The Reaction of Chlorite with Horseradish Peroxidase and Chloroperoxidase

ENZYMATIC CHLORINATION AND SPECTRAL INTERMEDIATES

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SUMMARY

Chloroperoxidase and horseradish peroxidase use NaClO₃ as both the oxidant and the halogen donor for the peroxidative chlorination of monochlorodimedone. Previous studies have shown that both horseradish peroxidase and chloroperoxidase can catalyze iodination reactions with hydrogen peroxide as the oxidant; however, only chloroperoxidase catalyzes chlorination reactions under these conditions. The chlorite chlorination reactions obey normal Michaelis-Menten kinetics with respect to chlorite. Free chloride ion is not required for the chlorite-promoted chlorination reaction and has no effect on the K_m values for chlorite or the V_max values for either enzyme. The V_max for the chlorination of monochlorodimedone by chloroperoxidase when chlorite is the substrate is approximately 3 times greater than when hydrogen peroxide is the oxidant, suggesting that chlorite is an exceptionally good substrate. The pH optimum for the chlorination reaction with horseradish peroxidase as catalyst is 4.1 and with chloroperoxidase a broad optimum from pH 2.25 to 3.0 is observed. Chloroperoxidase can also utilize chlorite as the oxidant for the oxidation of classical peroxidase substrates such as guaiacol, pyrogallol, and thiourea. Horseradish peroxidase reacts with chlorite to form a relatively stable product (half-life greater than 20 min) which is spectrally observable and indistinguishable from Compound I, the first detectable intermediate formed upon the reaction of horseradish peroxidase with peroxides. The reaction yielding the horseradish peroxidase-Compound I spectrum has a precise stoichiometry. The addition of increments of chlorite to horseradish peroxidase results in the rapid decomposition of the intermediate and regeneration of the spectrum of the native enzyme.

Incorporation studies using ^3Cl indicate that the chlorine atom derived from chlorite is incorporated directly into the substrate acceptor molecule during chlorination and does not undergo exchange with free chloride ion, even in the presence of exceedingly high levels of free halide. This suggests that the reaction of chlorite with both horseradish peroxidase and chloroperoxidase results in the formation of an enzyme-bound activated halogenating intermediate. These results, in conjunction with our previous studies on the chemical nature of Compound I, suggest that the activated halogenating intermediate may be represented as an enzyme-bound halogenium ion (−X⁺) or hypohalite ion (−OX⁻) coordinated to the heme prosthetic group.

Studies on the biosynthesis of caldariomycin in this laboratory led to the isolation of chloroperoxidase from the mold, Caldariomyces fumago (1). The purified enzyme is a monomer having a molecular weight of approximately 42,000, contains 1 mole of ferrirhodoporphyrin IX per mole of enzyme, and is quite similar in many of its physical properties to other protoheme peroxidases such as horseradish peroxidase, Japanese radish peroxidase and cytochrome c peroxidase (1). In the presence of hydrogen peroxide, halide anion (chloride, bromide, or iodide but not fluoride) and a suitable halogen acceptor, the enzyme catalyzes the peroxidative formation of a carbon-halogen bond as illustrated in Equation 1 (2).

\[ \text{AH} + X^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow AX + 2\text{H}_2\text{O} \]  

The halogen acceptor (AH) can be any good nucleophile having a suitably activated position available for attack by an electrophilic halogen species. Good organic acceptor molecules include β-keto acids, cyclic β-diketones and substituted phenols.

Reactions catalyzed by peroxidases involve the formation of a spectrally observable enzyme intermediate known as Compound I, which is thought to be the first product formed upon the reaction of the enzyme with peroxide (3). This intermediate was originally believed to represent an enzyme-substrate complex. George (4) subsequently proposed that Compound I actually represents a higher oxidation state of the enzyme. This proposal was based on the observation that a Compound I-type spectrum could be generated by the reaction of horseradish...
peroxidase with a wide variety of oxidants such as HIOCl, IOBr, NaClO₂, KBrO₃, and KIO₄. This proposal gained further support from the work of Schonbaum and Lo (5) who reported that the reaction of m-nitroperbenzoic acid with horseradish peroxidase to form Compound I proceeds with the concomitant release of m-nitrobenzoic acid, thereby ruling out the idea of an enzyme-substrate complex. George (4) also reported that several oxidants which do not contain the elements of hydrogen peroxide could nevertheless replace peroxide in the oxidation of guaiacol. We report here that sodium chloride (NaClO₂) serves not only as an alternate oxidant for peroxidases but also that chlorite can serve simultaneously as both a halogen donor and oxidant for the chlorination of MCD with both chloroperoxidase and horseradish peroxidase. Although horseradish peroxidase catalyzes iodination reactions (6, 7) it is unable to catalyze chlorination reactions with peroxide as the oxidant. Therefore, the results reported in this paper represent the first detection of a chlorinating activity for horseradish peroxidase. Horseradish peroxidase and chloroperoxidase both form special intermediates with chlorine which are quite similar to the respective Compound I spectra for each enzyme. The radioisotope-labeling studies described in this paper suggest the involvement of an enzyme bound activated halogen intermediate which is subsequently incorporated into the chlorinated substrate. A mechanism for peroxidase-catalyzed halogenation and a structure for the halogenating intermediate are discussed on the basis of these results.

EXPERIMENTAL PROCEDURE

Enzyme Preparations—Chloroperoxidase was isolated from Caldariummyces fumago and purified as reported previously (1). The preparations used in this paper had a specific activity of at least 2000 units per mg of protein in the standard chlorination assay and exhibited Rₜ values of at least 1.4, indicating greater than 95% purity. Protein concentrations were determined by the method of Lowry et al. (8).

Crude horseradish peroxidase (Rₜ = 0.3), obtained from Sigma Chemical Co., was purified by a modification of the procedure described by Shannon et al. (9). The crude enzyme material was dialyzed against 5 mM sodium acetate buffer, pH 4.4, and dialyzed overnight against 2 liters of the same buffer. The protein solution was centrifuged for 10 min at 40,000 x g in a Sorvall centrifuge at 4°. The supernatant fraction was layered onto a carboxymethyl cellulose column which had been equilibrated with 5 mM sodium acetate buffer, pH 4.4. The column was eluted and the effluent fractions were pooled in the manner described by Shannop et al. (9). The fractions were further purified by passage over a DEAE-cellulose column as previously described (9). The purified iso-zymes were concentrated by dialysis against 90% saturated ammonium sulfate followed by centrifugation at 40,000 x g for 15 min. The resulting precipitates were dissolved in 1 mM potassium phosphate buffer, pH 7, and dialyzed against the same buffer to remove residual ammonium sulfate. The isozymes were stored at 4° at protein concentrations of at least 5 mg per ml. Horseradish peroxidase concentrations were determined using the molar absorbance index for the various isozymes reported by Shannon et al. (9). Fraction A-1 had an Rₜ value of 3.2, Fraction B had an Rₜ value of 3.15, and Fraction C had an Rₜ value of 3.28. These Rₜ values compare favorably with those reported for homogeneous A (4.19), B (3.37), and C (3.42) fractions.

Enzyme Assays—The standard assay for enzymatic chlorinating activity involves the conversion of MCD to DCD and has been described previously (1). The standard assay reaction mixture contained 300 μmoles of potassium phosphate buffer (pH 2.8), 60 μmoles of potassium chloride, 0.3 μ mole of MCD, and a suitable aliquot of enzyme in a final volume of 3 ml. Unless otherwise stated the reaction was initiated by the addition of the oxidant, either hydrogen peroxide or sodium chloride. The rate of conversion of MCD to DCD was measured in a Gilford recording spectrophotometer by measuring the loss of absorbance at 276 nm.

The peroxidation of guaiacol and pyrogallol was also measured spectrophotometrically. The rate of formation of tetraguaiacol was measured at 470 nm (10) and the rate of formation of purpurinolin from pyrogallol was measured at 430 nm (10). The assay reaction mixtures contained 300 μmoles of phosphate buffer (pH 4.8), substrate (20 μmoles of guaiacol or 60 μmoles of pyrogallol), and a suitable aliquot of enzyme in a final volume of 3 ml. The reaction was initiated by the addition of either 6 μmoles of hydrogen peroxide or 6 μmoles of chlorite.

The halide-dependent oxidation of thiourea was also followed spectrophotometrically at 235 nm (11). The reaction mixture contained 300 μmoles of potassium phosphate buffer (pH 4.8), 60 μmoles of potassium chloride when hydrogen peroxide was used as the oxidant, 0.6 μ mole of thiourea, and a suitable aliquot of enzyme in a final volume of 3 ml. The reaction was initiated by the addition of 6 μmoles of either hydrogen peroxide or chlorite.

Synthesis of [3Cl-Labeled Sodium Chlorite—Sodium hypochlorite rapidly exchanges chlorine atoms with chloride in aqueous solution (12). The subsequent conversion of [3Cl-labeled hypochlorite to chlorite (13) produced converted sodium chlorite (14). This exchange reaction was carried out by mixing 1.2 ml of a 5% aqueous solution of sodium hypochlorite (0.8 mmole) in a two-neck round-bottom flask, adjusting the pH to 8 with dilute sulfuric acid and then adding 0.1 ml of [3Cl-labeled sodium chloride (5.78 μCi, 0.021 mmole). The flask was heated to 100° in an oil bath for 1 hour to form sodium chlorite. After cooling, 40 mg (0.14 mmole) of oxalic acid and 40 μl of 36Cl labeled sodium chloride were added and the flask was fitted with a receiver containing 3 ml of water which was cooled in an ice bath and protected from the light. The reaction mixture was heated to 100°, and the product, ClO₂, was flushed with nitrogen into cold water. After 45 min the flask was cooled and an additional 40 mg of oxalic acid and 40 μl of sulfuric acid were added. The mixture was warmed noonto 50° and the final chlorite was measured by collection of Cl₂ gas. A 0.5% aqueous NaClO₂ solution was shaken with 60 mg of Ba(OH)₂·8H₂O (0.19 mmole) and 30 μl of hydrogen peroxide (30%, 0.026 mmole) until the yellow color disappeared. The barium carbonate was filtered, washed and the filtrate was treated with 44 mg of sodium sulfate and boiled. After cooling, the barium sulfate was filtered off and the filtrate was evaporated on a flash evaporator. The resulting precipitate was dissolved in 5 ml of water and was stored frozen and protected from light.

Assay of [3Cl-Labeled Sodium Chlorite—Radioactive chlorite was assayed by measuring the conversion of MCD to DCD in the presence of chloroperoxidase. The assay mixture contained 300 μmoles of potassium phosphate buffer (pH 3), 0.3 μ mole of MCD, a suitable amount of enzyme (excess), and increasing amounts of sodium chlorite in a final volume of 3 ml. The final absorbance values for the various assay mixtures were plotted as a function of the amount of sodium chlorite used. The concentration of chlorite was calculated from the linear portion of the curve by comparison to a standardized solution of commercial sodium chlorite. Spectral Measurements—Optical spectra were recorded at room temperature on a Cary 15 spectrophotometer using cells with path lengths of 0.1 cm and precise concentrations of 0.3 to 0.6 mg per ml. Materials—MCD was synthesized as described previously (2). Thiourea was obtained from Mann Chemicals, pyrogallol from Allied Chemicals, and ethyl hydrogen peroxide from Ferozan. Sodium chloride and guaiacol were obtained from M C and B Manufacturing Chemists. The concentration of sodium chloride in aqueous solutions prepared from the commercial reagent was determined by iodometric titration with thiosulfate. These titrations indicated that the commercial chlorite was of high purity.

D E A E and CM cellulose were obtained from Sigma Chemical Co. Both were washed with base and acid before use (14). Silica gel thin layer chromatography sheets containing a fluorescent indicator were obtained from Eastman Organic Chemicals. [3Cl-Labeled chloride ion was obtained from Ameraham-Searle. All
other chemicals were reagent grade and were obtained from commercial sources.

RESULTS

Enzymatic Chlorination of MCD Using Chlorite—In confirmation of previous results, chloroperoxidase catalyzes the chlorination of MCD when hydrogen peroxide serves as the oxidant and chloride serves as the source of the halogen. These results are shown in Fig. 1 which plots the decrease in absorbance at 278 nm associated with the conversion of MCD to DCD. It can also be seen that chloroperoxidase is able to use sodium chlorite as both the halogen donor and the oxidant for the chlorination of MCD as measured in this assay system. Horseradish peroxidase is unable to catalyze the chlorination of MCD with peroxide and chloride as substrates as shown in Fig. 1, however, horseradish peroxidase readily catalyzes the halogenation reaction when chloride is the substrate (Fig. 1).

Identification of Dichlorodimedone as Product of Chlorite Reaction—The observation that the 278 nm absorption band of MCD disappears when chloroperoxidase and horseradish peroxidase are incubated with chlorite does not necessarily prove that DCD is the product of the reaction. MCD can be oxidized in the absence of halide ions to an unidentified species which no longer absorbs at 278 nm (15). In the regular chlorination assay with hydrogen peroxide as the oxidant, this oxidative side-reaction occurs; however, the rate of oxidation is vanishingly small compared to the rate of halogenation and can be ignored for purposes of routine assay. With chloride as both oxidant and source of halogen, it became important to identify the product of the reaction. The identity of DCD as the product of the chlorite reaction with both horseradish peroxidase and chloroperoxidase was established by thin layer chromatography of the reaction products as shown in Table I. In Solvent System A, the product formed in the chlorite reaction exhibits an $R_f$ value of 0.68, which is identical with that of authentic DCD. In Solvent System A, MCD has an $R_f$ value of 0.19. In Solvent System B, the chlorite product has an $R_f$ value of 0.84, which is the same as that for authentic DCD and differs significantly from that of MCD (0.65). Co-chromatography of the chlorite reaction product with DCD in both solvent systems confirms the conclusion that DCD is the product of the reaction. Similar results were obtained when horseradish peroxidase was substituted for chloroperoxidase as the catalyst in the chlorite reaction. A minor product which does not contain chlorine atoms derived from chlorite can also be detected in the chlorite reaction. The minor product, which has an $R_f$ value of 0.81 in Solvent System A, is probably an oxidation product of MCD. It behaves quite differently than does MCD on thin layer chromatograms but it is not labeled with $^{35}$Cl when Na$^{35}$ClO$_2$ is used as a substrate.

The titration of MCD by sodium chlorite in the presence of chloroperoxidase shows that 1 mole of chlorite promotes the conversion of approximately 1.3 moles of MCD to yield principally DCD and a small amount of the unidentified product. Identical results were obtained when horseradish peroxidase was substituted for chloroperoxidase in the titration experiment.

$^{35}$Cl Incorporation Studies—The results of the incorporation studies using Na$^{35}$ClO$_2$ as the halogen donor are shown in Table II. When $^{35}$Cl-labeled chlorite serves both as oxidant and halogen donor, $^{35}$Cl-labeled DCD was obtained as the product of the reaction. No exchange of the chlorine in MCD with labeled chlorite was observed when either horseradish peroxidase or chloroperoxidase served as the halogenation catalyst (Table II). This finding rules out the possibility that labeled DCD could arise via an enzymatic or nonenzymatic exchange reaction. When an excess of unlabeled chloride was added to the reaction mixture (up to a 200-fold excess over chlorite) there was no dilution of the $^{35}$Cl label in DCD with either horseradish peroxidase or chloroperoxidase as shown in Table II. These

![Graph](image_url)

Fig. 1. The chlorination of MCD catalyzed by horseradish peroxidase and chloroperoxidase. The reaction mixture contained 300 μmoles of potassium phosphate buffer (pH 3), 60 μmoles of potassium chloride when indicated, 0.3 μmole of MCD, 0.5 μg of chloroperoxidase or horseradish peroxidase, and 6 μmoles of either sodium chloride or hydrogen peroxide in a total volume of 3 ml. The reactions were initiated by the addition of enzyme. In Curve A, horseradish peroxidase, hydrogen peroxide, and chloride were present; in Curve B, horseradish peroxidase and sodium chloride; in Curve C, chloroperoxidase, hydrogen peroxide, and chloride ion; and in Curve D, chloroperoxidase and sodium chlorite.

![Table](image_url)

**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rp Value Solvent A</th>
<th>Rp Value Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic MCD</td>
<td>0.19</td>
<td>0.65</td>
</tr>
<tr>
<td>Authentic DCD</td>
<td>0.68</td>
<td>0.84</td>
</tr>
<tr>
<td>Chloroperoxidase + H$_2$O$_2$ + Cl product</td>
<td>0.68$^a$</td>
<td>0.84$^b$</td>
</tr>
<tr>
<td>Chloroperoxidase + NaClO$_2$ product</td>
<td>0.68$^a$</td>
<td>0.84$^b$</td>
</tr>
<tr>
<td>Horseradish peroxidase + NaClO$_2$ product</td>
<td>0.68$^a$</td>
<td>0.84$^b$</td>
</tr>
</tbody>
</table>

$^a$ Additional minor spots at 0.24 and 0.81.
$^b$ Additional minor spot at 0.75.

Additional chemicals were reagent grade and were obtained from commercial sources.
The reaction mixture contained 3 mmoles of potassium phosphate buffer (pH 3.5 for chloroperoxidase and pH 4.2 for horseradish peroxidase), 3 μmoles of MCD, and 5 μg of either chloroperoxidase or horseradish peroxidase in a total volume of 30 ml. The reaction was initiated by the addition of 1.5 μmoles (26,000 dpm of 3Cl-labeled) sodium chloride (see "Experimental Procedure" for preparation). The reaction was followed spectrophotometrically by measuring the decrease in absorbance at 278 nm. Upon completion of the reaction, the mixture was extracted five times with 20-ml aliquots of chloroform. The chloroform extracts were pooled, dried on anhydrous sodium sulfate, filtered, and evaporated. The residue was dissolved in 1 ml of chloroform and counted. In addition a 0.1 ml aliquot of this solution was spotted on thin layer chromatographic plates and chromato-"graphed in Solvent A (see Table I). The reaction products were co-chromatographed with authentic samples of MCD and DCD. The spots were visualized by ultraviolet light, cut out, and counted in a scintillation counter in order to establish that all the counts were incorporated into DCD.

Table II

Incorporation of 3Cl-labeled chlorite into DCD

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chloride added</th>
<th>DCD formation</th>
<th>3Cl incorporated into DCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>μmoles</td>
<td>%</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>None</td>
<td>0.775</td>
<td>1.69</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.708</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.210</td>
<td>2.64</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1.331</td>
<td>2.90</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>None</td>
<td>0.727</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.775</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.728</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.682</td>
<td>1.53</td>
</tr>
</tbody>
</table>

The results indicate that the reaction of chlorite with chloroperoxidase and horseradish peroxidase results in the direct formation of a halogen containing enzyme bound intermediate which does not equilibrate with added chloride ions. These results rule out a reaction of chloride with enzyme to produce a normal Compound I intermediate with the liberation of hypochlorite which could then react nonenzymatically with MCD to form DCD. If this were the case, the addition of chloride to the reaction mixture should depress the incorporation of 3Cl-labeled chlorite into DCD. Independent experiments indicate that the rate of exchange of chlorine atoms between hypochlorite and chloride ions is much faster than the rate of chlorination of MCD by hypochlorite since the addition of an aliquot of unlabeled sodium hypochlorite to a solution containing MCD and 3Cl-labeled chlorite results in quantitative incorporation of 3Cl into DCD.

In chloroperoxidase reactions, mixtures which contain excess halogen acceptor (MCD) over donor (chlorite), the addition of chloride to the reaction mixture results in an increased yield of DCD (see Lines 3 and 4, Table II). This is not the case when horseradish peroxidase is used as the catalyst (Line 5, Table II). These results suggest that the reaction of chloroperoxidase with chlorite yields a halogenating intermediate (which contains 2 of the oxidizing equivalents originally associated with chlorite) and at the same time releases the other 2 oxidizing eq originally associated with chlorite in some manner which native chloroperoxidase can subsequently use to oxidize chloride. The results can be readily interpreted by assuming that chloroperoxidase reacts with the released oxidizing equivalents to generate Compound I, which in turn can react with chloride ions to produce a second halogenating intermediate. Since horseradish peroxidase cannot oxidize chloride, it cannot carry out this second reaction.

It should be noted that all the reactions of 3Cl-labeled chlorite with chloroperoxidase were carried out at pH 3.5 instead of the lower optimal pH value. Chlorite at pH values below 3.5 decomposes yielding, among other products, chloride ion (16).

Table III

Kinetic constants for chlorination of MCD by chloroperoxidase and horseradish peroxidase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Vmax (μmoles/min/mg)</th>
<th>Km (μmoles/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroperoxidase</td>
<td>H2O2 + Cl-</td>
<td>2600</td>
<td>6.6 x 10^-5</td>
</tr>
<tr>
<td></td>
<td>NaClO2</td>
<td>6750</td>
<td>2.2 x 10^-4</td>
</tr>
<tr>
<td></td>
<td>NaClO2 + Cl-</td>
<td>6600</td>
<td>2.4 x 10^-4</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>NaClO2</td>
<td>370</td>
<td>1.5 x 10^-4</td>
</tr>
<tr>
<td></td>
<td>NaClO2 + Cl-</td>
<td>360</td>
<td>2.0 x 10^-4</td>
</tr>
</tbody>
</table>

Michaeis-Menten Constants for Enzymatic Chlorination with Chlorite—As can be seen from the Lineweaver-Burk plots in Fig. 2, both enzymes exhibit normal kinetics with respect to chlorite utilization. The kinetic constants for chlorination with chloride are given in Table III. In the chloroperoxidase-catalyzed reaction, the Km for chlorite is greater by a factor of 3 over the Km for peroxide in the corresponding peroxide-chloride chlorination reaction. The Vmax for the chlorite chlorination reaction with chloroperoxidase is approximately 3 times greater than the Vmax for the peroxide-chloride chlorination reaction. The Km for chlorite with horseradish peroxidase is a factor of 10 less than that for peroxide in horseradish peroxidase catalyzed peroxidation reactions (17), and is little affected by chloride ion. The Vmax is essentially identical in the horseradish peroxidase oxidative reactions. No direct comparison can be made between

Fig. 2. Lineweaver-Burk plots for the chloroperoxidase and horseradish peroxidase chlorite reaction. The enzymes were assayed in the usual reaction mixture at pH 3.2 instead of 2.8 and the substrate concentrations were varied as indicated. The assay was carried out at pH 3.2 since horseradish peroxidase exhibited a slow loss of activity at pH 2.8. The reaction was initiated by the addition of 0.5 μg of chloroperoxidase (A) or 2 μg of horseradish peroxidase (B). Concentrations are expressed in molarities and velocities are expressed as changes in absorbance at 278 nm per min.
Table III, free chloride ion is not required in these peroxidation with horseradish peroxidase than does peroxide. Chlorite displays a greater $K_m$ for chlorination reaction.

Both enzymes. Chlorite is a good substrate for both enzymes. Chlorite displays a greater $V_{max}$ for chlorination with chloroperoxidase than does the normal hydrogen peroxide-chloride donor system and chlorite exhibits a smaller $K_m$ for peroxidation with horseradish peroxidase than does peroxide.

As seen in Table III, free chloride ion is not required in these chlorite reactions and the presence of chloride has no significant effect on the $V_{max}$ or $K_m$ for chlorite with either enzyme.

### Table IV

**Utilization of Chlorite by Chloroperoxidase for Peroxidative Oxidation Reactions**

The oxidation of pyrogallol, guaiacol, and thiourea was followed spectrophotometrically as described under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Substrate</th>
<th>H$_2$O</th>
<th>Chlorite</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogallol</td>
<td>1.8</td>
<td>2.28</td>
<td>A/min</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>2.30</td>
<td>1.20</td>
<td>A/min</td>
</tr>
<tr>
<td>Thiourea</td>
<td>0.60</td>
<td>1.92</td>
<td>A/min</td>
</tr>
</tbody>
</table>

Chlorite and peroxide-chloride for chlorination with horseradish peroxidase because horseradish peroxidase does not catalyze the peroxide-chloride chlorination reaction.

By kinetic constant criteria, chlorite is a good substrate for both enzymes. Chlorite displays a greater $V_{max}$ for chlorination with chloroperoxidase than does the normal hydrogen peroxide-chloride donor system and chlorite exhibits a smaller $K_m$ for peroxidation with horseradish peroxidase than does peroxide. As seen in Table III, free chloride ion is not required in these chlorite reactions and the presence of chloride has no significant effect on the $V_{max}$ or $K_m$ for chlorite with either enzyme.

**Halogenation Activity as Function of pH**—In Fig. 3, the chlorination activity is plotted as a function of pH for (a) chloroperoxidase with hydrogen peroxide and chloride ion as substrates; (b) chloroperoxidase with sodium chloride serving both as the oxidant and halogen donor; and (c) horseradish peroxidase with sodium chloride as the substrate. For chloroperoxidase the optimum pH for chlorination using chlorite is quite similar to the pH optimum for chlorination with peroxide and chloride. In both cases the pH optimum lies somewhere between pH 2.5 and 2.75 (18). For horseradish peroxidase, the pH optimum for chlorination with chlorite is approximately 4.2.

The chlorite reaction can serve as an alternative to the $^{35}$S-Iodide-horseradish peroxidase system, for labeling cell surface structures. As noted in Fig. 3, the combination of chloroperoxidase and horseradish peroxidase provides a large pH range (approximately pH 2.6 to 6.6) in which to attempt surface labeling reactions using $^{35}$Cl-labeled chlorite.

**Utilization of Chlorite for Peroxidative Reactions**—Chlorite can replace peroxide as the oxidant for several of the reactions catalyzed by chloroperoxidase as illustrated in Table IV. Although the replacement of peroxide by chlorite in the oxidation of guaiacol results in a decreased rate, the rate is still appreciable and indicates that chlorite can serve as an alternate oxidant. When chlorite is used as the oxidant in the pyrogallol assay, an increase in the rate of oxidation of pyrogallol is observed. The chloroperoxidase-catalyzed oxidation of thiourea exhibits an absolute requirement for halide anion when hydrogen peroxide serves as the oxidant (11). However, no such requirement is observed with chlorite and, in addition, the rate of the reaction is approximately twice that observed with peroxide as the oxidant, once again indicating the efficacy of chlorite as an oxidant for peroxidase reactions.

**Formation of Horseradish Peroxidase and Chloroperoxidase Intermediates**—The addition of chlorite to horseradish peroxidase results in the rapid formation of a relatively stable (half-life of more than 20 min) spectral intermediate as shown in Fig. 4a. The spectrum of this intermediate compared favorably with that of horseradish peroxidase Compound I formed by reaction of the enzyme with ethyl hydrogen peroxide, as can be seen in Fig. 4a. The chlorite product exhibits peaks at 404, 525, 548, and 645 nm compared to 402, 527, 550, and 645 nm for Compound I. The isobestic points are at 420, 450, and 540 nm and compare favorably with the 420, 450 and 541 nm isobestic points generated for Compound I. The chlorite-produced intermediate can be quantitatively reduced back to the native ferric peroxidase form by the addition of reducing agents such as potassium ferrocyanide or cysteine. The addition of hydrogen peroxide to the chlorite adduct results in the formation of a new spectral species which has a spectrum similar to that of horseradish peroxidase Compound II. As shown in Fig. 4b, the addition of chlorite to chloroperoxidase results in the rapid
formation of a spectral intermediate characterized by a general loss of absorbance in the Soret band. The spectrum of this intermediate is quite similar to that of chloroperoxidase Compound I reported by Thomas et al. (19). The chloroperoxidase intermediate does not exhibit the stability of the horseradish peroxidase-chlorite adduct and rapidly decomposes to a form which has the spectral properties of the native enzyme. The half-life of the chloroperoxidase chlorite intermediate is less than 30 s. All attempts to stabilize the intermediate to permit further studies and allow titration experiments have been unsuccessful.

Titration of Horseradish Peroxidase—The addition of aliquots of a solution of chlorite to horseradish peroxidase causes stepwise changes in the absorption spectrum as shown in Fig. 5. The various spectra exhibit clear isosbestic points at 420, 450, and 540 nm, indicating that side products are not formed in significant amounts at any stage in the reaction. The extreme stability of these intermediates permitted a spectral titration with chlorite. The changes in absorbance at 403 nm determined from titration experiments similar to the one shown in Fig. 5 are plotted as a function of the amount of chlorite added to the reaction mixture in Fig. 6. The results indicate essentially a stoichiometric reaction of chlorite with horseradish peroxidase to form the spectral equivalent of Compound I. The equivalence point corresponds to approximately 0.56 mole of chlorite per mole of enzyme. Since chlorite possesses 4 oxidizing eq per mole, each enzyme molecule can be considered to have under-
Peroxidase isozymes and gone a formal 2-electron oxidation analogous to that involved in the formation of Compound I by reaction with peroxide.

**Reaction of Chlorite with Different Samples of Horseradish Peroxidase**—A number of horseradish peroxidase isozymes and horseradish peroxidase samples of varying degrees of purity were surveyed for their ability to react with chlorite and form spectral intermediates similar to Compound I. These results are shown in Table V. Samples of the “B” and “C” isozymes, which were considered to be reasonably pure on the basis of their R, values, exhibited spectra essentially identical with Compound I upon reaction with chlorite. However, a less pure sample of the “C” isozyme and an impure preparation of the “A” isozyme gave predominantly Compound II-type spectra under these same conditions.

**Reaction of Horseradish Peroxidase with Other Oxidants**—The spectral intermediates formed upon the reaction of various oxidants with a pure sample of the “C” isozyme (R, ~ 3.42) are shown in Table VI. As previously described by George (4), reaction of horseradish peroxidase with hydrogen peroxide or hypochlorite results in the formation of significant amounts of Compound II. The formation of Compound I by reaction of horseradish peroxidase with ethyl hydrogen peroxide has already been described (3). The reaction of m-chloroperoxybenzoic acid with horseradish peroxidase is also known to result in the formation of Compound II (5). In addition to chlorite, potassium bromate, and potassium periodate also give Compound I on reaction with horseradish peroxidase. The addition of a reducing agent such as potassium ferrocyanide results in complete regeneration of the spectrum of the native ferriperoxidase in every case except for the intermediate formed by the reaction of horseradish peroxidase with hypochlorite.

**Reaction of Horseradish Peroxidase with Hypochlorite**—The spectral intermediates formed upon the reaction of horseradish peroxidase with chloride or hypochlorite exhibit strikingly different properties, as shown in Table VII. Although some formation of Compound I is observed upon reaction of horseradish peroxidase with hypohalite, Compound II is the predominant species formed, even at a very low level of hypochlorite added. Attempts to titrate the enzyme with hypochlorite in a manner similar to the titration with chlorite resulted in a titration which slowly tailed off and exhibited no real end point. In addition, greater than 4 moles of hypochlorite were required per mole of enzyme in order to observe an 80% conversion of the native enzyme to spectral intermediates, suggesting that the reaction of hypochlorite with horseradish peroxidase does not result in the stoichiometric formation of a spectral intermediate as observed with chlorite. Also, unlike the chlorite product, the hypochlorite intermediate cannot be quantitatively reduced back to the native ferriperoxidase by ferrocyanide.

**DISCUSSION**

The results presented in this paper clearly establish the role of chlorite as a chlorination substrate for both horseradish per-
oxidase and chloroperoxidase. Perhaps in the case of chloroperoxidase, this finding is not too surprising. The original discovery of chloroperoxidase resulted from efforts to find an enzyme capable of catalyzing enzymatic chlorination reactions (20, 21), and subsequent work has shown that chloroperoxidase is capable of catalyzing peroxidative halogenation reactions using hydrogen peroxide as the oxidant and either chloride, bromide, or iodide ions as the halogen donors (2). In a sense, chlorite could be considered as a dual source of hydrogen peroxide and chloride for the enzyme. However, the finding that horseradish peroxidase is an excellent catalyst for enzymatic chlorination with chlorite deserves special attention because horseradish peroxidase clearly is inert with respect to chlorination in the presence of chloride ion and hydrogen peroxide. On the other hand, horseradish peroxidase readily catalyzes enzymatic iodination reactions using hydrogen peroxide and iodide ion (6, 7). Thus with horseradish peroxidase, chloride extends the range of halogenation reactions catalyzed by this enzyme.

In the case of enzymatic halogenation reactions utilizing hydrogen peroxide and halide ions, an enzyme-bound halogenium ion has been postulated as the active halogenating intermediate for both chloroperoxidase (22) and horseradish peroxidase (23). Extension of this concept to the chlorite reaction predicts that chlorite reacts with both of these enzymes to produce an enzyme-Cl\(^+\) species. Therefore, in terms of reaction mechanism, it becomes important to characterize and define the chlorite reaction in some detail, especially in relation to the normal peroxidative reactions catalyzed by horseradish peroxidase and chloroperoxidase.

**Stoichiometry Considerations**—Sodium chlorite contains 4 oxidizing eq as opposed to the 2 oxidizing eq present in a molecule of hydrogen peroxide. It is apparent from the results of the titration studies between chlorite and horseradish peroxidase, that all 4 oxidizing eq are available for the formation of the typical horseradish peroxidase Compound I spectral intermediate. Previous studies have firmly established the fact that 2 oxidizing eq are associated with the spectral intermediate, horseradish peroxidase Compound I (24–26). Since it is difficult to envisage a mechanism whereby 2 molecules of enzyme react with 1 molecule of chlorite to simultaneously consume 4 oxidizing eq, it appears more fruitful to consider two successive reactions, each of which consumes 2 oxidizing eq in discussions concerning the mechanism of the chlorite reaction. The same type of spectral stoichiometry has also been observed for the titration of cytochrome peroxidase with chloride (27). These results were interpreted in terms of a higher oxidation state of peroxidase.

**Mechanistic Considerations**—Any discussion of mechanism must address several questions. As discussed above, a two-step mechanism for utilizing all 4 chlorite oxidizing eq must be considered. In addition, the mechanistic considerations must account for the generation of an active halogenating intermediate and must consider the formation of 2 eq of Compound I per chlorite molecule. Each of these facets of the chlorite reaction will be considered separately.

One possible way of visualizing a two-step reaction for chlorite in the chlorination reaction would be to consider the reaction of the hydrolytic products of chlorite with enzyme and substrate acceptor (AH) in the following manner:

\[
\text{NaClO}_3 + \text{H}_2\text{O} \rightarrow \text{HOOH} + \text{NaOCl} \quad (2)
\]

\[
\text{HOOH} + \text{enzyme} \rightarrow \text{Compound I} \quad (3)
\]

\[
\text{NaOCl} + \text{AH} \rightarrow \text{A-Cl} + \text{NaOH} \quad (4)
\]

In this hypothesis, the peroxidase would first catalyze the hydrolytic reaction (Equation 2). The enzymatic hydrolysis of chlorite would then furnish a continuous supply of hydrogen peroxide for the generation of Compound I (Equation 3) and a chemical chlorinating species, hypochlorite, would be available for the nonenzymatic chlorination of MCD (Equation 4). This formulation partially meets the requirements of the over-all chlorite reaction, however, two important pieces of experimental data appear to rule out this reaction sequence. First, sodium chlorite exchanges rapidly with chloride ion (Equation 5), faster in fact than hypochlorite can chlorinate MCD (Equation 6). When the enzymatic

\[
\text{NaOCl} + ^{36}\text{Cl}^- \rightarrow \text{NaOCl} + \text{Cl}^- (5)
\]

\[
\text{NaOCl} + \text{MCD} \rightarrow ^{36}\text{Cl}^-\text{DCD} + \text{NaOH} (6)
\]

chlorite reaction was carried out in the presence of \(^{36}\text{Cl}\)-labeled chlorite and unlabeled chloride ion, no dilution of label in the \(^{36}\text{Cl}\)-labeled product, DCD, could be detected. However, when the chemical chlorination of MCD was carried out with unlabeled hypochlorite in the presence of \(^{36}\text{Cl}\)-labeled chlorite, \(^{36}\text{Cl}\) was incorporated into DCD. This latter finding shows that hypochlorite exchanges faster with chloride than it chlorinates MCD. These experiments rule out the formation of free hypochlorite as a chemical halogenating species in the enzymatic reaction. Since an enzyme-bound hypochlorite would be equivalent to the postulated enzyme-bound Cl\(^+\) species, no useful purpose could be served by further considering this possibility. Secondly, the results show that reaction of horseradish peroxidase with the hydrolysis products of chlorite (hypochlorite and hydrogen peroxide) leads to the generation of a Compound II spectral species, not Compound I. Contrarily, the incubation of horseradish peroxidase with chlorite produces a very stable Compound I spectrum. These experimental findings invalidate the hypothesis that the first step of the chlorite reaction may involve the enzymatic hydrolysis of chlorite to hypochlorite and hydrogen peroxide.

A slight variant of the hydrolysis hypothesis would involve the reaction of the peroxidase with chlorite to form Compound I (utilizing 2 oxidizing eq) and release hypochlorite as a product which could serve as the halogenating agent. However, as discussed above, the \(^{36}\text{Cl}\) studies rule out free hypochlorite as an intermediate in the reaction.

An alternate hypothesis would have the 1st enzyme molecule react directly with chlorite and produce an enzyme-bound electrophilic chlorinating intermediate (containing 2 oxidizing eq) and release the other 2 oxidizing eq as hydrogen peroxide. Hydrogen peroxide would then react with a 2nd molecule of enzyme and generate a classical Compound I intermediate. Without specifying the exact chemical nature of the halogenating intermediate, the postulated sequence of events would be:

\[
\text{Enzyme} + \text{NaClO}_3 + \text{H}_2\text{O} \rightarrow \text{enzyme-OCl} + \text{NaOOH} \quad (7)
\]

\[
\text{Enzyme} + \text{NaOOH} \rightarrow \text{Compound I} \quad (8)
\]

This formulation would satisfy the \(^{36}\text{Cl}\)-labeling studies and the other parameters of the over-all chlorite reaction. By analogy to the substrate derived oxygen atom in Compound I (28–30) which does not exchange with \(\text{H}_2\text{O}\), the chlorine atom in the enzyme-OCl intermediate would not be expected to exchange with chloride ion. This second formulation would also explain the observation that chloroperoxidase but not horseradish peroxidase utilizes mixtures of chlorite and chloride for DCD formation from chloride. In the chloroperoxidase-chlorite reac-
tion, the addition of chloride to the reaction mixture essentially doubles the amount of DCD formed from MCD in the presence of chloride alone. In the presence of chloride and chloride (with excess MCD present) not only would the enzyme-OCl formed in Equation 7 be available for the chlorination of MCD but also Compound I (formed in Equation 8) would be available to oxidize chloride and produce a second enzyme-OCl intermediate. In the case of horseradish peroxidase, this second enzyme-OCl intermediate could not be formed, because as previously discussed, horseradish peroxidase cannot oxidize chloride ion.

The formation of 2 eq of Compound I per chlorite molecule could easily be explained by assuming that the active halogenating species formed by the initial reaction of enzyme with chlorite (see Equation 1) is unstable and decomposes to Compound I and chloride ion in the absence of a halogen acceptor. Preliminary spectral evidence in support of this hypothesis has recently been obtained. When horseradish peroxidase is mixed with chloride under stopped flow conditions and the Soret region of the absorption spectrum is rapidly scanned (approximately 40 times per s), the conversion of native enzyme (Soret$_{max}$ = 403 nm) to a new species having an absorption maximum at 417 nm can be detected. The 417 nm intermediate rapidly decomposes to a Compound I spectral intermediate. The details concerning the formation and decomposition of this intermediate will be published separately.

**Chemical Nature of Chlorinating Intermediate Formed from Chlorite**—Before considering the structure of the halogenating intermediate we must first examine the current status of the structure of Compound I. Dolphin et al. (31) have presented persuasive evidence indicating that Compound I is best represented as an Fe(IV)-porphyrin π-cation radical species. These workers have shown that the electrochemical 2 electron oxidation of Co(II)-porphyrin model compounds yields products which have spectral properties similar to Compound I. Dolphin et al. (31) show that the diethyl etherate salt of Co(III)-octaethylporphyrin π-cation radical exhibits optical absorption properties which resemble horseradish peroxidase Compound I while the dibromide salt of the same compound shows visible absorption bands which closely resemble catalase Compound I. This information, combined with recent studies from this laboratory which show that chloroperoxidase Compound I contains only a single oxygen atom derived from substrate peroxide (28-30) suggest that Compound I can be best represented by the structures shown in Fig. 7 (Structures A and B). In this formulation, the oxygen atom derived from substrate sits as a hydroxyl (or O$^-$) ligand on the Fe(IV) porphyrin cation radical. Based on this information concerning the structure of Compound I it is useful to consider whether or not the proposed structure for Compound I with chloride ion which would generate a sensible halogenating intermediate and whether or not this same intermediate could arise from a reaction of native enzyme with chlorite. In answer to the first question, two reactions of Compound I with chloride ion can be considered. The Fe(IV)OH-π-cation radical could react with chloride in a simple displacement reaction in which chloride ion replaces the hydroxide ion as the iron ligand (Fig. 7, Structure C). Chloride ion, once it had been established as an iron ligand on the oxidized heme group could behave as a Cl$^-$ leaving group with concomitant reduction of the Fe(IV)-π-cation radical back to the oxidation state of the heme in the resting enzyme. A second possibility would be the reaction of Compound I with chloride ion to generate the equivalent of a hypochlorite ligand (—OCl) on an Fe(III) heme. Either of these proposed derivatives should be excellent chlorinating species.

The application of the sum of the various possibilities discussed in the preceding paragraphs to the chlorite reaction produces a mechanism in which the enzyme and chlorite combine to directly form a halogenating intermediate. The chlorite halogenating intermediate would be identical with the halogenating intermediate derived from the reaction of chloroperoxidase Compound I and chloride ion. The reaction of chlorite with Fe(III) protoporphyrin IX for the generation of an Fe(IV)-Cl protoporphyrin cation radical is given in Equation 9, the formation of an Fe(III)-OCl protoporphyrin intermediate is given in Equation 10.

\[
\text{Equation 9: } \text{NaClO}_2 + \text{Fe(III) protoporphyrin IX} \rightarrow \text{Fe(IV)-Cl protoporphyrin cation radical} + \text{NaOCl}
\]

\[
\text{Equation 10: } \text{NaClO}_2 + \text{Fe(III) protoporphyrin IX} + \text{H}_2\text{O} \rightarrow \text{Fe(III)-OCl protoporphyrin} + \text{NaO}_2\text{H}
\]

In the reaction of chlorite with enzyme, the formation of an —OCl ligand is much more attractive than the formation of a —Cl ligand because of the structure of chlorite (O—Cl—O). Thus at the present time although it is impossible to absolutely choose one intermediate over the other, the —OCl ligand appears much more attractive.

**Comments on Inability of Horseradish Peroxidase to Oxidize Chloride Ion**—Although both chloroperoxidase and horseradish peroxidase catalyze chlorination reactions with chlorite and both enzymes catalyze iodination reactions using peroxide and iodide, only chloroperoxidase catalyzes chlorination reactions with peroxide as the oxidant. These experimental observations can be accounted for by the hypothesis illustrated in Fig. 8. Although both peroxidases can react with chlorite to form directly the active halogenating intermediate, only chloroperoxidase Compound I can react with chloride to form a chlorinating intermediate. The inability of horseradish peroxidase to form an active chlorinating species using peroxide and chloride

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most probably must be due to slight differences in the oxidation-reduction potential of the Compound I forms of horseradish peroxidase and chloroperoxidase. It would be difficult to invoke enzyme specificity as the basis for this differential behavior. Lactoperoxidase can oxidize bromide and iodide, but not chlorine; thus according to this theory lactoperoxidase Compound I would possess an oxidation-reduction potential intermediate between chloroperoxidase and horseradish peroxidase.

**Reaction of Oxidizing Agents with Impure Fractions of Horseradish Peroxidase**—In contrast to the formation of the Compound I spectrum when highly purified samples of horseradish peroxidase react with chlorite, impure horseradish peroxidase reacts with chlorite to yield significant amounts of Compound II. George (4), in early studies, reported that horseradish peroxidase and cytochrome c peroxidase react with a wide variety of oxidants (sodium chlorite, chlorine dioxide, potassium bromate, and potassium periodate) to give intermediates having spectral properties similar to those formed upon reaction with peroxides. However, George was unable to demonstrate the quantitative conversion of horseradish peroxidase to Compound I and reported a mixture of Compounds I and II with Compound II predominating. Similar observations on the predominance of Compound II were explained by Chance (25) as the result of the presence of endogenous donor which reacted with Compound I to form Compound II. Chance demonstrated that the endogenous donor could be removed by incubation of the enzyme with 2 to 3 eq of peroxide.

As shown in this paper, a sample of horseradish peroxidase which is essentially pure, based on the criteria of Shannon et al. (9), reacts with sodium chlorite to give quantitative conversion to a stable intermediate which is spectrally indistinguishable from horseradish peroxidase Compound I. However, with less pure samples of horseradish peroxidase, significant amounts of Compound II were observed. These results suggest that the formation of Compound II as observed by George, was due to the presence of endogenous donor in his enzyme preparation. George worked with horseradish peroxidase preparations which had an Rf value of 2.7 (29). In these studies we also observed the quantitative formation of Compound I in the presence of potassium bromate and periodate, contrary to the results of George. As discussed earlier, the predominant formation of a Compound II-type intermediate upon the reaction of highly purified horseradish peroxidase with hypochlorite suggests the involvement of a different type of reaction. In addition, the reaction with hypochlorite is not stoichiometric; at least 4 eq of hypochlorite are required for the conversion of 80% of the native enzyme to the intermediate. Also, the addition of reducing agents to the hypochlorite intermediate does not give quantitative conversion back to native enzyme.

In brief summary, chlorite serves as a substrate for enzymatic chlorination and the reaction of chlorite with chloroperoxidase and horseradish peroxidase generates the spectral equivalent of Compound I. Studies with halogen acceptors indicate that the chlorite reaction generates both an enzyme-bound halogenating intermediate and a classical peroxidase Compound I, most probably in equivalent amounts when stoichiometric amounts of enzyme and chlorite are employed.

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