The Reaction of Chloroperoxidase with Chlorite and Chlorine Dioxide*

Shahram Shahangian and Lowell P. Hager
From the Roger Adams Laboratory, Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

Chloroperoxidase catalyzes the dismutation of chlorite-forming chloride, chlorite, chlorine, and oxygen as products. The yields of chloride dioxide determined. The kinetic parameters for the chlorite reaction have been determined. The value of the formation of chloride dioxide from chlorite was about 70,000 s⁻¹.

Chloroperoxidase (EC 1.11.1.10, chloride/hydrogen peroxide oxidoreductase) was first purified in attempts to characterize an enzymatic system involved in the biosynthesis of caldariomycin by the mold, Caldariomyces fumago (1). Chloroperoxidase is a nonglycine hemoeprotein of 42,000 molecular weight and contains one ferriprotoporphyrin IX per molecule as its heme prosthetic group (2). It has been shown that chloroperoxidase catalyzes classical peroxygenation and dismutation reactions in addition to halogenation reactions. Thus, chloroperoxidase reactions may be either dependent on a suitable halide ion (Cl⁻, Br⁻, or I⁻) or unaffected by their presence (3–9). Oxidative halogenation reactions also can be either halide ion dependent (3) when hydrogen peroxide provides the oxidation equivalents or halide ion independent (8) when chlorite serves both as a source of halogen and oxidation equivalents. It appears that the halogen acceptor in chloroperoxidase reactions can be any good nucleophile with a suitably activated position available for attack by an electrophilic halogen species. β-Ketoacids, cyclic β-diketones, phenols, and phenolic esters have all been found to serve as good acceptors. Peroxidation reactions also can be either dependent or independent of halide ions (4, 7). Several dismutation reactions are not dependent upon halide ion and are accompanied by oxygen evolution. Hydrogen peroxide, substituted peroxides, and peracids are all substrates for dismutation (7).

This paper shows that chloride and chlorine dioxide are also dismutation substrates for chloroperoxidase.

The mechanism of the chloride-dependent chlorination reaction catalyzed by horseradish peroxidase has been studied in some detail (8, 10, 11). A mechanism involving chemical chlorination via hypochlorite formation has been ruled out (8) for both chloroperoxidase and horseradish peroxidase. Based on the early results obtained with a rapid scanning spectrophotometer in conjunction with a stopped flow apparatus, it was concluded that a chlorinating enzyme species was formed in the reaction of horseradish peroxidase with chlorite (10, 12). However, more recent experiments have shown that the intermediate observed in the reaction of chlorite and horseradish peroxidase has an optical absorption spectrum identical with that of compound II (11). In contrast, in the reaction between chloroperoxidase and chlorite, an optical absorption spectrum similar to that of the native enzyme is observed in the time span 3 ms to 1 s after mixing.

**EXPERIMENTAL PROCEDURES**

Chloroperoxidase was prepared from C. fumago and purified essentially as before with minor modifications (13). After the second ethanol fractionation step, the precipitated chloroperoxidase was dissolved in a minimal amount of 0.04 M potassium phosphate buffer, pH 5.2. This solution was then dialyzed overnight against one change of 2 liters of the same buffer. The dialyzed solution was layered onto a pre-equilibrated DEAE-cellulose column (3.5 x 27 cm), medium mesh, and eluted with a linear gradient from 0.04 M to 0.1 M potassium phosphate buffer, pH 5.8. This procedure yielded chloroperoxidase fractions having $R_Z(A_{280}/A_{250})$ values of 1.4 or better. The overall enzyme yield was typically 75%. All enzyme preparations used in the experiments reported in this paper had $R_Z$ values of at least 1.4. The specific activity of the samples ranged from 1600 to 2100 units/mg (3). Chloroperoxidase preparations were stored at 4–6°C in 0.1 M potassium phosphate buffer, pH 3.9. Chloroperoxidase concentrations were measured spectrophotometrically at 385 nm using an extinction coefficient of 86 mM⁻¹ cm⁻¹.

Sodium chlorite was purchased from Matheson, Coleman and Bell. Iodimetric assays showed the salt to be better than 99% pure. Aqueous chlorine solutions decompose in the light (14) and at low pH values (15). Hence, all chlorine solutions were freshly prepared in black-painted flasks at concentrations of 10 to 100 mM in glass-distilled water.

Chlorine dioxide was generated by the addition of acetic acid to aqueous solutions of sodium chloride as described earlier (11). Chlorine dioxide concentrations were determined using a value of 1.23 mmol·cm⁻³ for the extinction coefficient of chlorine dioxide at 359 nm. This extinction coefficient was based on iodometric assays carried out according to the procedure of Hollenberg et al. (8). Sodium hypochlorite (0.4 mM) at pH 8.0 was exchanged with 0.12 mmol of Na²¹Cl (56 μCi). The resulting Na²¹Cl-labeled sodium hypochlorite (43 μCi) was heated at 100°C for 1 h to form Na²¹Cl-labeled sodium chloride. The sodium chloride was converted to Na²¹Cl chlorine dioxide as described previously (8). Three aliquots of 0.12 mmol of oxalic acid and 0.36 mmol of sulfuric acid were sufficient for a maximal yield of chlorine dioxide. The resulting Na²¹Cl chlorine dioxide was displaced from the reaction mixture with a fine stream of nitrogen and collected in five traps arranged in series.
Each trap, chilled in an ice bath, contained 4.0 ml of 0.1 M sodium phosphate buffer, pH 2.75. The formation of chlorine dioxide could be visually detected by its characteristic greenish yellow color. All of the reactions forming chlorine dioxide were carried out in a hood in subdued light.

Chloride concentrations were determined colorimetrically using the mercuric thiocyanate method as described by Vogel (17). Corrections of up to 5% were made for the presence of chloroperoxidase, which interfered slightly in this assay.

A Beckman LS 8000 scintillation counter was used to count the radioactive fractions. Typically, a 20-μl radioactive aliquot was added to 5 ml of a scintillation cocktail prepared according to Patterson and Greene (18). The ratio of counts per minute to disintegrations per minute was constant (0.85) throughout all of the fractions as indicated by internal 4Cl standards.

All ordinary spectrophotometric work was performed with either a Beckman Acta C III or a Cary 219 spectrophotometer. A Durum stopped flow instrument was used for the transient state studies. It was interfaced to a DEC P-T-11 computer in conjunction with a Texas Instruments silent 700 ASR printer. Voltage readings were converted to their corresponding absorbance values and were recorded at set time intervals.

An oxygen electrode, model 5391 from Yellow Springs Instrument Co., was used in combination with a Gilson model K oxygraph for measuring oxygen formation. The electrode response was calibrated with hydrogen peroxide and catalase. The values for oxygen released were directly proportional to hydrogen peroxide concentrations over the concentration range 0.09 to 6 mM. The calibration curve was chosen to be the least squares line fitted to pass through the origin. The oxygen electrode membrane and the contacting KCl solution were changed daily in order to attain maximal response and give the best reproducibility.

A Metrohm-Brinkmann 103 pH meter with a combination glass electrode was used for pH measurements.

Monochlorodimedone was synthesized as described earlier (3). Dowex 2-X8 was from Baker Chemical Co. DEAE-cellulose was obtained from Sigma Chemical Co. All solutions were made in glass-distilled water using reagent grade chemicals obtained from commercial sources.

RESULTS

Dismutation of Chlorite and Chlorine Dioxide—The addition of catalytic amounts of chloroperoxidase to freshly prepared, acidic (pH 2.75) solutions of chlorite gave rise to the immediate appearance of a greenish yellow solution indicative of chlorine dioxide formation. Both the rate and extent of chlorine dioxide formation from chlorite were found to be pH dependent. Fig. 1 shows that the pH optimum for the formation of chlorine dioxide from chlorite was approximately pH 2.75 when catalytic quantities of enzyme were used with substrate quantities of chlorite. The number of moles of chlorine dioxide evolved for each mole of chlorite decomposed by chloroperoxidase under various conditions was highly dependent upon the ratio of chlorite to enzyme as shown in Table I. This variable yield of chlorine dioxide from chlorite led to the discovery that chloroperoxidase could catalyze the decomposition of chlorine dioxide and also that chlorine dioxide would inactivate the enzyme. Various studies showed that relatively low concentrations of chlorine dioxide were quite toxic for chloroperoxidase. For example, the reaction of 11 nmol of chloroperoxidase with 970 nmol of chlorine dioxide leads to the destruction of both reactants. As shown in Fig. 2, characteristic optical absorption peaks for both chlorine dioxide (λmax = 359 nm) and chloroperoxidase (Soret λmax = 400 nm) disappear. Parallel experiments also show a concomitant loss of enzyme activity. Consequently, attempts were made to minimize the inactivation of chloroperoxidase by chlorine dioxide in order to characterize the stoichiometry of the chlorite dismutation reaction. Some success in this direction was accomplished by removing chlorine dioxide from the reaction mixture by bubbling a stream of nitrogen gas through the reaction mixture and by repeated addition of chloroperoxidase.

TABLE I

The indicated amounts of chloroperoxidase and sodium chlorite were mixed and incubated at room temperature until chlorine dioxide formation ceased. The formation of chlorine dioxide was followed spectrophotometrically according to the method described under "Experimental Procedures." The total volume of the reaction mixture was 1 ml.

<table>
<thead>
<tr>
<th>Chloroperoxidase</th>
<th>Chlorite</th>
<th>Ratio (mol chlorine dioxide formed/mol chlorite used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol</td>
<td>μmol</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.0</td>
<td>0.15</td>
</tr>
<tr>
<td>20</td>
<td>2.0</td>
<td>0.23</td>
</tr>
<tr>
<td>20</td>
<td>1.0</td>
<td>0.34</td>
</tr>
<tr>
<td>240</td>
<td>2.0</td>
<td>0.29</td>
</tr>
<tr>
<td>240</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>240</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>1000</td>
<td>4.0</td>
<td>0.11</td>
</tr>
<tr>
<td>1000</td>
<td>2.0</td>
<td>0.07</td>
</tr>
<tr>
<td>1000</td>
<td>1.0</td>
<td>0.04</td>
</tr>
</tbody>
</table>

![Fig. 1. Effect of pH on the formation of chlorine dioxide from chlorite.](image1)

Fig. 1. Effect of pH on the formation of chlorine dioxide from chlorite. The complete reaction mixture contained 0.1 M sodium phosphate/citrate buffer at the pH value indicated, 5 mM sodium chlorite, and 6.2 mM chloroperoxidase in a total volume of 1 ml. Incubation was at room temperature. The rate (●—●) and extent (○—○) of chlorine dioxide production were monitored at 359 nm.

![Fig. 2. Spectral changes in response to the addition of excess chlorine dioxide to chloroperoxidase.](image2)

Fig. 2. Spectral changes in response to the addition of excess chlorine dioxide to chloroperoxidase. The curves trace the absorbance of 11.2 μM chloroperoxidase (——), of 0.97 mM chlorine dioxide (— —), and of a mixture containing 11.2 μM chloroperoxidase and 0.97 mM chlorine dioxide (——) in 1 ml of 0.1 M sodium phosphate buffer, pH 2.75. The absorbance of the chloroperoxidase-chlorine dioxide solution was recorded 5 min after mixing.
Product Analysis for the Dismutation of Chlorine Dioxide—Chloride was identified as one of the products of chlorine dioxide decomposition. The stoichiometry between chlorine dioxide decomposition and chloride ion formation was established by incubating various concentrations of chlorine dioxide (0.1 mM to 2 mM) with sufficient chloroperoxidase so that all of the chlorine dioxide was decomposed. The moles of chloride formed for each mole of chlorine dioxide decomposed was found to be 0.27 ± 0.02 (see Table II).

Since only about 30% of the chlorine atoms in chlorine dioxide could be accounted for by chloride ion formation, chlorine oxides having oxidation states greater than 4 were tested as potential product(s) of the dismutation reaction. A qualitative test for the presence of chlorate was positive (19), while a sensitive color test for perchlorate was found to be negative (19).

In order to precisely quantitate the reaction, 36Cl-labeled chlorine dioxide was decomposed and the products of the reaction were fractionated on a Dowex 2 column. Fig. 4 shows the radioactive elution profile where three separate peaks can be recognized. The first peak constituted only 0.2% of the applied radioactivity. The fractions in peak 1 also had a significant absorbance at 400 nm, strongly suggesting that this minor radioactive peak corresponded to 36Cl-labeled enzyme. The second peak contained approximately 29% of the applied radioactivity. An authentic 36Cl-labeled sodium chloride marker solution eluted at this same peak position, providing convincing evidence that this second peak represented chloride ion. The third peak was relatively broad and accounted for approximately 68% of the applied radioactivity. Authentic sodium chlorite was fractionated on the column and showed an elution profile identical with that of the third radioactive peak.

These results show that almost all of the radioactivity, approximately 98%, could be accounted for in terms of two products, chloride and chlorite. However, the relative amount of the oxidized product, chlorate, was not sufficient to balance all of the reducing equivalents associated with the reduced product, chloride ion. We, therefore, searched for other oxidized products. An oxygen electrode was used to test for oxygen production during the decomposition of chlorine dioxide. The oxygraph showed responses for oxygen formation which were directly proportional to the initial concentrations.

<table>
<thead>
<tr>
<th>Chlorine dioxide</th>
<th>Chloride</th>
<th>Chloride:Chlorine dioxide Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μM</td>
<td>29</td>
<td>0.29</td>
</tr>
<tr>
<td>200 μM</td>
<td>53</td>
<td>0.27</td>
</tr>
<tr>
<td>500 μM</td>
<td>140</td>
<td>0.28</td>
</tr>
<tr>
<td>1000 μM</td>
<td>250</td>
<td>0.25</td>
</tr>
<tr>
<td>2000 μM</td>
<td>520</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**Table II**

**Formation of chloride from chlorine dioxide**

Chlorine dioxide at the indicated concentrations was incubated with chloroperoxidase in 0.1 M sodium phosphate buffer, pH 2.75. The total volume of the reaction mixture was 1 ml. Chloroperoxidase was added in amounts which were sufficient to completely decompose all of the chlorine dioxide in the reaction mixture. The formation of chloride as a product of the reaction was measured by the mercuric thiocyanate reaction (17).

**Fig. 3. Utilization of chlorine dioxide as a substrate for chloroperoxidase.** The complete system contained 2.5 mM sodium phosphate, pH 2.75, 1 mM chlorine dioxide, and increasing amounts of chloroperoxidase in a total volume of 1 ml. The zero time absorbance scan (—) between 320 and 400 nm was done in the absence of enzyme. Subsequent scans were made at approximately 1-min intervals after the addition of chloroperoxidase to yield the following final concentrations of enzyme: 2.2 μM (— — —); 2.9 μM (— —); 3.7 μM (—).
of chlorine dioxide. The moles of oxygen produced from each mole of chlorine dioxide consumed were determined from a standard O₂ calibration curve. Fig. 5 shows two plots resulting from these assays. One plot shows the total micromoles of O₂ evolved as a function of the initial concentration of chlorine dioxide. The other plot shows the moles of O₂ formed per mol of chlorine dioxide decomposed as a function of the initial chlorine dioxide concentration. This ratio is seen to have the fairly constant value of 0.17 ± 0.01. Chlorine dioxide decomposition was found to have an optimum pH of approximately 2.75 as shown in Fig. 6.

Dismutation of Chlorite: Quantitative Product Analysis—In order to quantitate product formation from the enzymic decomposition of chlorite, ³⁵Cl-labeled chlorite was reacted with aliquots of chloroperoxidase in 0.1 M sodium phosphate buffer, pH 2.75. During the course of the reaction, all of the chlorite and chlorine dioxide were decomposed. The reaction products were applied on a Dowex 2 column and eluted under precisely the same conditions used for the fractionation of the products of the chlorine dioxide reaction. Fig. 7 shows the radioactive elution profile. Again, three peaks could be discerned. The first peak could again be attributed to ³⁵Cl-labeled enzyme and accounted for only 0.2% of the total applied radioactivity. The second peak was coincident with an authentic chloride ion peak as before and contained approximately 42% of the applied radioactivity. The third, relatively broad peak fractionated in a manner analogous to authentic chlorate. The chlorate peak contained 58% of the applied radioactivity. Thus, as found with the products of the chlorine dioxide reaction, two peaks accounted for approximately 98% of the chlorine present in the added substrate, chlorite.

Oxygen evolution was also measured during chlorite decomposition. Since previous experiments had shown that chlorine dioxide was an intermediate product of the chlorite reaction and that chloroperoxidase catalyzes the decomposition of chlorine dioxide with oxygen formation, we expected molecular oxygen to be formed as a product of the chlorite reaction. In these experiments, relatively high enzyme concentrations were used in order to assure complete destruction of both chlorite and chlorine dioxide. Fig. 8 shows the results of an experiment in which 1, 2, 4, 10, and 20 μmol of chlorite were

FIG. 5. Detection and quantitation of oxygen as a product of the chlorine dioxide reaction. The complete system contained 0.1 M sodium phosphate buffer, pH 2.75, the indicated levels of chlorine dioxide, and sufficient amounts of chloroperoxidase (10 to 100 nmol) to ensure the complete dismutation of the chlorine dioxide. The reactions were carried out in a total volume of 1.5 ml. Oxygen analyses were carried out according to the method described under "Experimental Procedures."

FIG. 6. Effect of pH on the dismutation of chlorine dioxide. The complete system contained 80 μM chlorine dioxide and 320 nM chloroperoxidase in 1 ml of 0.1 M sodium phosphate/citrate buffer at the indicated pH value. Changes in chlorine dioxide concentrations were monitored by measuring the change in absorbance at 359 nm.

FIG. 7. Chromatography of the products of the chlorite reaction. The complete system contained 2.2 μmol of ³⁵Cl-labeled chlorite (0.2 μCi) and 0.12 μmol of chloroperoxidase (added in four aliquots at approximately 3-min intervals) in 1.2 ml of 0.1 M sodium phosphate buffer, pH 2.75. Following an incubation period of 5 min at 23 °C, the reaction mixture was applied to a Dowex 2 column and chromatographed according to the procedure described in Fig. 4. One-mi fractions were collected. The total radioactivity (---) and the concentration of sodium nitrate (---) are plotted as a function of the fraction number. A total of 2.86 × 10⁵ dpm were applied to the column and 2.80 × 10⁴ dpm were recovered.

FIG. 8. Quantitation of oxygen as a product of the chlorite reaction. The complete system contained 0.1 M sodium phosphate buffer, pH 2.75, the indicated levels of chlorite, and sufficient levels of chloroperoxidase (0.1 to 1 μmol) to completely decompose all of the added chlorite and the chlorine dioxide formed in the reaction. The total volume of the reaction was 1.5 ml. Oxygen evolution was monitored by the method described under "Experimental Procedures."
decomposed and the evolution of oxygen was measured with an oxygen electrode. The moles of $O_2$ produced per mole of chlorite dismutated were determined to be $0.13 \pm 0.01$.

Chloride ion was also identified and quantitated as a product of the chlorite reaction using the mercuric thiocyanate reaction. The moles of chloride ion formed per mole of chlorite decomposed were determined at four different chlorite concentrations covering a 15-fold range. In the mercuric thiocyanate assay, the moles of chloride ion formed per mole of chlorite added were found to be $0.43 \pm 0.02$, in full agreement with the value of 0.42 obtained from the $^{36}$Cl-chlorite experiments.

**Kinetics of Chlorine Dioxide Formation from Chlorite**—Chlorine dioxide formation was monitored in the stopped flow apparatus after mixing chloroperoxidase with varying concentrations of chlorite. Reliable initial rates of chlorine dioxide formation from chlorite could only be obtained from stopped flow data since enzyme inactivation and chlorine dioxide decomposition could only be avoided by measuring chlorine dioxide production during the first 100 ms of the reaction. For these experiments, chlorine dioxide formation was measured at 375 nm using an experimentally determined extinction coefficient of $1.05 \text{ M}^{-1} \text{ cm}^{-1}$. When 10 mM solutions of chloroperoxidase in 0.1 M sodium phosphate buffer, pH 2.75, were mixed with equal volumes of chlorite ranging in concentration from 2 to 200 mM, a series of rate curves was generated. Table III lists the kinetic parameters obtained using various kinetic plots (20, 21). Fig. 9 shows the Lineweaver-Burk plot of this data.

**Attempts to Detect a Transient State Enzymic Intermediate in the Chlorite and Chlorine Dioxide Reaction**—A stopped flow spectrophotometer was used to record the optical absorption spectra of any transient state intermediates formed by the reaction of chloroperoxidase with either chlorite or chlorine dioxide. For these studies, a 3.8 $\mu$L solution of chloroperoxidase in 0.1 M sodium phosphate buffer, pH 2.75, was mixed with an equal volume of 28 $\mu$L sodium chlorite or 34 $\mu$L chlorine dioxide. For the chlorite reaction, transient changes in absorbance as a function of time were monitored at 2.5-nm intervals in the Soret region of the spectrum (377 to 450 nm) and at 5-nm intervals from 455 to 700 nm. The optical absorbance spectrum of the enzyme at 3, 100, and 1000 ms after mixing was constructed by combining the rate data recorded at the different wavelengths. Fig. 10 compares the Soret spectrum of the native enzyme with its Soret absorbance 100 ms after the addition of chlorite. Except for a loss of about 10% of the absorbance at 400 nm and a slight increase in absorbance in the 425- to 475-nm range, the Soret absorption of the chlorite-treated enzyme resembled closely that of the native enzyme. Although the data are not plotted in Fig. 10, the Soret absorbance spectrum of the enzyme 3 ms after mixing (the dead time of the instrument) was essentially identical with the spectrum taken at 100 ms. One second after mixing, the Soret spectrum of the chlorite-treated enzyme had reverted back to that of the native enzyme. Furthermore, the visible spectrum of the enzyme did not change after the reaction with chlorite. Attempts to detect the formation of an intermediate in the reaction of chloroperoxidase with chlorine dioxide also were not successful. There was a general irreversible decrease of approximately 15% in the Soret absorbance upon mixing enzyme and chlorine dioxide. Higher levels of chlorine dioxide lead to enzyme destruction as shown earlier in Fig. 2.

---

1 Due to the fluctuating intensity of the lamp at 359 nm, 375 nm was used to monitor chlorine dioxide in the stopped flow spectrophotometer.

---

**Table III**

<table>
<thead>
<tr>
<th>Method of plot</th>
<th>$k_1$ (mM$^{-1}$ s$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lineweaver-Burk</td>
<td>12</td>
<td>70,000</td>
</tr>
<tr>
<td>Eadie-Hofstee</td>
<td>12</td>
<td>68,000</td>
</tr>
<tr>
<td>Wolf</td>
<td>13</td>
<td>72,000</td>
</tr>
<tr>
<td>Hyperbolic</td>
<td>13</td>
<td>71,000</td>
</tr>
</tbody>
</table>

---

**Fig. 9.** Lineweaver-Burk plot for the formation of chlorine dioxide from chlorite. The complete system contained varying levels of chlorite and 5 nM chloroperoxidase in 0.2 ml of 0.05 M sodium phosphate buffer, pH 2.75. The reaction was carried out at room temperature. The rate of chlorine dioxide formation was measured by the increase in absorbance at 375 nm as a function of time during the first 100 ms of the reaction.

**Fig. 10.** Transient state spectra of chloroperoxidase after reaction with chlorite. The complete system contained 1.9 $\mu$L chloroperoxidase and 14 $\mu$L sodium chloride in 0.2 ml of 0.05 M sodium phosphate buffer, pH 2.75. The curves trace the Soret absorbance of chloroperoxidase before (--) and 100 ms after (---) the addition of chlorite to the reaction mixture. Each point on the curve was constructed from a separate reaction mixture.

**DISCUSSION**

Like horseradish peroxidase, chloroperoxidase acts to dismutate chlorite-forming chloride, chlorine dioxide, chlorate, and oxygen (11). However, unlike horseradish peroxidase,
Chlorite Dismutation

chloroperoxidase is also an effective catalyst for the dismutation of chlorine dioxide. In their studies on the action of horseradish peroxidase on chlorite, Hewson and Hager (11) did not detect a reaction between chlorine dioxide and horseradish peroxidase. Following the discovery of the ability of chloroperoxidase to catalyze the dismutation of chlorine dioxide, we investigated the possible utilization of chlorine dioxide by horseradish peroxidase. Horseradish peroxidase has some activity with respect to chlorine dioxide but is not nearly as active as chloroperoxidase.2 Under optimal conditions for each enzyme (pH 2.75 for chloroperoxidase and pH 5.5 for horseradish peroxidase), the rate and extent of chlorine dioxide decomposition were about an order of magnitude lower with horseradish peroxidase. Hewson and Hager (11) have reported chloride ion yields from the horseradish peroxidase-catalyzed chlorite dismutation reaction which are slightly higher than the theoretical value predicted if the dismutation was exclusively to chloride and chlorine dioxide. In the Hewson and Hager (11) study, the chlorine dioxide yield was correspondingly lower. These observations may be easily explained by a partial dismutation of chlorine dioxide to chloride and other chlorine-containing products in the case of horseradish peroxidase reaction.

The broad diversity of products, especially the obvious involvement of several multioxide-reduction steps, strongly suggests a complicated reaction sequence for the dismutation of chloride by chloroperoxidase. However, two major reaction sequences can be recognized by assuming the intermediacy of chloride dioxide in the overall reaction. In the first step, chloroperoxidase acts on chlorite to produce chlorine dioxide and chloride ion according to the following equation:

\[
5 \text{ClO}_2^- + 4 \text{H}^+ \rightarrow \text{Cl}^- + 4 \text{ClO}_2 + 2 \text{H}_2\text{O}
\] (1)

This reaction as formulated would be precisely analogous to the observed dismutation of chloride by horseradish peroxidase (11). The subsequent decomposition of chlorine dioxide to chloride, chlorate, and oxygen can be written in a balanced equation as follows:

\[
18 \text{ClO}_2^- + 9 \text{H}_2\text{O} \rightarrow 5 \text{Cl}^- + 13 \text{ClO}_3^- + 3 \text{O}_2 + 18 \text{H}^+
\] (2)

The stoichiometric parameters predicted by equation 2 are listed in Table IV and are compared with the corresponding experimental values obtained for the reaction of chloroperoxidase with chlorine dioxide. It will be noted that there is a very good agreement between the theoretical and experimental values. It should also be noted that the photodecomposition of chlorine dioxide yields chlorite, chlorate, and oxygen according to equation 2 (22). Thus, in terms of the decomposition of chlorine dioxide, chloroperoxidase catalysis and the absorption of light energy produce the same overall reaction. The sum of equations 1 and 2 yields the following balanced equation for the complete dismutation of chloride by chloroperoxidase:

\[
45 \text{ClO}_2^- \rightarrow 19 \text{Cl}^- + 26 \text{ClO}_3^- + 6 \text{O}_2
\] (3)

Table V compares the stoichiometric parameters predicted by equation 3 with the experimental values obtained under conditions which promoted the complete decomposition of chlorite. The agreement between the theoretical and experimental values is again quite good.

Attempts to detect a transient state enzymic intermediate in the reaction of chloroperoxidase with chloride and chlorine dioxide were not very rewarding. In the corresponding reaction between horseradish peroxidase and chlorite, intermediates having optical spectra identical with both compound I and II could be detected. Since both chloride and chlorine dioxide are good oxidizing agents, the formation of an oxidized enzyme intermediate might be expected. On the other hand, the simplest reaction sequence for the production of chlorine dioxide in a reaction between enzyme and chlorite would be the 1-electron reduction of the enzyme and the 1-electron oxidation of chloride to chlorine dioxide. Since chloroperoxidase possesses a heme prosthetic group, the conversion of the native enzyme to a reduced species would most likely involve the conversion of the ferric enzyme to its ferrous form. Furthermore, since the optical spectra of the ferrous and compound I and II forms of chloroperoxidase have been established, the formation of any one of these species could, in theory, be readily detected. However, the transient state kinetic studies failed to detect any of these unique enzyme species in the reaction of chloroperoxidase with chlorite. In the time range between 3 and 1000 ms after reaction with chlorite, the optical spectrum of the enzyme resembled very closely that of the native enzyme. It is quite possible that the formation and breakdown of an intermediate in the reaction of chloride with enzyme are very fast and take place during the mixing time (0 to 3 ms). Since the formation of chlorine dioxide from the reaction of chlorite with chloroperoxidase is a very fast reaction (the pseudo-first order rate constant is 70,000 s⁻¹), it is reasonable to assume that any enzyme intermediates would be formed and decomposed very quickly. Conversely, it is possible that we do observe the formation of an intermediate which has an optical spectrum which closely resembles that of the native enzyme. Any intermediate which maintained the iron atom of the heme prosthetic group of the enzyme in the +3 valence state could, in theory, resemble the native enzyme in terms of its optical absorbance in the Soret and visible region. Further work will obviously be required in order to resolve this question.

REFERENCES

![Table IV](image)

<table>
<thead>
<tr>
<th>Stoichiometric parameter</th>
<th>Experimental value</th>
<th>Theoretical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mol Cl⁻ formed)/(mol ClO₂ used)</td>
<td>0.27 ± 0.02*</td>
<td>0.28</td>
</tr>
<tr>
<td>(Mol ClO₃⁻ formed)/(mol ClO₂ used)</td>
<td>0.69</td>
<td>0.72</td>
</tr>
<tr>
<td>(Mol O₂ formed)/(mol ClO₂ used)</td>
<td>0.17 ± 0.01</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Based on five mercuric thiocyanate determinations and the ³⁵Cl elution profile.
* Based on eight oxygraph determinations.

![Table V](image)

<table>
<thead>
<tr>
<th>Stoichiometric parameter</th>
<th>Experimental value</th>
<th>Theoretical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mol Cl⁻ formed)/(mol ClO₂ used)</td>
<td>0.43 ± 0.02*</td>
<td>0.42</td>
</tr>
<tr>
<td>(Mol ClO₃⁻ formed)/(mol ClO₂ used)</td>
<td>0.57</td>
<td>0.58</td>
</tr>
<tr>
<td>(Mol O₂ formed)/(mol ClO₂⁻ used)</td>
<td>0.13 ± 0.01</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Based on four mercuric thiocyanate determinations and the ³⁵Cl elution profile.
* Based on five oxygraph determinations.

\º S. Shahangian and L. P. Hager, unpublished observations.
Chlorite Dismutation