39 VANADIUM-DEPENDENT REDOX ENZYMES: VANADIUM HALOPEROXIDASE

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The two types of naturally occurring vanadium-containing proteins that have been identified to date are the vanadium haloperoxidases and vanadium nitrogenase. In addition to these, there are many other examples of vanadium in biological systems or biological-type roles for vanadium, including vanadate as a phosphate analog in both the inhibition and activation of phosphate enzymes, vanadium compounds as insulin mimics, vanadate-sensitized photocleavage and photooxidation of proteins, and vanadium in ascidians and amavadin, among others; the reader is referred to two books covering the bioinorganic chemistry of vanadium, including reviews of these topics: *Vanadium and Its Role in Life*, edited by Sigel and Sigel (1995) and *Vanadium in Biological Systems: Physiology and Biochemistry* edited by Chasteen (1990). The scope of this chapter will be limited to the vanadium haloperoxidases, vanadium bromoperoxidase, and vanadium chloroperoxidase. Vanadium nitrogenase is covered in the entry on the nitrogenases.

Introduction to Vanadium Haloperoxidases

General Considerations

Haloperoxidases are enzymes that catalyze the oxidation of a halide (i.e., chloride, bromide or iodide) by hydrogen peroxide, a process which can result in the concomitant halogenation of organic substrates (R—H) (equation 1) or the production of hypochlorous acid depending on the haloperoxidase (van Schijndel et al., 1994).

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

The nomenclature for the haloperoxidases has traditionally been based on the most electronegative halide which can be oxidized by hydrogen peroxide. (Fluoride is not a substrate because hydrogen peroxide does not have the potential to oxidize it.) Thus chloroperoxidase (ClPO) catalyzes the oxidation of chloride, bromide, and iodide by hydrogen peroxide; bromoperoxidase (BrPO) catalyzes the oxidation of bromide and iodide by hydrogen peroxide; while iodoperoxidase catalyzes the oxidation of only iodide by hydrogen peroxide. These enzymes would be better named on the basis of their physiological function; however, in most cases the physiological function is not known with certainty (see below). The physiological reactivity probably depends on the concentration and nature of the halide, hydrogen peroxide, pH, and the organic substrate.

Both vanadium-containing haloperoxidases and iron–heme-haloperoxidases (e.g., chloroperoxidase from the fungus *Caldaromyces fumago* (Hager et al., 1966) and those from mammalian haloperoxidases (Neidleman and Geigert, 1986) such as eosinophil peroxidase, myeloperoxidase, salivary peroxidase, and lactoperoxidase are well known; however, this article will focus solely on the vanadium haloperoxidases.[1] Bacterial haloperoxidases which lack a metal cofactor are also known (Hecht et al., 1994; van Pee et al., 1987; Weisner et al., 1988).

Vanadium haloperoxidases have been isolated from all classes of marine algae (Butler and Walker, 1993), terrestrial fungi (van Schijndel, 1993a), and a lichen (Plat et al., 1987). The marine haloperoxidases are thought to function in the biosynthesis of the numerous halogenated marine natural products, which range from relatively simple volatile halogenated hydrocarbons (e.g., $CHBr_3$, CH_2Br_2, $CHBr_2Cl$; Gschwend et al., 1985) to halogenated phenols (Fenical, 1975), terpenes and indoles (Faulkner, 1993), to name just a few classes (Figure 1). Some halogenated natural products have been shown to play a defensive role, displaying, for example, antifungal, antimicrobial, and ichthyotoxic activities.

Standard Assay for Haloperoxidase Activity

The standard assay for haloperoxidase activity is the halogenation of monochlorodimedone (MCD; 2-chloro-5,5-dimethyl-1,3-dimedone) using hydrogen peroxide as the oxidant of the halide (Scheme 1; Hager et al., 1966).

The halogenation of MCD is followed spectrophotometrically at 290 nm, which monitors the loss of MCD in the enol form (ε = 20 000 l mol^{-1} cm^{-1}). The dichlorodimedone or bromochlorodimedone species absorb negligibly at 290 nm (i.e., ε ~ 100 l mol^{-1} cm^{-1}). Bromoperoxidase and chloroperoxidase activities are expressed as μmoles MCD brominated per min per mg of enzyme (i.e., units(U)

[1]A non-heme iron bromoperoxidase was initially reported in certain species of marine red algae (Itoh et al., 1985, 1987b). These enzymes have now been shown to be vanadium bromoperoxidases (Krenn et al., 1989b; Everett et al., 1990b).

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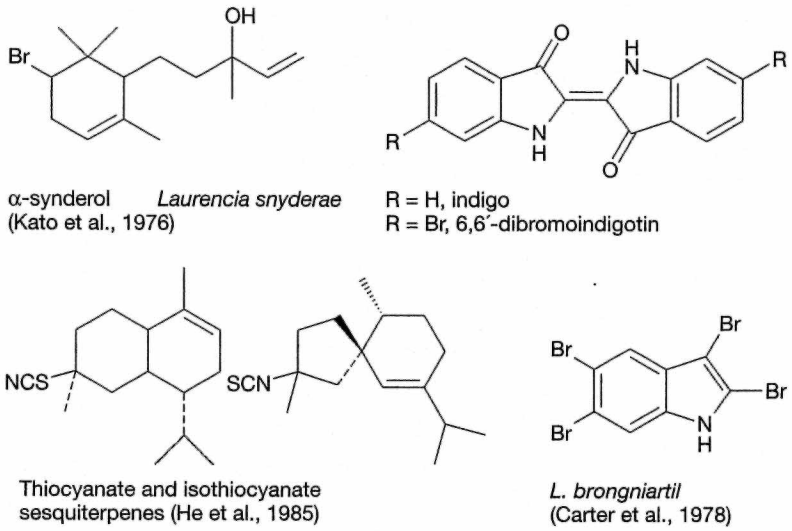
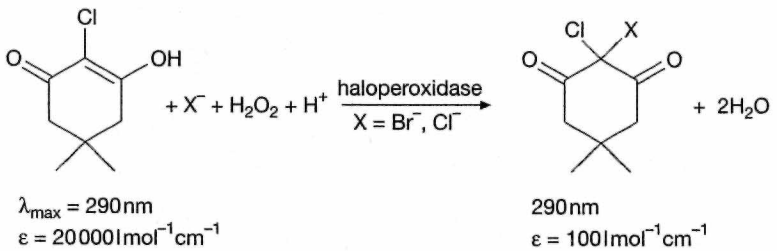


Figure 1 Selected halogenated or pseudohalogenated marine natural products.



Scheme 1

mg^{-1}). The formation of bromochlorodimedone and dichlorodimedone has been verified (Manthey and Hager, 1981; Soedjak, 1991). Iodination of MCD does not occur. The early work on V-BrPO employed the oxidation of iodide by hydrogen peroxide (Vilter, 1984), forming triiodide (I_3^-) which was followed spectrophotometrically at 353 nm (ε = 26 400 l mol^{-1} cm^{-1}) (equation 2).

$$3\,I^- + H_2O_2 + 2\,H^+ \xrightarrow{\text{V-BrPO}} I_3^- + 2\,H_2O \qquad (2)$$

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 (equation 3) and reduction of triiodide by hydrogen peroxide (equation 4).

$$3\,I^- + H_2O_2 + 2\,H^+ \xrightarrow{\text{uncatalyze}} I_3^- + 2\,H_2O \quad (3)$$

$$I_3^- + H_2O_2 \longrightarrow O_2 + 3\,I^- + 2H^+ \quad (4)$$

Structural Features of Vanadium Haloperoxidases

The structure of vanadium chloroperoxidase (V-ClPO), isolated from the fungus *Curvularia inaequalis*, has been reported (Messerschmidt and Wever, 1996). V-ClPO is a 67 kDa protein which catalyzes the oxidation of chloride by hydrogen peroxide (see below). The x-ray structure (2.11 Å resolution) revealed a pentagonal bipyramidal vanadium(V) site ligated by histidine-496, azide, and three nonprotein oxygen atoms (Plate 8). The azide ligand resulted from the azide-containing crystallization buffer and is reportedly replaced by hydroxide in the native enzyme (Messerschmidt and Wever, 1996). Thus V-ClPO is comprised of vanadate coordination by one protein ligand (i.e., His-496). The negative charge of the vanadate ion is balanced, in part, by multiple hydrogen-bonding between the vanadate oxygen atoms and the protein. Lys-353, Arg-360, Arg-390, Ser-402, and the amide nitrogen of Gly-403 are

hydrogen-bonded to the vanadate oxygen atoms, stabilizing vanadate coordination to the protein.

The overall secondary structure of V-ClPO is α-helical (Plate 9; Messerschmidt and Wever, 1996).

Two four-helix bundles form the main structural motifs of the tertiary structure; vanadate is coordinated at the top of one of these helical bundles in a broad channel (Figure 2). One half of the channel is lined by hydrophobic residues: Pro-47, Pro-211, Try-350, Phe-393, Pro-395, Pro-396, and Phe-397. The other half of the channel is predominantly polar with several main-chain carbonyl oxygens and an ion pair formed between Arg-490 and Asp-292 (Messerschmidt and Wever, 1996). His-404, present in the active-site channel in close proximity to the vanadate ion, is thought to function in acid–base catalysis; it must be protonated for H_2O_2 to bind to V-ClPO (van Schijndel et al., 1994, and see below). In addition Messerschmidt and Wever (1996) propose a putative chloride binding site formed by Trp-350 and Phe-397 as well as His-404. Such a hydrophobic site is analogous to chloride binding sites in other proteins, e.g., haloalkane dehalogenase (Verschueren et al., 1993) and certain amylases (Machius et al., 1995).

While the full sequence of V-BrPO (*Ascophyllum nodosum*) has not been reported, there is sequence similarity between V-ClPO and V-BrPO, including the regions containing the histidine ligand, His-496, the acid/base histidine, His-404 in the active-site channel, and four of the five amino acid residues which hydrogen-bond to the vanadate oxygen atoms (i.e., Arg-360, Ser-402, Gly-403, and Arg-490; Messerschmidt and Wever, 1996; Simons et al., 1995; Vilter, 1995). The fifth hydrogen-bonding amino acid, Lys-353 in V-ClPO, is an asparagine in V-BrPO. Trp-350 is present in both V-ClPO and V-BrPO; however, Phe-397 is replaced by a histidine residue, leading to speculation regarding the basis of the halide selectivity of each enzyme.

In light of the sequence similarity of V-BrPO (*A. nodosum*) and V-ClPO, it seems reasonable to assume the vanadium(V) site in V-BrPO (*A. nodosum*) can be depicted as vanadate coordination to the protein by one histidine ligand and multiple hydrogen bonds between the vanadate oxygen atoms and positively charged protein side-chains and a main-chain amide nitrogen in a trigonal bipyramidal geometry (see Figure 2; Messerschmidt and Wever, 1996). In addition, the bond valence sum analysis (Butler and Clague, 1995; Carrano et al., 1994)[2] and

[2]Bond valence sum (BVS) analysis is an empirical correlation between the oxidation state of the transition metal ion and its coordination geometry and bond lengths (Liu and Thorp, 1993).

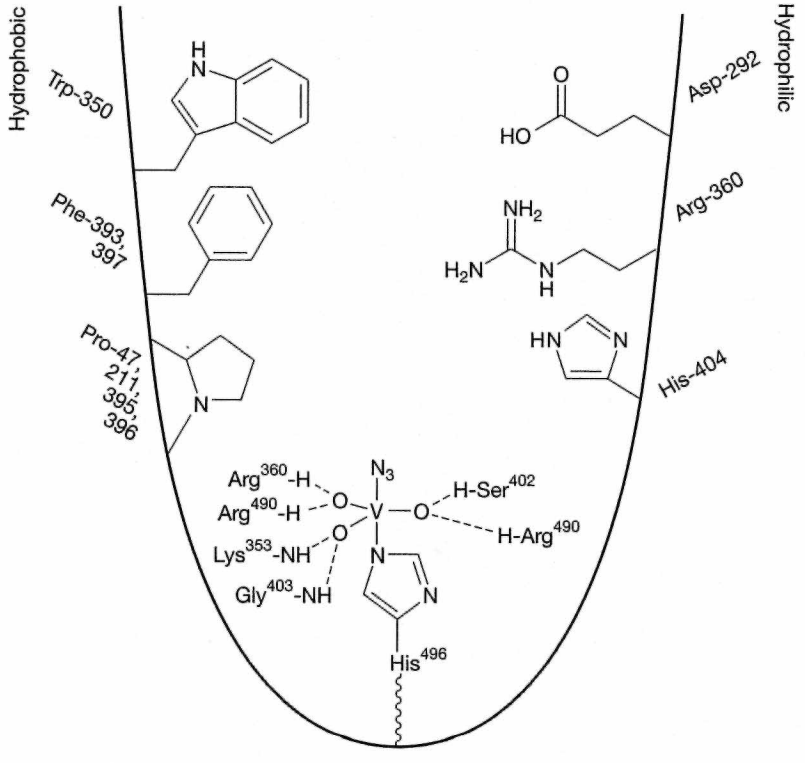


Figure 2 V–ClPO active site.

the extended x-ray absorption fine structure (EXAFS) analysis (Arber et al., 1989) of V-BrPO (*A. nodosum*) are consistent with a trigonal bipyramidal vanadium site in V-BrPO. Crystallization of V-BrPO (*A. nodosum*) was reported some time ago, although refined structural data have not been reported yet; the crystals defracted to 2.4 Å resolution (Muller-Fahrnow et al., 1988).

The native vanadium(V) states of V-BrPO and V-ClPO can be reduced, forming the respective vanadium(IV) derivatives (Arber et al., 1989; van Schijndel et al., 1993a). The EPR spectra of both V-BrPO and V-ClPO are nearly identical and show an axially symmetric vanadyl site (deBoer et al., 1987a; van Schijndel et al., 1993a). The **EXAFS** spectrum (see Lexicon) of reduced V-BrPO (*A. nodosum*) is consistent with formation of vanadyl-BrPO in which the V(IV) site is coordinated by a single terminal oxide ligand at 1.63 Å, and five O(N) ligands, of which three are at 1.91 Å and two are at 2.11 Å (Arber et al., 1989). Electron spin echo results of this derivative indicate that a nitrogen ligand is coordinated in the equatorial plane (deBoer et al., 1988). The x-ray structure of native V^{V}-ClPO, however, shows axial coordination of HIS-496. Thus it is possible that the coordination environment of the VO^{2+} derivative differs from that of the vanadium(V) state or that the coordination geometry of V-ClPO differs from that of V-BrPO. V-BrPO is ESR silent under turnover conditions (i.e., in the presence of Br^- and H_2O_2).

Vanadium Bromoperoxidase

Characteristics of V-BrPO

Vanadium bromoperoxidases are all acidic glycoproteins (deBoer et al., 1986a; Krenn et al., 1989a) with very similar amino acid composition (Wever et al., 1988), molecular mass charge (pI 4–5), and vanadium content. The subunit molecular mass of V-BrPO is ~65 kDa. As isolated V-BrPO contains a substoichiometric ratio of vanadium per subunit. However, a content of 1 gram-atom of vanadium per subunit can be achieved by addition of excess vanadate and subsequent removal of adventitiously bound vanadium(V) by dialysis (deBoer et al., 1986b; Muller-Fahrnow et al., 1988; Soedjak and Butler). Isozymes of vanadium bromoperoxidases from *A. nodosum* have been isolated which differ in carbohydrate content (Everett et al., 1990b; Krenn et al., 1989a). 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 is located in both the cortical and surface protoplasts of *Macrocystis pyrifera* (Butler et al., 1990), *Laminaria saccharina*, and *Laminaria digitata* (Jordan et al., 1990). In addition, the biosynthesis of V-BrPO in the protoplasts of *Laminaria saccharina* has been demonstrated using [^{35}S]methionine tracer studies (Jordan et al., 1990).

In light of the very simple vanadium site in V-ClPO, i.e., vanadate coordination to the protein by one histidine ligand, and the proposal that the vanadate site in V-BrPO (*A. nodosum*) is very similar, it is interesting to note that incubation of V-BrPO in phosphate buffer produces the inactive apoprotein derivative (Soedjak et al., 1991). Inactivation by phosphate occurs much faster at low pH (~4) than at neutral or higher pH values. Phosphate inactivation does not occur in the presence of hydrogen peroxide, which suggests that the mechanism of vanadium(V) release is through a phosphate–vanadate complex; the formation of such a complex could be inhibited by peroxide coordination to vanadium(V) (Soedjak et al., 1991).

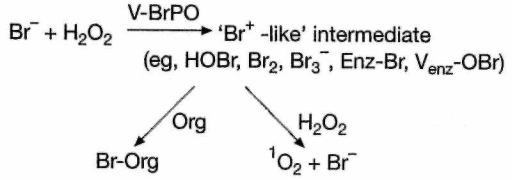
The activity of the apo-BrPO can be fully restored by addition of vanadate (deBoer et al., 1987a). Addition of other metal ions did not restore bromoperoxidase activity (Vilter, 1984). Vanadium(V) is only fully incorporated in the absence of phosphate (deBoer et al., 1987a; Soedjak et al., 1991). Like phosphate, molybdate, arsenate, tetrafluoroaluminate, and tetrafluoroberrylate inhibit coordination of vanadium(V) to apo-BrPO (Tromp et al., 1991).

V-BrPO is particularly stable to strong oxidants such as singlet oxygen ($^1O_2$, $^1\Delta_g$), and oxidized bromine and chlorine compounds (HOBr, HOCl, etc.) (Everett et al., 1990b). 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 and water, or 40% (v/v) 1-propanol and water (deBoer et al., 1987b). In addition, V-BrPO retains activity after immobilization on solid support media, e.g., photocross-linked to DEAE Cellulofine (Itoh et al., 1987a).

Reactivity of V-BrPO

Halogenation and Halide-assisted Disproportionation of Hydrogen Peroxide V-BrPO catalyzes peroxidative halogenation reactions (Vilter, 1984; Soedjak and Butler, 1990a; Wever et al., 1985) and the halide-assisted disproportionation of hydrogen peroxide (Everett and Butler, 1989). In the first step, the enzyme catalyzes the oxidation of the halide by hydrogen peroxide, producing an intermediate that is a two-electron-oxidized halide species; hypobromous acid, bromine, tribromide, or an enzyme-bound bromonium ion equivalent are all consistent with the

in vitro reactivity of the enzyme. (At this point the organic substrate is not considered to be bound to the enzyme; see below for further consideration.) 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 2 for bromide (Everett and Butler, 1989; Everett et al., 1990a).



Scheme 2

V-BrPO-catalyzed bromination is shown to be an electrophilic (Br^+) as opposed to a radical ($Br\bullet$) process (Soedjak et al., 1995) through product analysis of the bromination of 2,3-dimethoxytoluene (DMT). Only a single product, ring-brominated 2,3-dimethoxytoluene, was observed at pH 6.5 and pH 4, indicative of electrophilic (Br^+) bromination. 2,3-Dimethoxybenzyl bromide, the product expected from a radical ($Br\bullet$) bromination pathway, was not observed (Soedjak et al., 1995). No products are observed in the absence of enzyme because the oxidation of bromide by hydrogen peroxide in the neutral pH range is very slow.

In the case of bromide, the dioxygen formed is in the singlet excited state ($^1O_2$;$^1\Delta_g$) which was identified spectroscopically by characteristics of the near-infrared emission (Everett et al., 1990b). Singlet oxygen ($^1\Delta_g$) is a well established product of the oxidation of H_2O_2 by HOBr, HOCl (Kanofsky, 1989), and bromamines (Kanofsky, 1989). Recent $H_2{}^{18}O_2$ tracer studies now establish that each atom of oxygen in dioxygen originates from the same molecule of hydrogen peroxide, which is also consistent with singlet oxygen production (Soedjak et al., 1995).

The rate of dioxygen formation in the absence of an organic substrate and the rate of bromination of monochlorodimedone (at > 75 μmol l^{-1} MCD) are the same, which supports the mechanism in Scheme 2; the reactive intermediate is common to both pathways and is formed in a rate-limiting step (Everett and Butler, 1989). 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 (Soedjak et al., 1995). In addition, competitive dioxygen formation is strongly enhanced at higher pH.

Enzyme Kinetics The steady-state kinetic analysis of the rate of MCD bromination (Everett et al., 1990a; deBoer and Wever, 1988) and dioxygen formation (Everett et al., 1990a; Soedjak and Butler, 1991) catalyzed by V-BrPO from *A. nodosum*, *M. pyrifera*, and *F. distichus* fit a substrate-inhibited **bi–bi ping-pong** kinetic mechanism (see Lexicon), in which the substrates bromide and hydrogen peroxide are also noncompetitive inhibitors at certain pH values (Everett et al., 1990a; Soedjak et al., 1995). The kinetic parameters (K_m^{Br}, K_m^{H2O2}, K_{ii}^{Br}, K_{is}^{Br}) obtained in the dioxygen formation reaction and the MCD bromination reaction agree within a factor of 2, providing further evidence that the rate-limiting steps are the same for both the bromination of MCD and the bromide-assisted disproportionation of hydrogen peroxide (Everett et al., 1990a). At pH 6.5, the K_m^{Br} and K_m^{H2O2} are ~22 mmol l^{-1}, and ~100 μmol l^{-1}, respectively (taken as the average of the K_m values for the MCD bromination reaction and the dioxygen formation reaction (Everett et al., 1990a)). Bromide inhibition is strongest at pH 5–5.5 for V-BrPO from the three sources examined (Everett et al., 1990a; Soedjak and Butler, 1991).

Hydrogen peroxide also inhibits V-BrPO. The inhibition could only be investigated using the bromide-assisted dioxygen formation reaction because the kinetics of MCD bromination reaction were complicated by competing dioxygen formation at the high concentrations of hydrogen peroxide required for inhibition. Hydrogen peroxide inhibition increases with increasing pH (i.e., 312 mmol l^{-1} at pH 5.5, to 64 mmol l^{-1} at pH 8) (Soedjak et al., 1995). The inhibition is noncompetitive at all pH values; K_{ii}^{H2O2} is equal to K_{is}^{H2O2}, showing that bromide does not affect the inhibition by H_2O_2 (Soedjak et al., 1995). The inhibition is fully reversible under the conditions of the initial steady-state kinetic experiments (i.e., short reaction times) (Soedjak et al., 1995).

The steady-state kinetic studies on V-BrPO (*A. nodosum*) reveal that an active-site residue with a pK_a of ~5.7 is required to be deprotonated prior to binding of hydrogen peroxide (deBoer and Wever, 1988). Under peroxide inhibition conditions, the steady-state kinetic constants indicate that an ionizable group with a pK_a between 6.5 and 7 is involved in the inhibition (Soedjak et al., 1995). It seems reasonable to assume, given these pK_a values, that this residue is histidine, i.e, specifically the histidine in V-BrPO analogous to His-404 in V-CIPO.

Fluoride is a competitive inhibitor of H_2O_2 binding in both the MCD bromination reaction and the bromide-assisted disproportionation of hydrogen peroxide (Everett et al., 1990a). Fluoride inhibition (K_i^F) is noncompetitive with respect to bromide (Everett et al., 1990a).

Reversible Inactivation of V-BrPO by Hydrogen Peroxide As discussed above, H_2O_2 is a fully reversible, noncompetitive inhibitor of V-BrPO when the steady-state kinetics are observed during the initial portion of the reaction (e.g., first 10 min at the highest concentrations of H_2O_2). If the reaction proceeds for longer times, the specific activity of V-BrPO decreases. Between pH 6 and 8, the inactivation that occurs on prolonged turnover can be fully reversed by the addition of vanadate (Soedjak et al., 1995). The increase in activity upon addition of vanadate suggests that protein-bound vanadium is released under turnover at high concentrations of hydrogen peroxide, forming the apoenzyme derivative. At pH 4 and 5, inactivation also occurred under turnover; however, this turnover was not reversed on addition of vanadate (Soedjak et al., 1995).

Irreversible Inactivation of V-BrPO under Turnover at Low pH and the Formation of 2-Oxohistidine The irreversible inactivation of the V-BrPO (*A. nodosum*) that occurs under turnover conditions at low pH (i.e., 15 nmol l^{-1} to 100 mmol l^{-1} H_2O_2, 0.1 mol l^{-1} KBr, ~15 nmol l^{-1} V-BrPO in 0.1 mol $^{-1}$ citrate, pH 4 or 5) was found to produce 2-oxohistidine as identified by HPLC using electrochemical detection (Winter and Butler, 1996). Formation of 2-oxohistidine required all the components of turnover (i.e., bromide, hydrogen peroxide, and V-BrPO) as well as low pH; inactivation did not occur, nor was significant 2-oxohistidine formed in the presence of hydrogen peroxide alone. The oxidation of histidine did not occur by singlet oxygen generated by V-BrPO, because neither 2-oxohistidine nor inactivation occurred under the conditions in which singlet oxygen is produced quantitatively by V-BrPO. The addition of aqueous bromine to N^{α}-benzoylhistidine at low pH formed N^{α}-benzoyl-2-oxohistidine. Several functional mimics of V-BrPO (*cis*-VO^{2+} in strong acid; $MoO(O_2)_2(oxalato)^{2-}$ at pH 5) catalyzed the oxidation of N^{α}-benzoylhistidine to N^{α}-benzoyl-2-oxohistidine mediated by bromide oxidation by hydrogen peroxide. Furthermore, when hypobromite was added to N^{α}-benzoylhistidine in the presence of hydrogen peroxide at neutral pH, conditions under which HOBr would react first with H_2O_2 to produce singlet oxygen, N^{α}-benzoyl-2-oxohistidine was not formed. Thus, the oxidation of histidine in V-BrPO is mediated via oxidized bromine species (Scheme 3).

Irreversible inactivation V-BrPO was also accompanied by release of vanadium (Winter and Buder, 1996).

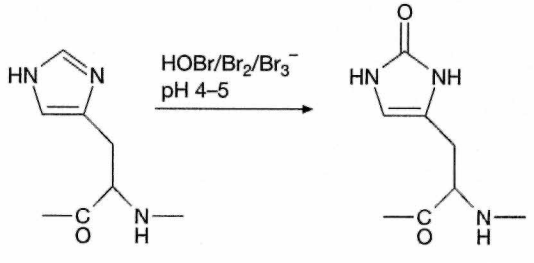
Halide Reactivity In addition to the oxidation of bromide and iodide discussed above, V-BrPO catalyzes the oxidation of chloride (Soedjak and Butler, 1990a), as well as pseudohalides such as thiocyanate, azide and cyanide (Soedjak, 1991; Walker and Butler, 1996). The overall reactivity of V-BrPO with bromide is similar to that of V-BrPO with chloride. The rate of chlorination of MCD is equivalent to the rate of the chloride-assisted dioxygen formation reaction (see Scheme 2, substituting chlorine for bromine).

The overall reactivity of V-BrPO with bromide and chloride does differ, however, in the presence of amines (primary, secondary, and tertiary) (Soedjak and Butler, 1990a). The rate of dioxygen formation is much slower in the presence of amines owing to the formation of chloramines, which can be observed spectrophotometrically by their characteristic UV absorption maxima (equation 5).

$$NH_2R + Cl^- + H_2O_2 + H^+ \rightarrow NHRCl + 2H_2O \qquad (5)$$

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 (Soedjak and Butler, 1990a), but because the rate of chlorination of the organic substrate by the chloramine is very rapid, we cannot determine whether MCD chlorination proceeds through a chloramine intermediate.[3] Haloamine intermediates may be important if active-site intermediates are formed during the halogenation reaction.

The pseudohalides The reduction potentials for the oxidation of I^-, Br^-, and Cl^- span a range from 0.53 to 1.36 V (vs NHE) (Table 1). The potentials for the oxidation of the pseudohalides fall within or below



Scheme 3

[3]Bromamine formation was not observed with V-BrPO using hydrogen peroxide as the oxidant (Soedjak and Butler, 1991) because bromamines are rapidly reduced by hydrogen peroxide, forming singlet oxygen and bromide (Kanofsky, 1989). Bromamines were observed using acyl peracids in place of hydrogen peroxide (Soedjak and Butler, 1991); see the section on peroxide reactivity.

Table 1 Reduction potentials (vs NHE) for the oxidation of halide, thiocyanate and cyanide

Cl^-	1.36 V
Br^-	1.05 V
NCS^-	0.77 V
I^-	0.53 V
CN^-	0.375 V

this range, suggesting that vanadium haloperoxidases may also catalyze the oxidation of these anions. Indeed, both cyanide ($\varepsilon^0 = 0.375$ V) and thiocyanate ($\varepsilon^0 = 0.77$ V) inhibit bromination catalyzed by V-BrPO through preferential oxidation of the pseudohalide (Wever and Kustin, 1990; Walker and Butler, 1996). ^{13}C NMR spectroscopy of the oxidation of $KS^{13}CN$ (133.4 ppm vs TMS) by H_2O_2 catalyzed by V-BrPO shows the formation of several oxidized thiocyanate species, including the putative dithiocyanate 'ether' (NCS—O—SCN; 127.6 ppm), which is unstable, hypothiocyanate ($OSCN^-$; 128.6 ppm), thiooxime ($SCNO^-$ or SCNOH; 156.5 ppm), as well as bicarbonate (HCO_3^-; 160.2 ppm). In addition, V-BrPO catalyzes the thiocyanation of 1,3,5-trimethoxybenzene and 1,2-dimethylindole to 1-thiocyanato-2,4,6-trimethoxybenzene and 1,2-dimethyl-3-thiocyanatoindole, respectively. Azide, on the other hand, is a mechanism-based inactivator of V-BrPO (Everett, 1990; Soedjak, 1991).

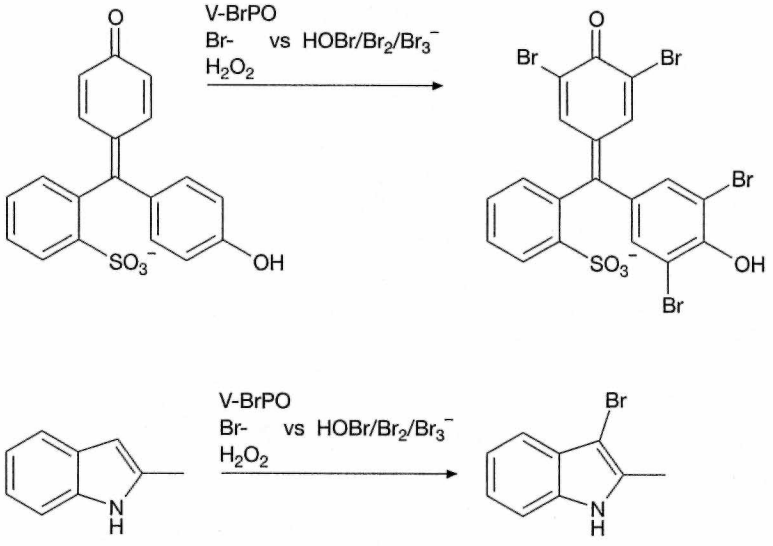
Peroxide Reactivity In place of hydrogen peroxide, V-BrPO can use acyl peracids (i.e., peracetic acid, *m*-chloroperoxybenzoic acid, *p*-nitroperoxybenzoic acid, phenylperacetic acid) as the oxidant of the halide, but not alkylhydroperoxides (i.e., ethyl hydroperoxide, cuminyl hydroperoxide, *t*-butyl hydroperoxide) (Soedjak and Butler, 1990b). The peracid reactivity results from direct use of peracid and not from hydrogen peroxide which could be formed from peracid hydrolysis (Soedjak and Butler, 1990b). One result of the acyl peroxide-containing reactions is the formation of bromamine species when the reaction is carried out in the absence of an organic substrate and in the presence of an amine (including buffers or amino acids) (Soedjak and Butler, 1990b). Because acyl peroxides are not efficient two-electron reductants, the BrNHR species can build up in solution. Bromamines have been proposed as possible intermediates in other haloperoxidase reactions (Kanofsky, 1989; Neider and Hager, 1985; Soedjak and Butler, 1990b). Dioxygen is not formed in the acyl peroxide-containing reactions 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 (several minutes).

Mechanistic Considerations

Nature of the Oxide Halogen Intermediate and Organic Substrate Binding to V-BrPO The nature of the oxidized intermediate as enzyme bound or released and the role of organic substrate binding to V-BrPO is a topic of much interest (Butler and Walker, 1993). Under optimum reaction conditions (i.e., pH 6.5), oxidized bromine species (e.g., HOBr, Br_2, Br_3^-, Enz-Br) cannot be detected because reaction of these species with excess H_2O_2 to produce $^1O_2$ or with organic substrates is too fast. Through competitive kinetic studies comparing the reactivity of V-BrPO with HOBr, we have demonstrated that the nature of the halogenating species produced by V-BrPO depends on the nature of the organic substrate (Tschirret-Guth and Butler, 1994). V-BrPO does not release an oxidized bromine species (e.g., HOBr, Br_2, Br_3^-) in the presence of certain indole derivatives, as established by competitive bromination against phenolsulphonephthalein (phenol red) (Scheme 4) (halogenated indoles are marine natural products).

V-BrPO preferentially brominates 2-methylindole, as shown by a lag phase in the appearance of bromophenol blue. The lag phase is proportional to the concentration of 2-methylindole, although after the lag phase, the rate of bromination of phenol red is independent of the 2-methylindole concentration. By comparison, a lag phase is not observed in the competitive bromination of 2-methylindole and phenol red by HOBr; under these conditions, bromination of 2-methylindole and phenol red occur concurrently and an increase in the 2-methylindole concentration leads to a decrease in the appearance of bromophenol blue. This differential reactivity between V-BrPO and HOBr suggests that released HOBr is not the active brominating species in the V-BrPO-catalyzed reactions of 2-methylindole, a situation arising from indole binding to V-BrPO. Fluorescence quenching of 2-phenylindole by V-BrPO established that the indole binds to V-BrPO; the binding constant from the Stern–Volmer analysis is 1.1×10^5 l mol^{-1} (Tschirret-Guth and Butler, 1994).

Further evidence that the enzyme-catalyzed bromination of indoles is not mediated by enzyme-released HOBr was established from comparison of the rate of V-BrPO-catalyzed bromide-assisted disproportionation of H_2O_2 (forming O_2) in the presence and absence of 2-methylindole versus the rate of oxidation of H_2O_2 by HOBr (forming O_2) in the presence and absence of 2-methylindole (Tschirret-Guth and

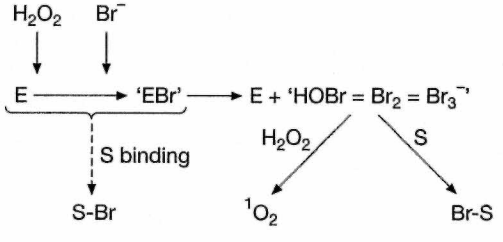


Scheme 4

Butler, 1994). In the enzyme reaction, indole bromination (i.e., the k_1[indole] pathway in Scheme 2) is favored over H_2O_2 oxidation (i.e., the $k_2[H_2O_2]$ pathway in Scheme 2), whereas in the nonenzymatic reaction (HOBr + H_2O_2 ± indole), H_2O_2 was preferentially oxidized by HOBr (Tschirret-Guth and Butler, 1994). The same kinetic behavior is observed for 2-phenylindole (Tschirret-Guth, 1996).

These results are the first to demonstrate organic substrate binding to V-BrPO, and that bromination of indoles by V-BrPO is not consistent with enzyme-released HOBr. A mechanistic scheme involving substrate binding is shown in Scheme 5 (Tschirret-Guth and Butler, 1994); 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 releases 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 indole bromination.

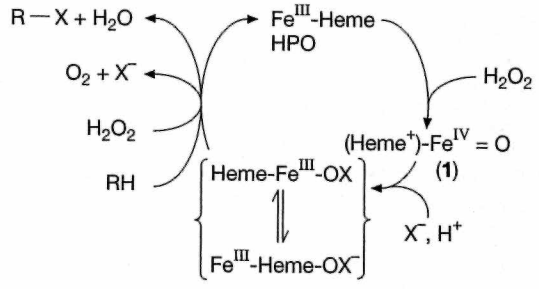
The Reaction Sequence and Role of Vanadium The first step of the V-BrPO catalytic cycle appears to be coordination of hydrogen peroxide to vanadium(V). Tromp and colleagues (1990) have reported a small decrease in absorbance (0.03 absorbance units for 20 μmol l^{-1} V-BrPO) between 300 and 340 nm upon addition of H_2O_2 to V-BrPO; upon subsequent addition of bromide, the original UV spectrum reappeared, consistent with bromide oxidation by an enzymatic peroxovanadium(V) species. In a different set of experiments, it was found that bromination of MCD did not occur when excess bromide was added to relatively high concentrations of V-BrPO (i.e., 20 μmol l^{-1}) and 50 μmol l^{-1}MCD (Soedjak, 1991). Thus V-BrPO does not oxidize bromide in the absence of a peroxide source (hydrogen peroxide or an acylperoxide) (Soedjak, 1991). Several questions still remain about the actual mechanism of oxidation of bromide. For example, it has not been established whether the halide coordinates to vanadium(V) prior to oxidation or whether the halide attacks the coordinated peroxide directly. Because the enzyme kinetics show



Scheme 5

saturation in bromide and chloride (Everett et al., 1990b; deBoer and Wever, 1988), a halide binding site exists, but the nature of this site remains to be determined.

Given the number of stable oxidation states available to vanadium, it is somewhat surprising that no direct evidence for an electron transfer role of vanadium has been detected in V-BrPO. The reaction is consistent with a Lewis acid role of vanadium(V), in which coordination of hydrogen peroxide to vanadium(V) serves to activate peroxide toward halide oxidation. The vanadyl-BrPO state is probably not an important component in the catalytic cycle because VO^{2+}-BrPO does not have bromoperoxidase activity and because an EPR signal is not observed during turnover conditions (deBoer et al., 1987a). The Fe-heme-containing haloperoxidases, including Fe-heme bromoperoxidase (Manthey and Hager, 1985), function as two-electron redox catalysts. (Scheme 6).



Scheme 6

Hydrogen peroxide oxidizes the Fe-heme moiety by two electrons, forming compound (**1**); compound (**1**) 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 (**1**) ((heme^{+})-Fe^{IV}=O), does not oxidize bromide directly.

Specific Activities The specific activities for peroxidation of chloride, bromide, and iodide differ substantially and depend on pH and halide and hydrogen peroxide concentrations as well as the source of the enzyme. The pH optimum for V-BrPO-catalyzed bromination of MCD 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 halides are also inhibitors. The specific bromoperoxidase activity varies depending on the source of V-BrPO: V-BrPO (*A. nodosum*), 170 U mg^{-1} at pH 6.5, 2 mmol l^{-1} H_2O_2, 0.1 mol l^{-1} Br^-, 50 μmol l^{-1} MCD, 0.2 mol l^{-1} Na_2SO_4; V-BrPO (*M. pyrifera*), 1730 U mg at pH 6; V-BrPO (*F. distichus*), 1580 U mg^{-1} (pH 6.5) (Soedjak and Butler, 1990b). The specific chloroperoxidase activity for V-BrPO (*A. nodosum*) is 0.76 U mg^{-1} (under conditions of 1 mol l^{-1} KCl, 2 mmol l^{-1} H_2O_2, 50 μmol l^{-1} MCD in 0.1 mol l^{-1} citrate buffer, pH 4.5) which is ~200 times less than the maximum specific bromoperoxidase activity (Soedjak and Butler, 1990a). The iodoperoxidase activity was measured by the formation of triiodide (λ_{max} = 350 nm). The maximum activity is reported at ~pH 6.3 under conditions of 0.8 mmol l^{-1} H_2O_2, 6.06 mmol l^{-1} KI in phosphate buffer (Vilter et al., 1983). The reported specific activity is 400 U mg^{-1} (Vilter, 1984); the reactivity of iodide has not been investigated as extensively as has the reactivity with bromide.

Vanadium Chloroperoxidase

Characteristics of V-ClPO

V-ClPO (*Curvularia inaequalis*) is a 67 488 Da protein consisting of 609 amino acid residues, as determined from the DNA sequence analysis (Simons et al., 1995). This protein lacks disulfide bonds, although two cysteine residues are present as free thiols. Two putative *N*-glycosylation sites were identified, but the protein is not glycosylated. V-ClPO is secreted from *C. inaequalis*. As isolated it may contain a variable content of vanadium, depending on the concentration of vanadate in the growth medium; however, one vanadium(V) per subunit can be achieved by addition of excess vanadate to the growth medium or to the purified protein (van Schijndel et al., 1993a, b).

Like V-BrPO, V-ClPO is stable in the presence of organic substrates, to elevated temperatures, and in the presence of high concentrations of strong oxidants (e.g., HOCl) (van Schijndel et al., 1994).

Reactivity of V-ClPO

V-ClPO catalyzes the chlorination of MCD using hydrogen peroxide as an oxidant of chloride. The specific chloroperoxidase activity is 7.5 U mg^{-1} (μmoles MCD chlorinated per min per mg enzyme) at pH 5.0 under conditions of 1 mmol l^{-1} H_2O_2, 5 mmol l^{-1} KCl, 50 mmol l^{-1} MCD in 0.1 mmol l^{-1} sodium acetate buffer (van Schijndel et al., 1993a). The $K_m^{Cl^-}$ is 0.25 mM at pH 4.5 (van Schijndel et al., 1993a). $K_m^{H_2O_2}$ decreased linearly between pH 3 to pH 5 with a slope of −1 (van Schijndel et al., 1994). Chloride is both a substrate and an inhibitor. At pH 3.1, chloride

inhibition is competitive: $K_i^{Cl^-}$ is 6 mmol l^{-1}; at pH 4.1 chloride inhibition becomes noncompetitive (van Schijndel et al., 1994). Nitrate also inhibits V-ClPO, competitively with respect to chloride and uncompetitively with respect to hydrogen peroxide at pH 5.5. The $K_i^{nitrate}$ is 2 mmol l^{-1} at pH 5.5.

In contrast to the reactivity of V-BrPO, V-ClPO generates HOCl, which can build up in solution and be detected spectrophotometrically (van Schijndel et al., 1994). In addition, HOCl could be separated from the reaction solution by ultrafiltration (30 000 Da molecular mass cut off membrane) (van Schijndel et al., 1994). Both of these experiments were carried out at pH 4.5, which means that the reduction of HOCl with excess H_2O_2 producing dioxygen is not efficient under the conditions of the experiment (i.e., 200 μmol l^{-1} H_2O_2, 1 mmol l^{-1} Cl^-, 27–64 nmol l^{-1} V-ClPO, pH 4.5). Since only ~50 μmol l^{-1} HOCl was detected starting with an initial concentration of 200 μmol l^{-1} H_2O_2, it was inferred that some reduction of HOCl by H_2O_2 could have occurred (van Schijndel et al., 1994).

Conclusions

The overall reactivities of V-BrPO and V-ClPO are similar in that each catalyzes the oxidation of halides by hydrogen peroxide. The active sites of these two enzymes are like to be similar, characterized by vanadate coordination to the protein by only one histidine residue. The kinetic studies of each enzyme suggest that a residue with a pK_a of 5.7–6.5 is important for reactivity; this residue has been identified as a histidine (i.e., His-404 in V-ClPO) (Messerschmidt and Wever, 1996). However, the basis of the preferential oxidation of chloride by V-ClPO and of bromide by V-BrPO remains a very interesting question. Halide saturation is observed in the enzyme kinetics with both V-BrPO and V-ClPO for both bromide and chloride, yet the halide binding site is not known with certainty. The favorable reactivity and stability of these enzymes make them attractive candidates for biocatalytic conversions. Recent results show that V-BrPO is a promising signal-generating catalyst in ELISA (enzyme-linked immunosorbent assays) (Friedman et al., 1995; Winter, 1996).

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