Chloroperoxidase

VII. CLASSICAL PEROXIDATIC, CATALATIC, AND HALOGENATING FORMS OF THE ENZYME*

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SUMMARY

The substrates for chloroperoxidase can be divided into three groups. Group 1 substrates react with chloroperoxidase in the presence of peroxide and a halogen anion (chloride, bromide, or iodide) to form stable halogenated products. Group 2 substrates are oxidized in a halogen anion-dependent reaction. The pH optima for the reactions of chloroperoxidase with both Groups 1 and 2 substrates are below 4. Group 3 substrates are oxidized in the absence of a halogen anion. This category includes classical peroxidase substrates such as guaiacol, pyrogallol, and leucomalachite green. The pH optima for Group 3 reactions extend over a broad range from pH 4 to 7, depending on the particular substrate being tested.

Chloroperoxidase also catalyzes reactions previously thought to be peculiar to catalase. In the absence of donors, chloroperoxidase reacts catalytically with hydrogen peroxide to form oxygen. At pH 4.5, the second order rate constant for this reaction is $2 \times 10^4 \text{M}^{-1} \text{sec}^{-1}$. Although this represents only about 2% of the rate observed with catalases, it is several hundred times more effective than horseradish peroxidase. The enzyme also oxidizes ethanol to form acetaldehyde, a reaction not catalyzed by horseradish peroxidase. Evidently the four-subunit structure of catalase but not both. In this respect, chloroperoxidase appears to bridge the gap in the range of catalytic activities that are classically used to distinguish between catalase and peroxidase.

A previous report from this laboratory (1) has described the crystallization and physical properties of the enzyme chloroperoxidase. The enzyme has a molecular weight of about 42,000, contains about 25% carbohydrate, and has ferrirhomboporphyrin IX as the heme prosthetic group. In these respects, the enzyme is similar to other plant peroxidases. Chloroperoxidase can catalyze the formation of a carbon-halogen bond, starting from halide ion (chloride, bromide, or iodide), hydrogen peroxide, and a suitable acceptor molecule (2).

In this paper, we report on a variety of reactions catalyzed by chloroperoxidase. The substrates involved in these reactions can be conveniently divided into several categories, based on their dependence on halide ion. The halide-dependent reactions appear to be associated with an acidic form of the enzyme, and the halide-independent reactions with a neutral enzyme form. In the latter category are a number of reactions that have previously been described (1, 2). Chloroperoxidase appears to bridge the gap in the range of catalytic activities that are classically used to distinguish between catalase and peroxidase.

EXPERIMENTAL PROCEDURE

Enzyme Preparation—The isolation of crystalline chloroperoxidase has been previously reported (1). The experiments reported in this paper were performed with preparations having a specific activity of 1200 to 1600.

Methods—The standard assay for the halogenating activity of chloroperoxidase involving the conversion of 1,1-dimethyl-4-chloro-3,5-cyclohexanediene (monochlorodimedone) to 1,1-dimethyl-4,4-dichloro-3,5-cyclohexanediene (dichlorodimedone) has been previously described (2).

The peroxidation reactions of substrates which required the presence of halogen anion were carried out in the following standard assay mixture: 300 μmoles of potassium phosphate buffer (pH 2.75), 60 μmoles of potassium chloride, 6 μmoles of hydrogen peroxide, and a suitable aliquot of chloroperoxidase plus the substrate to be tested in a total volume of 3 ml. All of the substrates were assayed spectrophotometrically by measuring the disappearance of an absorption peak characteristic of the reduced substrate or the appearance of an absorption peak characteristic of the oxidized product

The classical peroxidase activity of chloroperoxidase toward...
guaiacol and pyrogallol was measured in a spectrophotometric assay which contained 300 μmoles of potassium phosphate buffer (pH 4.8), 6 μmoles of hydrogen peroxide, enzyme, and substrate (20 μmoles of guaiacol or 60 μmoles of pyrogallol) in 3 ml of solution. The oxidation was measured by following the formation of tetraguaiacol at 470 μM (3) and the formation of purpuraginol at 430 μM (3). The oxidation of guaiacol was also measured under the same conditions described by Chance and Maehly for determination of the rate constant for the reaction of guaiacol with horseradish peroxidase Compound I (3). The final assay mixture contained 1.0 pmole of guaiacol, 0.4 μmole of hydrogen peroxide, 30 μmoles of potassium phosphate buffer, and chloroperoxidase (1.5 × 10⁻⁴ M) in a total volume of 3 ml. The luminol light-emitting reaction was followed by plotting the phototube response as a function of time with a Sanborn high speed recorder. Acetaldehyde was determined enzymatically as a product in the peroxidation of ethanol by chloroperoxidase and in the reaction between ethyl hydrogen peroxide and chloroperoxidase. These reactions were initiated by the addition of chloroperoxidase (0.3 μM final concentration) to the appropriate peroxide in the presence or absence of ethanol. The same incubation mixtures minus chloroperoxidase were used as controls. After about 10-min incubation, each reaction mixture (or aliquot thereof) was made 2.3 × 10⁻⁴ M in NADH in 0.1 M phosphate buffer, pH 6.0. The absorbance of NADH at 340 μM was recorded and liver alcohol dehydrogenase was added. The amount of acetaldehyde present was calculated from the loss of NADH absorption (ε₃₄₀ = 6.2 mm⁻¹ cm⁻¹).

Materials—Luminol was obtained from Eastman Organic Chemicals and was twice recrystallized from 3 N sulfuric acid before use. Leucomalachite green was a gift from American Cyanamid Company and was recrystallized three times from ethanol before use. Thiourea, thiouracil, and uric acid were purchased from Mann, ascorbic acid from Nutritional Biochemicals, NADH from Sigma, pyrogallol from Allied Chemicals, and guaiacol from Matheson. Guaiacol was redistilled before use. Hydrogen peroxide was purchased from Barium and Chemical, Inc., and was standardized periodically by iodometric titration. Ethyl hydrogen peroxide was obtained from Gallard-Schlechter Chemical Manufacturing Corporation, and was standardized by titration with ferrocyanide in the presence of cytochrome c peroxidase (6). Crystalline liver alcohol dehydrogenase and cytochrome c peroxidase were generously supplied by Dr. T. Yonetani (Johnson Research Foundation). m-Chloroperbenzoic acid (93 to 95% by assay) was a gift from Dr. G. R. Schonbaum (Johnson Research Foundation) and was dissolved in t-butanol directly before use. Monochlorodimedone was synthesized by the previously published method (2).

**RESULTS**

A number of substrates have been surveyed for their activity as electron donors in peroxidations catalyzed by this enzyme. The substrates which are acted upon by chloroperoxidase can be conveniently divided into three classes as shown in Table I. Group 1 substrates include those compounds which serve as halogen acceptors for chloroperoxidase. Thus, the β-diketone, monochlorodimedone, has been shown to be chlorinated, bro-

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<tr>
<td>50,000</td>
<td>β-Keto acids (7)</td>
<td>50,000</td>
<td>Thiourea (9)</td>
<td>10⁶ M⁻¹ sec⁻¹</td>
<td>Ethanol</td>
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<tr>
<td>60,000</td>
<td>Cyclic β-diketone (2)</td>
<td>60,000</td>
<td>Thiouracil (9)</td>
<td>2,300</td>
<td>Guaiacol</td>
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<tr>
<td>&gt;10,000</td>
<td>Phenols and phenolic esters (8)</td>
<td>1.8 × 10⁶ M⁻¹ sec⁻¹</td>
<td>Uric acid</td>
<td>2.3 × 10⁶ M⁻¹ sec⁻¹</td>
<td>Guaiacol (3)</td>
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<td>Hydrogen peroxide</td>
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<td>Leucomalachite green</td>
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<td></td>
<td>NADH</td>
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<td>Pyrogallol</td>
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The general procedure used in assaying substrates was described under "Methods." The reactions involving Group 1 substrates have been previously reported (2, 7, 8). The amounts of substrates in the Group 2 reaction mixtures and the wave lengths at which the reactions were measured were: luminol, 1.5 pmoles, 410 μM; hydrogen peroxide, 20 μmoles, 230 μM; thiourea, 0.4 μmole, 250 μM; thiouracil, 0.4 μmole, 280 μM; leucomalachite green, 6 μmoles, 610 μM; uric acid, 0.5 μmole, 290 μM; and NADH, 0.48 μmole, 340 μM. For the halide-independent reactions (Group 3), the amounts of substrates and the wave lengths at which the spectrophotometrically measured reactions were followed were: hydrogen peroxide, 20 μmoles, 230 μM; guaiacol, 20 μmoles, 470 μM; and pyrogallol, 60 μmoles, 430 μM. The activity with guaiacol was also measured under the conditions used by Chance and Maehly for horseradish peroxidase (3); 1 μmole of guaiacol, 0.4 μmole of hydrogen peroxide, and 30 μmoles of potassium phosphate (pH 7.0), in a total volume of 3 ml. The luminol reaction mixture contained 1.5 μmoles of luminol. The light-emitting reaction was followed by plotting the phototube response as a function of time with a Sanborn high speed recorder. Acetaldehyde was determined as the product of ethanol oxidation by assay with alcohol dehydrogenase (see "Methods"). Under the conditions used, reactions were independent of the substrate concentration, except for the reaction with ethanol, with hydrogen peroxide, and with guaiacol. The rates of these reactions are expressed as second order velocity constants, while all of the other rates are given in terms of turnover number for the enzyme (per min).
minimized, and ioxidated when incubated with chloroperoxidase, hydrogen peroxide, and the corresponding halide ion (2). These halogenation reactions have a pH optimum of 2.75 under the standard assay conditions (2).

Group 2 compounds are those substrates which are oxidized by chloroperoxidase in a reaction which has the same requirements as does the halogenation reaction, i.e., an absolute requirement for chloride, bromide, or iodide and a pH optimum below 4. The halogen anion requirement for peroxidation of Group 2 compounds cannot be met by fluoride, thiocyanate, cyanide, or formate.

Into the last category falls the group of substrates toward which chloroperoxidase exhibits classical peroxidase activity (Group 3). Peroxidation of Group 3 substrates does not require the presence of halide, and the pH optimum is very broad, ranging between pH 4 and 7, depending on the substrate under consideration. In this pH range, luminol is peroxidized in the typical light-emitting reaction (10). Guaiacol and pyrogallol are peroxidized in a typical peroxidatic fashion, and exhibit broad pH optima, ranging from 4 to 6. In addition, at these higher pH values, chloroperoxidase exhibits catalatic activity which is not dependent on the presence of a halide.

**pH Profiles for Group 1, 2, and 3 Substrates**—The pH optimum for representative substrates from each of these groups is shown in Fig. 1, in which the rate of chlorination of monochlorodimedone (Group 1 substrate), the rate of oxidation of uric acid in the presence of chloride (Group 2 substrate), and the rate of classical peroxidation of guaiacol (Group 3 substrate) are plotted as a function of pH. As can be seen from these curves, the chlorination reaction (Curve 1) and the halide-dependent oxidation of uric acid (Curve 2) have the same pH optimum. It should also be noted that the rate of chlorination of monochlorodimedone is identical with the rate of the halide-dependent oxidation of uric acid in terms of turnover number for the enzyme. The halogen anion-independent peroxidation of a Group 3 substrate, guaiacol (Curve 3), shows the same type of broad pH curve exhibited by horseradish peroxidase with this type of substrate (12). The rate of the chloroperoxidase-catalyzed chlorination reaction and the rate of the halide-dependent oxidation reaction are 20 times greater than the rate of classical peroxidation of guaiacol by chloroperoxidase. When compared on a weight basis to horseradish peroxidase, the classical peroxidase activity of chloroperoxidase with guaiacol is 10 times lower (3).

Some compounds appear as substrates in more than one group. For example, most of the substrates in the first and second categories are peroxidized in the absence of halide ion. However, these reactions are very slow in comparison to the halide-dependent reactions. Thus, monochlorodimedone is peroxidized to some unknown product in the absence of halide. The rate of this reaction is about 0.3% the rate of the chlorination reaction in the presence of 20 mM sodium chloride. During the course of the halide-independent oxidation of monochlorodimedone, the extinction coefficient of monochlorodimedone drops from 12 cm⁻³ M⁻¹ to less than 0.2 cm⁻³ M⁻¹. The extinction loss in the presence of halide is about the same, but in this case is due to the gem dihalogenated product (2).

Luminol is also placed in Group 2 because chloroperoxidase, when supplemented with chloride, bromide, or iodide, catalyzes the oxidation of luminol in a non-light-emitting reaction. This halogen anion-dependent oxidation reaction, which can be measured by an increased absorbance at 410 nm, has a pH optimum at 2.75 under the normal assay conditions. However, in the pH range 4 to 7, chloroperoxidase catalyzes the oxidation of luminol in a typical light-emitting reaction (10). The light-emitting luminol oxidation does not require the presence of a halogen anion (Group 3 reaction).

**Catalatic Activity of Chloroperoxidase**—Hydrogen peroxide is listed as both a Group 2 and a Group 3 substrate. Chloroperoxidase catalyzes the catalatic decomposition of hydrogen peroxide both in the presence and absence of halogen anion. Fig. 2A shows the formation of oxygen as observed by the oxygen electrode when 500 nmoles of hydrogen peroxide were mixed with 0.24 μM chloroperoxidase. The data indicate that over 210 nmoles of oxygen were formed. The same stoichiometry was observed when catalase was substituted for chloroperoxidase, in agreement with the equation

\[ 2H_2O_2 \rightarrow O_2 + 2H_2O \]  

**Equation 1**

Since the response time of the oxygen electrode is long, kinetic studies of the catalatic reaction were more conveniently followed spectrophotometrically by the loss of hydrogen peroxide absorbance in the ultraviolet. The catalatic reaction was found to have a first order dependence on the concentration of hydrogen peroxide at both pH 3.4 and 6.0. From the slope of the first order plots and the enzyme concentration, the second order rate constant could be calculated for the catalatic reaction (Equation 2).

\[ k = \frac{X_0}{X} \ln \frac{X}{[Enz]} \]  

**Equation 2**

In this equation, \( t \) is elapsed time, \( X_0 \) is the initial hydrogen peroxide concentration, \( X \) is the hydrogen peroxide concentration at time \( t \), and \([Enz]\) represents the concentration of chloroperoxidase. At pH 6, \( k = 7 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1} \) and, at pH 3.4, \( k = 1.1 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1} \). The pH dependence of the catalatic reaction is shown in Fig. 3, Curve 2. This curve shows a pH
Chloroperoxidase. VI

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A hydroque

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I

0 1 2 3 4 5

TIME (minutes)

Fig. 2. Formation of oxygen by chloroperoxidase (CPO) with different oxidants. All reactions were run in 3 ml of 0.1 M potassium phosphate buffer, pH 6.0. In A, chloroperoxidase (2.4 X 10^-7 M final concentration) was added to 500 nmoles of hydrogen peroxide. In B, chloroperoxidase (2.4 X 10^-7 M final concentration) was added to 2100 nmoles of m-chloroperbenzoic acid. For C, the addition of chloroperoxidase (1.5 X 10^-7 M final concentration) to 4800 nmoles of ethyl hydrogen peroxide was preceded by two additions of catalase to get rid of a small contaminant of hydrogen peroxide present in the ethyl hydrogen peroxide.

The pH dependence of the rate of chloroperoxidase-catalyzed hydrogen peroxide decomposition. Curves 1 (△ △) and 2 (○ --- ○) show the rates of catalytic activity in the presence and absence of chloride, respectively. These rates were measured as described in Table I with phosphate buffer of the indicated pH value. Curve 3 (●●●) is the difference between Curves 1 and 2. The rates were calculated with an extinction coefficient of 67 M^-1 cm^-1 for hydrogen peroxide (3) at 230 mp.

Table II

Acetaldehyde and oxygen formation by chloroperoxidase

Oxygen formation was followed with an oxygen electrode in conjunction with a Honeywell recorder. The concentration of acetaldehyde was determined spectrophotometrically by measuring the amount of NADH oxidized by an aliquot of the reaction mixture in the presence of alcohol dehydrogenase.

<table>
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<tr>
<th>Experiment</th>
<th>Reactants</th>
<th>Products</th>
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<tbody>
<tr>
<td></td>
<td>H2O2</td>
<td>CH3CH2OH</td>
</tr>
<tr>
<td>1</td>
<td>330</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>330</td>
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<tr>
<td>3</td>
<td>330</td>
<td>28.0</td>
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Fig. 3, Curve 1, shows the pH dependence of the catalatic reaction in the presence of 20 mm sodium chloride. Curve 2 plots the difference between the catalatic rates in the presence and absence of chloride. This curve, which represents the chloride-dependent catalatic activity of chloroperoxidase, shows the same pH optimum as does the chlorination reaction under similar conditions (compare Fig. 1). Higher concentrations of chloride were found to stimulate the catalatic activity even more. At 0.1 M sodium chloride, the rate constant for the catalatic reaction was found to be 1.8 X 10^6 M^-1 sec^-1 at pH 3.5.

Oxygen Evolution from Other Peroxides—Chloroperoxidase was also found to have catalatic activity with ethyl hydrogen peroxide. In Fig. 2, Curve B, 1 X 10^-8 M bovine liver catalase was added to 4.8 pmoles of ethyl hydrogen peroxide in 0.1 M phosphate buffer, pH 6.0. Since this sample of ethyl hydrogen peroxide was contaminated by a small amount of hydrogen peroxide, the evolution of 54 nmoles of oxygen was observed. A second addition of an equivalent or greater amount of catalase failed to catalyze further oxygen evolution. When chloroperoxidase was then added (final concentration, 1.4 X 10^-7 M), the slow evolution of oxygen was observed. The amount of oxygen finally formed was 180 nmoles. The total oxygen evolution accounts for only a very small proportion of the ethyl peroxide initially present. The remaining equivalents of ethyl hydrogen peroxide were found to be converted into acetaldehyde.
amounts of oxygen were formed. Increasing concentrations of ethanol resulted in decreased yields of oxygen, with resultant increase in the amount of acetaldehyde formed.

Oxygen was also evolved when m-chloroperbenzoic acid was added to chloroperoxidase (Fig. 2, Curve C). Neither catalase nor horseradish peroxidase would substitute for chloroperoxidase in this reaction. Heat-denatured chloroperoxidase also had no catalatic activity with any of the peroxide compounds.

Oxidation of Iodide to Iodine—It has been previously reported that chloroperoxidase catalyzes the peroxidation of iodide ion to form iodine (2). The pH dependence of the rate of this reaction is shown in Fig. 4. This bimodal pH curve is very similar to the pH dependence of the catalatic activity in the presence of chlorite (see Fig. 3) and again probably reflects the halide-independent and -dependent activities of the enzyme. In Fig. 5, the rates of iodine formation are shown at pH 2.75 (Curve 1) and at pH 4.8 (Curve 2). At pH 2.75, the initial rate of iodine formation was linear for at least 15 sec, while, at pH 4.8, there was a definite lag period. The length of the initial lag period was inversely proportional to enzyme concentration. The difference in kinetics may reflect a mechanistic difference in these two pH ranges.

Fig. 4. Dependence of the rate of iodine formation on pH. The reaction mixture contained 300 μmoles of phosphate of the designated pH, 3 μmoles of potassium iodide, 3 μmoles of hydrogen peroxide, and 0.15 μg of chloroperoxidase in a total volume of 3 ml. The reaction was followed by the increase in absorbance at 350 μm due to the formation of triiodide. The measurement of rate was taken at a point at which the course of the reaction was linear (see Fig. 6). The rates in terms of micromoles per min were calculated according to the method of Hosoya (15). At these concentrations of iodide and hydrogen peroxide, a correction for the nonenzymatic oxidation of iodide was unnecessary.

Fig. 5. Dependence of the time course of iodide peroxidation on pH. Curves 1 (---) and 2 (----) show the formation of iodine at pH 2.75 and pH 4.8, respectively. The reaction mixtures were the same as in Fig. 5.

DISCUSSION

The classical distinction between enzymes of the peroxidase and catalase type has been based on their reactivity with hydrogen peroxide. In the presence of catalase, hydrogen peroxide is rapidly decomposed to form oxygen. Peroxidases have less than 0.01% of the reactivity of catalase in this same reaction (16). These enzymes also differ in their specificities for donors. Peroxidase can catalyze the oxidation of ferrocyanide and ascorbate, whereas catalase does not. On the other hand, catalase can catalyze the peroxidation of ethanol and formic acid, while peroxidase is inert toward these compounds (16). In addition to being distinguished by their substrate specificities, peroxidases and catalases also have greatly different physical parameters. Catalases from many different sources, including mammalian and bacterial, have molecular weights between 220,000 and 250,000, and contain four subunits per molecule (3). Peroxidases, in general, are monomeric, have one heme group, and possess molecular weights of about one-fourth that of catalase. In addition, all of the peroxidases (with the exception of cytochrome c peroxidase) contain a carbohydrate portion. Horseradish peroxidase and Japanese radish peroxidase contain approximately 18% (17) and 28% (18) carbohydrate, respectively. Catalases lack a carbohydrate component.

In this paper, we have shown that chloroperoxidase bridges some of the classical differences between these two classes of enzymes. With regard to physical and chemical composition, chloroperoxidase is similar to the plant peroxidases. Chloroperoxidase has previously been shown to have a molecular weight of 42,000 with only one heme group per molecule, and contains 28% carbohydrate. Although chloroperoxidase has the single subunit structure of peroxidases, it manifests a significant amount of catalatic activity. In the absence of halide, the rate constant for the destruction of hydrogen peroxide by chloroperoxidase is 2 × 10^4 M^-1 sec^-1 at pH 4.5. Although this represents only a few per cent of the rates observed with catalases (16), it is several hundred times larger than the value observed with other peroxidases. In the presence of 0.1 M sodium chloride, the chloroperoxidase catalatic rate constant is 1.8 × 10^4 M^-1 sec^-1 at pH 3.5, or about 20% of the maximal rate observed with catalases (per heme group) (16).

Other activities of chloroperoxidase show characteristics similar to both peroxidase and catalase. Chloroperoxidase has been shown to oxidize ethanol to acetaldehyde. Preliminary results indicate that formate is also oxidized by the enzyme. Both of these reactions have been considered to be peculiar to catalase. The enzyme also oxidizes typical peroxidase substrates such as guaiacol, pyrogallol, and ascorbate. The rate with guaiacol as substrate is approximately 10% that observed with horseradish peroxidase under similar conditions. In terms of turnover numbers, the enzyme is a poor catalase when compared to other catalases, and a poor peroxidase when compared to horseradish peroxidase. In this respect, chloroperoxidase has catalytic properties intermediate between the classical enzymes. If it is assumed that enzymes catalyzing similar reactions have similar active sites, then it follows that chloroperoxidase has an active site which represents a composite of the active sites of catalase and peroxidase. This would imply that the active sites of catalase and peroxidase are not very different, and that small changes determine whether an enzyme functions better as a catalase or a peroxidase. It also implies
that the four-subunit structure of catalase may have little to do with its high catalatic activity.

Rather than being due to similarities in active sites, the range of reactions catalyzed by chloroperoxidase may simply represent a lack of enzyme specificity. The initial peroxide compound of chloroperoxidase (19) could be more accessible for reaction with substrates than the peroxide compounds of peroxidase or catalase. Several facts support this "lack of specificity" concept.


2. The catalatic activity of chloroperoxidase is pH-dependent, whereas that of catalase is pH-independent over a wide range (13).

3. The spectral complexes of chloroperoxidase with ligands do not show intermediate behavior between catalase and peroxidase, as would be expected if the heme sites were similar. For example, the Soret bands of the azide and cyanide complexes of chloroperoxidase (1) are shifted 12 to 19 nm to the red with respect to the corresponding complexes of catalase and peroxidase (20).

The reactions catalyzed by chloroperoxidase show two different pH optima. The classical (halide-dependent) peroxidatic and catalatic activities of this enzyme have an optimum at about pH 5, while the halide ion-dependent reactions have a much lower pH optimum. Iodide ion, a common donor for peroxidases, has maximal rates of peroxidation at both pH 3 and 5. However, the kinetics of iodine formation is different in these two pH regions. At pH 5, there is a time lag preceding the maximal rate of iodine formation, while at pH 3 the rate of iodine formation is linear.

The above facts are consistent with chloroperoxidase having two active forms, "acidic" and "neutral," which are responsible for these catalytic activities. The transition from the neutral to the acidic form of the enzyme occurs between pH 5 and 3. The most likely candidate for titration in this pH region is a carboxyl group.

It was noted that the rate of chlorination of monochlorodimethane (Group 1 substrate) was found to be identical with the rate of chloride-dependent oxidation of uric acid (Group 2 substrate). Other substrates in these two groups have also been found to have the same rate. The simplest interpretation of these results is that the rate-limiting step in these reactions is the formation of an active halogenating species. It has been proposed previously on the basis of anisole isomer ratios obtained in the chlorination reaction that the active intermediate was equivalent to a halogenium ion (Cl+) (8). The interaction of Group 1 substrates with the active halogen species leads to the formation of a covalent carbon-halogen bond, while Group 2 substrates form unstable halogenated intermediates which decompose by hydrolysis or by reaction with a 2nd molecule of substrate (9).

The formation of oxygen from ethyl hydrogen peroxide and from m-chloroperbenzoic acid is quite interesting from a mechanistic standpoint. In the catalatic reaction of catalase, isotope experiments have shown that both atoms in a product oxygen molecule originate from the same molecule of hydrogen peroxide (21). For such a reaction, one may propose a direct oxidation of peroxide by an oxidized enzyme molecule (Equation 5).

\[ \text{Enz}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Enz}^{2+} + 2\text{H}^+ + \text{O}_2 \]  

However, to get oxygen from ethyl hydrogen peroxide, it is quite difficult to write a similar mechanism without proposing the existence of unstable intermediates, such as CH_3CH_2O^-. Organic reactions involving the decomposition of organic peroxides to yield oxygen are second order in the organic peroxide, indicating a bimolecular reaction (22). Assuming that the enzymatic reaction is similar to known organic reactions, one would have to propose that the enzyme provides a site for the appropriate collision of 2 molecules of the peroxide. This would imply that the initial peroxide compound of chloroperoxidase contains either all of the elements of the peroxide or at least a reactive oxygen atom. The latter is more likely, as Schonbaum has shown that formation of the initial peroxide compound of horse-radish peroxidase by m-nitroperbenzoic acid is accompanied by the simultaneous release of m-nitrobenzoate (23). The origin of the oxygen obtained in the experiments with chloroperoxidase and m-chloroperbenzoic acid is currently under investigation.

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