Marine Haloperoxidases

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I. Introduction

A. Halogenated Compounds in the Marine Environment

Ocean water is approximately 0.5 M in chloride, 1 mM in bromide, and 1 μM in iodide. Given the high halogen content, it is not surprising that marine organisms have developed means to incorporate halogens into their metabolites. Many of these halogenated compounds are thought to be involved in chemical defense roles to keep predators away from a particular organism. In many cases these compounds are also of pharmacological interest due to their biological activities, which include antifungal, antibacterial, antineoplastic, antiviral (e.g., anti-HIV), antiinflammatory, and other activities.

The halogenated marine natural products encompass a very wide range of compounds. Some are simple volatile halohydrocarbons produced in very large quantities, such as bromoform, dibromomethane, methyl iodide, bromo- and chloroanisoles, etc.[1-4] Others include phenolic derivatives in which the biosynthesis of these compounds can be readily envisaged, as in aeroplysinin-1 (see **1** in Figure 1), an antimicrobial metabolite from sponges,[5] or 14-debromoprearaplysillin (**2**) from the marine sponge *Druinella purpurea*,[6] both of which could originate from tyrosine (see below), or

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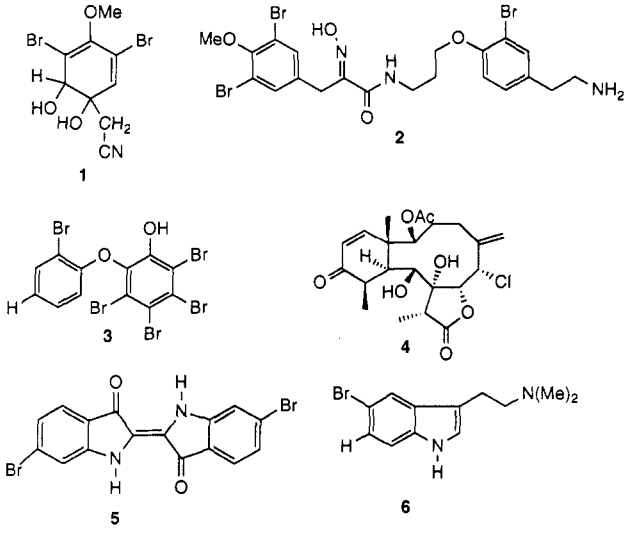


Figure 1. Examples of marine natural products with important biological activities.

the antimicrobial compound 2-(2′-bromophenoxy)-3,4,5,6-tetrabromophenol (**3**) from the sponge *Dysidea*.[7] Numerous chiral chlorinated and brominated terpenoids have also been isolated, including solenolide E (**4**), a diterpene which is an antiinflammatory and antiviral compound isolated from a gorgonian.[8] One of the more famous brominated marine natural products is tyrian purple (**5**), isolated from a marine mollusk. Other brominated and chlorinated indoles, e.g., 5-bromo-*N*,*N*-dimethyltryptamine (**6**), from a marine sponge,[9] are also known. A comprehensive summary of halogenated compounds is contained in a series of reviews of marine natural products by Faulkner.[10]

Marine natural products chemists have long invoked the role of haloperoxidases in the biogenesis of the halogenated marine natural products. Bromoperoxidase and iodoperoxidase activity have been well documented in marine algae since the turn of the century.[11,12] Haloperoxidases have been found in virtually all classes of marine organisms. Early on, Hewson and Hager established that *many* species of marine algae have bromoperoxidase activity in aqueous algal extracts.[13] Rhodophyta were the richest in both bromoperoxidase activity and lipid halogen content; Pheophyta were the poorest. Marine haloperoxidases have now been isolated and purified to homogeneity; thus the scope of this review article will be the reactivity and characterization of the marine haloperoxidases.

B. Marine Haloperoxidases

Haloperoxidases are enzymes that catalyze the oxidation of a halide (i.e., chloride, bromide, or iodide) by hydrogen peroxide, a process which results in the concomitant halogenation of organic substrates.

$$\text{Org-H} + \text{X}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{X-Org} + 2\text{H}_2\text{O}$$

The nomenclature for the haloperoxidases has traditionally been based on the most electronegative halide which is able to be oxidized by H_2O_2 catalyzed by the enzyme. Thus chloroperoxidase catalyzes the oxidation of chloride, bromide, and iodide by hydrogen peroxide; bromoperoxidase catalyzes the oxidation of bromide and iodide by hydrogen peroxide, while iodoperoxidase catalyzes the oxidation of only iodide by hydrogen peroxide. A better system of nomenclature would be based on the enzyme's physiological role, although at this point, that is hard to define given that the concentrations of halides, hydrogen peroxide, and nature of the endogenous organic substrate are not known in many marine organisms.

1. Types of Marine Haloperoxidases

Haloperoxidases have been isolated from all classes of marine algae and many other marine organisms (Table I); in addition haloperoxidase activity has been detected in many other species of algae[13] and in other marine organisms. Two types of marine haloperoxidases have been identified: (1) vanadium bromoperoxidase (V-BrPO), a non-heme enzyme, and (2) FeHeme bromoperoxidase (FeHeme-BrPO). In some cases, a marine haloperoxidase has been isolated, but the vanadium or FeHeme content has not been determined, as in the marine snail *Murex trunculus*.[30] In addition to these enzymes, other terrestrial haloperoxidases are known, including the first discovered haloperoxidase, chloroperoxidase, the FeHeme enzyme from the fungus *Caldaromyces fumago*,[31] mammalian haloperoxidases such as eosinophil peroxidase[32] and myeloperoxidase[32] found in human white blood cells, salivary and lactoperoxidase found in saliva and tears,[32] and bacterial haloperoxidases (bromoperoxidase from *Streptomyces*[33] and chloroperoxidase from *Pseudomonads*[34]) for which the identity of the active-metal species, if any, has not been determined. Recently a new enzyme, methyl transferase, was isolated from marine alga (*Endocladia muricata*) that catalyzes the formation of methyl chloride.[35] Methyltransferase uses *S*-adenosylmethionine as the carbon source.

Table I. Sources of Marine Haloperoxidases

	ref(s)
vanadium bromoperoxidase	
I. Rhodophyta (red algae)	
Corallina officinalis	14
Corallina pilulifera	15, 16
Corallina vancouveriensis	17
Ceramium rubrum	18
II. Phaeophyta (brown algae)	
Alaria esculenta	19
Ascophyllum nodosum	20
Chorda filum	21
Fucus distichus	22
Laminaria digitata	23
Laminaria sacchirina	21
Macrocystis pyrifera	22
III. Chlorophyta (green algae)	
Halimeda sp.	24
FeHeme bromoperoxidases	
I. Rhodophyta	
Cystoclonium purpureum	25
Rhodomela larix	26
II. Chlorophyta	
Penicillus capitatus	27, 28
Penicillus lamourouxii	28
Rhipocephalus phoenix	28
III. Marine worm	
Notomastus lobatus	29
Ptychodera flavin laysanica	26
Thelepus setosus	26

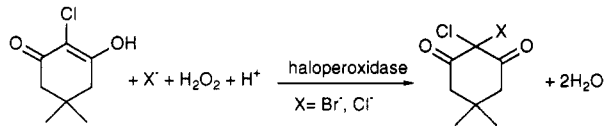


Figure 2. Halogenation of monochlorodimedone (MCD) catalyzed by haloperoxidases.

2. Standard Assay for Haloperoxidase Activity

The standard assay for haloperoxidase activity is the halogenation of monochlorodimedone (MCD; 2-chloro-1,3-dimedone) using hydrogen peroxide as the oxidant of the halide (Figure 2).[31]

The halogenation of MCD is followed spectrophotometrically at 290 nm, which monitors the loss of MCD in the enol form ($\epsilon = 20\,000\ M^{-1}\ cm^{-1}$). Bromoperoxidase activity is expressed as micromoles of MCD brominated per minute per milligram of enzyme (i.e., units/mg). The early work on V-BrPO employed the oxidation of iodide by hydrogen peroxide,[20] forming triiodide (I_3^-) which was followed spectrophotometrically at 353 nm ($\epsilon = 26\,400\ M^{-1}\ cm^{-1}$). However, this reaction is less desirable for quantitation of haloperoxidase activity because of competing side reactions, such as the nonenzymatic oxidation of iodide by hydrogen peroxide and reduction of triiodide by hydrogen peroxide.

The formation of bromochlorodimedone and dichlorodimedone have been verified by chromatographic analysis and comparison with an authentic sample of each.[24,27] Bromochlorodimedone is not stable at neutral pH over long periods of time, but it is stable at low pH. Thus the bromoperoxidase reactions run at neutral pH were adjusted to pH 3 at the end of the reaction in order to isolate bromochlorodimedone.[24]

II. Vanadium Bromoperoxidase

A. Characteristics of V-BrPO

Vanadium bromoperoxidases are all acidic proteins[37,68] with very similar amino acid composition[38] (see Table II), molecular weight, charge (pI 4-5), and vanadium content. The subunit molecular weight of V-BrPO is ca. 65 000. As isolated V-BrPO contains only about 0.4 vanadium atoms per subunit. However, a content of one gram-atom of vanadium per subunit can be achieved by addition of excess vanadate and subsequent removal of adventitiously bound vanadium(V) by dialysis.[21,22,37] While the physical characteristics of V-BrPO isolated from marine algae are all very similar, some differences in reactivity have been observed, such as specific activity (see below). V-BrPO (*Ascophyllum nodosum*) has been crystallized, although refined structural data have not been reported yet.[39] The crystals defract to 2.4-Å resolution. Four molecules are present per asymmetric subunit.

Table II. Percent Amino Acid Composition of Marine Haloperoxidases

amino acid	*A. nodosum* V-BrPO	*L. saccharina* V-BrPO	*C. pilulifera* V-BrPO	*P. capitatus* FeHeme-BrPO
Asx	14.0	12.9	11.4	12.8
Thr	6.9	6.4	4.9	7.1
Ser	5.0	5.8	6.7	5.4
Glx	10.2	9.1	10.2	11.9
Pro	4.9	5.2	5.3	0.5
Gly	9.8	8.9	7.8	4.1
Ala	11.1	8.0	10.0	6.0
Val	6.2	5.5	6.5	8.3
Met	1.0	2.0	1.4	0.7
Ile	4.0	2.7	5.9	6.3
Leu	8.5	9.4	9.2	10.1
Tyr	1.7	3.1	2.2	2.3
Phe	5.7	3.5	5.7	9.9
Lys	1.6	3.7	4.3	1.1
His	1.2	1.7	1.4	1.2
Arg	4.0	4.7	1.4	7.1
Cys	1.7	5.5	5.1	4.6
Trp	nd	nd	0.8	nd
ref	63	63	63	27

Isozymes of vanadium bromoperoxidases from *A. nodosum* have been isolated which differ in carbohydrate content.[17,37] The more abundant bromoperoxidase based on isolated yield, V-BrPO-I, was found in the thallus and the other bromoperoxidase, V-BrPO-II, was reported to be present on the thallus surface.[37] A previous study reported that V-BrPO is present in two different locations of *A. nodosum*, one in the cell walls of the transitional region between the cortex and medulla of the thallus and the other in the cell wall of the thallus surface.[40] More recent experiments demonstrate that vanadium-dependent bromoperoxidase activity is present in both the cortical and surface protoplasts of *Macrocystis pyrifera*,[41] *Laminaria saccharina*, and *Laminaria digitata*.[42] The biosynthesis of V-BrPO in the protoplasts of *Laminaria saccharina* has been shown using [^{35}S]methionine.[42]

1. Isolation and Purification of V-BrPO

Isolation and purification of V-BrPO from *A. nodosum* begins by homogenization of the macroalga in an equal volume of 0.2 M Tris-sulfate (pH 8.3), followed by centrifugation.[17,21,69] The pellet is collected and reextracted until the enzymatic activity of the reextracted pellet has decreased significantly. The extracts (typically 20–60 L for 1–4 kg of algae) are then pooled and the alginic acid precipitated by making the solution 0.1 M in calcium chloride. The calcium alginate is conveniently removed by centrifugation using a continuous flow centrifuge; the supernatant volume is then reduced in a hollow fiber concentrator prior to protein precipitation by 80% ammonium sulfate. The ammonium sulfate precipitate is then collected and dialyzed against 0.1 M Tris-sulfate (pH 8.3) before batch loading onto an anion exchange column (e.g., DEAE). Active fractions from the anion exchange column are pooled and subjected to a series of gel filtration columns (e.g., sepharose Cl-6B, sephacryl S200). The activity and purity of the enzyme are monitored throughout the course of the purification procedure by the MCD assay and SDS-PAG electrophoresis, respectively. An improved isolation procedure based primarily on a two-phase extraction system (i.e., poly(ethylene glycol) and aqueous potassium carbonate) has been described for V-BrPO from *Laminaria digitata* and *Laminaria saccharina*.[23,43] This procedure, however, is not effective in the isolation of V-BrPO from *A. nodosum*, which has been the principal source of V-BrPO for the mechanistic studies that have been reported to date.

2. The Vanadium Site

The resting oxidation of vanadium in V-BrPO is vanadium(V). From extended X-ray absorption fine structure (EXAFS) analysis, the vanadium site is believed to be a distorted octahedron coordinated by

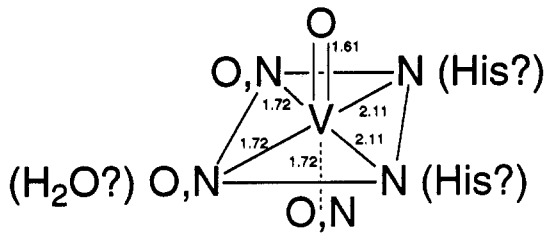


Figure 3. The proposed active site of V-BrPO from EXAFS.[44]

a single terminal oxide ligand at 1.61 Å, three unknown light-atom donors ca. 1.72 Å, and two nitrogen donors at 2.11 Å (possibly histidine nitrogens[44]) (Figure 3). The EXAFS spectrum of V-BrPO is independent of the presence of H_2O_2 and Br^-. V-BrPO can be reduced to VO^{2+}-BrPO as shown by the appearance of an ESR signal.[44] The EXAFS spectrum of the reduced enzyme is consistent with formation of vanadyl–BrPO in which the V(V) site is coordinated by a single terminal oxide at 1.63 Å, and 5 O(N) ligands, of which three are at 1.91 Å and two are at 2.11 Å[44] (Figure 3). Electron spin echo results of the V^{IV}-BrPO derivative indicates that a nitrogen ligand is in the equatorial plane.[45] V-BrPO is ESR silent under turnover conditions (i.e., in the presence of Br^- and H_2O_2).

The standard procedure for removal of active-site vanadium(V) has been to incubate V-BrPO in 0.1 M phosphate–citrate buffer pH 3.8, containing 10 mM EDTA. These conditions remove over 95% of the vanadium, producing the inactive apo-BrPO derivative.[20,46] The essential component of the apoprotein preparation is the phosphate, without which vanadium is not completely removed and the enzyme is not completely inactivated.[47] In fact phosphate in the absence of EDTA is sufficient for preparation of apo-BrPO;[47] inactivation by phosphate is much faster at low pH (pH $\approx$ 4) than at neutral or higher pH's. Interestingly, phosphate inactivation does not occur in the presence of hydrogen peroxide;[47] this result suggests that the mechanism of vanadium(V) release is through a phosphate–vanadate complex of which the formation would be inhibited by peroxide coordination to V(V).

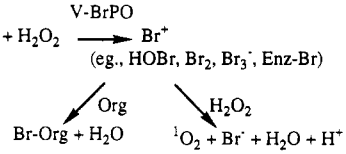
The activity of the apoderivative can be fully restored by addition of vanadate.[46] Addition of other metal ions did not restore bromoperoxidase activity.[20] Vanadium(V) is only fully incorporated in the absence of phosphate.[46,47] Like phosphate, molybdate, arsenate, tetrafluoroaluminate (AlF_4^-), and tetrafluoroberrylate (BeF_4^{2-}) are also reported to be competitive inhibitors of the coordination of vanadium(V) to apo-BrPO.[48]

B. Reactivity of V-BrPO

1. Halogenation and Halide-Assisted Disproportionation of Hydrogen Peroxide

V-BrPO catalyzes peroxidative halogenation reactions[20,22,49] and the halide-assisted disproportionation of hydrogen peroxide.[50] In the first step, the enzyme catalyzes the oxidation of the halide by hydrogen peroxide producing an intermediate which is a two-electron-oxidized halogen species; for bromide hypobromous acid, bromine, tribromide, or an enzyme-bound "bromonium ion equivalent" are all consistent with the *in vitro* reactivity of the enzyme. In the second step, the oxidized intermediate can halogenate appropriate organic substrates or react with another equivalent of hydrogen peroxide, forming dioxygen, as depicted in Scheme I for bromide.[50,51]

Scheme I. Summary of the General Reactivity of V-BrPO with Bromide



In the case of bromide, the dioxygen formed is in the singlet excited state ($^1O_2$,$^1\Delta_g$) which was identified spectroscopically by the near-infrared emission characteristics.[17] Singlet oxygen ($^1\Delta_g$) is a well-established product of the oxidation of H_2O_2 by HOBr, HOCl,[55] and bromamines.[55] Recent $H_2{}^{18}O_2$ tracer studies now establish that each atom of oxygen in O_2 originates from the same molecule of H_2O_2 which is also consistent with singlet oxygen production.[24]

The rate of dioxygen formation in the absence of an organic substrate and the rate of bromination of monochlorodimedone (MCD; at >75 μM) are the same, which supports the mechanism in Scheme I; the reactive intermediate is common to both pathways and is formed in a rate-limiting step.[50] Moreover k_1[MCD] is competitive with $k_2[H_2O_2]$, because the sum of k_1[MCD] and $k_2[H_2O_2]$ in the presence of MCD is equal to $k_2[H_2O_2]$ in the absence of an organic halogen acceptor.[24]

V-BrPO also catalyzes peroxidative chlorination reactions and the chloride-assisted disproportionation of hydrogen peroxide.[53] The chloroperoxidase reactivity of V-BrPO was not recognized originally,[54] presumably due to the much slower rate of chloride oxidation than bromide oxidation catalyzed by V-BrPO. Like the enzyme-catalyzed reactions with bromide, the rates of chlorination of MCD and the chloride-assisted dioxygen formation reaction are equivalent, *except* in the presence of amines (primary, secondary, and tertiary).[53] The rate of dioxygen formation is much slower in the presence of amines due to the formation of chloramines which can be observed spectrophotometrically by their characteristic UV absorption maxima:

$$NH_2R + Cl^- + H_2O_2 + H^+ \rightarrow NHRCl + 2H_2O$$

Chloramine formation is observed because chloramines do not readily oxidize hydrogen peroxide. Identical rates of MCD chlorination in the presence and absence of amine are observed[53] (Table III), but because chloramines chlorinate very rapidly, it cannot be determined whether MCD chlorination proceeds through a chloramine intermediate.

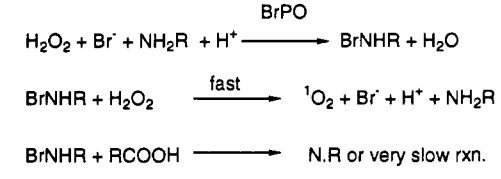
One might also ask if bromamines can be observed in the bromoperoxidase reaction. Bromamine formation would not be observed using hydrogen peroxide because bromamines are rapidly reduced by hydrogen

Table III. Comparison of the Rates of MCD Chlorination and Cl^--Assisted Dioxygen Formation in the Presence and Absence of an Amine

[taurine], mM	$-d[MCD]/dt$, μM/min	$d[O_2]/dt$, μM/min
0	4.62 (0.07)	4.61 (0.01)
1	4.62 (0.07)	1.31 (0.09)

peroxide, forming singlet oxygen and bromide.[55] By replacing hydrogen peroxide with acyl peroxides (e.g., peracetic acid and others; see below), bromamine formation can be detected in the absence of organic substrates. Because acyl peroxides are not efficient two-electron reductants, the Br^+NHR^- species can build up in solution[22] (Scheme II). Bromamines have been

Scheme II. Summary of the Reactivity of Bromamines with Hydrogen Peroxide and Acyl Peroxides



proposed as possible intermediates in other haloperoxidase reactions.[5,55]

2. Specific Activities

The specific chloroperoxidase, bromoperoxidase, and iodoperoxidase activities differ substantially and depend on pH, halide concentration and hydrogen peroxide concentrations. The pH for maximum specific activity for bromination of MCD by V-BrPO is generally higher than that for chlorination, but direct comparisons are difficult because the pH maximum can be shifted over several pH units by varying the ratio of halide to hydrogen peroxide and because both of these substrates can also act as inhibitors. The specific activity of MCD bromination for V-BrPO isolated from *A. nodosum* is 170 units/mg (at pH 6.5, 2 mM H_2O_2, 0.1 M Br^-, 50 μM MCD, 0.2 M Na_2SO_4). The specific activities for the *M pyrifera* and *F. distichus* enzymes are 1730 units/mg (pH 6, other conditions same as above) and 1580 units/mg (pH 6.5, other conditions same as above), respectively;[22] the reasons for the significantly higher specific activities of these enzymes compared to V-BrPO from *A. nodosum* is not yet known. The specific chloroperoxidase activity is 0.76 unit/mg (under conditions of 1 M KCl, 2 mM H_2O_2, 50 μM MCD in 0.1 M citrate buffer, pH 4.5) which is ca. 200 times less than the maximum specific bromoperoxidase activity.[53]

3. Enzyme Kinetics

Detailed steady-state analyses of the rate of MCD bromination[51,56] and dioxygen formation[51,57] catalyzed by V-BrPO from *A. nodosum*, *M. pyrifera*, and *F. distichus* fit a substrate-inhibited bi-bi ping-pong kinetic mechanism, in which the substrates bromide and hydrogen peroxide are also noncompetitive inhibitors at certain pH's (see Table IV).[51,24] The kinetic parameters (K_m^{Br}, $K_m^{H_2O_2}$, K_{ii}^{Br}, K_{is}^{Br}) obtained in the dioxygen formation reaction and the MCD bromination reaction agree within a factor of ca. 2, providing further evidence that the rate-limiting steps are the same in both the bromination of MCD and the bromide-assisted disproportionation of hydrogen peroxide reactions.[51] Bromide inhibition is strongest at pH 5–5.5 for V-BrPO from the three sources examined.[51,57] The inhibition constants for hydrogen peroxide ($K_{ii}^{H_2O_2}$, $K_{is}^{H_2O_2}$) could only be determined for the bromide-assisted dioxygen formation reaction since high concentrations of hydrogen peroxide were required. Under these conditions the kinetics of MCD bromination were complicated by the competing dioxygen formation reaction. Hydrogen peroxide inhibition is strongest at pH 7–8. H_2O_2 inhibition is noncompetitive at all pH's and $K_{ii}^{H_2O_2}$ is equal to $K_{is}^{H_2O_2}$, showing that bromide does not affect the inhibition by H_2O_2.[24] Fluoride is a competitive inhibitor of H_2O_2 binding in both the MCD bromination reaction and the bromide-assisted disproportionation of hydrogen peroxide.[51] Fluoride inhibition (K_i^F) is uncompetitive with respect to bromide.[51]

Table IV. Summary of Kinetic Constants for V-BrPO (*A. nodosum*)

		pH	ref
$K_m^{H_2O_2}$	284 μM	5.25	51
K_m^{Br}	10.5 mM	5.25	51
K_{ii}^{Br}	332 mM	4.55	51
K_{is}^{Br}	730 mM	4.55	51
$K_{ii}^{H_2O_2}$	63 μM	7.0	24
$K_{is}^{H_2O_2}$	67 μM	7.0	24
K_i^F	1.11 mM	6.5	51

The steady-state kinetic studies also showed that an active-site residue with a pK_a of ca. 5.7 is required to be deprotonated prior to binding of hydrogen peroxide.[56] The group was attributed to histidine or possibly a bound water molecule.

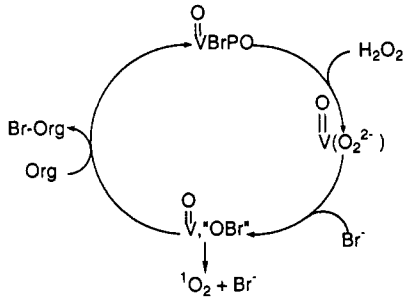
4. Peroxide Selectivity

The catalytic activity of V-BrPO is supported by peracids (i.e., peracetic acid, *m*-chloroperoxybenzoic acid, *p*-nitroperoxybenzoic acid, and phenylperacetic acid) to catalyze the bromination of organic substrates.[22] Unlike the reactions using hydrogen peroxide, the uncatalyzed rate of bromide oxidation by peracetic acid at pH 6.5, i.e., the pH range used for V-BrPO studies, is appreciable, however, the V-BrPO-catalyzed reactions are much faster, particularly in the higher pH range.[22] Dioxygen is not formed from peracids because peracids do not readily reduce the oxidized bromine species (e.g., HOBr, Br_2, Br_3^-, BrNHR) in the time frame of the enzymatic reactions (i.e., several minutes) (see Table III). It has been established that peracid reactivity actually arises from direct use of peracid and not from hydrogen peroxide which could be formed by the hydrolysis of the peracids.[22] Alkyl hydroperoxides, including ethyl hydroperoxide, cuminyl hydroperoxide, and *tert*-butyl hydroperoxide are not used by V-BrPO to catalyze bromination reactions.[22]

5. Mechanistic Considerations

Spectrophotometric analysis of the V-BrPO reaction has provided evidence that hydrogen peroxide binds first to V-BrPO, most likely by coordination to the vanadium.[58] Tromp et al. have reported a small absorbance decrease (0.03 absorbance units for 20 μM V-BrPO) between 300 and 340 nm upon addition of H_2O_2 to V-BrPO;[58] the original UV spectrum reappears upon addition of bromide, i.e., turnover conditions. In a different set of experiments, we have established that stoichiometric bromination of MCD does not occur by addition of excess bromide to relatively high concentrations of V-BrPO (i.e., 20 μM) and 50 μM MCD. Thus V-BrPO does not oxidize bromide in the absence of a peroxide source (hydrogen peroxide or an acyl peroxide).[24] Taken together, these two results indicate that hydrogen peroxide coordinates first, followed by bro-

Scheme III. Catalytic Cycle for V-BrPO Showing Coordination of Hydrogen Peroxide before Oxidation of Bromide[a]



[a] The intermediate, $V^{V}O$, "OBr", is not meant to indicate whether the oxidized bromine species is enzyme bound or released.

mide oxidation (Scheme III). One can envisage several mechanisms of halide oxidation by the peroxovanadium(V) species. The halide could coordinate to vanadium before oxidation or the halide could attack bound peroxide.

The detailed role of the active-site vanadium ion, i.e., whether it functions as an electron-transfer catalyst of bromide oxidation or a Lewis acid catalyst (and remains in the 5^+ oxidation state), is not known. There is considerable precedence for both types of reactions, as in the two-electron reduction of vanadium(V) tetraglyme by bromide (electron-transfer catalysis)[59] and in the oxygenation of organic substrates by oxygen atom transfer from vanadium(V) peroxides (Lewis acid catalysis).[60] The vanadyl-BrPO state is probably not an important component because VO^{2+}-BrPO does not have bromoperoxidase activity and because an EPR signal is not observed during turnover conditions. Most other peroxidases are FeHeme-containing systems, which function as two-electron redox catalysts (see below). Hydrogen peroxide oxidizes the FeHeme moiety by two electrons, forming compound I.[61] Compound I oxidizes the halide ion, forming the active halogenating species. This mechanism cannot be operative in V-BrPO because the vanadium is already in its highest accessible oxidation state. Moreover, native V-BrPO (protein-V^{V}=O), which might be viewed analogous to compound I (Heme$^+$Fe^{IV}=O) does not oxidize bromide directly. ^{51}V NMR studies of functional model compounds show that a long-lived reduced oxidation state is not observed.[62]

C. Stability of V-BrPO

V-BrPO has been shown to be an exceptionally stable enzyme which is not inactivated by high concentrations of singlet oxygen or oxidized bromine species.[17] V-BrPO is capable of turning over multiple aliquots of hydrogen peroxide in the bromide-assisted catalase reaction producing stoichiometric yields of singlet oxygen ($^1O_2$,$^1\Delta_g$) without inactivation (Table V). By contrast the FeHeme haloperoxidase enzymes, lactoperoxidase and fungal chloroperoxidase, are inactivated in the halide-assisted disproportionation of hydrogen peroxide (Table V). The inactivation of the FeHeme haloperoxidases is due to the species produced during turnover (i.e., $^1O_2$, HOBr/Br_2/Br_3^-) primarily although a small amount of inactivation occurs by hydrogen peroxide in the absence of halide.[17]

V-BrPO also has appreciable thermal stability and stability in many organic solvents. For example V-BrPO does not lose activity when stored at room temperature for a month in 60% (v/v) acetone, methanol, or ethanol or 40% (v/v) 1-propanol.[63] V-BrPO also retains activity after immobilization on solid support media, e.g., photocross-linked to DEAE cellulofine.[64]

Table V. Stability of Haloperoxidases on Turnover of Second Aliquot of Hydrogen Peroxide in the Bromide-Assisted Disproportionation of Hydrogen Peroxide Producing Singlet Oxygen[17]

enzyme	control 1 1st aliquot H_2O_2	$^1O_2$ yield from 2nd aliquot H_2O_2
V-BrPO	1.00 (0.01)	1.07 (0.01)
FeHeme-LactoPO	1.00 (0.05)	0.00 (0.01)
FeHeme-ClPO	1.00 (0.05)	0.47 (0.02)

D. Comment on the Putative Non-Heme Iron Bromoperoxidase

A non-heme iron bromoperoxidase was reported to be isolated from certain species of Rhodophyta.[65,66] An unusual characteristic was the low specific activity which has now been shown to be due to a small amount of vanadium contained in these enzymes[15,16] despite the presence of excess iron and magnesium. In these enzymes, the metal ions can be completely removed and the apoenzyme can be fully reconstituted by addition of vanadate.[15,17] The original enzyme isolation procedure was carried out in phosphate buffer.[65] Given what is now known about phosphate displacement of bound vanadium(V),[47] the low specific activity of the *Corallina* enzymes can be understood in terms of phosphate displacement of endogenous vanadium(V).

III. FeHeme Bromoperoxidase

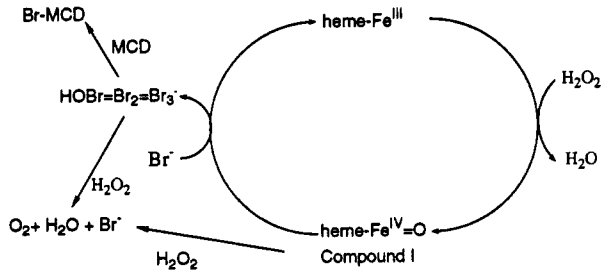
A. Characteristics of FeHeme Bromoperoxidase

FeHeme bromoperoxidase (FeHeme-BrPO) was isolated from several marine sources (see Table I) in the early 1980s by Baden and Corbett,[28] Ahern,[26] and Manthey and Hager,[27,36,61] and more recently from a marine snail[30] and worm[29] (see below). The most thoroughly characterized of these enzymes is the one isolated from the green alga, *Penicillus capitatus*.[27,36,61] FeHeme-BrPO is a dimeric protein (MW 97 600 determined by equilibrium ultracentrifugation) comprised of two identical subunits (55 000 determined from SDS PAG electrophoresis) each containing a tightly bound ferriprotoporphyrin IX moiety.[27] The FeHeme moiety is present in a high-spin electronic configuration.[61] The amino acid composition of FeHeme-BrPO shows that it is rich in the acidic residues (Table II), as was also observed for V-BrPO and other peroxidases.[27]

B. Reactivity of FeHeme Bromoperoxidase

FeHeme-BrPO catalyzes peroxidative halogenation reactions, halide-assisted disproportionation of hydrogen peroxide, direct catalase activity, and free-radical peroxidation reactions.[27] In the peroxidative halogenation reactions, the FeHeme moiety functions by cycling between native enzyme (Fe^{III}Heme) and compound I (Heme$^+$-Fe^{IV}=O; the two-electron oxidized state above the native enzyme) as shown in Scheme IV. Compound I oxidizes a halide (I^-, Br^-, or Cl^-[36]) by two

Scheme IV. Summary of the General Reactivity of FeHeme-BrPO with Bromide



electrons, which in the case of Br^- forms an equilibrium mixture of HOBr, Br_2, and Br_3^-. Kinetic studies show that the rate of Br_3^- formation catalyzed by FeHeme-BrPO is twice as fast as the rate of MCD bromination[36] which indicates that the active brominating species is not enzyme bound, but rather released into solution, as depicted in Scheme IV.

Saturation kinetics were not observed for bromide which was interpreted by the authors as indicative of the absence of a specific bromide binding site or one with a very low affinity.[36] This kinetic behavior differs from V-BrPO in which bromide is also an inhibitor; thus both bromide saturation rate behavior and bromide inhibition are observed.[51,56] The steady-state kinetics of MCD bromination catalyzed by FeHeme-BrPO as a function of pH show that protonation of an active site residue with a pK_a of 6.4 and deprotonation of another residue with a pK_a of 5.3 are required for optimal activity. Fluoride is a partial inhibitor of FeHeme-BrPO.[36]

Initially it was thought that FeHeme-BrPO could not catalyze peroxidative chlorination reactions. However, peroxidative chlorination and the chloride-assisted disproportionation of hydrogen peroxide is observed[36] but in a low pH regime where FeHeme-BrPO does not catalyze bromide oxidation.[27] Chlorination was established by isolation of dichlorodimedone. The nature of the oxidized chlorine intermediate, as enzyme-bound or released, has not been established.[36]

FeHeme-BrPO also catalyzes the direct disproportionation of hydrogen peroxide, forming dioxygen (i.e., direct catalase activity),[27] although the overall activity is only 1% of that of catalase. Like V-BrPO, halide-assisted catalase activity is observed in the presence of bromide[27] and chloride.[36] Alkyl peroxides and acyl peroxides can be used in place of hydrogen peroxide to support halogenation reactions;[27] dioxygen formation is not observed with these peroxides contrary to the reactivity of fungal FeHeme CIPO.[27] In fact ethyl hydroperoxide, peracetic acid, and *m*-chloroperoxybenzoic acid have been used to generate stable solutions of the compound I state.[61]

In addition to haloperoxidase activity, FeHeme-BrPO catalyzes classical peroxidase reactions, such as the single-electron oxidation of phenolic compounds (pyrogallol, guaiacol, and hydroquinone),[27] *o*-dianisidine,[27] and MCD[61] in the absence of halide, contrary to the reactivity of V-BrPO which does not carry out these reactions. In these reactions, compound I is reduced by one electron to compound II (Heme-Fe^{IV}=O) and the organic radical. Compound III (the oxyferrous or ferric superoxide states) is also observed by reaction of compound II with excess hydrogen peroxide.[27,61]

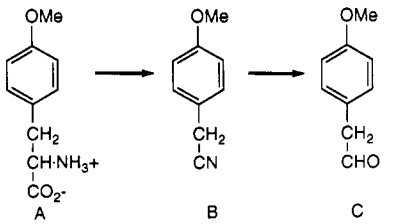


Figure 4. Oxidation of *p*-methoxytoluene (A), to *p*-methoxyphenylacetonitrile (B), and *p*-methoxyphenylacetaldehyde (C).

The order of addition of reactants in the MCD bromination reaction was found to affect the overall reaction profile.[61] Initiating the reaction with bromide showed linear steady-state MCD bromination assays over time. Initiation of the reaction with hydrogen peroxide showed a fast initial rate followed by a slower steady state rate equal to that of the bromide-initiated reactions.[61] The slower rate was shown to be due to the formation of the inactive compound III (Heme-Fe^{II}-O_2 or Heme-Fe^{III}-O_2^-). Compound II (Heme-Fe^{IV}=O) is formed first by the one-electron oxidation of MCD by compound I; compound III is then formed by reduction of compound II with excess hydrogen peroxide.[61] Compound III can be reduced to native enzyme.[61]

1. Biosynthetic Investigations

Hager has investigated biosynthetic origins of halohydrocarbon formation.[67] Crude extracts of *P. capitatus* as well as the purified FeHeme-BrPO from this alga catalyze the formation of 1-bromo-2-heptanone, 1,1-dibromo-2-heptanone, and 1,1,1-tribromo-2-heptanone from 3-oxooctanoic acid, hydrogen peroxide, and bromide. 1,1,1-Tribromo-2-heptanone then hydrolyzes in a nonenzymatic reaction, producing bromoform. Halogenated ketones have been isolated from numerous species of algae (for a review see ref 4). Bromomethane and dibromomethane, which are found in the red alga *Bonnemaisonia hamerifera*, are not produced by crude extracts of *P. capitatus* acting on 3-oxooctanoic acid.

FeHeme-BrPO from *P. capitatus* has been shown to catalyze the conversion of α-amino acids and peptides to the decarboxylated nitriles and aldehydes.[5] The conditions required are those of the normal enzyme activity, i.e., bromide, hydrogen peroxide, and α-amino acid. This reaction was discovered by presence of endogenous isobutyronitrile in crude bromoperoxidase preparations. Nieder and Hager showed that isobutyronitrile could arise from valine via the formation of the *N*-bromo and *N,N*-dibromo amino acid derivatives.[5] *p*-Methoxytoluene (A), is also converted to *p*-methoxyphenylacetonitrile (B), and *p*-methoxyphenylacetaldehyde (C) (Figure 4). Such reactivity may form the basis of the biogenesis of aeroplysinin-1 (see 1 in Figure 1), a brominated compound isolated from certain marine sponges.

2. Other FeHeme Haloperoxidases

Recently a new FeHeme chloroperoxidase was isolated from the marine worm, *Notomastus lobatus*.[29] This protein (MW 174 000) is comprised of four flavoprotein subunits (MW 30 000), tentatively identified as containing FAD, two FeHeme subunits (MW 15 500) and two other FeHeme subunits (MW 11 500). All components are required for activity, since neither

the FeHeme or flavoproteins have chloroperoxidase activity alone.

IV. Future Directions

One of the most interesting, yet unsolved problems in the area of marine halogenation, is the biogenesis of the chiral halogenated marine natural products. An attractive feature of an enzyme-bound haloamine (chloroamine or bromoamine), or other enzyme-bound halogenated moiety, would be the possible generation of chiral halogenated compounds, through direct halogenation by an enzyme-bound halonium ion. One factor affecting the stability of haloamines is the presence of excess halide. For example bromonium ions are readily displaced by bromide forming bromine or tribromide, particularly at lower pH. The critical factor would be the reactivity of the putative enzyme-bound halogenated intermediate toward halogenation, hydrolysis, or displacement by excess halide. Another possibility to account for chiral halogenation via HOBr (or equivalent) would be if the precursor substrate was bound by the haloperoxidase or another enzyme such that only one face of the substrate could be halogenated.

Previously proposed mechanisms of the biosynthesis of certain chlorinated compounds have invoked electrophilic bromination of alkenes followed by passive chloride attack.[32] While this mechanism could explain the origin of adjacent brominated and chlorinated carbons, it does not readily account for chlorine-only containing compounds. Thus, with the discovery of chloroperoxidase activity of the vanadium[53] and Fe-Heme[27] enzymes, the origin of specific chlorinated marine natural products can now be addressed.

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