A Kinetic Study of the Reaction between Cytochrome *c* Peroxidase and Hydrogen Peroxide. Dependence on pH and Ionic Strength†

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ABSTRACT: The rate of the reaction between cytochrome *c* peroxidase and hydrogen peroxide was investigated using the stopped-flow technique. The apparent bimolecular rate constant was determined between pH 3.3 and pH 11 as a function of ionic strength. The pH dependence of the apparent bimolecular rate constant can be explained by assuming that two ionizable groups on the enzyme strongly influence the rate of the reaction. At 0.1 *M* ionic strength, a group with a pK_a of 5.5 must be unprotonated and a group with a pK_a of 9.8 must be protonated for the enzyme to react rapidly with hydrogen peroxide. The apparent acid dissociation constants depend upon the ionic strength. The true bimolecular rate constant has a value of $(4.5 \pm 0.3) \times 10^7\ M^{-1}\ sec^{-1}$ and is independent of ionic strength.

During our studies of the oxidation of ferrocyanide by compounds I and II of cytochrome *c* peroxidase (Jordi and Erman, 1974a,b) we observed that the rate of the reaction between the native enzyme and hydrogen peroxide was dependent upon the pH. This was unexpected since catalase (Chance, 1952; Jones and Suggett, 1968), horseradish peroxidase (Marklund et al., 1974), and lactoperoxidase (Maguire et al., 1971) all react with hydrogen peroxide in rapid, essentially pH independent reactions. In addition, one of the interesting differences in heme protein reactivity is the difference in reaction rate with hydrogen peroxide between such enzymes as the peroxidases and catalase and the nonenzymatic proteins such as methemoglobin (Dalziel and O'Brien, 1954) and metmyoglobin (George and Irvine, 1956). Even though these proteins contain the same prosthetic group, the latter two react with hydrogen peroxide about five orders of magnitude more slowly than the peroxidases and catalase. Furthermore, the reactions between hydrogen peroxide and methemoglobin and metmyoglobin are pH dependent.

Since the cytochrome *c* peroxidase–hydrogen peroxide reaction has attributes of both groups of heme proteins, we decided to investigate the reaction in greater detail to determine whether additional insight into the reactivity differences can be obtained.

Experimental Procedure

Isolation of cytochrome *c* peroxidase and preparation of enzyme solutions were the same as previously described (Jordi and Erman, 1974a). Stock solutions of hydrogen peroxide were standardized with potassium permanganate (Kolthoff and Belcher, 1957). Dilute solutions of hydrogen peroxide were prepared just prior to the kinetic measurements using volumetric techniques.

Phthalate, acetate, phosphate, borate, and glycinate buffers were used in the appropriate pH regions. Total buffer concentrations were usually 10 m*M*, but values as low as 0.3 m*M* were used at the lowest ionic strength. Ionic strength was adjusted using potassium nitrate. The kinetics of the reaction were independent of the buffer when different buffers were used at the same pH and ionic strength.

Kinetic measurements were performed on a Durrum-Gibson stopped-flow spectrophotometer at 25.0 ± 0.1°. The reaction was observed at 424 nm, the maximum difference in the absorption spectra of cytochrome *c* peroxidase and its first oxidized derivative (Yonetani, 1965). To increase the pH range beyond that in which the enzyme is stable, pH 4 to about pH 8, enzyme in unbuffered potassium nitrate solutions (pH ~5.3) was placed in one drive syringe. Buffered hydrogen peroxide solutions were placed in the second drive syringe. The pH was determined after mixing the two reactants.

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

The very rapid reaction between cytochrome *c* peroxidase and hydrogen peroxide could only be investigated over a relatively narrow range of concentrations by the stopped-flow technique. Cytochrome *c* peroxidase concentrations were usually near 0.5 μM and hydrogen peroxide concentrations ranged from 0.5 to 25 μM. Under conditions where the hydrogen peroxide concentration was at least ten times larger than that of the enzyme, the formation of compound I followed first-order kinetics indicating the reaction is first order with respect to the enzyme. The observed pseudo-first-order rate constants are directly proportional to the hydrogen peroxide concentration (Figure 1), indicating the reaction is also first order with respect to hydrogen peroxide. No evidence for saturation was detected. The apparent reaction can be written as

$$\mathrm{CcP} + \mathrm{H_2O_2} \xrightarrow{k_1^{app}} \mathrm{CcP\text{-}I} \qquad (1)$$

CcP represents the native ferric state of the enzyme and CcP-I represents compound I of cytochrome *c* peroxidase, oxidized two equivalents above the ferric state.

Values of k_1^{app} were determined as a function of pH and ionic strength. The results are shown in Figure 2. The

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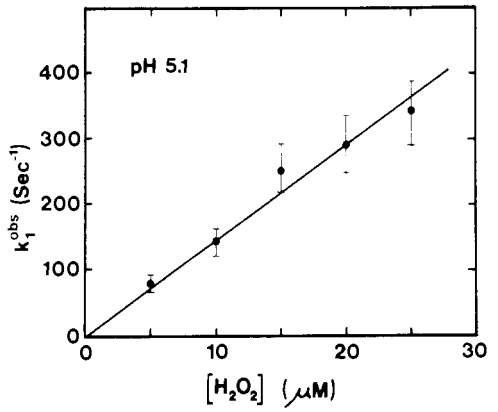


FIGURE 1: Plot of the observed pseudo-first-order rate constant as a function of hydrogen peroxide concentration. Reaction conditions: 0.42 μM cytochrome c peroxidase–10 mM acetate buffer (pH 5.1) adjusted to 0.10 M ionic strength with potassium nitrate.

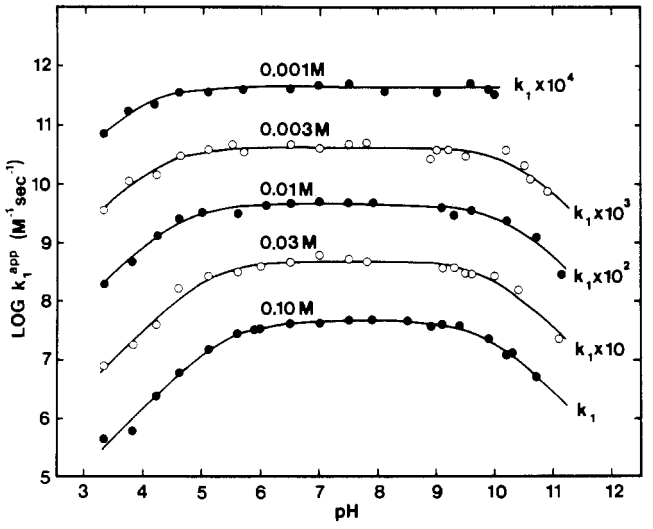


FIGURE 2: Plot of log k_1^{app} as a function of pH and ionic strength. Note that the rate constants have been multiplied by factors of 10, 10^2, 10^3, and 10^4 at 0.03, 0.01, 0.003, and 0.001 M ionic strength, respectively, in order to offset the data.

values of k_1^{app} are constant in the neutral pH region but decrease at both high and low pH.

In addition to the second-order reaction between cytochrome c peroxidase and hydrogen peroxide, a second, slower reaction was observed below pH 6. The amplitude of this second reaction was small, but increased as the pH was lowered. It is thought that this reaction is due to a relatively slow isomerization of cytochrome c peroxidase from a minor acid form of the enzyme which cannot react with hydrogen peroxide to the major enzyme species which rapidly reacts with hydrogen peroxide.

Discussion

The rate of the reaction between hydrogen peroxide and catalase or peroxidase species previously investigated is essentially independent of pH in the region where the enzyme is stable. The decrease in k_1^{app} below pH 6 at 0.1 M ionic strength seen in Figure 2 is unique to cytochrome c peroxidase. The simplest mechanism consistent with the pH dependence of k_1^{app} is shown in Scheme I. Scheme I indicates that two ionizable groups on the enzyme affect the rate of reaction between hydrogen peroxide and the enzyme. Hydrogen peroxide can only react with the enzyme when the group with acid dissociation constant K_1 is unprotonated and the group with acid dissociation constant K_2 is protonated. The expression for k_1^{app} in terms of the parameters in Scheme I is given by

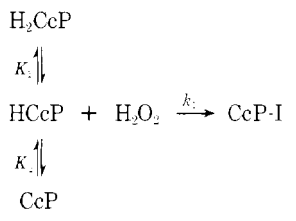
Table I: Values of k_1, K_1, and K_2 as a Function of Ionic Strength, 25°.

I (M)	k_1 ($\times 10^{-7}$ M sec)	K_1 ($\times 10^5$ M^{-1})	K_2 ($\times 10^{10}$ M^{-1})
0.10	4.6 ± 0.2	0.36 ± 0.04	1.5 ± 0.2
0.03	4.8 ± 0.3	0.78 ± 0.12	1.2 ± 0.2
0.01	4.4 ± 0.2	2.3 ± 0.4	0.68 ± 0.12
0.003	4.1 ± 0.3	5.3 ± 1.1	0.46 ± 0.11
0.001	4.4 ± 0.2	9.9 ± 1.9	

Table II: Rate Constants for the Reaction between Various Peroxidases and Hydrogen Peroxide.

Peroxidase	k_1 ($\times 10^{-7}$ M sec)	Ref
Cytochrome c peroxidase	4.5	This work
Lactoperoxidase	1.0	Maguire et al., 1971
Horseradish peroxidase		
Isozyme C2	1.5	Marklund et al., 1974
Isozyme A2	0.2	Marklund et al., 1974
Turnip peroxidase		
Isozyme A1	1.0	Hosoya, 1960
Isozyme A2	0.2	Hosoya, 1960
Isozyme D	0.54	Hosoya, 1960

Scheme I



The nonlinear least-squares program, KINET, was used to fit k_1^{app} as a function of pH to eq 2. The values of k_1, K_1,

$$k_1^{app} = k_1/([H^+]/K_1 + 1 + K_2/[H^+]) \quad (2)$$

and K_2, along with their standard error, are given in Table I as a function of ionic strength. The solid lines in Figure 2 were drawn according to eq 2 using the values for k_1, K_1, and K_2 given in Table I.

The rate constant, k_1, is independent of ionic strength as is to be expected for a reaction involving a neutral molecule such as hydrogen peroxide. The value of k_1 is somewhat larger than that for other peroxidases. Representative values from the literature are collected in Table II.

Cytochrome c peroxidase is unstable at pH values above about pH 8. The apparent ionization near pH 10, K_2, could be a consequence of denaturation. Alternatively, it could be a reflection of an acid–alkaline transition for cytochrome c peroxidase. The acid–alkaline transition occurs near pH 11 for horseradish peroxidase (Ellis and Dunford, 1969) and is thought to be due to ionization of a water molecule bound to the heme ion. There is also evidence that a conformational change of horseradish peroxidase is involved in the transition (Epstein and Schejter, 1972). Chance has indicated that the alkaline form does not react with hydrogen peroxide (Chance, 1949).

The ionization denoted by K_1 occurs within the pH stability range of the enzyme. The pK_1 value for this ionization decreases by almost 1.5 units as the ionic strength is reduced from 0.1 to 0.001 *M*. This is a much larger ionic strength dependence than normally observed for the ionizations of most simple compounds. The large ionic strength dependence of the apparent ionization constant could be due to several factors: specific ion effects, ionization coupled to a salt-dependent conformational change, or to interactions between the ionizable group and its environment.

We have observed a specific ion effect due to acetate on the steady state kinetic parameters for the oxidation of ferrocyanide at high ferrocyanide concentrations by cytochrome *c* peroxidase (Jordi and Erman, 1974b). At low ferrocyanide concentrations, the specific acetate effect is not observed, suggesting that acetate interacts with the enzyme–ferrocyanide complex, not the enzyme itself. Interchange of nitrate and phosphate has no effect on the steady-state oxidation of ferrocyanide. In addition we have not observed specific ion effects due to the interchange of phosphate and acetate in the reaction between cytochrome *c* peroxidase and hydrofluoric or hydrocyanic acid (Erman, 1974a,b) or in the reaction between cytochrome *c* peroxidase and hydrogen peroxide in this study. While specific ion effects due to nitrate have not been rigorously precluded, the previous studies suggest they would be unlikely under our experimental conditions. A salt-dependent conformational isomerization coupled to the ionization of a particular group could affect its apparent pK_a value. The conformational change would have to be rapid compared to the rate of reaction between the enzyme and cytochrome *c* peroxidase. Such a conformational change would be too fast to measure with our stopped-flow apparatus. Temperature-jump relaxation studies are planned along with an investigation of optical rotatory dispersion and circular dichroism of the enzyme in order to determine whether a fast, pH-dependent, conformational isomerization can be detected.

Finally, it may not be unusual for ionizable groups in proteins to have a large ionic strength dependence just due to their local environmental interactions. Parsons and Raftery, using a differential titration technique, determined the pK_a values for the side chain carboxyl groups of Asp-52 and Glu-35 at the active site of lysozyme as a function of ionic strength (Parsons and Raftery, 1972). The pK_a values for Asp-52 and Glu-35 decreased by 0.9 and 0.7 unit, respectively, as the ionic strength was reduced from 0.5 to 0.02 *M*. Shire et al. have also found that the acid dissociation constants of individual groups in metmyoglobin can vary substantially with pH and ionic strength (Shire et al., 1974).

The identity of the group with the acid dissociation constant K_1 is a matter of speculation. The similarities of the pK_a values and ionic strength dependence between this group and Asp-52 and Glu-35 of lysozyme suggest it could be a carboxyl group in the hydrophobic interior of cytochrome *c* peroxidase.

Cytochrome *c* peroxidase has a high aspartate and glutamate content in its polypeptide chain. In addition, the heme group has two propionic acid side chains at positions 6 and 7. Electron paramagnetic resonance spin-label studies suggest the propionic acid side chains are buried in the interior of the cytochrome *c* peroxidase (Asakura et al., 1969; Asakura and Yonetani, 1972). Esterification of one of the propionic acid groups reduces the activity of the enzyme to 5.6% of its original value using ferrocytochrome *c* as the substrate (Asakura and Yonetani, 1972). Esterification of both propionic acid side chains reduces the activity to less than 1% (Asakura and Yonetani, 1969). On the other hand, the activity of the protoheme dimethyl ester derivative toward ferrocyanide is about 40% (Asakura and Yonetani, 1969). This is to be expected if the esterification reduces the rate of the reaction between hydrogen peroxide and the enzyme. The reaction between hydrogen peroxide and cytochrome *c* peroxidase is rate determining under the conditions of the ferrocytochrome *c* kinetic assay (Yonetani and Ray, 1966) while it is not the rate-determining step in the ferrocyanide assay (Jordi and Erman, 1974a,b).

An ionizable group with an acid dissociation constant equal to that of K_1 strongly influences the association rate for the binding of both hydrofluoric and hydrocyanic acid to cytochrome *c* peroxidase (Erman, 1974a,b). It is probable that the same group influences both the hydrogen peroxide reaction and the ligand binding reactions. The similarity of the pH dependence of both reactions suggests that the ionizable group controls access to the heme site rather than directly participating in either reaction. Finally, the pH dependence of the hydrogen peroxide reaction supports the interpretation of the ligand binding data, that the enzyme is reactive when the ionizable group with acid dissociation constant K_1 is unprotonated and that hydrofluoric and hydrocyanic acid are the kinetically reactive species rather than the anionic forms. It still remains to be established whether the bound form of these ligands retains the proton or whether the proton dissociates upon binding.

It has recently been shown (Erman, 1975) that two ionizable groups affect the rate of oxidation of dicyanobis(1,10-phenanthroline)iron(II) by compound II of cytochrome *c* peroxidase. It appears likely that the two ionizable groups also affect the rate of oxidation by ferrocyanide (Jordi and Erman, 1974a). The pK_a values for the two ionizable groups in compound II are near 4.5 and 9.3. It is possible that the same two groups are affecting both the reaction between hydrogen peroxide and the native enzyme and the reaction between compound II and the ferrous complexes. If this is the case, it is interesting that the pH–rate profiles show an opposite relationship with respect to the acidic group. For the reaction with hydrogen peroxide, the reaction is fastest when the acidic group is unprotonated, while the preferred interpretation for the oxidation of the ferrous complexes by compound II is that the acidic group must be protonated.

An alternate, but less likely possibility, is that protonated forms of the two ferrous complexes are the reactive species. The same pH–rate profile would be generated by having the protonated ferrous complexes reacting with the enzyme when the acidic group is unprotonated just as in the hydrogen peroxide reaction. Both ferrocyanide (Jordan and Ewing, 1962) and dicyanobis(1,10-phenanthroline)iron(II) (Schilt, 1963) can be protonated although the pK_a of the latter compound in aqueous solution is unknown. If the pK_a of the dicyano complex is zero or less, a reasonable estimate, the mechanism involving its protonated form is impossible since it would require a pH independent rate constant larger than that of a diffusion controlled reaction to explain the observed rates.

In conclusion, it seems clear that the reactive form of cytochrome *c* peroxidase in its reaction with hydrogen peroxide is when the acidic group is unprotonated but that additional work is required to interpret the pH dependence of the oxidation of ferrous complexes by compound II of cytochrome *c* peroxidase.

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A Nuclear Magnetic Resonance Study of Nicotinamide Adenine Dinucleotide Phosphate Binding to *Lactobacillus casei* Dihydrofolate Reductase†

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ABSTRACT: The binding of $NADP^+$ to dihydrofolate reductase (EC 1.5.1.3) in the presence and absence of substrate analogs has been studied using ^1H and ^{13}C nuclear magnetic resonance (NMR). $NADP^+$ binds strongly to the enzyme alone and in the presence of folate, aminopterin, and methotrexate with a stoichiometry of 1 mol of $NADP^+$/mol of enzyme. In the ^{13}C spectra of the binary and ternary complexes, separate signals were observed for the carboxamido carbon of free and bound [^{13}CO]$NADP^+$ (enriched 90% in ^{13}C). The ^{13}C signal of the $NADP^+$–reductase complex is much broader than that in the ternary complex with methotrexate because of exchange line broadening on the binary complex signal. From the difference in line widths (17.5 ± 3.0 Hz) an estimate of the dissociation rate constant of the binary complex has been obtained (55 ± 10 sec^{-1}). The dissociation rate of the $NADP^+$–reductase complex is not the rate-limiting step in the overall reaction. In the various complexes studied large ^{13}C chemical shifts were measured for bound [^{13}CO]$NADP^+$ relative to free $NADP^+$ (upfield shifts of 1.6–4.3 ppm). The most likely origin of the bound shifts lies in the effects on the shieldings of electric fields from nearby charged groups. For the $NADP^+$–reductase–folate system two ^{13}C signals from bound $NADP^+$ are observed indicating the presence of more than one form of the ternary complex. The ^1H spectra of the binary and ternary complexes confirm both the stoichiometry and the value of the dissociation rate constant obtained from the ^{13}C experiments. Substantial changes in the ^1H spectrum of the protein were observed in the different complexes and these are distinct from those seen in the presence of NADPH.

Dihydrofolate reductase catalyses the NADPH-linked reduction of dihydrofolate to tetrahydrofolate, and is the target of a potent group of inhibitors of considerable chemotherapeutic interest (Blakley, 1969; Hitchings and Burchall, 1965). We are undertaking a detailed study of ligand binding to dihydrofolate reductase from a methotrexate-resistant strain of *Lactobacillus casei.*

We have already reported (Roberts et al., 1974) studies of the binding of the coenzyme (NADPH) and of a fragment of the substrate (*p*-aminobenzoyl-L-glutamate) to the

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