Cytochrome *c* Peroxidase Catalyzed Oxidation of Ferrocyanide by Hydrogen Peroxide. Transient State Kinetics†

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ABSTRACT: At low concentrations of ferrocyanide, the apparent bimolecular rate constants for the oxidation of ferrocyanide by compounds I and II of cytochrome *c* peroxidase have been measured over the pH range 4.0–9.5. The ionic strength dependence of the oxidation was measured from 0.1 to 0.005 M. Under all conditions, compound I oxidizes ferrocyanide about three times faster than compound II. Both the pH and ionic strength variation of the rate constants for the oxidation of ferrocyanide by each enzyme intermediate may be explained by the contribution of electrostatic interactions to a single intrinsic rate constant and to the variation of the activity coefficients of the reactants with charge and ionic strength. The intrinsic bimolecular rate constant has a value of $(3.8 \pm 0.8) \times 10^5$ M^{-1} sec^{-1} for the oxidation of ferrocyanide by I and $(1.4 \pm 0.3) \times 10^5$ M^{-1} sec^{-1} for the oxidation of ferrocyanide by II. The rates of ferrocyanide oxidation by I and II show saturation at high substrate concentrations. At pH 6.25 the limiting values of the apparent oxidation rate constants are 170 sec^{-1} for I and 30 sec^{-1} for II. The apparent Michaelis constants are 6 and 1.7 mM for I and II, respectively. The limiting values of the oxidation rates indicate that ferrocyanide complexes with both I and II. A study of the interaction of ferrocyanide with the native enzyme also indicates complex formation. At pH 6.3 the equilibrium dissociation constant is 1.9 mM, very similar to the values of the apparent Michaelis constants. In addition to complex formation, a unimolecular reaction is observed which is interpreted to be a transfer of a cyanide ligand from the bound ferrocyanide to the heme group of the enzyme.

Cytochrome *c* peroxidase, a yeast mitochondrial enzyme, catalyzes the oxidation of ferrocytochrome *c* by hydrogen peroxide, both *in vivo* (Erecinska *et al.*, 1973) and *in vitro* (Yonetani and Ray, 1966). At low concentrations of ferrocytochrome *c* the catalysis follows the classical peroxidase mechanism developed for horseradish peroxidase by Chance and George (Chance, 1952; George, 1952, 1953). Two oxidized cytochrome *c* peroxidase intermediates have been identified in the oxidation of ferrocytochrome *c* and ferrocyanide (Coulson *et al.*, 1971). At low substrate concentrations, the reaction sequence is given by Scheme I. CcP represents the native enzyme, CcP-I represents compound I of cytochrome *c* peroxidase which is oxidized 2 equiv higher than the native enzyme, CcP-II represents compound II of the enzyme, oxidized 1 equiv above the native enzyme, and Fe^{2+} and Fe^{3+} represent ferrous and ferric iron in either cytochrome *c* or the hexacyanide complex.

SCHEME I

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

$$\mathrm{CcP\text{-}I} + \mathrm{Fe^{2+}} \xrightarrow{k_2^{app}} \mathrm{CcP\text{-}II} + \mathrm{Fe^{3+}}$$

$$\mathrm{CcP\text{-}II} + \mathrm{Fe^{2+}} \xrightarrow{k_3^{app}} \mathrm{CcP} + \mathrm{Fe^{3+}}$$

At high concentrations, ferrocytochrome *c* binds to cytochrome *c* peroxidase (Mochan and Nicholls, 1971; Gupta and Yonetani, 1973).

The steady-state oxidation of ferrocytochrome *c* attains a limiting velocity at high substrate concentrations and has been interpreted on the basis of intramolecular electron transfer through the cytochrome *c*–enzyme complex (Yonetani and Ray, 1966; Nicholls and Mochan, 1971). In this case steps two and three in Scheme I are actually composed of separate binding and electron transfer steps. The oxidations of ferrocytochrome *c* by other peroxidases, including horseradish peroxidase, lactoperoxidase, and verdoperoxidase, all show saturation effects which can be interpreted on the basis of cytochrome *c*–enzyme complexes (Chance, 1950).

The oxidation of ferrocyanide by horseradish peroxidase indicates that ferrocyanide does not complex with the enzyme and that the oxidation is strictly bimolecular (Hasinoff and Dunford, 1970; Cotton and Dunford, 1973). On the other hand, the steady-state oxidation of ferrocyanide by cytochrome *c* peroxidase was characterized by saturation kinetics indicating complex formation (Yonetani and Asakura, 1968).

We are interested in investigating electron transfer reactions to and from the oxidizable sites of cytochrome *c* peroxidase and, in particular, to determine whether or not electron transfer can occur through the protein portion of the enzyme. As a first step, the pH dependence of the electron transfer reactions was investigated to determine whether acidic or basic groups in cytochrome *c* peroxidase can participate in the reactions. Since acidic or basic groups in both cytochrome *c* and cytochrome *c* peroxidase could influence the electron transfer rate, it would be difficult to differentiate the effects of the substrate from the enzyme. For this reason, we have chosen to initially investigate the cytochrome *c* peroxidase catalyzed oxidation of ferrocyanide by hydrogen peroxide.

Experimental Procedure

Cytochrome *c* peroxidase was isolated from commercial baker's yeast and crystallized by dialyzing against distilled, deionized water according to the method of Yonetani (Yonetani and Ray, 1965; Yonetani *et al.*, 1966a). The purity index (ratio of absorbance at 408 nm to that at 280 nm) was 1.18. Cytochrome *c* peroxidase concentrations were determined spectro-

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photometrically using an extinction coefficient of 93 mM^{-1} cm^{-1} at 408 nm.

Reagent grade $K_4Fe(CN)_6 \cdot 3H_2O$ and 30% H_2O_2 were used without further purification. The ferrocyanide solutions were prepared by weight with deoxygenated, distilled water and were discarded after 1 hr to minimize decomposition.

Acetate, phosphate, and borate buffers were used in the appropriate pH region. Total buffer concentration varied from 1 to 10 mM. Ionic strength was adjusted with KNO_3 after accounting for the contribution from the buffer and ferrocyanide ions.

Between pH 4 and 7, kinetic measurements were performed on a Durrum-Gibson stopped-flow spectrophotometer thermostated at 25°. Most of the studies were carried out by mixing the ferrocyanide, contained in one drive syringe, with preformed I, contained in the second drive syringe. Compound I was formed just prior to introduction into the stopped-flow apparatus by adding slightly less than a stoichiometric amount of hydrogen peroxide to cytochrome *c* peroxidase. In some experiments cytochrome *c* peroxidase and ferrocyanide were contained in one drive syringe and mixed with hydrogen peroxide in the second drive syringe.

Above pH 7, the reaction was slow enough to follow with a Cary Model 14 spectrophotometer thermostated at 25°. To initiate the reaction, enzyme or preformed I was added to the buffered reactants in the cuvet. In all cases the ferrocyanide concentrations were at least ten times greater than the total enzyme concentration resulting in pseudo-first-order reactions.

The net charge on cytochrome *c* peroxidase at 0.1 M ionic strength was determined by titration of the enzyme with nitric acid and potassium hydroxide. Enzyme solutions were prepared by dissolving crystals of cytochrome *c* peroxidase in deaerated 0.1 M potassium nitrate solutions. The pH of these solutions was within 0.05 pH unit of the isoelectric point, pH 5.25 (Yonetani, 1967). Potassium hydroxide was standardized against potassium acid phthalate and nitric acid solutions were standardized against the potassium hydroxide. Titrations were carried out on 3–5-ml aliquots of enzyme, with the surface of the solution flushed with nitrogen. The titrant was added with micrometer syringes and the pH read to the nearest 0.01 pH unit after each addition.

Analysis of the data was facilitated by the use of a nonlinear least-squares curve fitting program, KINET, obtained from Dr. J. L. Dye of Michigan State University (Dye and Nicely, 1971).

Results

In order to determine the pH limits for the study of ferrocyanide oxidation by cytochrome *c* peroxidase, the inactivation of the enzyme as a function of pH was investigated. The enzyme was incubated for 5 min at various pH values followed by measurement of the activity at pH 5.5. Under these conditions, the enzyme is stable between pH 4 and 7.5. Below pH 4 there is a rapid loss of activity to less than 1% at pH 3. On the alkaline side, the loss of activity is relatively slow, with about 80% remaining at pH 10 after 5 min.

The ferrocyanide oxidation rates could be studied at pH values greater than 7.5 by rapidly mixing unbuffered enzyme or I with buffered ferrocyanide solutions of sufficient concentration to make the oxidation faster than the enzyme inactivation. In this manner, rates for the ferrocyanide oxidation were obtained as high as pH 9.5, although with less accuracy than the values between pH 4 and 7.5.

The enzyme intermediates, I and II of cytochrome *c* peroxidase, have very similar absorption spectra at pH 8 (Coulson *et al.*, 1971). Below pH 8, II appears to be an equilibrium mixture of an Fe(III) species with a spectrum similar to the native enzyme and an Fe(IV) species with a spectrum similar to I. As a consequence, the isosbestic points between the native enzyme and I and II are very close together and are not suitable for observation of the interconversion of the individual enzyme species. Instead, the kinetics of the reduction of I to II and the reduction of II to the native enzyme by ferrocyanide were followed at 424 nm, the wavelength of maximum difference in absorption between I and II and the native enzyme.

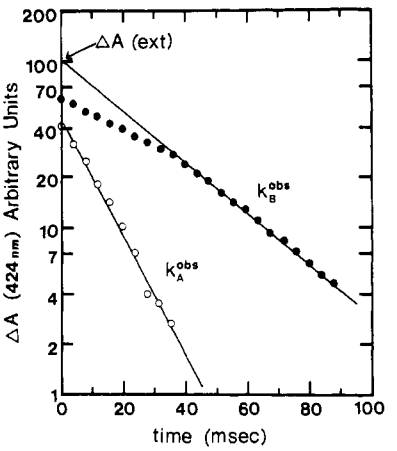

FIGURE 1: Change in absorbance at 424 nm as a function of time for the reduction of I by ferrocyanide: pH 5.0, 1.05 μM I, 42.2 μM ferrocyanide.

Figure 1 shows a typical plot of the logarithm of the change in absorbance at 424 nm as a function of time. It is biphasic and can be resolved into two first-order rates. The slower of the two reactions is denoted by k_B^{obsd} and the faster by k_A^{obsd}. In all cases studied, k_A^{obsd} is always about three times larger than k_B^{obsd}. Under these conditions, it is difficult to separate the two reactions and undoubtedly some systematic error has been introduced into the absolute value of the rate constants. However, the shape of the pH–rate profile should not be affected.

The similar values of k_A^{obsd} and k_B^{obsd} make it difficult to assign *a priori* the two experimentally observed pseudo-first-order rate constants with a particular reaction in the mechanism shown in Scheme I, *i.e.*, k_A^{obsd} could be the observed rate constant for the oxidation of ferrocyanide by either I or II. However, a consideration of the amplitude of the two reactions allows the proper assignment to be made.

Under the experimental conditions, where ferrocyanide is present in excess over the enzyme species, two pseudo-first-order rate constants can be defined for the mechanism shown in Scheme I. The amplitudes of the two reactions depend upon

$$k_2^{obsd} = k_2^{app}[Fe(CN)_6^{4-}] \quad (1)$$

$$k_3^{obsd} = k_3^{app}[Fe(CN)_6^{4-}] \quad (2)$$

the extinction coefficients and concentrations of I and II and the observed rate constants. Unfortunately, the extinction coefficient of II cannot be measured independently of the rate constants. However, one can utilize the amplitude of the reaction, the concentration of species, and the rate constants to calculate the extinction coefficient of II. If II is an equilibrium mixture of Fe(III) and Fe(IV) species as postulated (Coulson *et al.*, 1971), the extinction coefficient of II at 424 nm should vary between about 50 and 100 mM^{-1} cm^{-1}, the extinction coefficients of cytochrome *c* peroxidase and I at 424 nm, respectively.

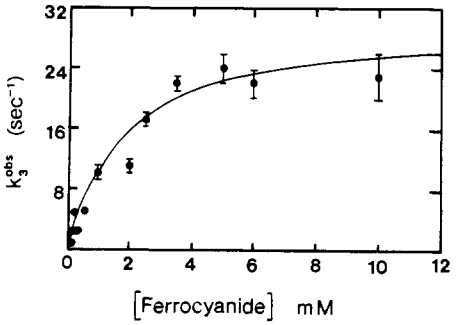

FIGURE 2: Plot of k_3^{obsd} as a function of ferrocyanide concentration: pH 6.25 ± 0.1, 1.05 μM II.

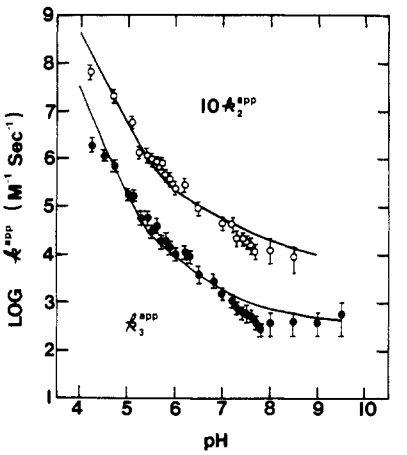

FIGURE 3: Plot of log k_2^{app} and k_3^{app} as a function of pH at 0.1 M ionic strength, 25°. Note that k_2^{app} has been multiplied by a factor of 10 to offset it from k_3^{app}. The solid lines are calculated from equations discussed in the text.

Assuming k_A^{obsd}, the larger of the two observed rate constants, equals k_2^{obsd} and k_B^{obsd} equals k_3^{obsd}, the calculated extinction coefficients for II at 424 nm vary from about 73 mM^{-1} cm^{-1} at pH 4.2 to 104 mM^{-1} cm^{-1} at pH 7.8, compatible with expectations. On the other hand, if the opposite assumption is made, the calculated values for the extinction coefficient of II vary from 23 mM^{-1} cm^{-1} at pH 4.2 to 162 mM^{-1} cm^{-1} at pH 7.8, clearly outside the range of expected values. On this basis, k_A^{obsd} is equated with k_2^{obsd}, the rate constant for the oxidation of ferrocyanide by I and k_B^{obsd} is equated with k_3^{obsd}, the rate constant for the compound II oxidation of ferrocyanide.

At relatively high concentrations of ferrocyanide, a third slower reaction is observed. This reaction makes it very difficult to obtain accurate values of k_2^{obsd} and k_3^{obsd} since its amplitude must first be subtracted from the change in absorbance in plots such as that shown in Figure 1 before k_2^{obsd} and k_3^{obsd} can be evaluated.

To determine whether or not ferrocyanide binds to the enzyme intermediates and whether or not the complex influences the electron transfer reactions, it is necessary to study the reaction at high ferrocyanide concentrations. At pH 6.25, values of k_2^{obsd} and k_3^{obsd} were determined as a function of ferrocyanide concentration up to 10 mM, the maximum allowable at constant ionic strength of 0.1 M. Below 0.2 mM ferrocyanide, only the two oxidation-reduction reactions were observed. Above 0.2 mM, the third reaction was present and its rate constant designated k_4^{obsd}. Values of k_2^{obsd}, k_3^{obsd}, and k_4^{obsd} as a function of ferrocyanide concentration are given in Table I. Where all three reactions are present, the analysis of the kinetic traces is subject to large error. As a consequence as many as 12 observations at a single ferrocyanide concentration were made at the higher ferrocyanide concentrations to reduce the standard error.

Both k_2^{obsd} and k_3^{obsd} show saturation at high ferrocyanide concentrations, indicating complex formation between ferrocyanide and both I and II. Values of k_3^{obsd} are plotted as a function of ferrocyanide concentration in Figure 2. Fitting k_2^{obsd} and k_3^{obsd} to eq 3 gave the following values for the apparent

$$k^{obsd} = \frac{k_{max}^{obsd}[S]_0}{K_m^{obsd} + [S]_0} \quad (3)$$

Michaelis constants and maximum rate constants: K_{m2}^{obsd}, 6.0 ± 1.1 mM; K_{m3}^{obsd}, 1.7 ± 0.5 mM; k_{max2}^{obsd}, 170 ± 20 sec^{-1}; k_{max3}^{obsd}, 30 ± 3 sec^{-1}.

Within experimental error, the third reaction is independent

TABLE I: Concentration Dependence of Observed Rate Constants at pH 6.25 ± 0.10.[a]

Ferrocyanide Concn (mM)	k_2^{obsd} (sec^{-1})	No. of obsd	k_3^{obsd} (sec^{-1})	No. of obsd	k_4^{obsd} (sec^{-1})	No. of obsd
10	100 ± 23	6	23 ± 3	9	0.7 ± 0.2	9
6.0	94 ± 20	3	22 ± 2	6	0.6 ± 0.1	6
5.0	74 ± 15	7	24 ± 2	12	0.5 ± 0.1	12
3.5	60 ± 14	6	22 ± 1	11	0.6 ± 0.2	11
2.5	55 ± 10	5	17 ± 1	10	0.6 ± 0.2	10
2.0	39 ± 15	6	11 ± 1	11	0.5 ± 0.1	11
1.0	20 ± 5	3	10 ± 2	3	0.4 ± 0.1	3
0.6	12 ± 2	3	5 ± 2	3	0.4 ± 0.1	3
0.4	6 ± 2	3	2.5 ± 1	3	0.6 ± 0.1	3
0.3	4.8 ± 1	3	3.1 ± 0.5	5	0.4 ± 0.1	3
0.2	3.5 ± 1	3	2.3 ± 0.5	5	0.4 ± 0.1	3
0.1	3.0 ± 0.5	2	0.97 ± 0.1	2		
0.05	1.4 ± 0.2	2	0.51 ± 0.05	2		
0.04	1.0 ± 0.1	2	0.41 ± 0.04	2		
0.03	0.8 ± 0.1	2	0.23 ± 0.02	2		

[a] 25°, 0.1 M ionic strength.

TABLE II: Ionic Strength and pH Dependence of the Apparent Rate Constant for the Oxidation of Ferrocyanide by I of Cytochrome *c* Peroxidase, 25°.

		k_2^{app} (M^{-1} sec^{-1}) at μ =			
pH	Buffer[a]	0.10	0.03	0.01	0.005
4.0	A	$(9.0 \pm 2.0) \times 10^6$ [b]		$(1.6 \pm 0.4) \times 10^8$	$(5.2 \pm 1.0) \times 10^8$
4.5	A	$(3.2 \pm 0.7) \times 10^6$ [b]	$(1.0 \pm 0.3) \times 10^7$	$(2.6 \pm 0.5) \times 10^7$	$(4.2 \pm 1.5) \times 10^7$
5.0	A	$(8.0 \pm 2.0) \times 10^5$ [b]	$(7.8 \pm 1.6) \times 10^5$	$(1.6 \pm 0.3) \times 10^6$	$(2.5 \pm 0.3) \times 10^6$
5.5	A	$(8.2 \pm 2.0) \times 10^4$	$(9.7 \pm 2.0) \times 10^4$	$(1.3 \pm 0.3) \times 10^5$	$(1.2 \pm 0.3) \times 10^5$
5.5	P	$(8.9 \pm 2.2) \times 10^4$	$(1.1 \pm 0.4) \times 10^5$	$(1.4 \pm 0.6) \times 10^5$	$(1.4 \pm 0.4) \times 10^5$
6.0	P	$(2.4 \pm 0.6) \times 10^4$	$(3.8 \pm 0.8) \times 10^4$	$(2.9 \pm 0.6) \times 10^4$	$(2.3 \pm 0.5) \times 10^4$
6.5	P	$(8.7 \pm 2.2) \times 10^3$	$(8.0 \pm 1.6) \times 10^3$	$(5.7 \pm 1.2) \times 10^3$	$(4.0 \pm 0.8) \times 10^3$
7.0	P	$(4.6 \pm 1.2) \times 10^3$	$(2.4 \pm 0.8) \times 10^3$	$(1.2 \pm 0.3) \times 10^3$	$(7.8 \pm 1.6) \times 10^2$
7.5	P	$(1.8 \pm 0.5) \times 10^3$	$(8.2 \pm 1.6) \times 10^2$	$(5.2 \pm 1.1) \times 10^2$	$(3.2 \pm 0.6) \times 10^2$
8.0	P	$(1.2 \pm 0.6) \times 10^3$	$(3.7 \pm 0.8) \times 10^2$	$(2.3 \pm 0.8) \times 10^2$	$(2.7 \pm 0.5) \times 10^2$

[a] Buffer key: A, acetate; P, phosphate. [b] Extrapolated or interpolated from data in Figure 3.

of ferrocyanide concentration with a value for k_4^{obsd} equal to 0.5 ± 0.1 sec^{-1}. As will be seen below, this third reaction is not an electron transfer reaction but is associated with an interaction of ferrocyanide and the native enzyme.

In view of the difficulty in obtaining values of k_2^{obsd} and k_3^{obsd} at high ferrocyanide concentrations, it was decided to limit the transient state kinetic studies to ferrocyanide concentrations below that at which k_4^{obsd} is detectable. In an accompanying paper (Jordi and Erman, 1974), steady-state reaction techniques are used to study the oxidation at high ferrocyanide concentrations. At low ferrocyanide concentration, k_2^{obsd} and k_3^{obsd} depend upon substrate concentration according to eq 1 and 2 from which the apparent bimolecular rate constants, k_2^{app} and k_3^{app}, can be evaluated.

Both k_2^{app} and k_3^{app} have been evaluated as functions of pH and ionic strength at 25°. The logarithms of k_2^{app} and k_3^{app} at 0.1 M ionic strength are plotted as a function of pH in Figure 3. It should be noted that k_2^{app} has been multiplied by a factor of 10 to offset it from k_3^{app}. Values of k_2^{app} and k_3^{app} as a function of ionic strength at selected pH values are given in Tables II and III, respectively.

Since ferrocyanide complexes with I and II, it is likely that it will also complex with the native enzyme. At pH 6.0, addition of ferrocyanide to cytochrome *c* peroxidase perturbs the spectrum of the enzyme. Figure 4A shows the difference spectrum in the visible region between the enzyme with various concentrations of ferrocyanide added and the enzyme in the absence of ferrocyanide. The absolute spectrum of the final mixture is shown in Figure 4B.

The interaction is not due to simple complex formation as seen by the lack of isosbestic points in Figure 4A. In addition, the kinetics of the interaction of ferrocyanide with cytochrome *c* peroxidase at pH 6.3 followed at 424 nm in the stopped-flow instrument shows three reactions, two of which are dependent on the ferrocyanide concentration. These reactions, denoted by the rate constants k_5^{obsd}, k_6^{obsd}, and k_7^{obsd}, are shown in Figure 5 as a function of ferrocyanide concentration. The faster reactions have the least accurate rate constants since the contributions from the slower reaction must first be subtracted in evaluating the data.

The value of k_7^{obsd} is 0.65 ± 0.2 sec^{-1}, independent of ferrocyanide concentration. Both k_5^{obsd} and k_6^{obsd} increase linearly with ferrocyanide concentrations with intercepts at zero ferrocyanide concentrations of 19 ± 5 and 1.2 ± 1.1 sec^{-1}, respectively. The slopes are $(1.0 \pm 0.2) \times 10^4$ and $(6 \pm 1) \times 10^3$ M^{-1} sec^{-1} for k_5^{obsd} and k_6^{obsd}, respectively.

TABLE III: Ionic Strength and pH Dependence of the Apparent Rate Constant for the Oxidation of Ferrocyanide by II of Cytochrome *c* Peroxidase, 25°.

		k_3^{app} (M^{-1} sec^{-1}) at μ =			
pH	Buffer[a]	0.10	0.03	0.01	0.005
4.0	A	$(3.7 \pm 0.8) \times 10^6$ [b]	$(1.7 \pm 0.3) \times 10^7$	$(6.0 \pm 1.6) \times 10^7$	$(8.1 \pm 1.6) \times 10^7$
4.5	A	$(1.1 \pm 0.3) \times 10^6$	$(3.1 \pm 0.6) \times 10^6$	$(6.4 \pm 1.5) \times 10^6$	$(1.1 \pm 0.2) \times 10^7$
5.0	A	$(1.8 \pm 0.5) \times 10^5$	$(2.9 \pm 0.6) \times 10^5$	$(6.8 \pm 1.4) \times 10^5$	$(9.8 \pm 2.0) \times 10^5$
5.5	A	$(2.6 \pm 0.5) \times 10^4$	$(2.4 \pm 0.6) \times 10^4$	$(4.1 \pm 0.7) \times 10^4$	$(3.7 \pm 0.8) \times 10^4$
5.5	P	$(3.4 \pm 0.9) \times 10^4$	$(5.0 \pm 2.2) \times 10^4$	$(5.4 \pm 1.6) \times 10^4$	$(5.1 \pm 1.2) \times 10^4$
6.0	P	$(9.6 \pm 2.4) \times 10^3$	$(1.4 \pm 0.3) \times 10^4$	$(1.1 \pm 0.3) \times 10^4$	$(7.8 \pm 1.6) \times 10^3$
6.5	P	$(3.8 \pm 1.4) \times 10^3$	$(3.1 \pm 0.6) \times 10^3$	$(1.9 \pm 0.4) \times 10^3$	$(1.3 \pm 0.3) \times 10^3$
7.0	P	$(1.5 \pm 0.4) \times 10^3$	$(6.9 \pm 1.4) \times 10^2$	$(5.0 \pm 1.0) \times 10^2$	$(3.0 \pm 0.6) \times 10^2$
7.5	P	$(6.3 \pm 2.5) \times 10^2$	$(3.0 \pm 0.6) \times 10^2$	$(1.6 \pm 0.3) \times 10^2$	$(1.2 \pm 0.2) \times 10^2$
8.0	P	$(4.0 \pm 2.0) \times 10^2$	$(1.2 \pm 0.4) \times 10^2$	$(7.5 \pm 2.4) \times 10^1$	$(8.6 \pm 1.8) \times 10^1$

[a] Buffer key: A, acetate; P, phosphate. [b] Extrapolated from data in Figure 3.

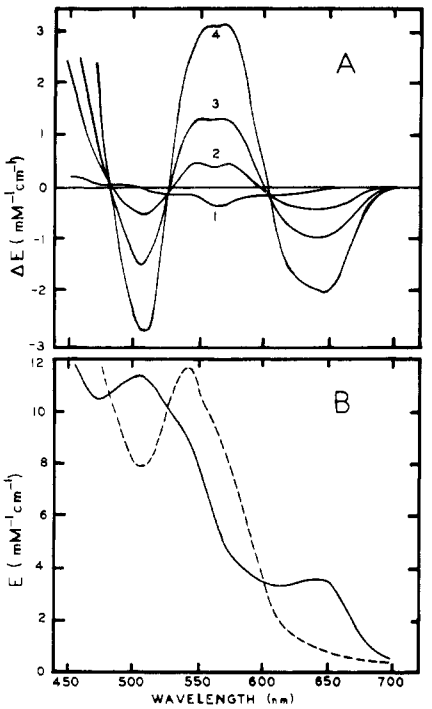

FIGURE 4: (A) Difference spectra of solutions of cytochrome *c* peroxidase with added ferrocyanide *vs.* cytochrome *c* peroxidase at pH 6.0. Cytochrome *c* peroxidase concentration is 24 μM; ferrocyanide concentrations are 1.25, 9.80, 21.5, and 81.6 mM for curves 1–4, respectively. (B) Absolute spectrum of cytochrome *c* peroxidase (solid line) and of cytochrome *c* peroxidase in the presence of 0.26 M ferrocyanide (dashed line).

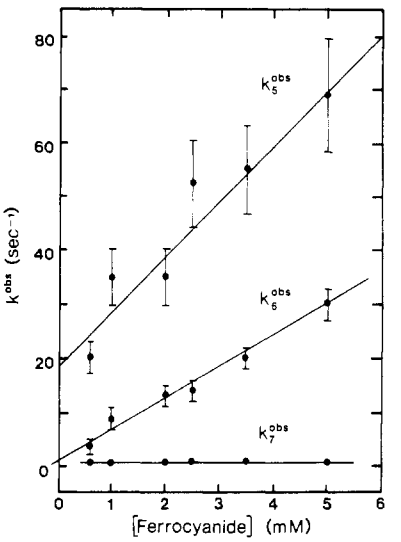

FIGURE 5: Plot of the rate constants for the three reactions observed when ferrocyanide is mixed with cytochrome *c* peroxidase at pH 6.3 as a function of ferrocyanide concentration.

Discussion

The spectral perturbation on mixing ferrocyanide and cytochrome *c* peroxidase indicates that these two compounds or other species in solution interact. The most likely interpretation for the concentration dependent pseudo-first-order rate constants shown in Figure 5 is that they represent the binding of ferrocyanide and cyanide with the enzyme. Cytochrome *c* peroxidase binds cyanide extremely tightly (Erman, 1974) and it is not unreasonable to expect the enzyme to react with the small amount of cyanide present in the ferrocyanide solutions (Emschwiller, 1953).

For reversible ligand binding reactions, the value of the intercept is equal to the dissociation rate constants. The dissociation rate constant for the cyanide-cytochrome *c* peroxidase complex at pH 6.3 is about 0.5 sec^{-1} (Erman, 1974). This value is within experimental error for the intercept of k_6^{obsd}, 1.2 ± 1.1 sec^{-1}, and k_6^{obsd} can be associated with the binding of cyanide to the enzyme. Equating k_5^{obsd} with the binding of ferrocyanide, the values for the association and dissociation rate constants are (1.0 ± 0.2) × 10^4 M^{-1} sec^{-1} and 19 ± 5 sec^{-1}, respectively. Using these values, the equilibrium dissociation constant can be calculated to be 1.9 ± 0.6 mM at pH 6.3.

The reaction which is independent of ferrocyanide concentration, k_7^{obsd}, must be an isomerization of the ferrocyanide-cytochrome *c* peroxidase complex, since no isomerization was observed in the study of the cyanide-cytochrome *c* peroxidase complex at pH 6.3 (Erman, 1974). A likely reaction for the process is that once ferrocyanide binds to the enzyme, probably at a positively charged site, there is a transfer of a cyanide ion from the bound ferrocyanide to the heme group of the enzyme. This is suggested by the final spectrum of the enzyme upon addition of high ferrocyanide concentrations, Figure 4B, which is very similar to the spectrum of the cytochrome *c* peroxidase-cyanide complex (Yonetani *et al.*, 1966b).

The third reaction observed in the oxidation of ferrocyanide by I and II at high ferrocyanide concentration is probably the same unimolecular reaction seen in the interaction of ferrocyanide with the native enzyme. Both k_4^{obsd} and k_7^{obsd} are independent of ferrocyanide concentration, both have the same value within experimental error, and both give rise to an increase in absorbance at 424 nm relative to the free enzyme. As free enzyme is produced during the oxidation of ferrocyanide, the excess ferrocyanide complexes with the enzyme and the slow unimolecular reaction is observed subsequent to the redox and binding steps.

The interpretation of k_2^{app} and k_3^{app} depends upon the reason for the limiting oxidation rates at high ferrocyanide concentrations. There is no doubt that ferrocyanide binds to cytochrome *c* peroxidase. The question arises as to whether electron transfer occurs in the complex or whether the complex is inactive and electron transfer occurs *via* bimolecular reactions between ferrocyanide and the enzyme intermediates.

If electron transfer occurs intramolecularly in the complex then the apparent bimolecular rate constants measured at low ferrocyanide concentrations are essentially the association rate constants for formation of the complex. If the complex is inactive then the apparent bimolecular rate constants are electron-transfer rate constants. Regardless of which mechanism is operative, the pH dependence of the apparent bimolecular rate constants should indicate whether acidic or basic groups on the enzyme influence the reaction.

The variation of k_2^{app} and k_3^{app} with pH may be due to differences in the reactivity of various protonated forms of the reactants. In view of the large ionic strength dependence of the apparent rate constants, it seems unlikely that an ionic strength of 0.1 M would be sufficient to completely suppress electrostatic effects. Corrections for electrostatic effects can be made from a knowledge of the net charge on the enzyme, the charge on the ferrocyanide, and the ionic strength dependence of the apparent rate constants.

According to transition state theory, the apparent rate constant is given by eq 4. ΔG^* is the free energy of activation per

$$k^{app} = (\bar{k}T/h)e^{-\Delta G^*/RT} \quad (4)$$

TABLE IV: Values of $\bar{Z}$, log k^0, and C.[b]

pH	$\bar{Z}$[a]	log k_2^{app}		log k_3^{app}	
		log k_2^0	C[b]	log k_3^0	C[b]
4.0	+17.4 ± 0.6	12.65 ± 0.30	16 ± 3	12.14 ± 0.35	18 ± 4
4.5	+9.7 ± 1.1	9.92 ± 0.18	11 ± 2	9.37 ± 0.20	12 ± 2
5.0	+3.6 ± 0.7	7.16 ± 0.02	10.3 ± 0.1	6.81 ± 0.02	7.3 ± 0.2
5.5	−2.2 ± 0.4	4.84 ± 0.03	2.3 ± 0.6	4.38 ± 0.02	2.5 ± 0.3
6.0	−4.4 ± 0.4	3.76 ± 0.03	−0.3 ± 0.7	3.31 ± 0.03	0.2 ± 0.5
6.5	−7.3 ± 0.2	2.27 ± 0.04	−0.6 ± 0.5	1.82 ± 0.02	0.3 ± 0.3
7.0	−9.5 ± 0.2	1.08 ± 0.06	0.4 ± 0.7	0.58 ± 0.12	0.6 ± 1.3
7.5	−12.2 ± 1.2	−0.05 ± 0.17	−2.2 ± 1.7	−0.49 ± 0.15	−2.4 ± 1.5
8.0	−14.1 ± 1.4	−0.87 ± 0.27	−2.8 ± 2.8	−1.36 ± 0.28	−2.7 ± 2.8

[a] Values from pH titration of native enzyme, 0.1 M ionic strength. [b] Defined in eq 14 of text.

mole, R is the gas constant, T is the absolute temperature, k is Boltzman's constant, and h is Planck's constant.

For ionic reactions in solution, the free energy of activation may be separated into three parts (eq 5) (Clark and Wayne,

$$\Delta G^* = \Delta G^*_0 + \Delta G_{es}^* + \Delta G_\mu^* \quad (5)$$

1969). ΔG^*_0 is the intrinsic free energy of activation. ΔG_{es}^* is the free energy of activation associated with electrostatic forces between the two reactant ions as they are brought together in the absence of solvating ions. ΔG_μ^* is the free-energy contribution due to interactions between the reactants and other charged species in solution, the ion atmosphere contribution.

The contribution due to the ion atmosphere, ΔG_μ^*, can be eliminated by determining the apparent rate constant at zero ionic strength. A bimolecular reaction can be written as

$$A + B \overset{K_{eq}}{\rightleftharpoons} X^* \overset{k'}{\longrightarrow} \text{products} \quad (6)$$

where A and B are the reactant ions and X* is the activated complex. The rate of the reaction will be given by eq 7. The

$$k^{app}[A][B] = k'(X^*) \quad (7)$$

equilibrium between activated complex and reactants is given by eq 8. The a's are the activities and the f's are the activity

$$K_{eq} = \frac{a_*}{a_A a_B} = \frac{[X^*]}{[A][B]} \frac{f_*}{f_A f_B} \quad (8)$$

coefficients. Combining eq 7 and 8 gives eq 9, where k^0 has re-

$$k^{app} = k^0 f_A f_B / f_* \quad (9)$$

placed $k'K_{eq}$ and is defined as the rate constant at zero ionic strength. The problem of extrapolating the apparent rate constant to zero ionic strength now becomes one of finding suitable expressions for the activity coefficients as a function of ionic strength.

The extended Debye-Huckel expression, eq 10, or semiem-

$$\log f_i = -AZ_i^2\sqrt{\mu}/(1 + Ba\sqrt{\mu}) \quad (10)$$

pirical expressions with a term proportional to ionic strength such as eq 11 have been used as extrapolation functions for the

$$\log f_i = -AZ_i^2\sqrt{\mu}/(1 + Ba\sqrt{\mu}) + C_i\mu \quad (11)$$

activity coefficients (Perlmutter-Hayman, 1972). In eq 10 and 11, A and B are constants with values of 0.509 and 0.329 in aqueous solutions at 25° when a, the distance of closest approach between the reactant ion and its ion atmosphere, is given in ångströms.

In our first attempts to fit the ionic strength dependence of k_2^{app} and k_3^{app} we used eq 10 for the activity coefficients of all species. For ferrocyanide Z_i is −4 and a has a value of 5 Å (Kielland, 1937). For I and II we assume the charge is equal to the average net charge on the native enzyme, $\bar{Z}$, and that the charge on the activated complex is $(\bar{Z} - 4)$. The distance of closest approach of the ion atmosphere is assumed to be the same for I, II, and the activated complex.

The value of a can be estimated from data in the literature. Ellfolk has shown from sedimentation and diffusion measurements that the frictional ratio for cytochrome c peroxidase is quite small, 1.03 (Ellfolk, 1967). Most of the deviation from unity is probably associated with hydration since, assuming spherical shape, the maximum hydration is quite small, 0.07 g/g of protein compared to a more typical value of 0.2 g/g of protein. Using a molecular weight of 34,000, water of hydration of 0.07 g/g of protein, and $\bar{v}_2$ of 0.733 cm^3/g, the radius of the hydrated sphere can be calculated to be 22 Å. An independent calculation, again assuming a sphere, based on unit cell dimensions from X-ray studies and occupancy of the unit cell also yields a value of 22 Å for the radius (Larsson *et al.*, 1970). About 2 Å, the average radius of the ions making up the ionic atmosphere, is added to the value of 22 Å for the distance of closest approach, giving a value of 24 Å for a.

Equation 12 is derived from eq 9 and 10 after the appropriate substitutions for the parameters are made.

$$\log k^{app} = \log k^0 - \frac{8.14\sqrt{\mu}}{1 + 1.64\sqrt{\mu}} - \frac{(4.07\bar{Z} - 8.14)\sqrt{\mu}}{1 + 7.90\sqrt{\mu}} \quad (12)$$

Using $\bar{Z}$ values given in Table IV, the variation of log k^{app} with ionic strength fits eq 12 very well between pH 5.5 and 7.0. Outside this pH range there is significant variation. One approach to obtain a better fit to the experimental values would be to allow either $\bar{Z}$ or a to be an adjustable parameter. When this is done, a better fit is obtained. If a is held constant at 24 Å and $\bar{Z}$ is allowed to vary, the absolute values of $\bar{Z}$ are smaller than the experimental values from the pH titration curve below pH 5.5 and above pH 7.0. If $\bar{Z}$ is maintained at the experimental values and a is allowed to vary, the value of a increases at high and low pH. Both of these trends are reasonable. The average net charge on the protein could be reduced at the pH ex-

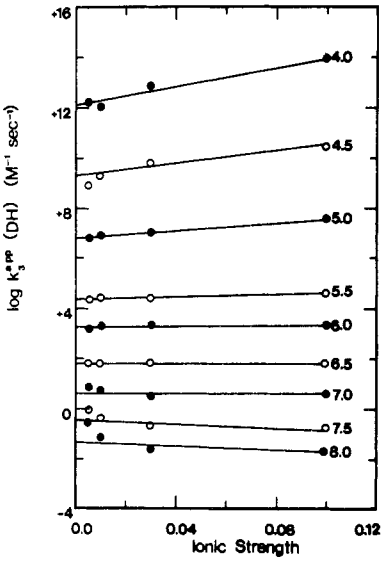

FIGURE 6: Plot of log k_3^{app}(DH), defined in eq 13 of text, *vs.* ionic strength.

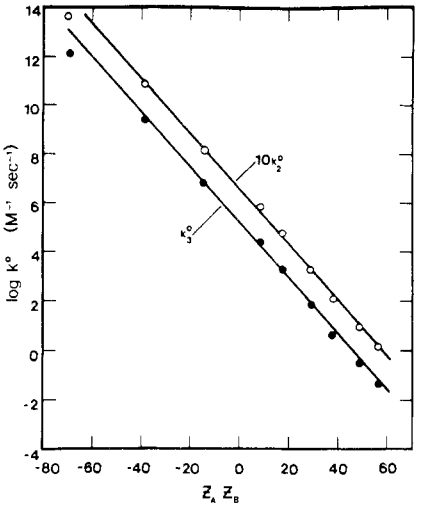

FIGURE 7: Plot of log k_2^0 and log k_3^0 *vs.* the product of the charges on ferrocyanide and I or II. Note k_2^0 has been multiplied by a factor of 10 to offset it from k_3^0.

tremes due to binding of buffer ions which should increase as the net charge on the protein increases. Also, a would tend to increase at the pH extremes as the electrostatic free energy of the molecule increases with increasing charge. The electrostatic free energy could be reduced by expansion of the molecule, one of the causes of protein denaturation at high and low pH. Since there are no experimental data on which to base a decision as to the more likely possibility, it was decided to use the semiempirical expression for the activity coefficients, eq 11. The parameters, C_i, would depend upon both effects. In addition, C_i would be dependent on such other factors as the variation of $\bar{Z}$ with ionic strength which has not been taken into account and other inadequacies of the model for the ionic strength dependence.

Before utilizing eq 11 for the activity coefficients it is useful to define log k^{app}(DH) according to eq 13. Log k^{app}(DH) is the

$$\log k^{app}(DH) = \log k^{app} + \frac{8.14\sqrt{\mu}}{1 + 1.64\sqrt{\mu}} + \frac{(4.07\bar{Z} - 8.14)\sqrt{\mu}}{1 + 7.90\sqrt{\mu}} \quad (13)$$

logarithm of the apparent rate constant corrected for the ionic strength variation expected from the extended Debye–Huckel expression for the activity coefficients. If the extended Debye–Huckel expression is adequate to explain the ionic strength dependence, then log k^{app}(DH) is equal to log k^0 as seen by comparison to eq 12.

Using eq 11 for the activity coefficients in eq 9, taking logarithms of both sides, and utilizing the definition of log k^{app}(DH) , eq 14 is obtained. C is equal to the sum of the

$$\log k^{app}(DH) = \log k^0 + C\mu \quad (14)$$

values of C_i for ferrocyanide and I or II minus the value of C_i for the activated complex in eq 11.

Log k^{app}(DH) was calculated according to eq 13 using values of k_2^{app}, k_3^{app}, and $\bar{Z}$ given in Tables II, III, and IV, respectively. Values of log k^{app}(DH) for k_3^{app} are shown in Figure 6 as a function of ionic strength. The fit to eq 14 is satisfactory. Values of log k^0 and C were calculated using a weighted least-squares method and these values are given in Table IV for both k_2^{app} and k_3^{app}. Between pH 6 and 7, the values of C are zero within experimental error indicating that the extended form of the Debye–Huckel equation gives a good approximation for the ionic strength dependence of k_2^{app} and k_3^{app}. At lower pH, C becomes increasingly positive and at higher pH, C becomes increasingly negative.

The values of the rate constants at zero ionic strength, if due to electrostatic interactions, should fit eq 15.

$$k^0 = \frac{\bar{k}T}{h} e^{-(\Delta G^*_0 + \Delta G_{es}^*)} \quad (15)$$

The simplest model for calculating ΔG_{es}* is that of two conducting spheres with radii r_A and r_B and charges Z_A and Z_B, which approach within a distance r_* in the transition state. For this model ΔG_{es}* equals $N_A Z_A Z_B e^2/Dr_*$, where N_A is Avogadro's number, e is the unit charge, and D is the dielectric constant of the reaction medium (Clark and Wayne, 1969). Substituting for ΔG_{es}* in eq 15, defining the intrinsic rate constant as $(\bar{k}T/h)e^{-\Delta G^*_0}$, and taking the logarithms of both sides gives eq 16. Figure 7 is a plot of log k^0 as a function of $Z_A Z_B$ where

$$\log k^0 = \log k^{int} - \frac{Z_A Z_B e^2}{2.303\bar{k}TDr_*} \quad (16)$$

Z_A equals -4, the charge on the ferrocyanide, and Z_B is equal to $\bar{Z}$ given in Table IV. Equation 16 is followed, with only a single point, at pH 4.0, showing significant deviation. A weighted least-squares fit to eq 16 gives values for log k_2^{int} of 5.57 ± 0.09, log k_3^{int} of 5.15 ± 0.08, and the slope of both lines is −0.112 ± 0.003. Using the bulk dielectric constant of water and the appropriate values for the other factors in eq 16, a value of 27.8 ± 0.7 Å can be calculated for r_*. This value is quite reasonable for the distance of closest approach of the enzyme and ferrocyanide in the transition state.

Reversing the process, *i.e.*, using the values of the intrinsic rate constant, $\bar{Z}$, and C as a function of pH, k_2^{app} and k_3^{app} can be calculated at 0.1 M ionic strength using the appropriate equations. The solid lines in Figure 3 were calculated in this manner. Although the fit is not perfect, it may be concluded that the major cause of the pH variation in the apparent oxidation rate constants at 0.1 M ionic strength is the electrostatic interaction between the ferrocyanide and oxidized enzyme intermediates.

This study has demonstrated that ferrocyanide binds to cyto-

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†

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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 1.2×10^3 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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