Vanadium haloperoxidases

Alison Butler

The nature of the oxidized halogen intermediate in vanadium bromoperoxidase has recently been shown to depend on the nature of the organic substrate. For example, in the presence of indoles, vanadium bromoperoxidase does not release a freely diffusible oxidized halogen intermediate (such as $HOBr{\pm}BR_2{\pm}Br_3^-$). Regioselective investigations are, therefore, now feasible.

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Current Opinion in Chemical Biology 1998, **2**:279–285

http://biomednet.com/elecref/1367593100200279

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Abbreviations

MCD	monochlorodimedone: 2-chloro-5,5-dimethyl-1,3-dimedone
V-BrPO	vanadium bromoperoxidase
V-ClPO	vanadium chloroperoxidase
V-HPO	vanadium haloperoxidase

Introduction

Haloperoxidases are enzymes that catalyze the oxidation of halides (iodide, bromide and chloride) by hydrogen peroxide, resulting in the halogenation of certain organic substrates (Equation 1) or the halide-assisted disproportionation of hydrogen peroxide forming dioxygen (Equation 2):

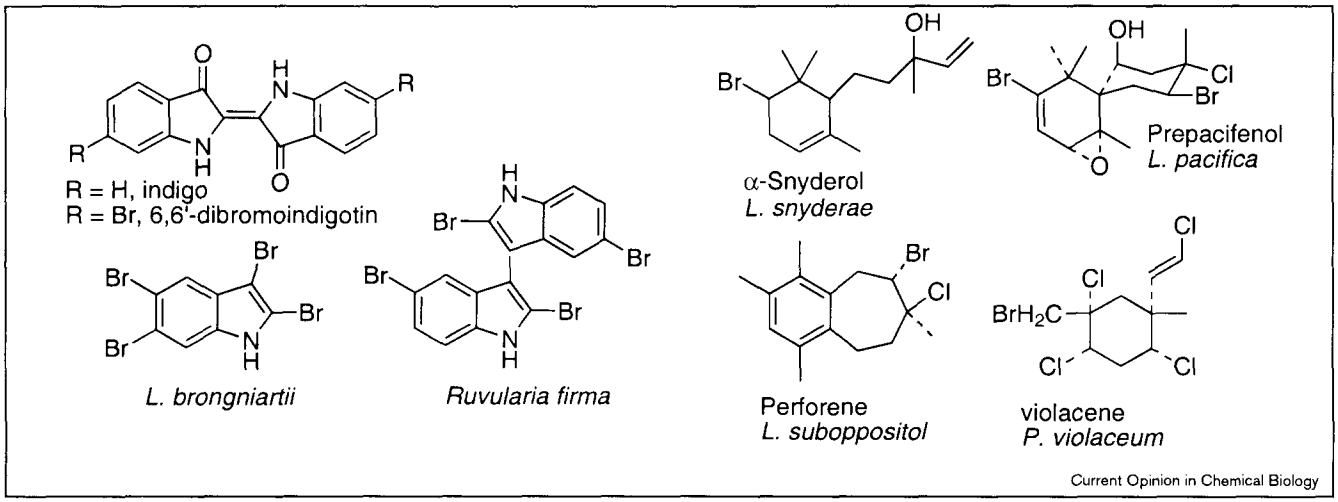
$$X^- + H_2O_2 + R{-}H + H^+ \rightarrow R{-}X + 2\,H_2O \quad (1)$$

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

Within the class of vanadium-haloperoxidase enzymes, both vanadium bromoperoxidases (V-BrPO), which are isolated mainly from marine algae, and vanadium chloroperoxidases (V-ClPO), which are isolated from certain terrestrial fungi, have been identified. Many halogenated natural products have been isolated from marine organisms. These compounds range from volatile halogenated hydrocarbons, for example, bromoform, chloroform, etc, which are produced in very large quantities, to chiral halogenated terpenes, acetogenins and indoles, among others, which are produced in smaller amounts, but which often have important biological activities (for example, antimicrobial properties, feeding deterrents, etc) or pharmacological properties (Figure 1). Halogenated indoles (e.g. the topsentins, diazonamides and jasplakinolides; Figure 2) appear to be particularly important targets for biosynthetic studies because of their potent anti-inflammatory and anti-cancer activities. The marine haloperoxidases are thought to be involved in the biosynthesis of these natural products. The function of the fungal V-ClPO (which is isolated from *Curvularia inaequalis*, for example, and other dematiaceous hyphomycetes) is thought to be in the degradation of the plant host cell wall through production of hypochlorous acid (HOCl) (see below). Halogenated natural products have not been identified in fungi that produce V-ClPO.

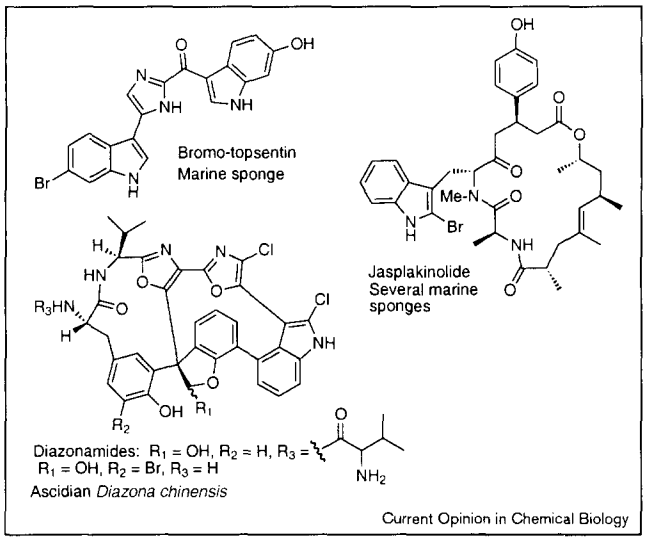
The overall reactivity of V-BrPO is described in figure 3 when MCD (2-chloro-5,5 dimethyl-1,3-dimedone), the classic substrate used to measure haloperoxidase, is used as

Figure 1



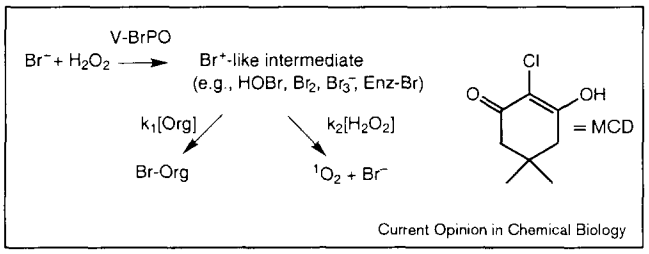
Selected examples of halogenated marine natural products.

Figure 2



Selected halogenated indole marine natural products of pharmacological interest.

Figure 3



A general scheme for V-BrPO reactivity. When the organic substrate, Org, is monochlorodimedone, MCD, k_1[MCD] is competitive with $k_2[H_2O_2]$. $^1O_2$ is formed in the bromide-assisted disproportionation of H_2O_2 (see reviews [1•,2,3•,4•,5]).

the organic substrate. For recent reviews on the vanadium haloperoxidases see [1•,2,3•,4•,5].

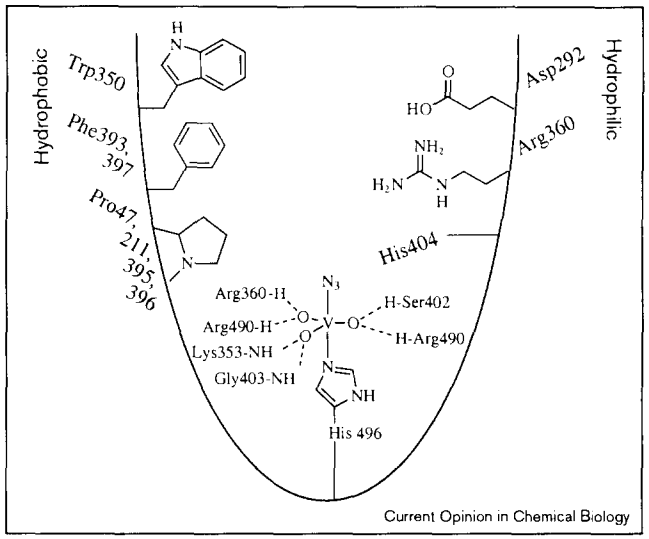
Figure 3 raises a number of questions, some of which have been addressed recently. What is the nature of the oxidized halogen intermediate? Is it bound to the enzyme or released from the active site? Does the organic substrate bind to V-BrPO? What are the critical amino acid residues for enzyme catalysis? What is the nature of the vanadium(V) site? This article will cover recent developments pertaining to these questions.

What is the nature of the vanadium(V) site?

Protein-bound vanadium(V) is required for activity in V-BrPO and V-ClPO. Phosphate can displace vanadium from V-BrPO and the activity can be fully restored on addition of vanadate in the absence of phosphate. This raises the question of whether these enzymes contain a vanadate ion (VO_4^{3-}) at the active site or whether, on binding, the vanadate oxygen atoms are protonated off, providing coordination sites for protein sidechain ligands (for example, oxygen donors such as serine, threonine, glutamic acid, aspartic acid or other donors such as histidine, etc).

The recently determined X-ray structure of the native form of V-ClPO (2.03 Å resolution [6••]) shows that two four-helix bundles form the main structural motif of *Curvularia inaequalis* V-ClPO [6••,7••]. Vanadate is coordinated at the top of one of these bundles in a broad channel that is lined on one half with predominantly polar residues and several mainchain carbonyl oxygen atoms (Figure 4). The other half of the channel is hydrophobic, containing Pro47, Pro211, Tyr350, Phe393, Pro395, Pro396, and Phe397.

Figure 4

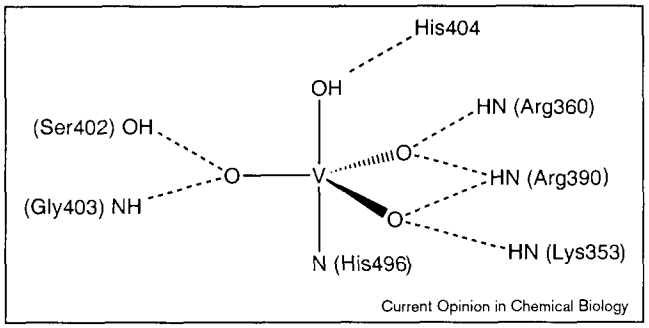


The V-ClPO active-site channel.

The vanadium site is remarkably simple. Essentially, vanadate is ligated to the protein by a single histidine ligand in a pentagonal bipyramidal geometry (Figure 5). Hydroxide (V–O 1.93 Å) and His496 (V–N 1.96 Å) occupy axial positions [6••]. Three oxygen atoms (V–O 1.65 Å) occupy the equatorial plane (Figure 5); however, unlike vanadate, this vanadium center lacks an oxide (V=O) ligand.

Multiple hydrogen bonds between the equatorial vanadate oxygen atoms and the positively charged protein residues Lys353, Arg360, Arg390, and Ser402, as well as the amide nitrogen proton of Gly403, stabilize vanadate coordination to the protein. In addition, the apical hydroxide is hydrogen bonded to His404 [6••], a residue implicated in catalysis and referred to as the acid-base histidine.

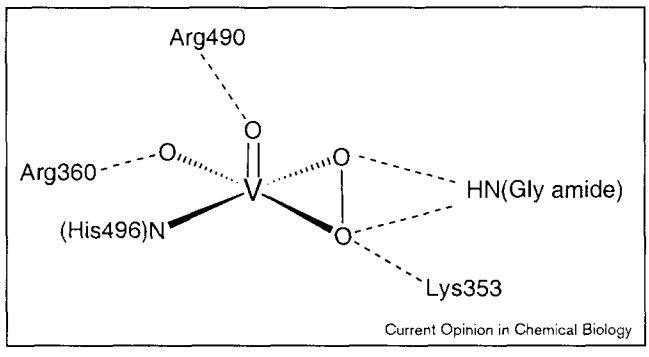
Figure 5



The vanadate site in V-ClPO.

The X-ray structure of the peroxide form of V-ClPO (2.24 Å resolution) reveals a distorted tetragonal pyramid in which vanadium(V) is coordinated by peroxide in a side-on bound fashion (1.87 Å V–O bond lengths; 1.47 Å O–O bond length), His496 (2.19 Å V–N bond length) and an oxygen atom (1.93 Å) in the basal plane and by an oxo ligand (1.60 Å) in the axial position (Figure 6) [6••]. His 404 is no longer hydrogen bonded to the vanadate. One of the peroxide oxygen atoms is hydrogen bonded to Lys353. The shortening of the apical V–O bond length from 1.93 Å to 1.60 Å upon coordination of peroxide has been interpreted as a shift from hydroxide coordination (V–OH) to oxide coordination (V=O).

Figure 6



The oxoperoxo-vanadium(V) site in peroxo-V-ClPO.

Structural features of V-BrPO have not been published, although V-BrPO from *Ascophyllum nodosum* and *Corallina officinalis* have been crystallized [8–10]. There is good sequence similarity between V-ClPO (*C. inaequalis*) and V-BrPO (*A. nodosum*), particularly in the active-site region. The ligand histidine, the acid-base histidine, and the amino acid residues that hydrogen bond to the equatorial vanadate oxygen atom are conserved [5,7••,11]. Extended X-ray absorption fine structure analysis [12] and bond valence sum analysis [13] are consistent with a trigonal bipyramidal vanadium site in *A. nodosum* V-BrPO. Taken together, these results suggest that the vanadium site in V-BrPO has a trigonal bipyramidal structure like that observed in V-ClPO, with coordination to one histidine ligand and multiple hydrogen bonds between the vanadate oxygen atoms and positively charged protein residues.

On the selectivity of vanadium haloperoxidase and the nature of the halogenating intermediate

Many halogenated marine natural products contain chiral carbon–halogen centers or are selectively halogenated or oxidized at sites not expected for reactions by aqueous halogen species (Figures 1,2). Thus it does not seem likely that a freely diffusible oxidized halogen intermediate is the active halogenating species. Other questions pertaining to the selectivity of the V-HPOs are whether the organic substrate binds to the enzyme and whether enzyme reactivity differs from that of aqueous bromine with the same substrate.

It was found recently that many organic substrates are brominated preferentially compared with MCD, the classic substrate used in the characterization of haloperoxidase activity [14•]. The extent of MCD bromination by V-BrPO in the presence of an equimolar competing substrate, many of which are related to likely precursors of halogenated marine natural products, is given in Table 1. Most of the indoles examined were brominated preferentially over MCD. Terpenes were also halogenated or oxidized preferentially over MCD.

Table 1

Substrate specificity of v-BrPO.

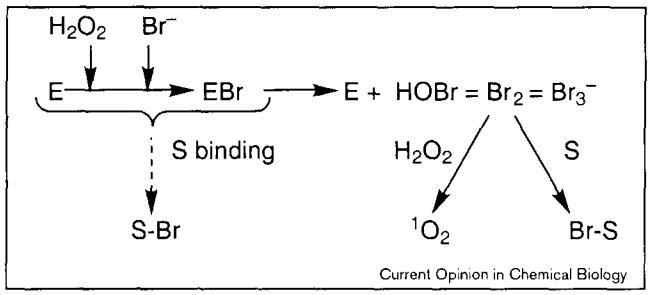
Competing substrate	MCD reacted (%)	Competing substrate	MCD reacted (%)
Cytosine	99.0	Indole-3-acetic acid	47.2
trans-Cinnamic acid	98.9	Farnesol	46.6
Phenol red	97.4	3-Methylindole	32.9
Indole-3-acrylic acid	62.0	5-Hydroxyindole	23.8
5-Aminoindole	60.1	2-Phenylindole	18.9
Indoxyl-β-D-glucoside	59.8	2-*tert*-Butylindole	5.8
1,3,5-trimethoxybenzene	57.0	2-Methylindole	0.5
Indole-3-methanol	47.6		

Reaction conditions: 970 μM H_2O_2, 50 mM KBr, 50 μM MCD, 50 μM competing substrate and 2 nM V-BrPO in 100 mM sodium phosphate buffer, pH 6.5 with 10% ethanol.

The selectivity observed in Table 1 may be achieved by substrate binding to V-BrPO which could arise from an enzyme-trapped oxidized halogen species. The oxidized halogen species has not been detected under optimal turnover conditions (pH 6.5) because it does not build up in solution owing to its fast reaction with organic substrates or with excess H_2O_2, producing O_2. In fact, competitive kinetic studies that compare the reactivity of the V-BrPO/H_2O_2/KBr system with HOBr, demonstrate

that the nature of the halogenating species produced by V-BrPO depends on the nature of the organic substrate [15]. For example, V-BrPO does not release an oxidized bromine species (e.g. HOBr, Br_2, Br_3^-) in the presence of certain indole derivatives, suggesting that these indoles bind to V-BrPO. Fluorescence quenching results further confirm that substituted indoles bind to V-BrPO [15]. The Stern–Volmer analysis revealed a binding constant of $1.1 \times 10^5\,M^{-1}$ for 2-phenylindole to *A. nodosum* V-BrPO (RA Tschirret-Guth and A Butler, unpublished results). A mechanistic scheme involving substrate binding is shown in Figure 7 [15]; 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, for example Br_2, Br_3^-). When the indole is present, it binds to V-BrPO, preventing release of an oxidized bromine species and leading to indole bromination.

Figure 7



The modified scheme for V-BrPO (shown as EBr) reactivity including substrate binding (S).

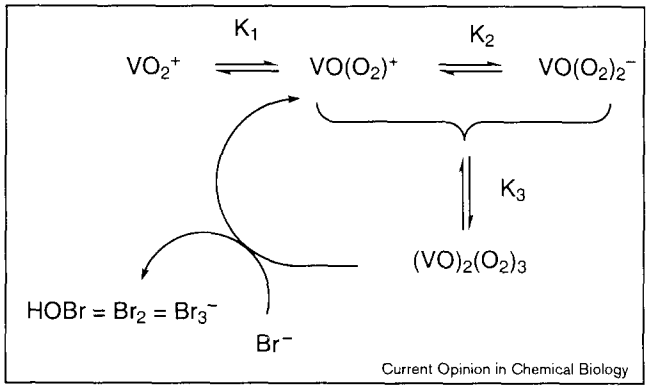
Thus regioselective and enantioselective halogenation catalyzed by V-BrPO is now an attractive goal to pursue; however, V-BrPO can effect the bromination of a variety of other organic substrates (for example, cytosine), albeit rather inefficiently as more hydrogen peroxide is consumed than halogenated product produced. For these substrates, the oxidized halogen intermediate could be released into solution.

A significant difference between the reactivity of *C. inaequalis* V-ClPO and *A. nodosum* V-BrPO is the apparent production of hypochlorous acid (HOCl) by V-ClPO under its optimal turnover conditions at pH 4.5 [16]. HOCl builds up in solution during turnover. It could be separated from the reaction solution by ultrafiltration. However, because the detected amount of HOCl was only about 25% of that expected, it was inferred that some reduction of HOCl by H_2O_2 could have occurred. Thus the overall reactivity in Figure 3 probably holds for V-ClPO with chloride.

Functional mimics of the vanadium haloperoxidases

Initial studies on functional mimics of V-BrPO were driven by the lack of spectroscopic techniques capable of observation of the vanadium(V) site in the enzyme. The results of these studies now point to the role of the critical amino acids for efficient catalysis, particularly at neutral pH. Early on it was found that acidic solutions of *cis*-dioxovanadium(V) (*cis*-VO_2^+) catalyzed the oxidation of halides by hydrogen peroxide resulting in halogenation of an organic substrate and the halide-assisted disproportionation of hydrogen peroxide [17–20]. Given the simple structure of vanadate coordination in V-ClPO, it was surprising initially that aqueous solutions of vanadate (HVO_4^{2-}/ $H_2VO_4^-$) at neutral pH did not catalyze the oxidation of bromide by hydrogen peroxide. Kinetic and mechanistic work on the catalysis by *cis*-VO_2^+ in acidic aqueous solution revealed that the active oxidant of bromide is a dimeric, triperoxo species (Figure 8) [18].

Figure 8



The catalytic cycle for peroxidative bromination catalyzed by *cis*-VO_2^+. The pH and concentration of hydrogen peroxide must be balanced so that both oxomonoperoxo and oxodiperoxo species are present in solution in order for the dimer to form [18]. The second-order rate constant for the oxidation of bromide by $(VO)_2(O_2)_3$ is independent of pH, although the overall rate depends on the acid concentration, which affects the concentration of the dimer species. At neutral pH and under conditions of at least two equivalents of H_2O_2 to vanadate, the oxodiperoxo species $VO(O_2)_2^-$ is formed exclusively; however, neither bromide nor chloride is oxidized under these conditions. The association constant for the dimer is rather small (K_3=9 M^{-1} at pH 0–2) which explains why it is not observed by ^{51}V NMR at low concentrations of vanadium under turnover [17].

The proposed structure of the dimer contains an asymmetrically bridged peroxide [21] in which one vanadium(V) center can activate the bridging peroxide for nucleophilic attack by the halide, thus implicating hydrogen-bonding activation of vanadium(V)-bound peroxide in V-HPO.

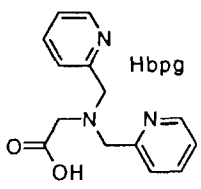
Owing to the simple vanadate site in V-ClPO, it is not surprising that other transition metal ions [22–26] and liganded vanadium complexes [4•,27–30] catalyze halide oxidation in acidic aqueous solution. In all of these systems, a monomeric metal peroxo species oxidizes the halide. The peroxo-V-BrPO complex has the largest rate constant for the oxidation of bromide (Table 2), most likely reflecting the influence of the 'acid/base' histidine which must be in its neutral, monoprotonated state (i.e. His404 in V-ClPO from *C. inaequalis*). In fact, under turnover conditions at low pH, V-BrPO is irreversibly inactivated by the oxidation of histidine to 2-oxohistidine [31•,32]. In addition, the X-ray structure of peroxo-V-ClPO shows Lys353 is hydrogen bonded to the bound peroxide [6••], which may assist the reductive cleavage of coordinated peroxide upon nucleophilic attack by bromide. None of the model complexes has this feature built in. The relatively large rate constants for the oxidation of bromide by $VO(O_2)(H_3heida)$ and $VO(O_2)$(Hbpg) (Table 2) which were observed in acetonitrile, but not water, may be a result of the protonation of the side on bound peroxide which occurs in acetonitrile, but not water [32]. The relatively large rate constant for rhenium over vanadium and molybdenum reflects the greater oxophilicity of high valent methylrhenium over vanadium or molybdenum.

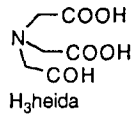
Table 2

Second-order rate constant for the oxidation of bromide by peroxo metal species.

Oxidant	Conditions	Rate constant ($M^{-1}s^{-1}$)	Ref
V-BrPO-(O_2)	pH 7.9*	2.78×10^3	[41]
	pH 4.0*	1.75×10^5	[41]
$MeReO_2(O_2)_2$	pH 0	350	[23]
$MeReO_2(O_2)_2$	pH 0	190	[23]
$VO(O_2)(H_3heida)$	$CH_3CN^\dagger$	280	[30]
$VO(O_2)$(Hbpg)	$CH_3CN^\dagger$	21	[30]
$(VO)_2(O_2)_3$	pH 0.7–2.0	4.1	[18]
$MoO(O_2)_2(H_2O)_2$	pH 1.0–5.1	1.5×10^{-2}	[22]
$MoO(O_2)_2(H_2O)(OH)^-$	pH 1.0–5.1	2.4×10^{-3}	[22]
$MoO(O_2)_2(C_2O_4)^{2-}$	pH 5.1 with 20% MeOH	9.2×10^{-3}	[22]
	pH 5.0	4.9×10^{-3}	[26]

*Lower limit as calculated by $k_{cat}/K_m^{H_2O_2}$. †Bromide oxidation did not occur in water.





Recently we discovered that titanium (IV) grafted mesoporous silicate materials catalyze peroxidative halogenation reactions at neutral pH [33••]. Mesoporous silicate materials contain large surface areas (1000–1600 m^2/g) and variable pore diameters (20–150 Å) [34], making them attractive candidates for catalytic applications. The reactivity at neutral pH or in organic solvents suggests that bound tmi-peroxide must be activated by hydrogen-bonding interactions from a neighboring silanol hydroxyl group or a titanolhydroxyl group (in the case of the Ti/MCM materials).

Phosphatase activity of apo(V)-chloroperoxidase

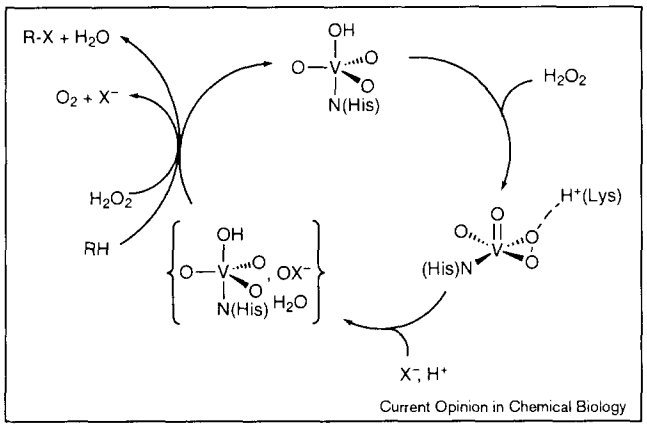
The amino acid sequence of vanadium chloroperoxidase was determined to be homologous with three families of acid phosphatases, which were previously considered unrelated [35••,36•,37•]. This sequence similarity raises questions about the phosphatase activity of apoV-ClPO and whether the acid phosphatases could coordinate vanadate and carry out peroxidative halogenation chemistry. Apo(V)-ClPO was found to have phosphatase activity, catalyzing the hydrolysis of *p*-nitrophenol phosphate (*p*-NPP) [35••]. In addition, *p*-NPP displaces vanadate from V-ClPO. At this point, haloperoxidase activity of vanadium-associated acid phosphatases has not been reported.

On the mechanism of the vanadium haloperoxidases

Our understanding of the catalytic activity of V-BrPO and V-ClPO is summarised in Figure 9. It also raises new questions. What controls the specific turnover activity, which can vary over 1000-fold depending on the source of the enzyme? What defines the basis of the halide specificity in V-BrPO and V-ClPO? The enzyme kinetics show saturation in halide for V-ClPO and V-BrPO, indicating that halide binds to the enzyme. Messerschmidt and Wever [7••] propose that the hydrophobic residues Trp350 and Phe397 form a chloride-binding site along with His404 in V-ClPO; a hydrophobic binding site for halides is observed in other proteins such as haloalkane dehalogenase [38] and certain amylases [39]. Trp350 is present in V-BrPO (*A. nodosum*), but Phe397 is replaced by a histidine residue in V-BrPO, which raises the question of whether these residues define the halide specificity.

Chloride coordination to the vanadium(V) center before oxidation by bound peroxide has been proposed [6••], yet halide ligands are particularly labile in aqueous solutions of vanadium(V) complexes. Moreover, the coordinatively saturated oxalateoxodiperoxomolybdenum(VI) complex catalyzes halide oxidation [22,26], suggesting that halide coordination is not necessarily a prerequisite for its oxidation. The state of the oxidized halogen intermediate immediately following halide oxidation is not known. Does a vanadium(V)/hypohalite complex form and what is the stability and reactivity like? Conte *et al.* cite evidence for a hypobromite-like vanadium(V) intermediate in their two-phase system [40]. In this regard, further investigations in the V-HPOs will be very interesting.

Figure 9



Summary of V-BrPO catalysis.

A look to the future

As we look to the future, studies on the origin and evolution of the vanadium haloperoxidases and related acid phosphatases can be anticipated [35••,36•,37•], in addition to studies on the haloperoxidase activity of vanadate-associated acid phosphatases. The biogenesis of the chiral halogenated marine natural products and the selectively halogenated or oxidized indole marine natural products has now become a tractable goal, given the selectivity of V-BrPO. Finally, the design of new functional mimics of the V-HPOs to test our understanding of the important structural features of the vanadium haloperoxidases and the important catalytic residues in these enzymes is also anticipated.

Acknowledgements

AB gratefully acknowledges grants from the National Science Foundation (CHE96-27374) and the AP Sloan Foundation. Partial support for this work is also sponsored by NOAA, US Department of Commerce under grant number NA66RGO447, project number R/MP-76 through the California Sea Grant College System and in part by the California State Resources Agency. The views expressed herein are those of the author and do not necessarily reflect the views of NOAA. The US Government is authorized to reproduce and distribute for governmental purposes.

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- •• of outstanding interest

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