The Reaction Mechanism of the Novel Vanadium-Bromoperoxidase  
A STEADY-STATE KINETIC ANALYSIS* 

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The reaction of vanadium-bromoperoxidase from the brown alga Ascophyllum nodosum with hydrogen peroxide, bromide, and 2-chlorodimedone has been subjected to an extensive steady-state kinetic analysis. Systematic variation of pH and the concentrations of these three components demonstrate that the reaction model includes four enzyme species: native bromoperoxidase, a bromoperoxidase-bromide inhibitory complex, a bromoperoxidase-hydrogen peroxide intermediate, and a bromoperoxidase-HOBr species. This latter intermediate did not display any direct interaction with the nucleophile reagent as oxidized bromide species (Br₂, Br₂, and/or HOBr) were the primary reaction products. The generation of oxidized bromine species was as fast as the bromination of 2-chlorodimedone. The enzyme did not show any specificity with regard to bromination of various organic compounds. Formation of the bromoperoxidase-bromide inhibitory complex was competitive with the reaction between hydrogen peroxide and enzyme. From the steady-state kinetic data lower limits for the second-order rate constants at various pH values were calculated for individual steps in the catalytic cycle. This pH study showed that native enzyme must be unprotonated prior to binding of hydrogen peroxide (second-order association rate constant of 2.5×10⁶ M⁻¹s⁻¹ at pH >6). The pKₐ for the functional group controlling the binding of hydrogen peroxide was 5.7 and is ascribed to a histidine residue. The reaction rate between bromide and enzyme-hydrogen peroxide intermediate also depended on pH (second-order association rate constant of 1.7×10⁶ M⁻¹s⁻¹ at pH 4.0).

The enzymic incorporation of halogens into organic compounds is known to be catalyzed by haloperoxidases. In recent years there has been considerable interest in this halogenation process and to date a variety of haloperoxidases have been identified. In particular, chloroperoxidase found in the mould Caldariomyces fumago, a bromoperoxidase from brown algae, and also from a lichen (7-10). Reconstitution experiments as well as several spectroscopic techniques have identified vanadyl(V) as the prosthetic group involved in the halogenation reaction catalyzed by these enzymes (8-11). Vanadium-containing bromoperoxidase from the brown alga Ascophyllum nodosum was able to brominate several organic compounds, such as 2-chlorodimedone (7), phenol red (12), and several barbituric acid derivatives (13).

Although a large number of organic compounds are known to be halogenated by haloperoxidases, a thorough understanding of the mechanism by which this occurs is still elusive. For instance heme-containing chloroperoxidase has been extensively studied by both transient-state (14, 15) and steady-state (16) kinetic experiments and rate constants for the different steps in the mechanism have been obtained. Evidence has been presented that halogenation occurs by an electrophilic substitution reaction (17, 18). However, the exact nature of the final halogenating intermediate, whether enzyme-bound or not, is still a matter of debate (16, 18-20).

Even less is known about the bromination mechanism for the haloperoxidases not containing a heme prosthetic group. It was proposed by Itoh et al. (21) that bromination by bromoperoxidase from C. pilulifera occurs specifically by an enzyme-bound bromonium (Br⁺) ion intermediate.

In a previous study we reported on the steady-state kinetics of the bromination catalyzed by vanadium-bromoperoxidase from the brown alga A. nodosum (7). In the present paper we have extended this study to identify the halogenating intermediate in the bromination reaction catalyzed by this vanadium-containing bromoperoxidase.

MATERIALS AND METHODS

Bromoperoxidase was purified from A. nodosum as described before (7, 11). Specific activity of the preparation used in the steady-state kinetic experiments was 120 nmol of 2-chlorodimedone (Sigma) brominated/min/mg of protein under standard assay conditions (7). Protein content was measured by the method of Lowry et al. (22) with bovine serum albumin as a standard.

The steady-state kinetic experiments were done by measuring the bromination of 2-chlorodimedone (50 μM) at 280 nm (Δε = 18.2 mm⁻¹cm⁻¹ at pH 4.0 and 19.9 mm⁻¹cm⁻¹ at pH values higher than 4.5; Ref. 23) on a Zeiss PMQ-2 spectrophotometer. The solutions were buffered at the desired pH using sodium acetate (pH 4.0-4.5), MESS (pH 5.2-6.7), or HEPES (pH 7.2-7.9) buffers at an ionic

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strength of 50 mM. Differences in enzymatic activity by using these buffers at identical pH values were not observed. Potassium bromide concentration was varied between 1- and 500 mM. Sodium sulfate was used to maintain a constant ionic strength of 550 mM when the potassium bromide concentration was varied. Hydrogen peroxide concentrations were varied between 1 mM and 20 mM. Dependent on reaction rates, bromoperoxidase was present between 1 and 30 nM. The experiments were done by measuring initial rates of 2-chlorodimedone consumption at a fixed concentration of potassium bromide and varying amounts of hydrogen peroxide in the pH range from 4.0 to 7.9. Within one series of measurements hydrogen peroxide was varied 25-fold to ensure at least a 3-fold rate dependence at a single enzyme concentration. All measurements were performed at 22°C. Mean values of the initial rates of three traces were used in the calculations.

Initial bromination rates (ν) as a function of hydrogen peroxide concentration were fitted as rectangular hyperbolae of the form

\[ \nu = \frac{\sqrt{[H_2O_2]}}{K + [H_2O_2]} \]

using a nonlinear, least squares regression analysis (24, 25). V and K are the kinetic parameters for maximum velocity at saturating substrate concentrations and half-saturation constant, respectively, at any given set of concentrations of the other substrate(s). V and K were determined by the iterative nonlinear procedure described by Wilkinson (24), and Bliss and James (25). Detailed information on these two parameters is given in the Appendix.

Concentrations of oxidized bromine species ([Br₂] + [Br] + [OBr⁻]) were determined spectrophotometrically by measuring the amount of tribromide ions (Br₃⁻) at 268 nm (ε = 43.0 M⁻¹ cm⁻¹; Ref. 26). The total concentration of oxidized bromine species ([=Br⁺]') was then calculated from

\[ [\text{Br}⁺']= [\text{Br}⁻] + \frac{1}{K_1 [K_2 [\text{Br}⁻][\text{H}⁺]} + \frac{1}{K_2 [K_1 [\text{Br}⁻][\text{H}⁺]} + \frac{1}{K_1 [\text{Br}⁻]} \]

where \( K_1 \) is 2.00-10⁻³ (M⁻¹), the protonation constant for hypobromous acid (27); \( K_2 \) is 1.4-10⁻⁴ (M⁻¹), the association constant for tribromide ion (19); and \( K_3 \) is 1.74-10⁻⁸ (M⁻²), the hydrolization constant for molecular bromine (28).

Hydrogen peroxide solutions were prepared from a 30% reagent grade solution (Merck). Concentrations were determined spectrophotometrically using an absorbance coefficient of 43.5 M⁻¹ cm⁻¹ at 240 nm (29).

The formation of tribromide ions by bromoperoxidase was studied with a Union Giken RA-401 rapid-scan spectrophotometer operating between 231 and 327 nm. The conditions after mixing were 100 mM sodium acetate (pH 4.2), 100 mM sodium sulfate, 50 mM potassium bromide, 300 pM hydrogen peroxide, and 130 nM bromoperoxidase.

The rate of formation of oxidized bromine species was compared with the rate of bromination of 2-chlorodimedone by measuring the absorbance changes at 268 and 290 nm, respectively, on the Union Giken RA-401 spectrophotometer using the stopped-flow device. The conditions after mixing were 50 mM sodium acetate (pH 4.2), 100 mM sodium sulfate, 50 mM potassium bromide, 930 pM hydrogen peroxide, 2-chlorodimedone, and 36 nM of bromoperoxidase. The organic compounds were present in the concentration range from 0.5 to 50 µM. The following extinction coefficients (ε, M⁻¹ cm⁻¹) were used: 17,200 at 308 nm for trans-4-hydroxyxinnamic acid, 18,540 at 268 nm for 5-phenylbarbituric acid, 9,470 at 270 nm for 2-thiouracil, and 18,200 at 290 nm for 2-chlorodimedone. Mean values of 6 or 7 traces were used in the calculations.

All chemicals used were of analytical grade.

**RESULTS**

When bromoperoxidase was added to solutions containing fixed levels of bromide but variable amounts of hydrogen peroxide, initial rates of 2-chlorodimedone bromination showed typical Michaelis-Menten type of rectangular hyperbolae (not shown). Even at hydrogen peroxide concentrations exceeding those of 20 mM, deviations from this hyperbolic behavior were not observed. As a consequence double-reciprocal plots of initial rates and hydrogen peroxide concentration were linear. This is shown in Fig. 1 for several fixed amounts of bromide. However, when hydrogen peroxide was fixed and bromide was varied, double-reciprocal plots of bromination rates and bromide concentration were hyperbolic concave-up (not shown). This indicates that enzymic activity is not inhibited by high concentrations of hydrogen peroxide, whereas excess bromide appreciably diminished bromination rates. The inhibition of bromoperoxidase at high levels of bromide has been noted before (7). Since the double-reciprocal plots of initial rates and hydrogen peroxide concentration at various fixed levels of bromide have common intercepts, the inhibition by bromide is competitive with respect to the reaction of hydrogen peroxide with enzyme (30). When the data of Fig. 1 were plotted in a Dixon plot (not shown) the point of intersection was in the second quadrant also pointing to a competitive type of inhibition by bromide (31).

The rectangular hyperbolae obtained by plotting initial rates (u) versus hydrogen peroxide concentration are characterized by the two parameters K and V. At fixed concentrations of the other substrate(s) K and V represent the half-saturation constant and maximum velocity at saturating levels of hydrogen peroxide, respectively. A detailed interpretation of these two parameters is given in the Appendix. When replots were made of 1/V versus 1/[Br⁻] and K/V versus [Br⁻] straight lines were obtained. This is shown in Figs. 2 and 3 for various pH values. In the Appendix it is shown that secondary plots of this type should be linear when two substrates react in an ordered mechanism with the enzyme. Moreover, the linear secondary plot of K/V versus [Br⁻] indicates that bromide is able to form an abortive binary complex with the enzyme and that this reaction is competitive with hydrogen peroxide adding to the enzyme in the first step of the mechanism, giving a productive binary complex (competitive substrate inhibition; Refs. 30 and 32). In the catalytic reaction sequence bromide, as the second substrate, then reacts with this hydrogen peroxide-enzyme complex.

From the slopes and intercepts of the secondary plots the different kinetic parameters can be calculated (see Appendix). Table I shows the Michaelis-Menten constants for hydrogen peroxide (K₉₀₂), and bromide (K₉₀⁻), the inhibition constant for bromide (K₉⁻), and also the maximum velocity/mol of...
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**FIG. 2.** Secondary double-reciprocal plot of the kinetic parameter V as a function of bromide concentration, at various values for the pH. X-X, pH 4.00; ○-○, pH 4.50; ●-●, pH 5.25; □-□, pH 5.90; ■-■, pH 7.27; □-□, pH 7.92. The vertical bars are the standard errors as obtained from the computer fits of the primary rectangular hyperbolae of bromination rate versus hydrogen peroxide concentration. See "Materials and Methods" and the Appendix for details.

**FIG. 3.** Dependence of K/V on the bromide concentration, at various values for the pH. X-X, pH 4.00; ○-○, pH 4.50; ●-●, pH 5.25; □-□, pH 5.90; ■-■, pH 7.27; □-□, pH 7.92. The vertical bars are the standard errors as obtained from the computer fits of the rectangular hyperbolae of bromination rate versus hydrogen peroxide concentration. See "Materials and Methods" and the Appendix for details.

**FIG. 4.** pH dependence of the lower limits of the second-order rate constants for the reaction between bromoperoxidase and hydrogen peroxide (k₅) and between bromoperoxidase-hydrogen peroxide intermediate and bromide (k₆). ●-●, k₅; ■-■, k₆, k₅ and k₆ were obtained by taking the fractions of kₛ/Kₘ and kₛ/Kₘ, respectively (see Table I).

**TABLE I**

| pH | K₈ Br⁻ | K₅ | K₆ | kₛ |
|----|--------|----|----|----|
|    | μM     | mM | mM | s⁻¹|
| 4.00| 3147   | 1.7| >1000| 300|
| 4.50| 1110   | 3.4| 417 | 278|
| 5.25| 162    | 5.1| 228 | 166|
| 5.90| 104    | 9.4| 748 | 138|
| 6.63| 42     | 9.3| >1000| 113|
| 7.27| 34     | 14.8| >1000| 94|
| 7.92| 22     | 18.1| >1000| 50|

* kₛ = v/e (s⁻¹); e, enzyme concentration (=concentration of active site; see Ref. 33).

enzyme (kₛ)² at saturating concentrations of the substrates, at various values for the pH. From the table it can be inferred that from the four kinetic parameters only the K₆ for hydrogen peroxide is highly affected by pH. The data show that in the pH range from 4 to 6 the affinity of the enzyme for hydrogen peroxide strongly increased and remained constant above pH 6. Both K₈ and kₛ are only slightly affected by pH. similar effects of pH (in the range from 4 to 6) on K₆ values for hydrogen peroxide and bromide and on the maximum reaction velocity have been noted before (7).

The effect of bromide as an inhibitor of the reaction is somewhat complex. The data show that the reaction of bromide with native bromoperoxidase depends on pH with a maximal inhibitory effect at about pH 5.3. Both at low pH (~4.0) and at high pH (about 7–8) an inhibitory complex of bromoperoxidase and bromide is hardly formed. Since the inhibition constants were obtained at bromide concentrations up to 500 mM, Kₛ values higher than this upper limit are not very accurate. This is also clear from Fig. 3: at high pH (>6.5) there is hardly any effect of bromide concentration on the slopes of the lines (see also the Appendix). Despite this inaccuracy in Kₛ values at both low and high pH it is clear that bromide preferentially reacts with a singly protonated form of bromoperoxidase. The increase in Kₛ at both low and high pH indicates that bromide ions do not appreciably react with doubly protonated or deprotonated forms of the native enzyme.

It was shown by Bloomfield et al. (34) that estimates for the lower limits of bimolecular rate constants can be obtained from steady-state kinetic experiments by dividing kₛ by K₆. This procedure was used in other peroxidase systems (35,36) to evaluate estimates of the rate constants of the substrates. When this method was applied to the bromoperoxidase/hydrogen peroxide/bromide system, the second-order rate constant for the combination of enzyme and hydrogen peroxide (k₅) increased from 9.5·10⁻⁶ M⁻¹ s⁻¹ at pH 4.0 to about 2.5·10⁻⁶ M⁻¹ s⁻¹ at pH > 6.0. When the log kₛ values determined by this method were plotted as a function of pH, it can be seen that in the pH range from 4 to 6 this increase was represented by a straight line with a slope of 0.83 (Fig. 4). Above pH 6, the graph of log kₛ versus pH was a horizontal line. The increase in complex-formation rate when the pH was raised from 4 to 6 indicates that hydrogen peroxide preferentially reacts with a deprotonated form of the enzyme. The slope of about +1 demonstrates that a single proton is involved in the formation of this primary collision complex (31). Since the pH for hydrogen peroxide is 11.6 (37), the possibility that bromoperoxidase combines with HO₂ can be excluded. The pH for the ionizable group on the enzyme controlling the binding of hydrogen peroxide is 5.7, as given by the point of intersection of the straight parts in Fig. 4.

Also the lower limit of the second-order rate constant for the combination of bromide with the enzyme-hydrogen peroxide complex (k₆) is pH-dependent. When kₛ is divided by K₆ it is clear that the estimated association rate constant

²Since bromoperoxidase was shown to contain one vanadium ion/ enzyme molecule (33), Kₛ is identical to the maximum turnover number (=maximum velocity divided by total enzyme concentration).
decreases from $1.75 \times 10^{-4}$ M$^{-1}$ s$^{-1}$ at pH 4.0 to $2.78 \times 10^{-3}$ M$^{-1}$ s$^{-1}$ at pH 7.9 for this bimolecular reaction. When log $k_r$ was plotted in a graph as a function of pH a straight line with a slope of about $-0.4$ was found (Fig. 4). This demonstrates that the reaction of bromide with the enzyme-hydrogen peroxide complex is fastest at high bromide concentration.

When bromoperoxidase was rapidly mixed with hydrogen peroxide and bromide at pH 5.0 in the absence of 2-chlorodimedone, a large increase in absorbance in the UV-region at 268 nm was observed (Fig. 5). Tri bromide ions ($Br_3^-$) absorb light in this region of the optical absorption spectrum also with a maximum at 268 nm (26). Molecular bromine is known to be in rapid equilibrium with tribromide ions, and we could demonstrate that UV-spectra of solutions containing molecular bromine and bromide were identical with those of the enzymic product (not shown). Therefore, the large increase in absorbance at 268 nm generated by the bromoperoxidase/hydrogen peroxide/bromide system was ascribed to the enzymic formation of molecular bromine.

The determination of oxidized bromine species in aqueous solutions is complicated by the equilibria between molecular bromine, tribromide ion, and hypobromous acid (26). By measuring the absorbance of tribromide ions at 268 nm and by using the constants of equilibria between $Br_3^-/Br_2^-$, $Br_2^-/HOBr$, and $HOBr/OB^-\cdot$, true yields of oxidized bromine species could be obtained. Furthermore, oxidized bromine species easily react with hydrogen peroxide. This side reaction is slow at acid pH and at high bromide concentrations (26). Therefore, in order to quantify the amount of oxidized bromine species formed by bromoperoxidase, initial rates of $Br_3^-$ formation were studied at high enzyme concentrations on a stopped-flow spectrophotometer. Within experimental error the formation rate of oxidized bromine species ($74 \pm 6$ (s$^{-1}$)) was equal to the bromination rate of 2-chlorodimedone ($73 \pm 5$ (s$^{-1}$)) under similar conditions (see "Materials and Methods"). This shows that the presence of the organic substrate has no effect on the rate of formation of bromine species by the enzyme. Further, if one assumes that the reaction between bromine species and 2-chlorodimedone is fast, this observation suggests that enzymically generated bromine species are intermediates in the bromination reaction.

In the absence of an organic compound, oxidized bromine species react with hydrogen peroxide to yield singlet oxygen and bromide (28, 38). This reaction was confirmed by us for the bromoperoxidase/hydrogen peroxide/bromide system. Bromide ions were an absolute requirement for this reaction since evolution of oxygen was not observed when bromoperoxidase was incubated with hydrogen peroxide in the absence of bromide. The oxidation of hydrogen peroxide by oxidized bromine species strongly depends on pH (25, 38). At acid pH (>2) this reaction is extremely slow and the Br$_2^-$ generated can easily be observed. However, at neutral pH the reaction of $Br^-\cdot$ with hydrogen peroxide even at the lowest possible concentrations is so fast that it falls completely within the mixing time (<3 ms) of the stopped-flow spectrophotometer.

Bromoperoxidase has been used in the bromination of several organic compounds, including 2-chlorodimedone (7), phenol red (12), and several barbituric acid derivatives (13). Here we have investigated the enzymic bromination of several organic acceptor substances to verify whether the nature of the nucleophilic molecule is of importance to bromination rates. For this purpose we have compared bromination rates of 2-chlorodimedone, 5-phenylbarbituric acid, 2-thiouracil, and trans-4-hydroxycinnamic acid. Both 2-chlorodimedone and 5-phenylbarbituric acid are known to be converted to the corresponding 2-bromo- and 5-bromo-derivatives, respectively (7, 13). 2-Thiouracil is oxidized by molecular halogen species ($Cl_2$, $Br_2$, or I) to uracil disulfide (39, 40). We could confirm this observation and, moreover, we noted that UV-spectra of chemically synthesized uracil disulfide were identical with those of the reaction product of bromoperoxidase, hydrogen peroxide, and bromide with 2-thiouracil (not shown). Obviously uracil disulfide is the oxidation product of this enzymic conversion. Uracil disulfide was also formed when heme-containing chloroperoxidase, hydrogen peroxide, and 2-thiouracil were incubated with either chloride, bromide, or iodide (19, 37). The bromination of trans-4-hydroxycinnamic acid has extensively been studied by Yamada and coworkers (18) using heme-containing chloroperoxidase as the halogenating enzyme. They found that this cinnamic acid derivative was converted into trans-1-bromo-2(4-hydroxyphenyl)-ethylene. Trans-4-hydroxycinnamic acid displays an intense absorption band in the UV-spectrum with a maximum at 308 nm ($e = 21$ M$^{-1}$,cm$^{-1}$ at pH 4.0). When bromoperoxidase was incubated with trans-4-hydroxycinnamic acid, hydrogen peroxide, and bromide a rapid decrease of this band with an isosbestic point at 274 nm was observed. Similar changes in the UV-spectrum were obtained when trans-4-hydroxycinnamic acid was allowed to react with a solution of bromine (not shown). These results indicate that trans-4-hydroxycinnamic acid is converted into a single, monobrominated product on incubation with bromoperoxidase, hydrogen peroxide, and bromide.

To study the possible interaction of an enzyme-halogenating intermediate with these organic substrates, all the preceding steps in the mechanism (reaction of the enzyme with hydrogen peroxide and bromide) should not be rate limiting. This problem was solved by working at low pH where inhibition by bromide is very weak, and by using high concentrations of hydrogen peroxide and bromide, largely exceeding the $K_a$ values for these substrates. Moreover, at low pH the side reaction of hydrogen peroxide with oxidized bromine species is slow (26). However, under these conditions the organic compounds reacted with hydrogen peroxide and bromide even in the absence of enzyme. Therefore, we increased reaction rates by working at high enzyme concentrations. As a consequence initial rates of the disappearance of the organic compounds were very high, making the use of a stopped-flow spectrophotometer necessary. Fig. 6 shows the reaction rates by which the organic compounds were converted into their

![Fig. 5. Rapid-scan UV-spectra of bromoperoxidase after mixing the enzyme with hydrogen peroxide and bromide (pH 5.0). The traces were recorded at 0 (I), 172-182 (2), 293-302 (3), 472-482 (4), 562-569 (5), and 952-962 (6) ms after mixing. The slight absorbance at 280 nm at t = 0 is due to the protein. See "Materials and Methods" for details.](image-url)
bromination or oxidation reactions catalyzed by bromoperoxidase. The organic substance is not of importance for the rates of hydrogen peroxide for the enzymically produced oxidized bromine species. The graph clearly shows that the nature of the organic substance is not of importance for the rates of bromination or oxidation reactions catalyzed by bromoperoxidase. As proposed (13) the hyperbolic behavior probably reflects the competition between the organic compound and hydrogen peroxide for the enzymically produced oxidized bromine species.

**DISCUSSION**

In this study we have presented data on the steady-state kinetics of vanadium-bromoperoxidase. This was done by measuring bromination rates of the nucleophile 2-chlorodimedone at a variety of concentrations of both hydrogen peroxide and bromide, and at various values for the pH. From this analysis values for the half-saturation constant (K) and maximum velocity at saturating substrate concentrations (V) were obtained with hydrogen peroxide as the varied substrate. Thus, K and V represent the apparent Km for hydrogen peroxide and Vmax, respectively, at fixed bromide concentrations. True values for K[hbo] and Vmax as well as for Km could be obtained by further analysis of the primary kinetic parameters K and V. Since it was noted that bromide fulfills a dual role in the enzymic reaction (both as a substrate and as an inhibitor), K and V also include the effect of bromide binding to the native enzyme. As is shown in the Appendix, values for the dissociation constant of the bromide-native bromoperoxidase complex (Kbrate) could also be obtained from further analysis of K and V.

The results show that hydrogen peroxide and bromide (as a substrate) react with the enzyme in an ordered mechanism. Since the reaction between native bromoperoxidase and bromide (as an inhibitor) is competitive with the binding of hydrogen peroxide, it is obvious that hydrogen peroxide adds first to the enzyme and that bromide is the second substrate in the catalytic cycle. The mechanism considered to be a proper description for the bromination reaction catalyzed by bromoperoxidase is shown in Fig. 7a. This mechanism involves three important enzyme species in the catalytic cycle, i.e. native bromoperoxidase and two intermediate forms where the substrates hydrogen peroxide and bromide are bound to the enzyme. This two-substrate model, including the competitive substrate inhibition by bromide, was predicted by the linear 1/V versus 1/[Br⁻] and K/V versus [Br⁻] secondary plots.

The enzymic production of oxidized bromine species is equally fast as the bromination of 2-chlorodimedone. Furthermore, the conversion rates of 2-chlorodimedone, 5-phenylbarbituric acid, 2-thiouracil, and trans-4-hydroxycinnamic acid at high concentration, are equal. Obviously these compounds react equally well. These results are not expected when a ternary enzyme-halogenating intermediate-organic substrate complex is involved since entirely different organic substrates are brominated at the same rate. Rather this supports a reaction mechanism in which oxidized bromine species in a rate-determining step are generated by the enzyme. As was pointed out by Brown and Hager (17) and by Yamada et al. (18), halogen acceptor molecules should have carbon atoms with enough electron density to be attacked by halogenium ions (e.g. Cl⁺ or Br⁺). This criterion is met by 2-chlorodimedone, 5-phenylbarbituric acid, and 2-thiouracil which all have electron dense π-bonds. However, for trans-4-hydroxycinnamic acid the electron density of the π-bonds is reduced so that reaction with trans-4-hydroxycinnamic acid is known to be in rapid equilibrium with molecular bromine and tribromide ions (e.g. Cl or Br). In aqueous solutions hypobromous acid is known to be in rapid equilibrium with molecular bromine and tribromide ions according to equation b in Fig. 7 (26).

Joh et al. (21) were not able to detect any appreciable

![FIG. 6. The effect of nucleophile concentration (AH) on the initial rates of bromination of 2-chlorodimedone ( ), 5-phenylbarbituric acid ( ), and trans-4-hydroxycinnamic acid ( ) and on initial oxidation rates of 2-thiouracil ( ) (pH 4.0). Total enzyme concentration is represented by e. See "Materials and Methods" for details.](image-url)
generation of tribromide ions when they mixed bromoperoxidase from *C. pilulifera* with hydrogen peroxide (1 mM) and bromide (100 mM) at pH 6.0 and monitoring UV-spectra at 2 min intervals. Mainly on the basis of this observation, Itoh et al. (21) concluded that bromination occurred at the active site of the enzyme by the action of an enzyme-bound bromonium ion intermediate on the nucleophile. We could also not detect the release of tribromide ions into the solution upon using bromoperoxidase from *A. nodosum* under similar conditions probably due to a reaction between oxidized bromine species and hydrogen peroxide to yield singlet oxygen and bromide (Fig. 7d, Refs. 26 and 38). We noted that the enzymatic production of tribromide ions could only be observed at more acid pH on a short time-scale, e.g. on a rapid-scan/stopped-flow spectrophotometer (Fig. 5). Thus, the conditions for measuring molecular bromine intermediates should be carefully chosen and, therefore, the experiments of Itoh et al. (21) cannot be employed to assess whether this red seaweed bromoperoxidase has an active site for the specific bromination of organic substrates.

As is shown in Fig. 7a several protonation steps are considered to be involved in the mechanism of action of bromoperoxidase. From an analysis of the lower limits of the association rate constants of bromoperoxidase and hydrogen peroxide (k₄) at various values for the pH, it appeared that an ionizable group with a pKₐ of 5.7 is present near the active site, which when protonated prevents the reaction with hydrogen peroxide. In this regard it should be noted that such groups with pKₐ values varying from 4 to 6 controlling the binding of hydrogen peroxide are also present on heme-containing peroxidases. For example for cytochrome c peroxidase an acid/base group was found with a pKₐ of 5.5 (41). Also, studies on horseradish peroxidase (pKₐ 4.0, Ref. 42), myeloperoxidase (pKₐ 4.3, Ref. 43), and lactoperoxidase (pKₐ 6.0, Ref. 35) implied the existence of such an ionizable group. In all these peroxidases these values were explained by the presence of the histidine residue distal to the heme prosthetic group (44–46). Although we cannot exclude the protonation of a water molecule near the active site of bromoperoxidase, most likely a histidine residue is responsible for this functional group with pKₐ 5.7. From electron paramagnetic resonance spectroscopic experiments on reduced bromoperoxidase, it appeared that an acid/base group was present near the vanadium(IV) ion with a pKₐ of 5.4 (33). It is conceivable that it is this group which is responsible for the pH-controlled binding of hydrogen peroxide.

The binding of bromide, both as a substrate and as an inhibitor is also affected by the pH (Fig. 7a). The pH profile of the dissociation constant of (native) bromoperoxidase-bromide complex (Kₐ[N]) shows a bell-shaped curve typical of two protonation equilibria each with a different value for the pKₐ. A pH-dependent inhibition of haloperoxidases by halides is also a common phenomenon for the heme-containing enzymes (43, 47–49). An important difference with regard to the hemoproteins, however, is that bromide hardly binds to the very acid form of vanadium-bromoperoxidase (at pH ~ 4.0; E-2H⁺ species in Fig. 7a). The heme-enzymes, on the contrary, become more strongly inhibited by halides as the pH is lowered (43, 47–49).

The second-order rate constant as estimated for the binding of bromide as a substrate (k₂) decreases as the pH is raised. The plot of log k₂ versus pH gave rise to a straight line with noninteger slope (−0.4) suggesting that bromide ions do not simply react with one form of enzyme-hydrogen peroxide intermediate (31). Probably two or even three protonated forms of this primary collision product exist with pKₐ values equally well spread throughout this pH interval. With a preference for the more acid forms, bromide ions would then react with all these intermediates to yield oxidized bromine species as the primary enzymic products. The lower limit for k₂ at acid pH (1.7·10⁵ M⁻¹·s⁻¹) is in the order of magnitude as the second-order rate constant for the reaction of thiocyanate (SCN⁻) with compound 1 of lactoperoxidase (1.1·10⁵ M⁻¹·s⁻¹; Ref. 35 and 2·10⁷ M⁻¹·s⁻¹; Ref. 50) and also agrees well with the combination of chloride with compound 1 of fungal chloroperoxidase (1.9·10⁶ M⁻¹·s⁻¹; Ref. 2.1·10⁶ M⁻¹·s⁻¹ at pH 2.8; Ref. 14). The pH dependence of these rate constants and the complex inhibition pattern by bromide probably explains the bell-shaped curves of brominating activity versus pH, which are under certain conditions asymmetric (Fig. 2 in Ref. 7).

In conclusion it has been shown that the brominating activity of vanadium-bromoperoxidase from *A. nodosum* can be described by an ordered two-substrate (hydrogen peroxide and bromide) reaction model, including competitive inhibition by one of the substrates (bromide). Oxidized bromine species are the primary enzymic products. The experiments demonstrate that an enzyme-bound halogenating intermediate does not exist.

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APPENDIX

The rate equation for an enzyme reaction involving two substrates (A and B), including competitive inhibition by the second substrate (B) is given by (30):

\[
v = \frac{V_{\text{max}}}{K_a [A] [B] (1 + K_a [A] [B])}
\]

where, Kₐ, the Kₐ for substrate A; Kₐ, the Kₐ for substrate B; Kₐ, the dissociation constant for enzyme-B complex; and Vₐ, the maximum velocity at saturating concentrations of both A and B. At fixed levels of B, Equation a represents a rectangular hyperbola of the form

\[
v = \frac{V_{\text{max}} [A]}{K_a + [A]}
\]

where,

\[
V = \frac{V_{\text{max}}}{1 + K_a [B]}
\]

and

\[
K = \frac{K_a (1 + K_a [B])}{K_a [B]}
\]

Equations c and d can be transformed to

\[
\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K}{V_{\text{max}} [B]}
\]

and

\[
\frac{K}{V} = \frac{V_{\text{max}}}{K_a [B]}
\]
Therefore, plots of $1/V$ versus $1/[B]$ and $K/V$ versus $[B]$ should be linear. The steady-state kinetic parameters listed in Table I were calculated from Equations $e$ and $f$.

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