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Mechanistic considerations of the vanadium haloperoxidases

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Abstract

Haloperoxidases are enzymes which catalyze the oxidation of halide ions (i.e. chloride, bromide and iodide) by hydrogen peroxide. These enzymes usually contain the FeHeme moiety or vanadium as an essential constituent at their active site, however, a few haloperoxidases which lack a metal cofactor are known. This review will examine (1) the reactivity of the vanadium haloperoxidases, particularly the mechanism of halide oxidation by hydrogen peroxide, and the mechanism of halogenation and sulfoxidation, including the newly reported regioselectivity and enantioselectivity of the vanadium haloperoxidases; (2) the X-ray structure of vanadium chloroperoxidase, the vanadium(V) active site and the role of critical amino acid side chains for catalysis and (3) functional biomimetic systems, with specific relevance to the mechanism of the vanadium haloperoxidase enzymes.

Keywords: Vanadium haloperoxidases; Enzymes; Halide ions

1. Introduction and scope

Biological systems have evolved haloperoxidase enzymes to catalyze the oxidation of chloride, bromide and iodide by hydrogen peroxide. The majority of these enzymes in terrestrial systems contain the FeHeme moiety, including chloroperoxidase from the fungus *Caldariomyces fumago*, and myeloperoxidase, eosinophil peroxidase and lactoperoxidase from mammalian systems [1]. Other haloperoxidases which do not contain metal ions are also known, such as the chloroperoxidase in *Pseudomonas pyrrocina* [2]. In marine algal systems, the vanadium-containing haloperoxidases predominate [3–6], although a few examples of FeHeme haloperoxidases in marine algae in are known [8]. The vanadium haloperoxidases have also been isolated from some terrestrial fungi [7].

The production of halogenated natural products is widespread in marine organisms [9,10]. These compounds range from halogenated indoles (Fig. 1), terpenes (Fig. 1), acetogenins, phenols, etc., which often have important biological activities

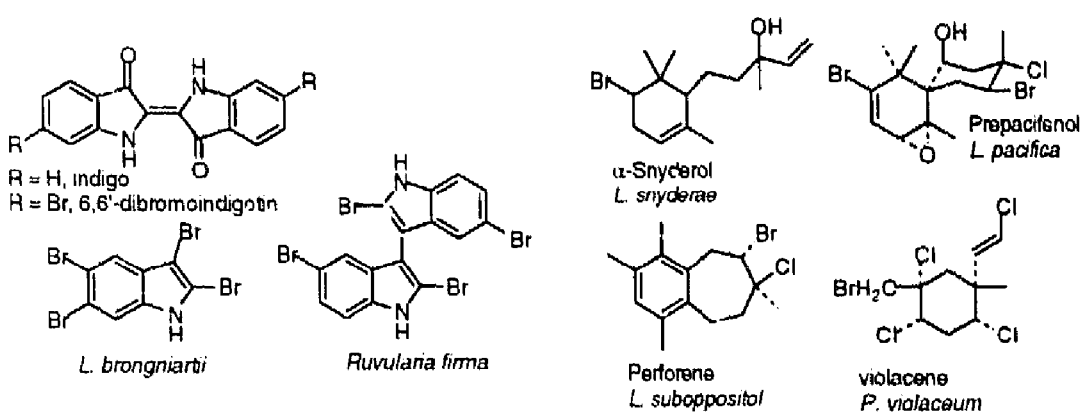


Fig. 1. Examples of halogenated indole and terpene natural products.

(e.g. antimicrobial properties, feeding deterrents, etc.) or pharmacological properties to volatile halogenated hydrocarbons (e.g. bromoform, chloroform, etc) which are produced on a very large scale.

The function of the marine haloperoxidases is thought to be in the biosynthesis of these natural products. On the other hand, halogenated natural products have not been isolated from the fungi (e.g. *Curvularia inaequalis* and other dematiaceous hyphomycetes) which produce vanadium chloroperoxidase (V–ClPO). Instead the function of the fungal V–ClPO is proposed to be in the degradation of the plant host cell wall through production of hypochlorous acid (HOCl). The reactivity differences between the fungal V–ClPO and the marine algal vanadium bromoperoxidase (V–BrPO) may reflect variations in their biological functions.

Several reviews of the vanadium haloperoxidase enzymes have appeared recently [1–6,11,12]. The emphasis of this review will be (1) to examine the reactivity of the vanadium haloperoxidases, particularly the mechanism of halide oxidation by hydrogen peroxide, and the mechanism of halogenation and sulfoxidation, including the newly reported regio- and enantioselectivity of the vanadium haloperoxidases; (2) to examine the X-ray structure of vanadium chloroperoxidase, the vanadium(V) active site and the role of critical amino acid side chains for catalysis and (3) to examine functional biomimetic systems, with specific relevance to the mechanism of the vanadium haloperoxidase enzymes. Recently the sequence of V–ClPO (*C. inaequalis*) was found to be similar to the sequence of the acid phosphatases [13–15]. In fact, the apo form of V–ClPO exhibits phosphatase activity [13]. The phosphatase activity of V–ClPO will not be reviewed here, however an interesting result of a phosphatase to which vanadium(V) was added will be presented in the section on sulfoxidation (Section 2.4).

2. Reactivity of the vanadium haloperoxidases

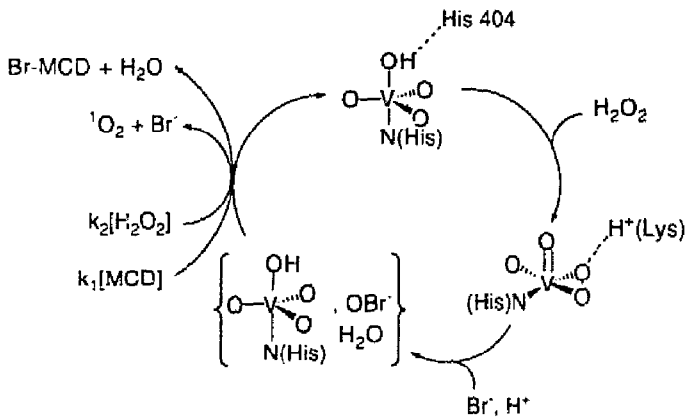
2.1. Halogenation and dioxygen formation

Vanadium haloperoxidases (V–HPO) catalyze halogenation reactions (Reaction (1)) and the indirect disproportionation of hydrogen peroxide in a reaction that requires the presence of a halide ion (Reaction (2)) [16,17]. The stoichiometry of the halogenation reaction shows that one proton is consumed per equivalent of halogenated product produced.

$$X^- + H_2O_2 + R{-}H + H^+ \rightarrow R{-}X + 2H_2O \quad (1)$$

$$X^- + 2H_2O_2 \rightarrow O_2 + 2H_2O + X^- \quad (2)$$

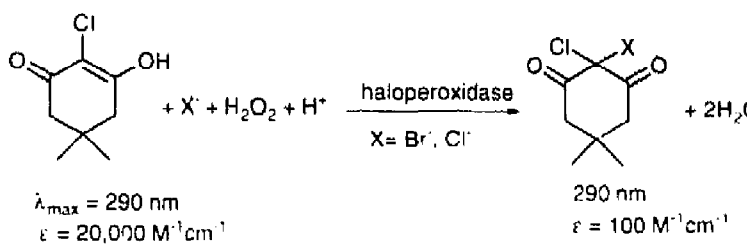
Both reactions proceed through an oxidized halogen intermediate that is two-electrons oxidized above the halide oxidation state [18]. The exact speciation of the oxidized intermediate is still not known, although for bromide, it is the equivalent of hypobromous acid, bromine, tribromide, or an enzyme-bound bromonium ion-type species. The actual speciation may vary considerably, depending on the



Scheme 1.

conditions (e.g. pH, substrate concentrations, etc.) and the nature of the organic substrate, as described more fully below.

The classic organic substrate used to evaluate and compare haloperoxidases from different sources is monochlorodimedone (2-chloro-5,5-dimethyl-1,3-dimedone; MCD: Reaction (3)).



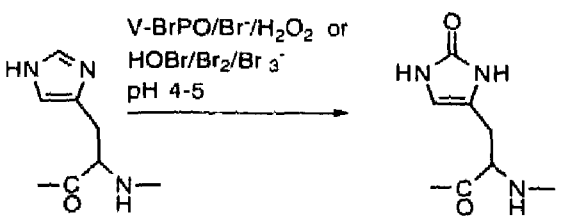
(3)

MCD has been used to investigate the enzyme kinetic mechanism of the vanadium haloperoxidases [17–20]. The kinetics of dioxygen formation has also been used to determine the kinetic parameters of V–BrPO [17,21]. The kinetic parameters, K_m^{Br}, $K_m^{H_2O_2}$, K_{ii}^{Br}, K_{is}^{Br}, determined for both the bromination of MCD and the bromide-assisted disproportionation of hydrogen peroxide are in agreement with each other [16,17], suggesting that both pathways proceed via a common intermediate, whose production is rate limiting. A catalytic cycle consistent with the enzyme-kinetic results and Reactions (1) and (2) is shown in Scheme 1. For MCD bromination, the k_1[MCD] pathway is competitive with the k_2[H_2O_2] pathway [16,17], however for other organic substrates, this may not necessarily be the case (see below). Competitive dioxygen formation is strongly enhanced at higher pH [18].

This scheme is also consistent with singlet oxygen ($^1O_2$; $^1\Delta_g$) formation in the halide-assisted disproportionation of hydrogen peroxide (i.e. Reaction (2) and Scheme 1) and electrophilic bromination catalyzed by V–BrPO (i.e. Reaction 1 and Scheme 1) [18]. Singlet oxygen was identified by direct spectral detection in the near IR [22] and mass spectral results showing that both oxygen atoms in singlet oxygen originate from the same molecule of hydrogen peroxide [13].

2.1.1. The effect of hydrogen peroxide concentration on enzyme activity

Hydrogen peroxide is a substrate for vanadium haloperoxidases, however it can also inhibit or inactivate V–BrPO under certain conditions [17,18]. Hydrogen peroxide is a fully reversible, noncompetitive inhibitor of V–BrPO when the steady state kinetics are observed during the initial portion of the reaction [18]. If the reaction proceeds for longer times, the specific activity of V–BrPO decreases. The inactivation that occurs on prolonged turnover at ca. neutral pH is the result of vanadate loss from V–BrPO. The activity can be fully restored by the addition of vanadate [18]. At lower pH (e.g. pH 4 and 5), however, inactivation also occurs under turnover which is again accompanied by the release of vanadium, but this inactivation is not restored by the addition of vanadate. The irreversible inactivation of V–BrPO was accompanied by the formation of 2-oxohistidine [23]. Inactivation of V–BrPO and formation of 2-oxohistidine require all the components of turnover (i.e. bromide, hydrogen peroxide and V–BrPO) in addition to low pH (Reaction (4)).



(4)

The inactivation of V–BrPO and the formation of 2-oxohistidine are not the result of oxidation by singlet oxygen produced by V–BrPO since neither inactivation nor 2-oxohistidine formation occur under conditions in which V–BrPO produces singlet oxygen quantitatively [23]. Singlet oxygen is only produced quantitatively at near neutral pH or higher, yet 2-oxohistidine only forms at or below pH 5. Aqueous bromine has also been shown to oxidize imidazole and histidine compounds to the 2-oxo derivative at pH $\leq$ 5, which is consistent with oxidation of the enzyme's histidines by the oxidized bromine intermediate generated during turnover [23]

2.2. Nature of the oxidized halogen intermediate and selectivity for organic substrates

It has not been possible to detect the enzyme intermediate directly because its reaction with organic substrates or with excess H_2O_2 to produce O_2 is faster than its formation, preventing its build up in solution. The nature of the oxidized intermediate as enzyme-bound or released may actually depend on the nature of the organic substrate and whether it binds to V–BrPO. This conclusion is the result of competitive kinetic studies, comparing the bromination of a variety of organic substrates by the V–BrPO H_2O_2 KBr system to that of bromination by 'aqueous bromine' (i.e. the equilibrium mixture of $HOBr = Br_2 = Br_3^-$) [24]. We have found that the kinetic profiles for the bromination of a variety of organic substrates, such

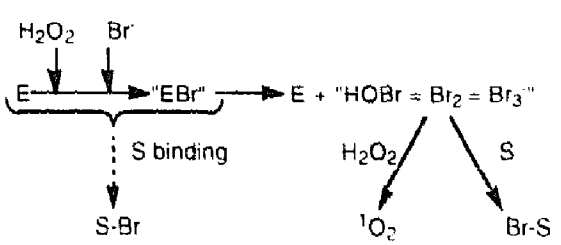
as indole and terpene derivatives, both of which are the precursors of many halogenated natural products, are consistent with bromination by an enzyme-trapped brominating intermediate [24]. Indole binding was confirmed by fluorescence quenching [24]: the binding constant of 2-phenylindole to V–BrPO (*A. nodosum*) was 1.1×10^5 M^{-1} determined from the Stern–Volmer analysis [25]. A mechanistic scheme involving substrate binding is shown in Scheme 2 [24].

V–BrPO binds H_2O_2 and Br^- leading to a putative 'enzyme-bound' or 'active-site trapped' brominating moiety, 'E–Br', which in the absence of an indole may release HOBr (or other bromine species, e.g. Br_2, Br_3^-). When indole is present, it binds to V–BrPO, preventing release of an oxidized bromine species and leading to more direct indole bromination.

2.2.1. The selectivity of V–BrPO

In addition to the competitive kinetic profiles and the fluorescence quenching described above, V–BrPO is also selective in its halogenation of organic substrates (e.g. indoles and terpenes [26]), which is consistent with direct enzyme halogenation by the enzyme. Of several indoles competed against equimolar MCD, e.g. 2-methylindole (2-MeI), 2-*tert*-butylindole, 2-phenylindole, 5-hydroxyindole, 3-methylindole, V-BrPO displayed its greatest selectivity with 2-MeI [26]. V–BrPO is also inhibited by other indoles or indole-like molecules. For example dioxygen formation in the V–BrPO-catalyzed bromide-assisted disproportionation of hydrogen peroxide (i.e. Reaction (2)) is inhibited by benzofuran and 2-*tert*-butylbenzofuran. Surprisingly, benzofuran inhibition is competitive with hydrogen peroxide [26].

Distinct sequential halogenation profiles are most pronounced for the competitive bromination of phenol red versus 2-MeI indole at lower pH values (e.g. pH 5.5–6.5; see Fig. 4 in reference [25]). At higher pH (e.g. > 7), the sharp delineation between the end of 2-MeI bromination and the beginning of phenol red bromination becomes more curved, although the general trend of a sequential process is still evident. These same trends have been observed with the competitive bromination and oxidation of luminol versus phenol red as a function of pH [27]. The basis of the pH dependence is not yet known, however, the increased basicity may decrease the affinity of V–BrPO for substrates; in addition the halide-assisted disproportionation of hydrogen peroxide becomes more favored at higher pH. In contrast to the V–BrPO-catalyzed reaction, the effect of pH on the reaction of aqueous bromine with phenol red and 2-MeI or phenol red and luminol is minimal [25].



Scheme 2. Reaction scheme showing substrate binding to V–BrPO.

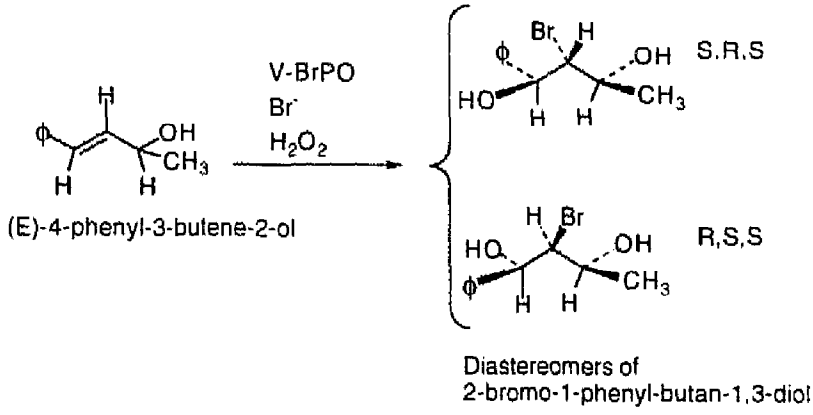


Fig. 2.

2.2.2. Regioselectivity

Many halogenated natural products in marine organisms contain chiral halogen–carbon centers. In fact, the biogenesis of these compounds is thought to involve the haloperoxidase enzymes. If haloperoxidases are involved, then one might expect the vanadium haloperoxidases to catalyze regioselective and stereoselective halogenation reactions. We have recently discovered regioselective bromination and oxidation of *tert*-butyl-indoles [28].

Diastereospecific bromohydrin formation from one cinnamyl substrate has also been reported [29]. In this paper, the authors examined the diastereomers produced on the bromination of (E)-4-phenyl-3-buten-2-ol catalyzed by V–BrPO, FeHeme ClPO (*C. fumago*) and *N*-bromoacetamide (NBA). Two products which are diastereomers of each other, i.e. S, R, S– (or R, S, R–) and R, S, S– (or S, R, R–) 2-bromo-1-phenyl-butan-1,3-diol (Fig. 2) were formed in each case, however the ratio of products differed between NBA and the two enzymes. When NBA effected bromohydrin formation the products were in a 75% 25% ratio of the S, R, S– (or R, S, R–) to R, S, S–(or S, R, R–) enantiomers. The corresponding ratio for V–BrPO was 66% to 34% (Fig. 2) and that for FeHeme ClPO was 61% to 39%. The authors have not reported further results or mechanistic proposals of this diastereoselectivity, however, the difference in the diastereospecific product formation of the enzymes versus NBA is consistent with an influence from the protein, such as substrate binding to the enzyme and bromination taking place in the active site.

2.3. Comparing the reactivity of V–BrPO to V–ClPO with bromide and chloride

The reaction mechanism of V–ClPO (*C. inaequalis*) does seem to differ from V–BrPO (*A. nodosum*) in regards to the nature of the oxidized halogen intermediate. Under turnover conditions (e.g. 0.2 mM H_2O_2, 1 mM Cl^-, 64 nM V–ClPO in 0.1 M phosphate buffer, pH 4.5), V–ClPO (*C. inaequalis*) produces HOCl which can be separated from the reaction mixture by ultrafiltration [20]. HOCl was also detected spectrophotometrically in the reaction solution. Both of these experiments

were carried out at pH 4.5 which is lower than the pH of the V–BrPO reactions (i.e. pH ca. 6.5). The build up of HOCl is consistent with the observation that the reduction of HOCl by excess H_2O_2 producing dioxygen is significantly slower at pH 4.5 compared to higher pHs. Since only ca. 50 mM HOCl was detected starting with an initial concentration of 200 mM H_2O_2, it was inferred that some reduction of HOCl by H_2O_2 could have occurred. Thus the overall reactivity in Scheme 1 likely holds for V–ClPO with chloride. Other than MCD, V–ClPO-catalyzed reactions have not been extensively explored with V–ClPO. In contrast to V–ClPO, oxidized halogen intermediates have not been isolated during the turnover of V–BrPO (*A. nodosum*) at pH 6.5. Production of the oxidized halogenating intermediate is rate limiting under optimum conditions for V–BrPO.

V–BrPO (*A. nodosum*) can catalyze the peroxidation of chloride, although a much larger concentration of chloride is required (>1 M [30]) compared to V–ClPO (0.2 mM Cl^- for standard turnover conditions). Similar to the V–BrPO (*A. nodosum*) reactivity with bromide, the rate of peroxidative chlorination of MCD is equivalent to the rate of the chloride-assisted disproportionation of hydrogen peroxide forming dioxygen (i.e. Scheme 1). However in the presence of primary, secondary or tertiary amines, the rate of dioxygen formation is much slower due to the formation of primary, secondary or tertiary chloramines, which do not readily oxidize hydrogen peroxide [30]. The formation of chloramines can be observed spectrophotometrically by their characteristic UV absorption maxima. In contrast to chloramine formation, bromamine formation at pH 6.5 is not observed with V–BrPO using hydrogen peroxide [31] since bromamines are rapidly reduced by H_2O_2 to singlet oxygen and bromide [32]. Using acyl peracids as the oxidant instead of hydrogen peroxide, bromamines were observed [31].

The rate of chlorination of MCD catalyzed by V–BrPO (*A. nodosum*) is the same in the presence and absence of an amine [30]. However, because the rate of chlorination of the organic substrate by the chloramine is very rapid, we were unable to determine whether MCD chlorination proceeds through a chloramine intermediate when an amine was present. Chloride oxidation by V–BrPO (*A. nodosum*) seems to be most favorable at about pH 5 under conditions of 1 mM H_2O_2, 1.5 M KCl, 50 μM MCD in 0.1 M citrate buffer, pH 5.0 [30].

2.4. Direct sulfoxidation by peroxo–V–BrPO: a newly reported reaction of V–BrPO

Until recently the only observed reactivity of V–BrPO required the presence of a halide and hydrogen peroxide. However, direct sulfoxidation catalyzed by V–BrPO in the absence of halide has now been reported [33]. V–BrPO from the coralline red alga *Corallina officinalis* oxidizes several bicyclic sulfides to the corresponding sulfoxide with a high enantioselectivity, i.e. e.e. up to 91% in the absence of an added halide source. Based on the recognition that indene was a good substrate for hydrobromination by V–BrPO [33] and that indoles have been shown to bind to V–BrPO [24–26], several bicyclic sulfides structurally related to indene and indole were investigated. 2,3-Dihydrobenzothiophene (**1**), thiochroman (**2**),

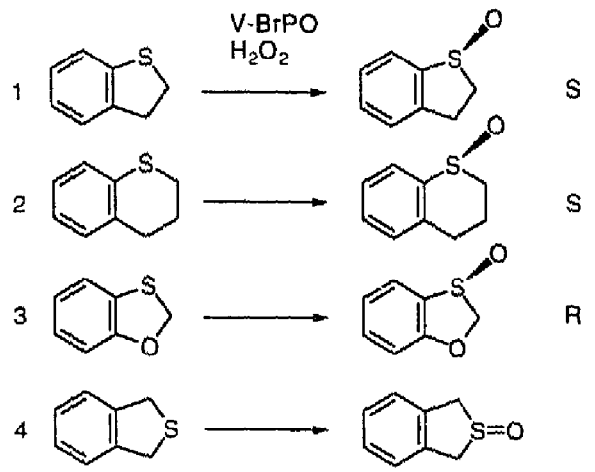


Fig. 3. Sulfoxidation catalyzed by V BrPO.

1,3-benzoxathiole (**3**) and 1,3, dihydrobenzo-[c]thiophene (**4**) are all oxidized to the corresponding sulfoxide as shown in Fig. 3.

With the exception of **4** which is symmetrical, the oxidation of all the other substrates occur stereospecifically. The sterochemical orientation of all of the sulfoxide products of **1**, **2**, and **3** are the same in Fig. 3, however, the **R** identity for the sulfoxide product of **3**, compared to the **S** identity of the sulfoxide product of **1** and **2** is a result of the nomenclature rules.

The concentration of hydrogen peroxide and its rate of addition to the reaction solution was found to significantly affect both the yield and enantioselectivity of sulfoxidation. When H_2O_2 is slowly added via syringe pump to **1** (2,3-dihydrobenzothiophene) over the course of 2 h, the yield (42%) was nearly doubled compared to initiating the reaction by adding the full aliquot of H_2O_2 all at once (yield 20%). In addition the yield from the spontaneous uncatalyzed sulfoxidation reaction was cut in half, and the e.e. increased from 65 to 87% [33]. When the rate of addition of H_2O_2 was decreased even further to 16 h, instead of 2 h, the yield and e.e. increased to 98 and 90%, respectively.

The slow rate of addition of H_2O_2 was also required to achieve high yields and enantioselectivity for sulfoxidation catalyzed by FeHeme chloroperoxidase (*C. fumago*). When H_2O_2 was added all at once, the FeHeme ClPO-catalyzed disproportionation of hydrogen peroxide, which produces dioxygen, was observed. However in the V–BrPO reaction, dioxygen formation is not observed in the absence of halide [16–18,33]. In fact even in the presence of **1** and bromide, dioxygen formation was not catalyzed by V–BrPO, which the authors attributed to the preferential oxidation of the sulfide over bromide [33]. Of course, as was also recognized, bromide could be oxidized preferentially, and sulfoxidation could occur via the oxidized bromine intermediate. The enantioselectivity, however, in the presence of bromide, was not reported [33]. We have observed sulfoxidation in the presence of bromide and hydrogen peroxide, however, without enantioselectivity [34].

These sulfoxidation reactions are the first report of direct oxidation by the peroxo–V–BrPO derivative as opposed to other oxidation reactions which are all mediated by an oxidized halogen intermediate. This reaction also confirms that oxidation is occurring within the active-site of the enzyme. Sulfide oxidation by oxoperoxovanadium(V) complexes is well known [35].

As mentioned briefly above, the sequence of V–ClPO (*C. inaequalis*) is related to the sequences of three acid phosphatase families [13–15]. Vanadate and vanadate oligomers, which can bind to phosphate sites in proteins, are well known inhibitors of certain acid phosphatases [36]. At this point, the haloperoxidase activity of vanadium(V)-incorporated acid phosphatases has not been reported, however, the apo form of vanadium chloroperoxidase (*C. inaequalis*) has been found to catalyze the hydrolysis of *p*-nitrophenol phosphate, forming *p*-nitrophenol [13]. *p*-Nitrophenol phosphate also displaces vanadium from V–ClPO. Phytase is a phosphatase which catalyzes hydrolysis of phytic acid to inositol and phosphate ion [37]. Of relevance to this review, is a recent report that vanadium(V)-incorporated phytase (*Aspergillus ficuum*) catalyzes the sulfoxidation of thioanisole by hydrogen peroxide, proceeding with 100% conversion and generating the S enantiomeric product preferentially (i.e. 50–60% e.e.). However, surprisingly it was also found that phytase in the absence of vanadate could also oxidize sulfide to sulfoxide by hydrogen peroxide [37]; the yield and enantioselectivity were somewhat less than in the vanadium-incorporated phytase sample, but nevertheless appreciable (e.g. as high as 81% yield and 51% e.e. at the highest concentration of phytase) The X-ray structure of phytase (*A. ficuum*) reveals an aspartate residue at the active site which the authors suggest could generate aspartyl peracid, thus providing the oxidant of sulfide [37]. It will be interesting to follow further progress of this reaction, particularly regarding the selectivity of the vanadate-free phytase. The haloperoxidase reactivity of the vanadium(V)-incorporated phytase derivative was not reported [37].

3. What does the X-ray structure of vanadium chloroperoxidase reveal about the reactivity of the vanadium haloperoxidases?

3.1. The X-ray structure of V–ClPO

The X-ray structure of V–ClPO (*C. inaequalis*) shows that the protein is cylindrical with approximate dimensions of 80 by 55 Å^2 [38]. Two four-helix bundles comprise the main α-helical structural motif [38]. The vanadium(V) site resides at the top of one of these bundles in a broad channel which is lined on one half with predominantly polar residues including an ion-pair between Arg-360 and Asp-292 and several main chain carbonyl oxygens (Fig. 4). The other half of the channel is hydrophobic, containing Pro-47, Pro-211, Tyr-350, Phe-393, Pro-395, Pro-396, and Phe-397.

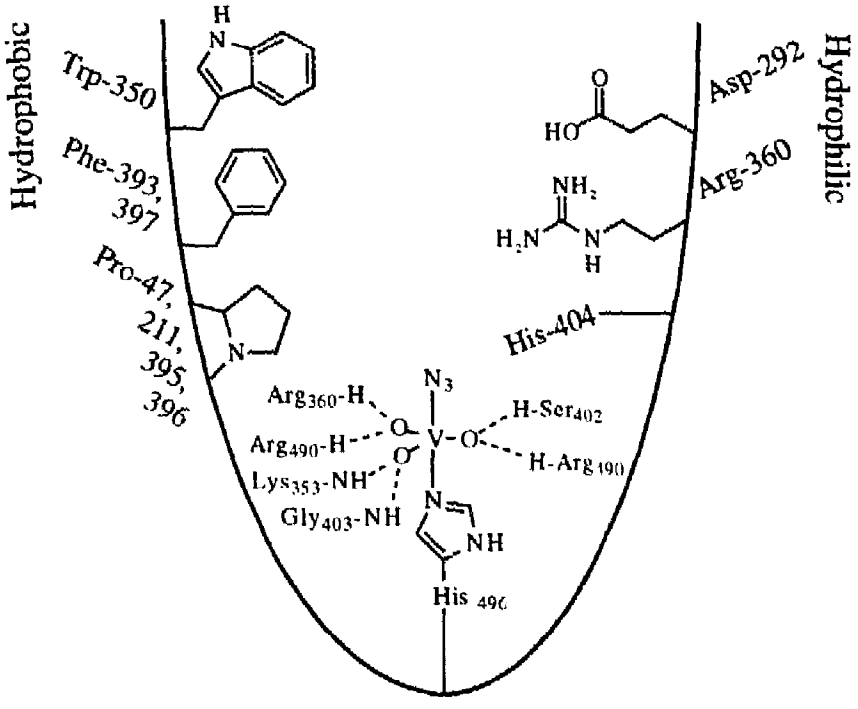


Fig. 4.

The vanadium(V) site is noticeably simple. Vanadium(V) is ligated by a single protein donar ligand, histidine 496, three oxygen atoms in an equatorial plane and an apical oxygen atom of a proposed hydroxide ligand [39]. The oxygen atoms are all hydrogen bonded to amino acid side chains or the peptide backbone of the protein, reducing the negative charge around the vanadium center (see Fig. 6(A)). This site is reminiscent of vanadate (HVO_4^{2-}) coordination to one histidine ligand in a trigonal bipyramidal geometry, although the axial oxide ligand is replaced by hydroxide. Actually, the first X-ray structure of V–ClPO to be solved was the azide-coordinated derivative, which was a result of crystallization of V–ClPO from azide-containing buffer. In this first structure, azide was found to be coordinated in an axial position as shown in Fig. 5.

The X-ray structure of the peroxide-bound derivative of V–ClPO reveals a distorted tetragonal bipyramidal vanadium site (Fig. 6(B)) [39]. Peroxide is coordinated in a side-on manner in the equatorial plane, along with an oxygen atom and His 496. An axial oxide ligand completes the pyramidal coordination geometry. The oxide ligand is hydrogen bonded to Arg 490; the coordinated peroxide is hydrogen bonded to a glycine amide backbone and Lys 353; the remaining oxygen atom is hydrogen bonded to Arg 360.

The X-ray structure of the apo form of V–ClPO is superimposable on the native structure, suggesting that the protein matrix is rigid and provides a preformed metal binding site [40]. A water molecule in apo-V–ClPO is bound in place of the vanadate ion in the native form. This water molecule is hydrogen bonded to His-496 and the amide nitrogen of Gly 403. The X-ray structure of the tungstate-

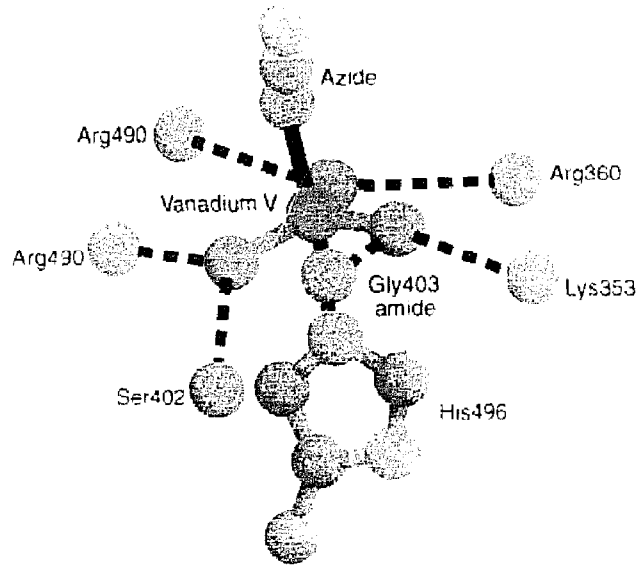
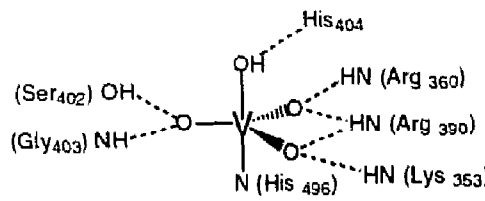
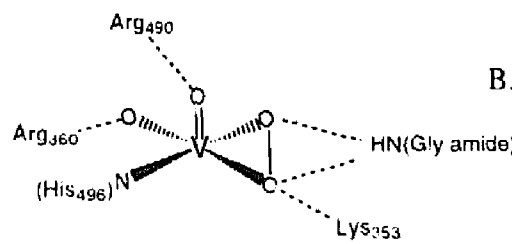


Fig. 5.

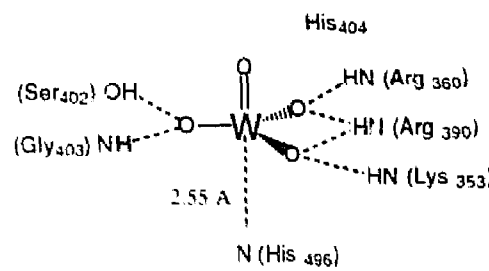
substituted V–ClPO reveals replacement of vanadate by tungstate, however His-496 is either not coordinated to W or only weakly coordinated, since the distance between the NE2 atom of His-496 and W is long, 2.55 Å (Fig. 6(C)). The tungstate



A. The native site



B. The peroxo site



C. The W-substituted site

Fig. 6.

oxygen atoms hydrogen bond to protein side chains and a main chain amide similar to native V–ClPO. Thus, coordination of vanadate and tungstate can be attributed to 'rack-induced bonding' of these metal ions to a rigid protein matrix [41,42]

3.2. Hydrogen bonding considerations and activation of vanadium(V)-bound peroxide

As discussed above, vanadium(V) is bound in a pentagonal bipyramidal geometry ligated by a single protein side chain, His 496. The three equatorial oxygen ligands and the axial hydroxide ligand are hydrogen bonded to multiple protein side chains or the protein backbone (see Figs. 4–6). His-404, which is present in the active site channel, must be deprotonated for H_2O_2 to bind to V–ClPO [7], and thus it is thought to function in acid–base catalysis. One might have expected to find His 404 hydrogen bonded to the vanadium(V)-bound peroxide, however, the X-ray structure of peroxo–V–ClPO, prepared by addition of H_2O_2 to crystals of native–V–ClPO revealed that Lys 353 was hydrogen bonded to one of the bound peroxide oxygen atoms. From a mechanistic stand point, the hydrogen bond of His 404 to the axial hydroxide in native V–ClPO and the hydrogen bond of Lys 353 to a V(V)-bound peroxide oxygen atom may be significant, although it is conceivable that His 404 could still activate the bound peroxide during turnover.

3.3. What controls the halide selectivity

Enzyme kinetics with V–BrPO and V–ClPO show saturation in bromide and chloride If halide binding occurs prior to the rate-limiting step, then saturation establishes that a halide binding site exists. If the step or steps involving halide occurs after the rate-limiting step, then a binding site cannot be established by kinetic means. Messerschmidt and Wever had proposed that the hydrophobic residues Trp-350 and Phe-397 form a chloride binding site along with His-404 in V–ClPO [38]. Halides are known to bind at hydrophobic sites in other proteins such as in haloalkane dehalogenase [42] and certain amylases [43]. The sequence of V–BrPO (*A. nodosum*) varies somewhat from V–ClPO in this region, in that in the V–BrPO (*A. nodosum*) sequence, a histidine takes the place of Phe-397 in V–ClPO. However in the sequences for V–BrPO from *Fucus distichus* and *C. pilulifera*, histidine is not present in this site [44,45]. Thus the origin of the halide specificity from this perspective is still open. The halide binding site could be at the vanadium(V) center, however, no difference in the exafs of V–BrPO (*A. nodosum*) was observed in the presence versus absence of bromide [46]. Moreover, it is not clear what the differences are in the vicinity of the vanadium site between V–ClPO and V–BrPO, particularly which could mediate the halide selectivity. Thus the origin of the halide selectivity remains one of the interesting, yet unanswered questions.

3.4. Preliminary structural studies of V–BrPO

V–BrPO from both *A. nodosum* and *Corallina officinalis* [47,48] have been crystallized, although the structures have not been reported. The full sequence of

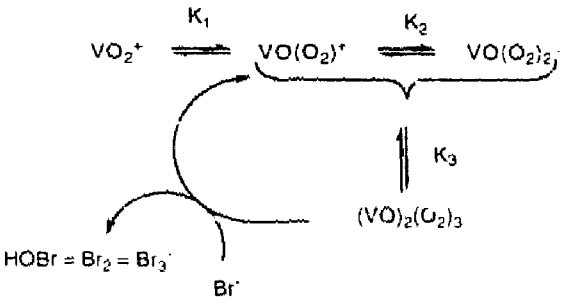
V–BrPO (*A. nodosum*) has also not been reported, but from the partial sequence of *A. nodosum* [6], and the sequences of V–BrPO from *C. pilulifera* [45] and *Fucus distichus* [44], it is clear that V–BrPO is similar to V–ClPO, particularly in the active site region. The similarities include regions containing the amino acid residues which hydrogen bond to the vanadate oxygens (i.e. Arg-360, Ser-402, Gly-403-amide backbone, and Arg-490; [The numbers refer to the sequence of V–ClPO from *C. inaequalis*]), the histidine ligand (His-496), and the acid–base histidine (His-404). Thus it seems likely that the vanadium site in V–BrPO is comparable to that in V–ClPO (i.e. Figs. 4–6).

4. What do biomimetic studies reveal about the mechanism of the vanadium haloperoxidases?

In addition to haloperoxidase enzymes, many transition metal complexes are also effective catalysts of the oxidation of halides by hydrogen peroxide. Some of these catalysts were discovered in the search for functional biomimics of the haloperoxidases, particularly to elucidate the mechanism of halogenation of organic substrates [49–64]. Several groups are now investigating the oxidation of halides by hydrogen peroxide, catalyzed by transition metal ions or their complexes [49–69]. Some groups report synthesis of halogenated compounds, without focusing on the mechanism of the oxidation of the halide by the metal–peroxide species or the mechanism of product formation [70,71]. Several recent reviews have covered the biomimetic chemistry of the vanadium haloperoxidases [11,12,72]. Thus the intent in this section is to focus on those results which shed light on the mechanism of the vanadium haloperoxidase reaction. The main questions pertain to (1) what factors promote efficient halide oxidation by a monoperoxovanadium(V) moiety under near neutral pH conditions?; (2) whether halide coordination to the vanadium center is required for halide oxidation? and (3) upon halide oxidation, does a vanadium–hypohalite complex persist long enough to halogenate or oxidize a substrate?

4.1. What factors promote efficient halide oxidation by a monoperoxovanadium(V) moiety under near neutral pH conditions?

Initial studies on functional mimics of V–BrPO were inspired by the lack of spectroscopic techniques capable of observation of the vanadium(V) site in the enzyme [49,50,53,54]. At that time, the X-ray structure of V–ClPO had not been solved, and thus the remarkably simple vanadate-like site had not been elucidated. Nevertheless, early investigations showed that *cis*-dioxovanadium(V), *cis*-VO_2^+, could catalyze peroxidative bromination and chlorination reactions and the bromide-assisted disproportionation of hydrogen peroxide in acid solutions [49]. Molybdate [52,67,68], tungstate [52] and methylrhenium trioxide [65,66] also catalyze the oxidation of halides by hydrogen peroxide, under generally acidic conditions. However, vanadate, HVO_4^{2-} / $H_2VO_4^-$ (i.e. the species of vanadium(V) present

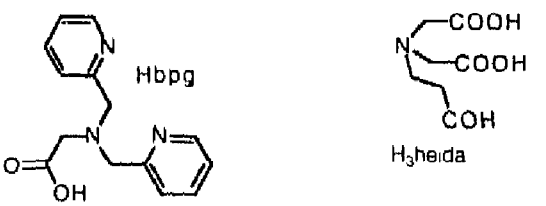


Scheme 3.

at neutral pH), was not catalytically competent at neutral pH to oxidize bromide. Vanadate readily coordinates two equivalents of peroxide at neutral pH, forming oxodiperoxovanadium(V), $VO(O_2)_2^-$, although it does not oxidize bromide. In acid solution, *cis*-VO_2^+ reacts with hydrogen peroxide, forming a mixture of oxo-monoperoxo–vanadium(V), i.e. $VO(O_2)^+$, and the diperoxo species, $VO(O_2)_2^-$, even in the presence of excess hydrogen peroxide [53]. The occurrence of both $VO(O_2)^+$ and $VO(O_2)_2^-$ turns out to be critical, because the sole oxidant of bromide is the dimer, $(VO)_2(O_2)_3$, formed by association of $VO(O_2)^+$ and $VO(O_2)_2^-$ (Scheme 3 [53]).

While the structure of $(VO)_2(O_2)_3$ is not known, it likely involves asymmetric coordination of a bridging peroxide [53]. Thus one can view the role of the 'second' vanadium(V) center as activating the peroxide for attack by bromide, similar to the proposed role of the 'acid–base' residue in V–BrPO.

Pecoraro has examined peroxidative bromination by a series of mixed carboxylate, alkoxide, amine or amide-containing ligands [56–58,72]. Vanadium(V) complexes of ligands such as Hbpg and H_3Heida coordinate peroxide but do not oxidize bromide in water. However, in acetonitrile, the rate of oxidation of bromide is appreciable, which has been attributed to activation by protonation of the bound peroxide. For comparison, the second order rate constant for oxidation of bromide by the oxoperoxovanadium(V) species, peroxo–V–BrPO (pH 7.9), peroxo–V–BrPO (pH 4), $VO(O_2)(H_3heida)$, $VO(O_2)$(Hbpg) and $(VO)_2(O_2)_3$ are 2.8×10^3, 1.8×10^3, 280, 21, and 4.1 $M^{-1}\,s^{-1}$, respectively.



4.2. Is halide coordination to the vanadium center required for halide oxidation?

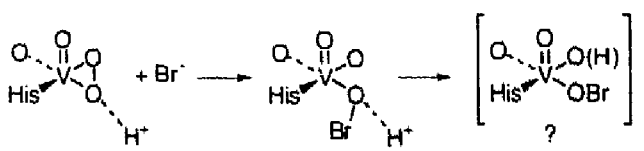
After the first step of peroxide coordination to vanadium(V) in V–HPO, the halide is oxidized. Subsequent to the elucidation of the X-ray structures for V–ClPO and the peroxo derivative, which reveal a potentially vacant coordination

site in each form. Messerschmidt and Wever have proposed that chloride binds to the vanadium(V) center [39,40]. At this point spectroscopic studies have not established whether the halide attacks the coordinated peroxide directly or whether it coordinates to vanadium(V) prior to oxidation (Fig. 7).

From mechanistic studies with the molybdenum(VI) functional mimics, comparing $MO(O_2)_2(H_2O)$ with $MO(O_2)_2(C_2O_4)^{2-}$, it appears that a vacant coordination site on the oxoperoxo metal species is not a prerequisite for halide oxidation [52]. Thus, the oxidation could occur via nucleophilic attack by halide on the coordinated peroxide.

4.3. Upon halide oxidation, does a vanadium–hypohalite complex persist?

After coordination of peroxide to the vanadium center and subsequent oxidation of the halide, the question arises whether a discrete vanadium hypohalite intermediate is formed for any appreciable length of time.



Such an intermediate could mediate the selective halogenation reactions which are beginning to be discovered, both in the enzyme and model complexes. In a two-phase acidic aqueous organic (CH_2Cl_2) system with ammonium vanadate, hydrogen peroxide and bromide, Conte and DiFuria observe a mixture of bromohydrin and dibromo products formed from styrene or other similar compounds [60,62]. They attribute bromohydrin formation to reaction from a vanadium(V)-hypobromite intermediate at the aqueous-organic interface and dibromo formation to halogenation by bromine (Br_2) in the organic layer [60]. Their main argument in favor of a reactive vanadium hypobromite intermediate is that, HOBr could not persist in the acidic conditions of the aqueous layer (pH 1, $HClO_4$). It is also known that the bromohydrins are not the result of hydrolysis of the dibromo product [60]. The stability of a metal–hypohalite complex would certainly be influenced by the concentration of the free halide. Thus studies on selectivity as a function of the concentration of halide and pH may be useful in implicating reaction from a metal–hypohalite complex.

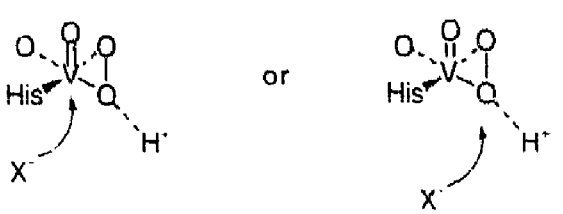


Fig. 7.

5. Summary

Vanadium(V) compounds are well known to coordinate peroxides and carry out a wide range of oxidation reactions [73]. Vanadium haloperoxidases coordinate peroxide, and oxidize chloride, bromide or iodide, as well as sulfides and pseudohalides [4,11,12,35]. The vanadium haloperoxidases are still the most efficient oxidants of halides among the vanadium catalysts so far investigated. Such efficiency is influenced by the protein active site and the role of certain amino acids in activation of vanadium(V)-bound peroxide for halide oxidation and also possibly in the selectivity of the halide. With the recent isolation of the genes for vanadium chloroperoxidase [7] and vanadium bromoperoxidase [44,45], it is likely that the roles of the critical amino acids will be investigated through site-directed mutagenesis studies. The favorable reactivity and robust stability of these enzymes makes them attractive candidates for biocatalytic conversions. With the emerging understanding of the mechanism of halogenation, the biogenesis of important marine natural products can be anticipated, as well as the development of catalysts for other important selective halogenation reactions.

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