Two new substrate classes that can be halogenated by haloperoxidase have been discovered. The enzymatic halogenation of alkynes yields α-halogenated ketones, and the enzymatic halogenation of cyclopropanes yields α,γ-halohydrins. The general reaction scheme proposed involves the initial formation of hypohalous acid as the key intermediate. This proposed mechanism, based upon observed differences in product selectivities, is opposite of that proposed previously, based upon observed differences in substrate selectivities.

The biosynthetic implications of these novel haloperoxidase reactions are also discussed.

Haloperoxidases such as chloroperoxidase (EC 1.11.1.10, chloride:hydrogen-peroxide oxidoreductase) and lactoperoxidase (EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase) have been extensively studied (1, 2). Chloroperoxidase can utilize chloride, bromide, and iodide ions as donors for enzymatic halogenation reactions, while lactoperoxidase can utilize only bromide and iodide ions.

In the absence of organic halogen-acceptor substrates, haloperoxidase catalyzes the peroxidation of halide ion to hypohalous acid. However, in the presence of organic substrates, it has been proposed that this molecular species is not formed as an intermediate in the enzymatic reaction. Instead, the formation of an enzyme-bound electrophilic halogenating species is proposed as the key intermediate (3). Observed differences in substrate specificities between enzymic and chemical halogenation reactions support this mechanism. With this proposed mechanism the oxidized species must be directly transferred from an enzyme intermediate to the organic halogen-acceptor substrate. Therefore, differences should be seen between enzymatic and chemical halogenation reactions not only in substrate specificities but also in product selectivities.

In this paper we report our study of the reaction of haloperoxidase on alkynes and cyclopropanes. The fact that alkynes are even substrates for haloperoxidase point against an enzyme-bound species being the key intermediate. A general reaction scheme involving hypohalous acid as the key intermediate is proposed to explain formation of all of the halogenated products.

EXPERIMENTAL PROCEDURES

Haloperoxidases—Chloroperoxidase (from Caldariomyces fumago), 2 mg of protein/ml and lactoperoxidase (from milk; 5 mg of protein/ml) were purchased from Sigma.

Substrates and Product Standards—Methyl acetylene, ethyl acetylene, and methyl cyclopropane gases were purchased from Matheson Gas Products (Lyndhurst, NJ). Phenyl acetylene, 1-phenyl-1-propane, a-bromo acetophenone (1), α-chloroacetone (6), and phenyl cyclopropane were purchased from Aldrich Chemical Company.

Enzymatic Reactions—The enzymatic reaction mixtures were incubated in 100-ml Pyrex flasks equipped with a magnetic stir bar and stirrer. Each mixture contained 400 μl of haloperoxidase, 20 mM potassium halide, and 25 μl of 300 mM phosphate buffer, at various pH values. Because each haloperoxidase has specific operational requirements, the following conditions were used: for chloroperoxidase, the pH of the buffer was 3.0 and the halides were Cl− and Br−; for lactoperoxidase, the pH of the buffer was 6.0 and the halide was Br−.

The gaseous substrates were continuously bubbled through the mixture (10 ml/min) during the reaction. For the liquid substrates, the concentration was 5 mM at the start of the reaction. Hydrogen peroxide was the last reagent added and had a 30 mM final concentration.

After initiation, the reaction was allowed to proceed for 15 min. All reactions were at room temperature and atmospheric pressure, not necessarily under optimized conditions, nor were reactions run to complete conversion of substrate.

Reaction Mixture Analysis—A aliquots of reaction mixtures (10 μl) were injected into a Finnigan 4021 gas chromatograph-mass spectrometer equipped with a coiled, glass column (1.8 m × 4 mm) packed with Tenax-GC (80/100 mesh). The carrier gas was helium, set at 25 ml/min. For rapid analysis of the many different reaction mixtures, the following temperatures were employed: the column temperature was programmed from 100 to 250 °C at a rate of 10 °C/min, and then held at 250 °C for 10 min; the injector and jet separator temperatures were set at 260 °C. The mass spectrometer was operated in electron impact mode at 70 eV. The mass range from m/z 40 to 400 was scanned every 2 s.

Confirmation of the identity of the reaction products was made by gas chromatograph retention time and mass spectral comparison with authentic standards, whenever available. Product titers ranged from 200 to 600 μg/ml.

RESULTS AND DISCUSSION

In an earlier report from this laboratory, it was shown that alkenes (i.e. carbon-carbon double bonds) are converted to α,β-halohydrins by haloperoxidase (4):

\[
\begin{align*}
\text{HOX} & \quad \text{haloperoxidase} \\
\text{C=CH} + \text{X}^- + \text{H}_2\text{O}_2 + \text{H}^+ & \quad \rightarrow \quad \text{C=CHX} + \text{H}_2\text{O}
\end{align*}
\]

The enzymatic formation of the wide range of halohydrin products was explained in terms of hypohalous acid (HOX) addition chemistry.

Hypohalous acid is known to also react on alkynes (i.e. carbon-carbon triple bonds) yielding mono- and dihalogenated ketones (5):

\[
\begin{align*}
\text{HOX} & \quad \text{haloperoxidase} \\
\text{C≡C}^- + \text{X}^- & \quad \rightarrow \quad \text{C≡C}^-\text{CH} + \text{C}^-\text{CX}^-.
\end{align*}
\]

With this information, alkyne substrates were used with hal-

(Received for publication, July 6, 1983)
Novel Haloperoxidase Substrates

TABLE I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Halide</th>
<th>Haloperoxidase</th>
<th>Products*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃C≡CH (methyl acetylene)</td>
<td>Cl⁻</td>
<td>Chloroperoxidase</td>
<td>CH₃COCH₂Cl (3)</td>
</tr>
<tr>
<td></td>
<td>Br⁻</td>
<td>Chloroperoxidase</td>
<td>CH₃COCH₂Br (7)</td>
</tr>
<tr>
<td>CH₂CH₂C≡CH (ethyl acetylene)</td>
<td>Br⁻</td>
<td>Lactoperoxidase</td>
<td>CH₂CH₂COCH₂Br (4)</td>
</tr>
<tr>
<td>CH₂C≡CCH₂ (1-phenyl-1-propyne)</td>
<td>Cl⁻</td>
<td>Chloroperoxidase</td>
<td>CH₂C≡C(CH₂)CH₃ (12)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are yield in milligrams.

FIG. 1. Proposed mechanism for the chemical reaction between phenyl acetylene and hypobromous acid (6).

operoxidase. We now report that haloperoxidases enzymatically convert alkynes into these same halogenated products (Table I). That haloperoxidase does react with both alkenes and alkynes means either that this enzyme is a unique enzyme (to our knowledge no other enzyme can carry out the same reaction on both an alkene and an alkyne) or this enzyme generates a chemical intermediate that can react upon both substrates.

A working model that can account for the formation of these halogenated products follows the chemistry of hypohalous acid addition across the carbon-carbon triple bond. Fig. 1 presents an addition sequence that has been proposed for the chemical reaction between phenyl acetylene and hypobromous acid. The enzymatic reaction between phenyl acetylene and lactoperoxidase, in the presence of bromide ion and dilute hydrogen peroxide, yielded these same two products:

\[
\text{OH} + \text{C≡CH} + \text{Br}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{1} + \text{2},
\]

FIG. 2. Gas chromatography-mass spectrometer analysis of reaction between methyl acetylene, chloroperoxidase, bromide ion, and hydrogen peroxide. Product A, bromoacetone (9); Product B, 1,1-dibromoacetone (4); Product C, 1,3-dibromoacetone (5). a, reconstructed ion chromatogram of reaction mixture; b-d, distinctive mass spectrum of each enzymatically formed product.

Alkynes which have acidic hydrogens adjacent to the triple bond (e.g., R-CH₂-C≡C-) gave an additional dibrominated product in the enzymatic reaction. For example, the reaction between methyl acetylene and chloroperoxidase, in the presence of bromide ion and hydrogen peroxide, gave the following three products:
Novel Haloperoxidase Substrates

\[ \text{CH}_3\text{-C}≡\text{CH} + \text{Br}^− + \text{H}_2\text{O}_2 + \text{H}^+ \xrightarrow{\text{chloroperoxidase}} \text{CH}_3\text{-C}≡\text{CHBr} + \text{Br}^− + \text{H}_2\text{O}_2 + \text{H}^+ \]

Fig. 2 illustrates how the gas chromatograph-mass spectrometer analysis permitted the rapid confirmation of the products formed. The proposed working model can also account for this mixture (see Fig. 3).

A previous report had indicated that haloperoxidases can further halogenate \( \alpha \)-halogenated ketones. Bromoperoxidase from the alga Penicillus capitatus carried out the following reaction, although conversions were less than 10% at each step (7):

\[ \text{CH}_3\text{(CH}_2\text{)}_2\text{C}≡\text{CHBr} \xrightarrow{\text{bromoperoxidase}} \text{Br}^−, \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{(CH}_2\text{)}_3\text{C}C\text{HBr}_2 \xrightarrow{\text{bromoperoxidase}} \text{CH}_3\text{(CH}_2\text{)}_3\text{C}C\text{Br}_2 \]

Attempts to convert 3 into either 4 or 5 by using 3 as the substrate in enzymatic reactions with chloroperoxidase and lactoperoxidase were not successful. Thus, ketone halogenation was not a significant reaction under our experimental conditions.

It was also observed that the ratio of monohalogenated product to dihalogenated product(s) was dependent upon the hydrogen peroxide level present in the reaction mixture (Table II). The proposed working model can account for this phenomenon: increasing amounts of \( \text{H}_2\text{O}_2 \) in the reaction mixture yield increasing amounts of enzymatically generated hypohalous acid, which can favor formation of those products that incorporate 2 eq of the acid (i.e., the dihalogenated products). Although previous studies have shown that the enzyme’s reactivity is a function of the \( \text{H}_2\text{O}_2 \) level present (8), this is the first report of product control by \( \text{H}_2\text{O}_2 \) level.

The discovery of haloperoxidase reaction on alkynes has widespread biosynthetic implications, especially in marine biology. Marine environments contain all the needed reagents for this enzymatic reaction to be carried out: abundant haloperoxidases, numerous alkyne substrates, and halide salts (9, 10). Haloperoxidase reactions on alkynes can explain the presence of \( \alpha \)-halogenated ketones frequently detected in marine organisms (11).

Cyclopropanes often simulate alkenes in their chemical properties and addition of hypohalous acid to cyclopropane is known to result in the formation of \( \alpha,\gamma \)-halohydrins (5):

\[ \text{C} \xrightarrow{\text{HOX}} \text{C} \xrightarrow{\text{Br}} \text{C} \]

**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Halide</th>
<th>Haloperoxidase</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl cyclopropane</td>
<td>Cl</td>
<td>Chloroperoxidase</td>
<td>( \text{CH}_3\text{(OH)}\text{(CH}_2\text{)}_2\text{Cl} ) (1-chloro-3-butanol)</td>
</tr>
<tr>
<td>Phenyl cyclopropane</td>
<td>Cl</td>
<td>Chloroperoxidase</td>
<td>( \text{OCH(OH)}\text{(CH}_3\text{)}_2\text{Cl} ) (3-chloro-1-phenyl-1-propanol)</td>
</tr>
<tr>
<td>Phenyl cyclopropane</td>
<td>Br</td>
<td>Lactoperoxidase</td>
<td>( \text{OCH(OH)}\text{(CH}_3\text{)}_2\text{Br} ) (3-bromo-1-phenyl-1-propanol)</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Effect of ( \text{H}_2\text{O}_2 ) level on the reaction between alkynes and haloperoxidases</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CH}_3\text{C}≡\text{CH} \xrightarrow{\text{chloroperoxidase}} \text{Cl}^−, \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{C}≡\text{CHCl} + \text{Cl}^− + \text{H}_2\text{O} )</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) initial</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>mg</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>150</td>
</tr>
</tbody>
</table>

**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Halide</th>
<th>Haloperoxidase</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl cyclopropane</td>
<td>Cl</td>
<td>Chloroperoxidase</td>
<td>( \text{CH}_3\text{(OH)}\text{(CH}_2\text{)}_2\text{Cl} ) (1-chloro-3-butanol)</td>
</tr>
<tr>
<td>Phenyl cyclopropane</td>
<td>Cl</td>
<td>Chloroperoxidase</td>
<td>( \text{OCH(OH)}\text{(CH}_3\text{)}_2\text{Cl} ) (3-chloro-1-phenyl-1-propanol)</td>
</tr>
<tr>
<td>Phenyl cyclopropane</td>
<td>Br</td>
<td>Lactoperoxidase</td>
<td>( \text{OCH(OH)}\text{(CH}_3\text{)}_2\text{Br} ) (3-bromo-1-phenyl-1-propanol)</td>
</tr>
</tbody>
</table>

**FIG. 4. Proposed reaction scheme for reaction between chloroperoxidase, bromide ion, hydrogen peroxide, and methyl cyclopropane.** [HOBr] is the presumed enzymatically generated hypohalous acid.
We now report that haloperoxidases also react with cyclopropanes, enzymatically converting them into the predicted halogenated products (Table III). The enzyme-mediated cyclopropane ring-opening follows Markovnikov’s rule, with the halogen going to the carbon with the most hydrogens and the hydroxyl group going to the carbon best able to stabilize a positive charge. The reaction between methyl cyclopropane and haloperoxidase in the presence of bromide ion and hydrogen peroxide illustrates this point (Fig. 4). Positional isomer a, formed from the more stable bromonium ion intermediate, was the only isomer detected in the enzymatic reaction (Fig. 5).

Several enzyme-mediated cyclopropane ring-openings and a hydroxylation are known (Table IV). To this list we now add the haloperoxidase conversion of cyclopropanes into α,γ-halohydrins.

In conclusion, although the mechanism of catalysis of the haloperoxidase enzymes is still a matter of discussion, the enzymatic reaction with alkynes and cyclopropanes appears to follow the chemistry that would be observed had the enzyme generated free hypohalous acid.

Acknowledgments—We express appreciation to our colleague Dr. Terry Lee for providing assistance in this study.

REFERENCES