous transient-state data (Jordi and Erman, 1974) as seen in Figure 6.

## Discussion

*Specific Ion Effects.* One of the most striking features in the pH dependence of the steady-state rate parameters is the large buffer effect on $V_m^{app}/e$ and $K_m^{app}$ at pH 5.5, Figure 2. A similar buffer effect was observed in the steady-state oxidation of

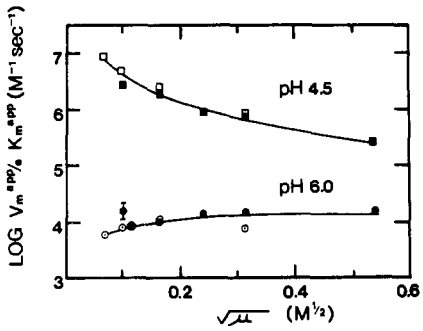


FIGURE 6: Plot of log $V_m^{app}/eK_m^{app}$ at pH 4.5 and 6 as a function of ionic strength. Symbols are the same as in Figure 3; the data for calculating the open symbols were obtained from Jordi and Erman (1974).

ferrocytochrome *c* by cytochrome *c* peroxidase (Yonetani and Ray, 1966). This suggests that the effect is due to specific interaction of the buffer with the common reactant, cytochrome *c* peroxidase. It is most likely an acetate effect since replacement of phosphate with nitrate in adjusting the ionic strength had no effect on the steady-state parameters. In addition, the enzyme was unstable when only acetate was used to adjust ionic strength at pH 4.5.

There is no significant specific ion effect on $V_m^{app}/eK_m^{app}$, Figure 3. This is consistent with the transient state study for the oxidation of low ferrocyanide concentrations by I and II of cytochrome *c* peroxidase (Jordi and Erman, 1974). In addition, no specific ion effect was observed in previous studies on the binding of small ligands to the heme site of cytochrome *c* peroxidase (Erman, 1974a,b).

*Ionic Strength Dependence.* It has previously been shown (Jordi and Erman, 1974) that the ionic strength dependence of $k_2^{app}$ and $k_3^{app}$ is that expected for a bimolecular reaction between the ferrocyanide ion and I and II. The ionic strength dependence of $V_m^{app}/eK_m^{app}$ is consistent with that interpretation as seen in Figure 6.

According to Scheme I, $V_m^{app}/e$ is a combination of two unimolecular rate constants. $V_m^{app}/e$ should be less dependent upon ionic strength than the bimolecular rate constants contained in $V_m^{app}/eK_m^{app}$. At pH 4.5 this is true as seen by a comparison of Figures 4 and 6. At pH 6, however, the maximum velocity is unexplicably more dependent on ionic strength. It should be pointed out that Scheme II does not resolve the dilemma. According to Scheme II, the ionic strength variation of $V_m^{app}/e$ is also due to unimolecular rate constants, the dissociation rate constants $k_{-32}$ and $k_{-34}$.

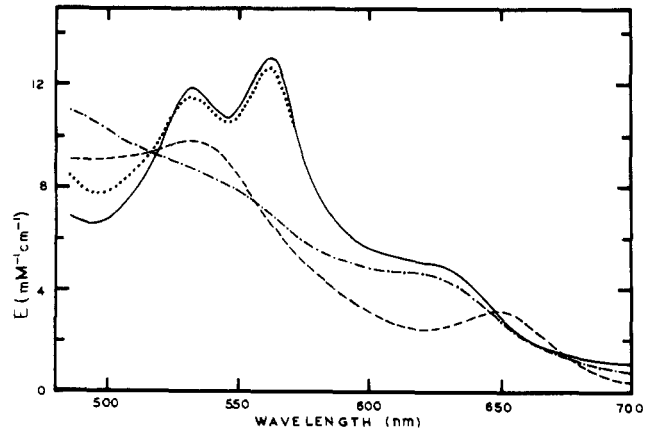


FIGURE 7: Dashed line, visible spectrum of cytochrome *c* peroxidase at pH 8. Solid line, visible spectrum of compound I at pH 8. Dotted line, spectrum taken during the steady-state reaction between 2.0 mM ferrocyanide and 0.48 mM hydrogen peroxide at pH 8. Dashed-dotted line, spectrum of cytochrome *c* peroxidase at pH 8 after complete reduction of 0.48 mM hydrogen peroxide in the presence of excess ferrocyanide.

TABLE I: Oxidation of Ferrocyanide by Hydrogen Peroxide as a Function of Hydrogen Peroxide Concentration.[a]

| [Initial Peroxide] ($\mu$M) | [Ferrocyanide] ($\mu$M) | Peroxide Not Used to Oxidize Ferrocyanide ($\mu$M) |
|---|---|---|
| 9 | 18 | 0 |
| 18 | 33 | 1.5 |
| 27 | 48 | 3 |
| 36 | 62 | 5 |
| 45 | 75 | 7.5 |
| 46 | 75 | 8.5 |

[a] pH 7.8, 25°, 1.2 $\mu$M cytochrome *c* peroxidase, 150 ± 30 $\mu$M ferrocyanide.

The ionic strength dependence of $K_m^{app}$ at pH 4.5 is consistent with expectations for the binding of a negatively charged ferrocyanide ion to the positively charged enzyme. The apparent association becomes stronger as the ionic strength decreases. At pH 6.0, $K_m^{app}$ is apparently dominated by the abnormal ionic strength dependence of the unimolecular rate constants seen in $V_m^{app}/e$ at this pH. The apparent association becomes stronger as the ionic strength decreases even though both reactants have net negative charges.

*Comparison of the Steady-State and Transient State Data.* The discrepancy between the steady-state and transient state data at low ferrocyanide concentrations above pH 5.5, Figure 3, does not appear to be due to inactivation of the enzyme. If this were the case, the steady-state rates would be expected to become slower as multiple turnovers of the enzyme occurred. Neither can the discrepancy be due to inhibition of the transient state oxidation by ferrocyanide, since the transient state studies were carried out at lower ferrocyanide concentrations than the steady-state studies.

A possible explanation is that at high pH a more reactive oxidant than II is formed during the steady-state process. An attempt was made to detect any new enzyme intermediate by observing the visible spectrum during the steady-state oxidation at pH 8. The results are shown in Figure 7.

The dashed line in Figure 7 is the spectrum of cytochrome *c* peroxidase and the solid line is the spectrum of I at pH 8. The dotted line is the spectrum taken during the steady-state reaction. There are no shifts in peak position, indicating that if a new intermediate is formed, it is present in very small quantities or that its spectrum is similar to that of I. However, there is evidence that hydrogen peroxide does interact with the enzyme to yield something other than I during the steady state. The dashed-dotted line in Figure 7 is the spectrum of cytochrome *c* peroxidase after complete reduction of the hydrogen peroxide. The spectrum is significantly altered from that of the native enzyme.

The altered spectrum of cytochrome *c* peroxidase at the end of the reaction is most likely due to oxidation of the enzyme by the hydrogen peroxide present during the steady state. Coulson and Yonetani have shown that addition of a tenfold excess of hydrogen peroxide to cytochrome *c* peroxidase at pH 4 and 7, in the absence of oxidizable substrate, oxidizes about four tyrosine residues in the enzyme (Coulson and Yonetani, 1972).

Further, as shown in Table I, not all of the hydrogen peroxide in the steady-state system is utilized to oxidize ferrocyanide at pH 7.8. In addition it has been observed that a large excess of hydrogen peroxide added to cytochrome *c* peroxidase at pH 8 causes rapid destruction of the heme group.

The above results suggest that at high pH, ferrocyanide is such a poor substrate for I and II that hydrogen peroxide can effectively compete with ferrocyanide in reactions with these enzyme intermediates, producing a number of side reactions including oxidation of the heme and amino acid residues of the protein. During the side reactions, an intermediate is most likely produced which is a better oxidant of ferrocyanide than II. This could account for the faster oxidation of ferrocyanide in the steady state compared to the single turnover transient state studies where only I and II are produced.

*pH Dependence.* The pH dependence of the steady-state parameters is difficult to interpret due to the specific ion effects, the abnormal ionic strength dependence of $V_m^{app}/e$ at pH 6, and the side reactions between enzyme and hydrogen peroxide at high pH. Certainly the pH dependence of $V_m^{app}/eK_m^{app}$, after correction for the side reactions, is consistent with the interpretation of $k_2^{app}$ and $k_3^{app}$ in the transient state (Jordi and Erman, 1974). The pH dependence is due primarily to the variation in electrostatic interaction between ferrocyanide and the enzyme intermediates as the net charge on the enzyme changes with pH.

$V_m^{app}/e$, essentially a unimolecular rate constant, should be relatively insensitive to electrostatic effects although the ionic strength dependence of $V_m^{app}/e$ at pH 6 casts some doubt on this expectation. Nevertheless, if $V_m^{app}/e$ is insensitive to the charge on the enzyme, then the strong pH dependence of $V_m^{app}/e$ suggests that a proton or a protonated form of the complex may be involved in the intramolecular electron transfer reaction. This is an interesting area for further investigation.

$K_m^{app}$ is relatively independent of pH, varying by only a factor of 10 over the pH range 4–8. This is due to the cancellation of the pH variation of the intramolecular electron-transfer rate constants in the numerator and the pH dependence of the bimolecular association rate constants in the denominator of the expression for $K_m^{app}$, eq 7.

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# The Metal Ion Acceleration of the Conversion of Trypsinogen to Trypsin. Lanthanide Ions as Calcium Ion Substitutes†

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ABSTRACT: The lanthanide ions are shown to be effective calcium ion substitutes in accelerating the conversion of trypsinogen to trypsin. The rate of activation of the zymogen in the presence of lanthanides is much greater than that of the calcium ion. In addition this increased activation takes place at nearly 100-fold lower concentrations of the lanthanide ions than with calcium ion. The effect of the lanthanide ions in accelerating the activation of the zymogen, like the calcium effect, is reflected in a decrease in the $K_m$ of the trypsin–trypsinogen interaction. Whereas a calcium concentration of 50 mM reduces the $K_m$ by a factor of 3 (over 4 mM $Ca^{3+}$), 0.5 mM $Nd^{3+}$ reduces the $K_m$ by a factor of 14. Inhibition of the activation occurs with concentrations of lanthanide ions ranging from $5 \times 10^{-4}$ to $10^{-3}$ M, depending on the particular lanthanide ion.

Within the last 10 years there has been substantial attention focused on specific interactions of proteins with metal ions. It has become apparent that the functions of metal ion–protein complexes are biologically important not only in a catalytic capacity, but also in a structural capacity. A majority of the work in this area has been conducted on systems which contain transition metal ions which are amenable to spectroscopic and magnetic investigations. Little has been accomplished on systems containing calcium ion since its rare-gas electronic configuration makes it difficult to probe by conventional spectroscopic techniques.

A solution to this inherent difficulty can be achieved by using lanthanide metal ions as substitutes for calcium (Birnbaum *et al.* 1970; Darnall and Birnbaum, 1970; Williams, 1970). In contrast to the calcium ion, the varied magnetic and spectral properties of the rare earth metal ions should make excellent spectroscopic probes of the metal ion binding sites in

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