Exploring the Chemistry and Biology of Vanadium-dependent Haloperoxidases

Published, JBC Papers in Press, April 10, 2009, DOI 10.1074/jbc.R109.001602

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Nature has developed an exquisite array of methods to introduce halogen atoms into organic compounds. Most of these enzymes are oxidative and require either hydrogen peroxide or molecular oxygen as a cosubstrate to generate a reactive halogen atom for catalysis. Vanadium-dependent haloperoxidases contain a vanadate prosthetic group and utilize hydrogen peroxide to oxidize a halide ion into a reactive electrophilic intermediate. These metalloenzymes have a large distribution in nature, where they are present in macroalgae, fungi, and bacteria, but have been exclusively characterized in eukaryotes. In this minireview, we highlight the chemistry and biology of vanadium-dependent haloperoxidases from fungi and marine algae and the emergence of new bacterial members that extend the biological function of these poorly understood halogenating enzymes.

Vanadium

Vanadium is a trace element that is widely distributed in nature. After molybdenum, vanadium is the second most abundant transition metal in the ocean, with a concentration of 35–50 nm (1) and up to 100 mg/kg in carbon-containing sediments of marine origin. In fresh water, the concentration is reported as 1.3 μg/liter (50 nm), and in the Earth’s crust, vanadium is present at 100 ppm (1, 2). Vanadium exists in many oxidation states, with V(V) being the most common in sea water (1–3). Only the V(III), V(IV), and V(V) oxidation states are involved, however, in biological systems, where vanadium has limited distribution as an essential mineral in organisms such as sea squirts and mushrooms and as a cofactor in metabolic enzymes. The most prevalent form of vanadium at neutral pH is the oxyanion vanadate, which is an oxidizing agent that is structurally and electronically similar to phosphate (1–3). Hence, vanadate and vanadate derivatives have been employed to interrogate a range of enzymes that interact with phosphorylated substrates (3). Interestingly, acid phosphatase enzymes have evolved to accommodate vanadate as a redox cofactor (4, 5).

Vanadium-containing Enzymes

To date, two classes of vanadium-containing enzymes have been identified: vanadium nitrogenases and vanadium-dependent haloperoxidases (V-HPOs).2 Nitrogenases are utilized by nitrogen-fixing bacteria to reduce dinitrogen to ammonia. Although this metalloenzyme system commonly contains a molybdenum-iron cofactor, some bacteria produce additional nitrogenases that are genetically distinct and instead contain V-Fe or Fe-Fe central metals (6–8). Vanadium nitrogenases have been identified from a diverse group of diazotrophic microorganisms and are synthesized under molybdenum-limiting conditions. On the other hand, V-HPOs have a larger distribution in nature, where they are present in macroalgae, fungi, and bacteria (1, 4–5, 9). These enzymes, which contain a ligated vanadate ion, oxidize halide ions to their corresponding hypohalous acids at the expense of hydrogen peroxide and are classified by the most electronegative halide they oxidize. Thus, vanadium chloroperoxidases (V-CIPOs) oxidize chloride, bromide, and iodide, whereas vanadium bromoperoxidases (V-BrPOs) oxidize only bromide and iodide.

Vanadium Haloperoxidases

Halogens are an abundant component of the Earth’s biosphere, so it is of little surprise that nature produces a profuse number of organohalogens (10). In addition to their therapeutic potential for human health, halogenated natural products have important biological functions for the producing organism, ranging from chemical defense to signaling molecules.

Nature has evolved several strategies for incorporating halogens into organic molecules (11). Together with the heme-dependent haloperoxidases, V-HPOs utilize hydrogen peroxide to convert a halide ion (X⁻) to a hypohalite (–O⁻X) intermediate that is chemically equivalent to an electrophilic “X⁺”. However, unlike the heme enzyme, there is no change in the oxidation state of the V-HPO metal center during the synthesis of the halogenating agent. Hence, V-HPOs do not suffer from oxidative inactivation during turnover and have received increasing attention as biocatalysts for pharmaceutical applications given their tolerance for organic solvents and high temperatures (12, 13), their ability to halogenate a range of organic compounds in a regio- and stereospecific manner (9, 14, 15), and their ability to oxidize organic sulfides in the absence of halides (16, 17).

The majority of naturally occurring organohalogens are of marine origin, and nearly all brominated natural products are produced by marine organisms (10). Some of the most frequently reported halometabolites are produced by marine red algae (Rhodophyceae) and include halogenated indoles, terpenes, acetylenes, phenols, and volatile hydrocarbons (9, 10, 15, 18, 19). Among the red, brown, and green algae, Rhodyphyta are...
Vanadium-dependent Haloperoxidases

FIGURE 1. Relatedness of V-BrPOs, V-CIPOs, and acid phosphatases identified in fungi, algae, and bacteria and some of their associated chemistry. Phylogenetic analysis was performed using ClustalW (56), and the unrooted neighbor-joining tree was visualized by TreeView. The scale bar indicates 0.1 changes per amino acid. Sequence identification codes include Ci_VClPO from C. inaequalis (accession number X64820), hypothetical Rb_VClPO from Rhodopirellula baltica SH1 (CAD37192), hypothetical Ss1_VClPO from Streptomyces sp. CNQ-525 (AB550486), hypothetical Ss3_VClPO from Streptomyces sp. CNQ-525 (AB550491), hypothetical Ss4_VClPO from Streptomyces sp. CNQ-525 (AB550492), hypothetical Cs_VClPO from Cellulophaga sp. MED134 (ZP_01050453), An_VBrPO from A. nodosum (P81701), Co_VBrPO from C. officinalis (AAM46061), C1p_VBrPO from C. pilulifera (BAA31261), C2p_VBrPO from C. pilulifera (BAA31262), hypothetical Sys_VBrPO from Synechococcus sp. CC9311 (YP_731869), Fd_VBrPO from Fucus distichus (AAC35279), Ld1_VBrPO from L. digitata (CAD37191), Ld2_VBrPO from L. digitata (CAD37192), PI_ACP acid phosphatase from Prevotella intermedia (AB017537), Kp_ACP acid phosphatase from Klebsiella pneumonia (AJ250377), St_ACP acid phosphatase from Salmonella typhimurium (X63599), Ps_ACP acid phosphatase from Providencia stuartii (X64820), and Eb_ACP acid phosphatase from Escherichia coli (AB020481). Halogenated natural products are α-sny- droler (2), β-sny-droler (3), γ-sny-droler (4), and A80915C (6). Proposed substrates for V-HPOs are (E)-(−)-nerolidol (1) and SF2415B1 (5).

the richest in bromoperoxidase activity, with the genus Corallina possessing the highest activity (20, 21). V-BrPOs are proposed to be responsible for the halogenation of natural products produced by marine algae (15, 18), and the first V-HPO to be isolated and characterized was the V-BrPO from the brown alga Ascophyllum nodosum in 1984 (22). To date, V-BrPOs have been characterized from all major classes of marine algae (23–28) as well as terrestrial lichen (29). Their proposed role in the biosynthesis of brominated cyclic sesquiterpenes from marine red algae was established through in vitro chemoenzymatic conversion of (E)-(−)-nerolidol (Fig. 1, 1) to yield the marine natural products α-sny-droler (2), β-sny-droler (3), and γ-sny-droler (4) (15).

Fungi belonging to the dematiaceous hyphomycetes were discovered to produce haloperoxidases distinct from the heme haloperoxidases that could function at elevated pH and temperature (30). A non-heme chloroperoxidase was identified from the fungus Curvularia inaequalis in 1987 (31) and 6 years later was characterized as a V-CIPO (32). A second V-CIPO was later isolated from the fungus Embellisia didymospora (33); however, no halogenated metabolites have been reported to date from either source. Rather, fungal V-CIPOs are suggested to function physiologically in the generation of hypochlorous acid to facilitate the oxidative degradation of plant cell wall lignocellulose to allow for fungal hyphen penetration (1, 5). A related in vivo role has been proposed for some macroalgal V-HPOs that are present on the plant surface in which the hypohalous acids function as anti-fouling agents against opportunistic bacteria (5). A recent study analyzing gene expression in the brown alga Laminaria digitata has shed light on how V-BrPOs and vanadium-dependent iodoperoxidases are up-regulated upon defense elicitation (34).

Recently, V-CIPO-encoding genes were identified in prokaryotes. The cloning and sequencing of the 43-kb napyradiomycin biosynthetic gene cluster from marine sediment-derived actinomycetes revealed three homologous V-CIPO genes (35, 36). Through heterologous expression in a surrogate bacterial host, the napyradiomycin biosynthetic gene cluster from Streptomyces sp. CNQ-525 was shown to be wholly responsible for the synthesis of the chlorinated meroterpenoids, in which the putative V-CIPOs may be responsible for the chlorination and cyclization of SF2415B1 (Fig. 1, 5) to A80915C (6) in a manner reminiscent of that of sny-droler biosynthesis in Corallina officinalis.

Structure Relationships

Crystal structures are available for three V-HPOs, namely V-CIPO from the fungus C. inaequalis (37–39), V-BrPO from the brown alga A. nodosum (40), and V-BrPO from the red alga C. officinalis (41). At the primary level, the proteins share \(<30\%\)
sequence identity and differ dramatically in reported monomeric state. The V-CIPO from *C. inaequalis* is a monomeric protein, whereas the V-BrPO from *A. nodosum* is a homodimer. Interestingly, the V-BrPO from *C. officinalis*, which is associated with snyderol (2-4) biosynthesis, is a homododecameric protein arranged in a 23-point group symmetry (41). Differences in monomeric states between the V-HPOs may be indicative of their biological function. However, each protein shares a core structure consisting of mainly α-helices with two four-helix bundles as the main tertiary structural motif (38–39, 41) and has an identical arrangement of amino acid residues at the vanadium active site itself.

The vanadium-binding site lies at the bottom of a 15–20-Å deep funnel-shaped channel located at the core of the four-helix bundle (9, 37, 40, 41). Vanadium is present as a V(V) ion and is ligated to the imidazole ring of a conserved histidine residue that anchors the cofactor in a trigonal bipyramidal fashion (Fig. 2A) (37, 40, 41). Three oxygen atoms of the cofactor are located in the equatorial plane, and the negative charges are compensated by an extensive hydrogen-bonding network with two four-helix bundles as the main tertiary structural motif (38–39, 41) and has an identical arrangement of amino acid residues at the vanadium active site itself.

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**Catalytic Cycle**

Active-site mutagenesis and steady-state kinetics studies of recombinant V-CIPO from *C. inaequalis* have shed light on the catalytic mechanism of the V-HPOs (Fig. 2B) (5, 38, 45, 46). Alanine scanning mutagenesis of the active-site residues Lys353, His404, Arg360, Arg490, and His353 (numbering from *C. inaequalis*) yielded mutant enzymes unable to oxidize chloride. However, the modified enzymes could still function as bromoperoxidases, albeit with decreased activity compared with native V-BrPOs (41, 45, 46). The H496A mutant lost the ability to bind vanate and could no longer function as a V-HPO, whereas the H494A mutation was shown to have a reduced affinity for the cofactor (45).

Steady-state kinetics show that V-HPOs catalyze the two-electron oxidation of halides in a substrate-inhibited Bi Bi ping-pong mechanism (1, 5, 9). Coordination of hydrogen peroxide to the vanadium center is the first step in catalysis, and His404, which activates the axial water molecule, must be deprotonated for H2O2 to bind (32). A crystal structure of the peroxo-V-CIPO shows that peroxide is coordinated in a side-on manner in the equatorial plane and distorts the vanadium site to a tetragonal bipyramidal geometry (38). After the binding of peroxide, His353 is no longer hydroxide-bonded to any oxygen atoms of the cofactor, and Lys353 makes direct contact with one of the oxygen atoms of the bound peroxide, further activating the bound peroxide through charge separation.

A halide ion is the second substrate that binds to the enzyme through nucleophilic attack on the partially positive oxygen atom (Fig. 2B). This binding breaks the peroxide bond and creates the nucleophilic OX 2− group, which leaves the coordination sphere as hypohalous acid after protonation by an incoming water molecule (46). If an appropriate nucleophile is present, the generated hypohalous acid intermediate will react with the organic substrate, giving rise to a halogenated compound. If not, the oxidized halogen intermediate will react with another equivalent of hydrogen peroxide to generate dioxygen in the singlet state and the halide (9). A new fluorescence microscopy-based method, which uses a fluorogenic derivative to monitor the formation and migration of HOBr from a V-BrPO active site, will shed light on where the actual halogenation of organic substrates occurs (47).

Active-site residues assisting with the selection and binding of the halide are still being elucidated. On the basis of crystal structure analysis, Phe357 and Trp360 (numbering from *C. inaequalis*) may participate in halide binding through their δ+ ring edge (48). V-BrPOs alternatively contain His and Arg residues at these positions, respectively (48). Recently, chlorinating activity was created in the *Corallina pilulifera* V-BrPO through a single amino acid substitution (47). The substitution of the Arg residue at position 350 with Trp or Phe increased the affinity of V-BrPO for chloride (49). The native V-BrPO from *A. nodosum* also contains a Trp residue corresponding to position 350, and it too was shown to exhibit chloroperoxidase activity (50). Therefore, this Trp residue is believed to participate in chloride binding.

Additional mutagenesis experiments on the His residue corresponding to Phe357 showed the importance of the residue in the oxidation of halides (51). The His residue was mutated to Ala in the V-BrPO from *C. officinalis*, which resulted in the enzyme’s inability to efficiently oxidize bromide; however, the mutant could still oxidize iodide (51). Interestingly, like the V-BrPOs, the three putative V-CIPOs from the napyradiomycin biosynthetic cluster also contain a His residue in the posi-
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...tion corresponding to Phe397 (35), and this residue may also play a role in substrate binding or selectivity.

Relationship to Phosphatases

Sequence alignment of V-CIPO from C. inaequalis and V-BrPOs from A. nodosum and C. officinalis and analysis of their high resolution crystal structures revealed that the amino acid residues required for binding the vanadate cofactor share homology with a family of acid phosphatases (4, 5, 52). Many of these enzymes are membrane-bound and include type 2 phosphatidic acid phosphatase, bacterial nonspecific acid phosphatases, and mammalian glucose-6-phosphatases (1, 4, 5, 52). Studies with vanadium-substituted bacterial class A nonspecific acid phosphatase, bacterial nonspecific acid phosphatase, and bacterial nonspecific acid phosphatase (45, 52), and its crystal structure was solved recently with a trapped phosphohistidine intermediate (54). Together, these data suggest an evolutionary relationship between the acid phosphatases and V-HPOs.

Phylogenetic analysis of V-BrPOs, V-CIPOs, and bacterial nonspecific acid phosphatases (Fig. 1) shows that the enzymes form clades based on proposed enzymatic function. However, the three putative V-CIPOs from the bacterium Streptomyces sp. CNQ-525 do not cluster with the fungal or other postulated bacterium-derived V-CIPOs, which is consistent with their proposed role in natural product biosynthesis in contrast to the other known V-CIPOs. With the rampant surge in genome sequencing, there has been an emergence of prokaryotic V-HPO homologs with the potential of possessing new biological functions that go beyond the chemistry affiliated with the eukaryotic enzymes (Fig. 1).

Chemoenzymatic Biotransformations

Although the biological function of fungal V-CIPOs has yet to be confirmed, marine algal V-BrPOs have been shown through in vitro chemoenzymatic conversions to catalyze the bromonium-assisted cyclization of terpenes and ethers (9, 15). Initial studies on the enzymatic halogenation of anisole and prochiral aromatic compounds by the C. pilulifera V-BrPO failed to show any regio- or stereospecificity (14), suggesting that the hypobromite intermediate was freely diffusible to facilitate the molecular reaction outside of the enzyme’s active site (9). However, more recent studies have convincingly shown that various V-BrPOs isolated from marine red algae (e.g. C. officinalis, Laurencia pacifica, and Plocamium cartilagineum) catalyze the asymmetric bromination/cyclization reactions of the sesquiterpene nerolidol (Fig. 1, 1) to the snyderol family of marine natural products (2-4) (15). Single diastereomers of β- and γ-snyderol were produced in the enzyme reaction, whereas in the synthetic reaction, two diastereomers of each were formed. This study established the likely role of V-BrPOs in the biosynthesis of brominated cyclic sesquiterpenes from marine red algae and further implies that other marine algal brominated natural products may similarly be constructed. Together with the biosynthesis of the bacterial napyradiomycins, these studies are beginning to contradict conventional wisdom that V-HPOs lack substrate specificity and regioselectivity (55). Although this may have been true for those V-HPOs that biologically function to produce hypohalous acids as antimicrobial agents, other V-HPOs are emerging to participate in the biosynthesis of complex halogenated natural products.

Future Perspectives

To date, V-HPOs have been identified in most branches of life, but have been characterized exclusively in eukaryotes. Although their biological roles are not yet fully elucidated, as more bacterial V-HPOs are identified through total genome sequencing projects, more tractable systems for exploring the in vivo role of these enzymes will be established. Newly identified V-HPOs may have new biological functions that go beyond the chemistry shown so far, and phylogenetic analyses of all known and newly identified V-HPOs may provide further insight on the evolutionary relationship between the V-HPOs and acid phosphatases.

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