

Handbook on METALLOPROTEINS

edited by

Ivano Bertini

*Magnetic Resonance Center
University of Florence
I-50019 Sesto Fiorentino, Italy*

Astrid Sigel

Helmut Sigel

*Institute of Inorganic Chemistry
University of Basel
CH-4056 Basel, Switzerland*



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Vanadium in Proteins and Enzymes

Alison Butler, Jayme N. Carter, and Matthew T. Simpson

Department of Chemistry and Biochemistry, University of California, Santa Barbara,
CA 93106-9510, USA

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1. INTRODUCTION

1.1. Coordination Chemistry of Vanadium

Vanadium compounds exist in oxidation states ranging from -3 , -1 and 0 , to $+1$, $+2$, $+3$, $+4$, and $+5$. Only the $+2$ to $+5$ oxidation states have been identified in aqueous solution, and of these only V(III), V(IV), and V(V) are found in biological systems. Coordination complexes of these ions span a beautiful array of colors from brilliant blue to deep yellow. Interconversion between the V(III), V(IV), and V(V) oxidation states is accessible at neutral pH, with reduction potentials of 0.99 V (vs. NHE) for vanadium(V) and 0.542 V for vanadium(IV) [1]. For additional information, the reader is referred to several recent books and reviews that cover the inorganic and bioinorganic chemistry of vanadium [2–6].

The oxyanion vanadate(V) ($\text{HVO}_4^{2-}/\text{H}_2\text{VO}_4^-$; $\text{p}K_a$ 8.3) is the most prevalent and stable species in aqueous solution at neutral pH. Vanadate undergoes polymerization reactions depending on its concentration, pH, and the nature of other ions present in solution. For example, $\text{H}_2\text{V}_2\text{O}_7^{2-}$, $\text{V}_4\text{O}_{12}^{4-}$, and decavanadate, $\text{H}_2\text{V}_{10}\text{O}_{28}^{4-}$, are formed at higher vanadate concentrations (e.g., $\geq$ mM) and at lower pH values. Of relevance to the vanadium haloperoxidase enzymes is the propensity of peroxide to coordinate to vanadate. Under neutral and alkaline conditions, hydrogen peroxide coordinates to vanadate, producing oxoperoxovanadates, with one to four coordinated peroxide ligands and peroxodivanadates, depending on the reaction conditions [7–10]. The coordination geometry adopted by nearly all oxoperoxovanadium(V) and oxodiperoxovanadium(V) small-molecule complexes is pentagonal bipyramidal [11].

The most common coordination geometry of V(II) and V(III) complexes is octahedral, while V(IV) and V(V) are much more flexible, adopting tetrahedral, trigonal bipyramidal, octahedral, and pentagonal bipyramidal geometries. The coordination chemistry of vanadium(IV) is usually considered to be dominated by the vanadyl, $\text{V}=\text{O}^{2+}$, group, while that of vanadium(V) is dominated by VO^{3+} and *cis*- VO^{2+} groups. Exceptions exist, such as “bare” (i.e., without oxo ligands) V(IV) and V(V) compounds are known, even with biological ligands (such as siderophores and siderophore analogues), but these complexes are not very stable in aerobic, aqueous solution [12,13].

1.2. Bioinorganic Role of Vanadium

Vanadium is widely distributed in nature. In the Earth's crust, vanadium is more abundant than many metal ions, at 100 ppm. In the ocean, vanadium is the second most abundant transition metal ion at 30–50 nM after molybdenum at 100 nM [14]. Vanadium shows a nutrient-like distribution profile with depletion in surface waters, where organisms grow, relative to deeper waters [14]. Given the relative abundance of vanadium in seawater, as well as the abundance of halides (e.g., 0.5 M Cl^- , 1 mM Br^- and $\mu\text{M I}^-$) and hydrogen peroxide, which is present at concentrations as high as μM in daylight hours due to photochemical events, it is not surprising that marine organisms have adapted to their chemical environment by evolving haloperoxidase enzymes.

Tunicates (order Ascidiacea), common sessile marine animals (also known as sea squirts), may have also adapted to their chemical environment by acquiring vanadium, yet the role of vanadium has eluded investigators since its discovery near the turn of the last century. The vanadium exists primarily in the trivalent oxidation state, although some tetravalent vanadium is also present. It is generally believed that tunicates concentrate vanadium from seawater through a reductive process. The reduction and possibly complexation is aided by an organic compound, tunichrome (Fig. 1), which is a modified tripeptide derived from three hydroxydopa residues [15–19].

2. VANADIUM ENZYMES WITH KNOWN STRUCTURE: VANADIUM HALOPEROXIDASES

Biological systems have evolved haloperoxidase enzymes to catalyze the oxidation of chloride, bromide, and iodide by hydrogen peroxide. The majority of these enzymes in

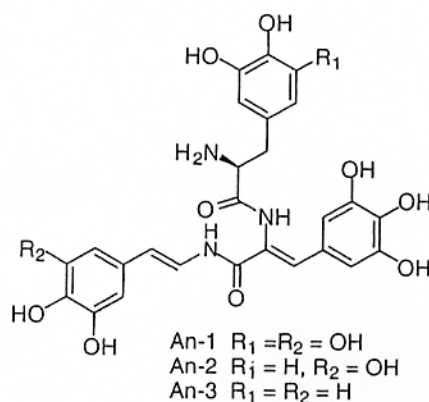
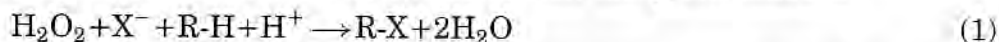


FIG. 1. Structures of selected tunichromes. "An" refers to species of *Ascidia nigra* from which these tunichromes are isolated.

terrestrial systems contain the Fe-heme moiety, including chloroperoxidase from the fungus *Caldariomyces fumago*, and myeloperoxidase, eosinophil peroxidase, and lactoperoxidase from mammalian systems. Other haloperoxidases that do not contain metal ions are also known, such as the chloroperoxidase in *Pseudomonas pyrrrocina* [20]. In marine algal systems, the vanadium-containing haloperoxidases predominate [21–24], although a few examples of Fe-heme haloperoxidases in marine algae and worms are known [25–27]. Hydrogen peroxide does not have the driving force to oxidize fluoride; hence, there are no fluoroperoxidase enzymes. Historically, haloperoxidases have been named based on the most electronegative halide that they can oxidize. Thus, chloroperoxidase catalyzes the oxidation of chloride, bromide, and iodide by hydrogen peroxide; bromoperoxidase catalyzes the oxidation of bromide and iodide; whereas iodoperoxidase can only catalyze the oxidation of iodide by hydrogen peroxide. Perhaps a better basis for the nomenclature of haloperoxidases would be a particular enzyme's physiological reactivity in vivo. However, in many cases the conditions under which the enzyme would function are not known, such as the halide content in a cell and the pH of the environment.

The overall reaction carried out by the vanadium haloperoxidases is:



where X^- is Cl^- , Br^- , or I^- and R-H is an organic substrate. For efficient halogenation, 1 mol of halide is oxidized by 1 mol of hydrogen peroxide, halogenating 1 mol of organic substrate (e.g., R-H, in the equation above) [28,29]. In this reaction, one proton, H^+ , is also consumed. In some haloperoxidase-catalyzed turnovers depending on the nature of the organic substrate, much more hydrogen peroxide is consumed than halo-organic compound produced. In this case, the oxidized halogen intermediate (i.e., X^+ in Fig. 2, below) is reduced by a second equivalent of hydrogen peroxide, producing singlet oxygen and the halide [30].

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 [31] (see Eq. (2)). The halogenation of MCD is followed spectrophotometrically at 290 nm, which monitors the loss of MCD in the enol form ($\epsilon = 20,000 \text{ M}^{-1} \text{ cm}^{-1}$). Haloperoxidase activity is expressed as micromoles MCD brominated or chlorinated per minute per milligram of enzyme (i.e., units per milligram). The early work on vanadium bromoperoxidase employed the oxidation of iodide by hydrogen peroxide [32], forming triiodide (I_3^-) which was followed spectrophotometrically at 353 nm ($\epsilon = 26,400 \text{ M}^{-1} \text{ cm}^{-1}$). However, this reaction is less

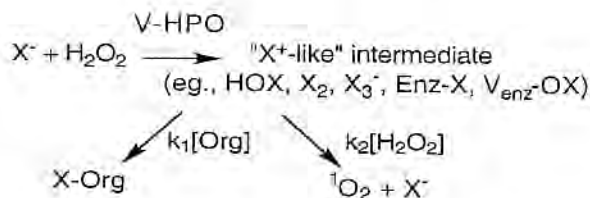
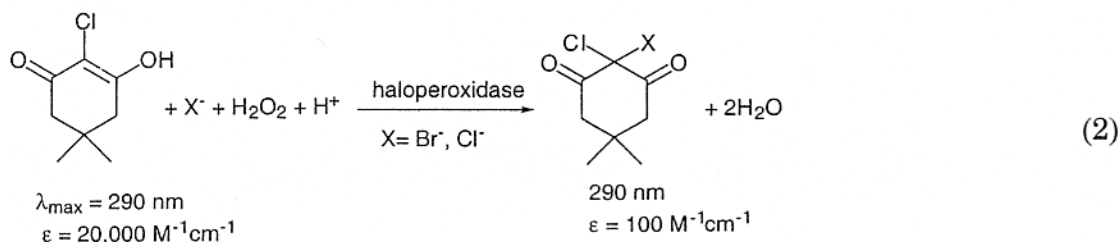


FIG. 2. Overall reaction scheme of vanadium haloperoxidases.

desirable for quantitation of haloperoxidase activity because of competing side reactions, such as the nonenzymatic oxidation of iodide by hydrogen peroxide and reduction of triiodide by hydrogen peroxide.



Extensive studies on the kinetics of halogenation by vanadium bromoperoxidase from several algae [33–35] and the fungal vanadium chloroperoxidase [36] have been carried out using MCD as the organic substrate, as well as kinetic studies of dioxygen formation [34,37] (Fig. 2). It is clear from these studies that the rate of oxidation of halide is rate limiting when MCD is the substrate, and that the $k_1[\text{MCD}]$ and $k_2[\text{H}_2\text{O}_2]$ pathways are competitive [28,34,38]. It should be noted that MCD may not be the best substrate to quantitate haloperoxidase activity for all enzymes. As we learn more about the active site channels of haloperoxidases from X-ray structures, we may discover some haloperoxidases that catalyze halogenation reactions of other substrates more efficiently than MCD [39,40], or that even fail to halogenate MCD at all. A recent review focusing on mechanistic aspects of the haloperoxidase reactivity is available [6].

2.1. Vanadium Chloroperoxidase

2.1.1. Occurrence and Putative Biological Role

The vanadium chloroperoxidases (V-ClPO, E.C. 1.11.1.10) have been isolated from the dematiaceous hyphomycete fungi *Curvularia inaequalis* [41] and *Embellesia didymospora* [42]. Other fungi in this class have also been shown to possess chloroperoxidase activity, in agreement with the initial screening reported by Hunter-Cevera and Sotos [43]. Some of these enzymes also cross-react immunologically with a polyclonal antibody to the *C. inaequalis* V-ClPO, including *Drechslera biseptata*, *D. subpapedorfii*, *E. didymospora*, and *Ulocladium chartarum*, suggesting that these fungi also produce V-ClPO [44].

Halogenated natural products have not been isolated from these fungi (unlike from marine algae that contain V-BrPO; see Sect. 2.2); thus, the biological role of V-ClPO is speculative. The fungi that produce the vanadium chloroperoxidase are phytopathogens and must penetrate the cell wall of the host plant. The proposed function of the fungal V-ClPO is in the degradation of the plant's cell wall through production of hypochlorous acid (HOCl), which is a strong oxidant. The apparent variation in the biological function of the fungal V-ClPO and the marine algal vanadium bromoperoxidase (V-BrPO) may in fact reflect reactivity differences between these two enzymes.

2.1.2. Molecular Structure: Overall Protein Structure and the Vanadium Site

The X-ray structure of V-ClPO (*C. inaequalis*) shows that the protein is cylindrical with approximate dimensions of 55 Å in diameter by 80 Å in length [45]. (See Table 1 for a summary of the X-ray structures of vanadium haloperoxidases (i.e., vanadium chloroperoxidase and derivatives and vanadium bromoperoxidase) deposited in the Protein Data Bank). One molecule is present per asymmetrical unit. The amino acid sequence predicts a protein containing 609 amino acids with a MW of 67,488, which has been confirmed by SDS-PAGE. V-ClPO contains two cysteine residues that are present as free thiols and not disulfide bridges. The secondary structure is dominated by α helices. Two 4-helix bundles comprise the main structural motif of the tertiary structure (Fig. 3) [45].

The vanadium(V) site resides at the top of one of the four-helix bundles in a broad channel that is lined on one half with predominantly polar residues, including an ion pair between Arg-490 and Asp-292 and several backbone 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.

TABLE 1

Summary of Vanadium Haloperoxidase Enzymes with X-ray Structures

PDB code	Source	Enzyme form or mutation	Date deposited	Resolution (Å)	Ref.
Vanadium Chloroperoxidase EC: 1.11.1.10					
1VNC	<i>Curvularia inaequalis</i>	(Native)	01-Sep-1995	2.10	45
1VNE	Recombinant	D292A	20-Jan-1999	2.15	49
1VNF	Recombinant	R360A	20-Jan-1999	2.35	49
1VNG	Recombinant	H404A	20-Jan-1999	2.20	49
1VNH	Recombinant	H496A	20-Jan-1999	2.11	49
1VNI	Recombinant	Holo	20-Jan-1999	2.15	46
1VNS	Recombinant	Apo	14-Jan-1999	1.66	46
Vanadium Bromoperoxidase					
1QHB	<i>Corallina officinalis</i>	Native	11-May-1999		—
1QI9	<i>Ascophyllum nodosum</i>	Native	14-Jun-1999	2.0	57

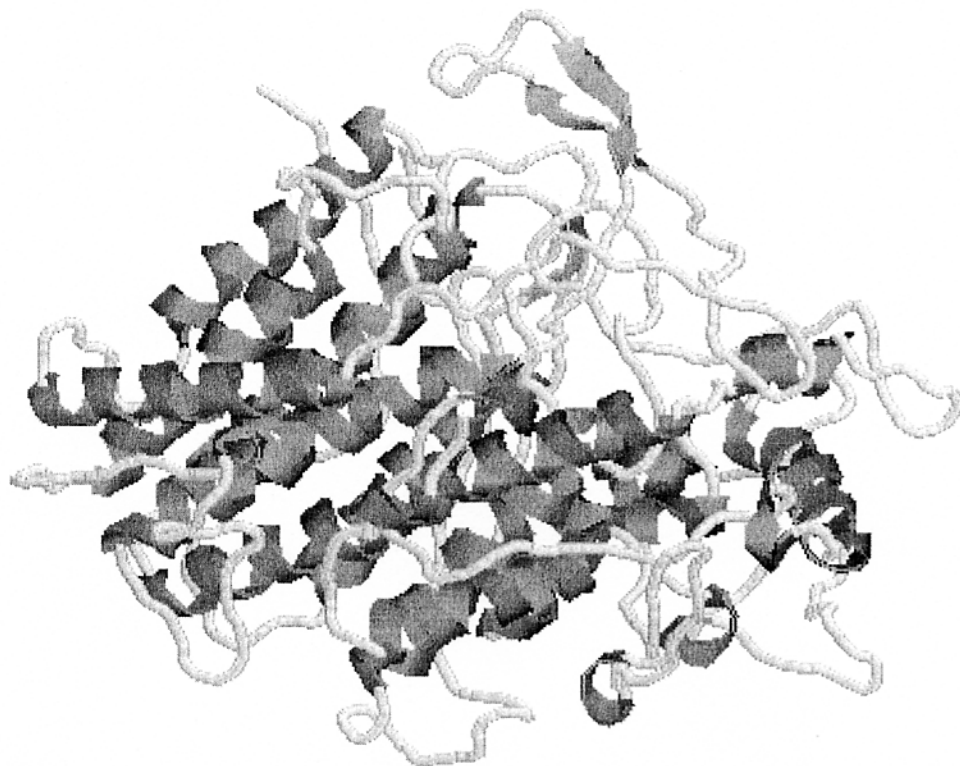


FIG. 3. Structure of vanadium chloroperoxidase from *Curvularia inaequalis*.

One of the more striking features of the vanadium site is its apparent simplicity, which resembles vanadate (HVO_4^{2-}) coordination to the protein by one histidine ligand, His-496, in a trigonal bipyramidal geometry [44]. The first X-ray structure of V-CIPO to be solved was the azide-coordinated derivative, which was a result of crystallization of V-CIPO from azide-containing buffer. In this first structure, azide was found to be coordinated to the vanadium(V) ion in an axial position as shown in Figs. 4 and 5. Three oxygen atoms are also coordinated in an equatorial plane and an apical oxygen atom of a proposed hydroxide ligand [46]. 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 vanadate center (see Fig. 4).

The structure of the recombinant V-CIPO crystallized in the absence of azide [46] revealed an apical O atom in place of azide at 1.93 Å, which was interpreted as being an OH ligand (Fig. 6). This apical oxygen ligand forms three hydrogen bonds of which two are to water molecules and one is to His-404 (2.97 Å). His-404 is an important residue because binding of peroxide is inhibited if this residue is doubly protonated.

The third structure to be solved was the peroxide-bound derivative of V-CIPO. The X-ray structure reveals a distorted tetragonal bipyramidal vanadium site (Fig. 7) [46] in which peroxide is coordinated in a side-on bound 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;

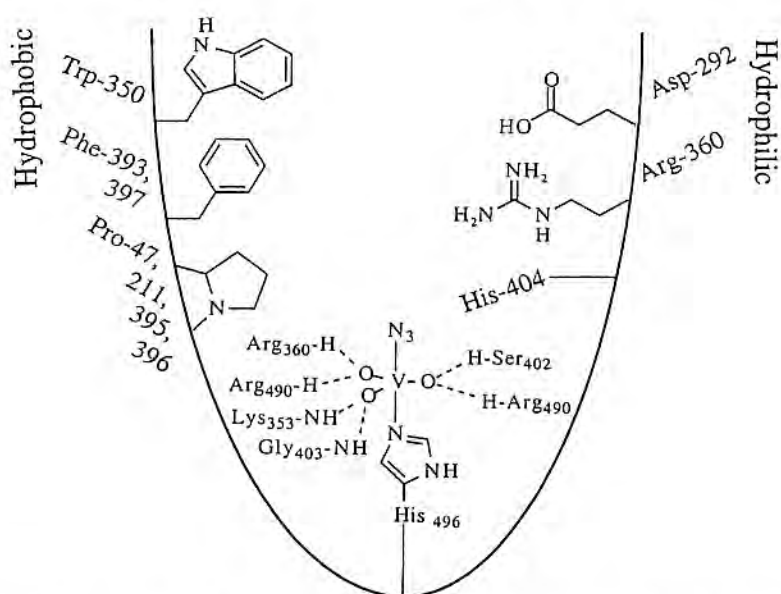


FIG. 4. Active site channel of V-CIPO from *Curvularia inaequalis* (constructed from [45]).

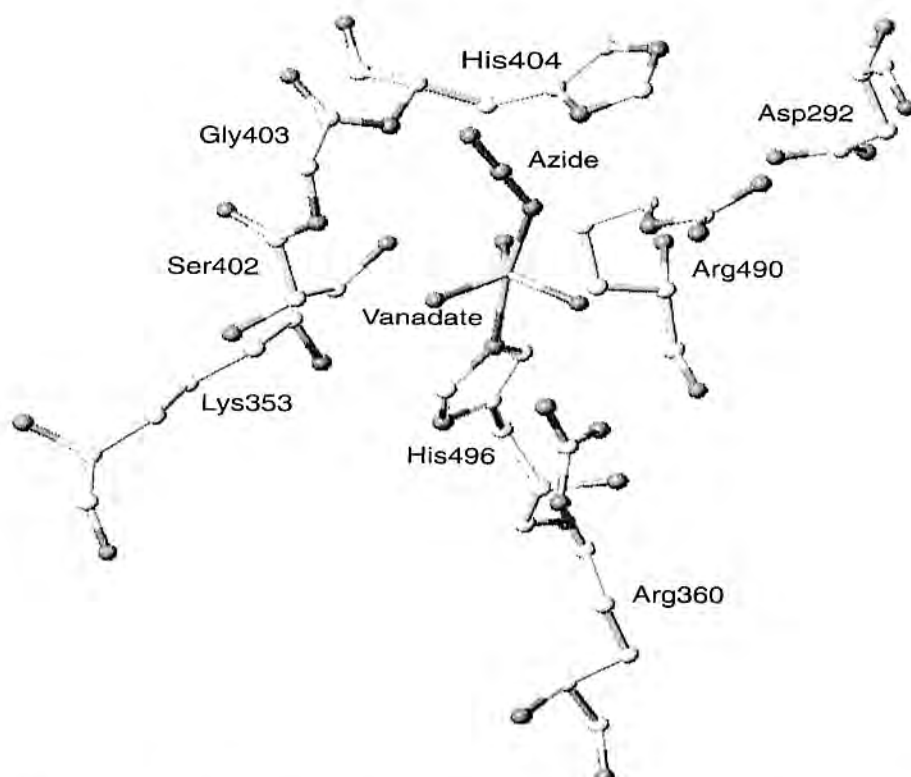


FIG. 5. The vanadium site of azide-bound V-CIPO from *Curvularia inaequalis*.

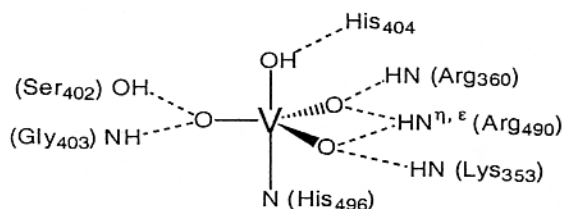


FIG. 6. The vanadium site of native V-CIPO from *Curvularia inaequalis*. (Adapted from [46].)

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-CIPO is superimposable on the native structure, suggesting that the protein matrix is rigid and provides a preformed metal binding site [46]. A water molecule in apo-V-CIPO 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 apo-V-CIPO (1.66 Å) heterologously expressed in yeast reveals a sulfate ion that is partially stabilized by electrostatic interactions and hydrogen bonds with positively charged side chain residues involved in vanadate interactions in the native structure [47].

The X-ray structure of the tungstate-substituted V-CIPO reveals replacement of vanadate by tungstate [47]; however, His-496 is either not coordinated to W or only weakly coordinated, since the distance between the $N^{\epsilon 2}$ atom of His-496 and W is long, i.e., 2.55 Å (Fig. 8). The tungstate oxygen atoms hydrogen-bond to protein side chains and a main chain amide similar to native V-CIPO, including a long H bond between the oxide and His-404 at 3.21 Å. Thus, coordination of vanadate and tungstate can be attributed to “rack-induced bonding” of these oxyanions to a rigid protein matrix [48].

The structures of several single-site mutants of V-CIPO (*C. inaequalis*) are also known [49,50]. These mutants are the result of replacement of His-496, the V-ligating histidine, with alanine (H496A); replacement of His-404, the putative acid-base histidine, with alanine (H404A); replacement of Arg-360, a residue involved in charge compensation of the vanadate site, with alanine (R360A); and replacement of Asp-292, the residue involved in a salt bridge with Arg-490, with alanine (D292A). The overall protein backbone structures of these mutants are the same as that of native V-CIPO, and virtually superimposable on one another except in certain regions of the vanadium site, as discussed below [49–51].

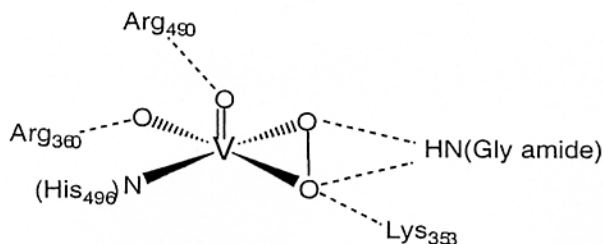


FIG. 7. The vanadium site of peroxo-V-CIPO from *Curvularia inaequalis*. (Adapted from [46].)

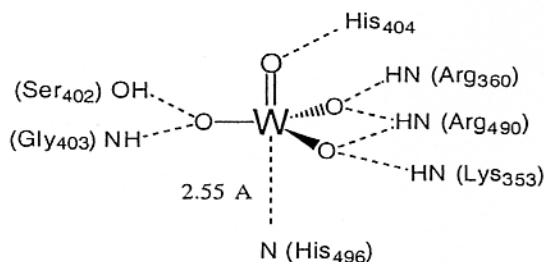


FIG. 8. The vanadium site of tungsten-substituted-ClPO from *Curvularia inaequalis*. (Adapted from [47].)

2.1.2.1. The H496A mutant [49]

The substitution of the ligand histidine His-496 by alanine does not prevent vanadate coordination; however, this mutant lacks haloperoxidase activity. Vanadate is now coordinated as a tetrahedral anion with three nonprotein oxygen ligands at 1.56 Å and one nonprotein oxygen ligand at 1.67 Å. All of the side chain residues that hydrogen-bond to the vanadium center in the native structure also form hydrogen bonds in this structure, including that between His-404 and the apical oxygen of the vanadate ion. The superposition of the vanadium sites H496A on the structure of the wild-type enzyme shows the differences in the coordination geometries of the vanadium (Fig. 9). The superposition also demonstrates the remarkable rigidity of the amino acids that frame the anion binding site (including His-404, Ser-402, Gly-403, Arg-360, Arg-490, Asp 292).

2.1.2.2. The H404A mutant [49]

The most significant structural change in the structure of the mutant in which alanine replaces His-404 is the broken salt bridge between Arg-490 and Asp-292 (Fig. 10) [49]. Arg-490 moves into the place of His-404, assuming hydrogen bonding interactions between other side chain residues in place of the vanadate oxygen atoms in the native structure. Vanadium still remains coordinated to His-496 (1.96 Å). The apical O atom sits 2.0 Å from the vanadium, corresponding to a mixture of water or hydroxide ligation, and the equatorial O atoms remain at 1.60 Å. The removal of His-404 completely abolishes chlorinating activity, which may be a direct result of the loss of the histidine residue or of the resulting rearrangements in the active site.

2.1.2.3. The D292A mutant [49]

Asp-292, which forms a salt bridge with Arg-490, helps to orient the side chain of Arg-490 so that it can also hydrogen-bond with two vanadate oxygen atoms at N^η and N^ε. In the mutant in which alanine replaces Asp-292, there is very little effect on the structure (Fig. 11). His-496 remains ligated to the vanadate ion, and vanadate remains in hydrogen-bonding contact with Lys-353, Ser-402, the amide backbone of Gly-403, and Arg-360 and Arg-490, each of which is hydrogen-bonded to two different oxygen atoms. Despite the similarity with native V-ClPO, this mutant has only 2% of the activity of the native enzyme.

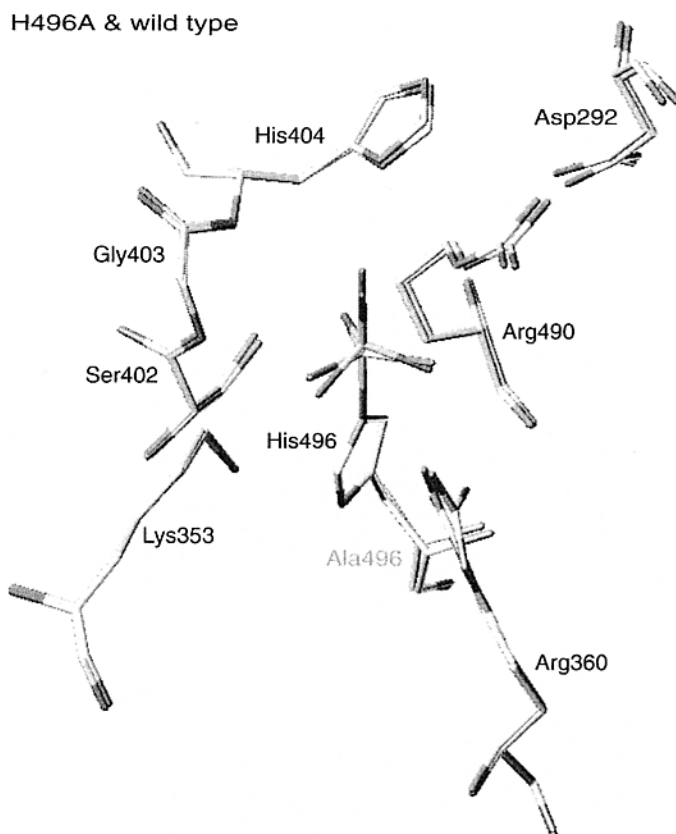


FIG. 9. Overlay of X-ray coordinates of the vanadium site of wild-type V-ClPO and the H496A mutant.

2.1.2.4. *The R360A mutant* [49]

Arg-360 is directly involved in charge neutralization of the HVO_4^- center through a single hydrogen bond to one of the vanadate oxygen atoms. In the mutant in which alanine is substituted for arginine, one might expect as a result of the change in charge compensation a significant reorientation about the vanadate to achieve charge neutralization. Somewhat surprisingly, however, the superposition of the vanadium site of R360A on native V-ClPO shows little change in the position of the residues other than the lack of Arg-360 (Fig. 12). The chloroperoxidase activity of this mutant is about 6% of that of native V-ClPO, illustrating the importance of the charge-neutralizing effect of Arg-360.

In summary, it is surprising how similar the overall structures are of native V-ClPO, the apo and metal-substituted derivatives, and the mutant enzymes, yet seemingly small changes effect significant consequences in reactivity [49]. The protein forms a very rigid active site matrix which is framed to bind oxyanions through charge neutralization by hydrogen bonding to the side chain residues of Lys-353, Arg-490, and Arg-360. His-496 is essential to promote the trigonal bipyramidal geometry about the vanadium center. His-404 seems to play an important role in peroxide coordination. In addition, hydrogen bonds from Ser-402 and the amide backbone of Gly-403 may also be essential, although not tested at this point.

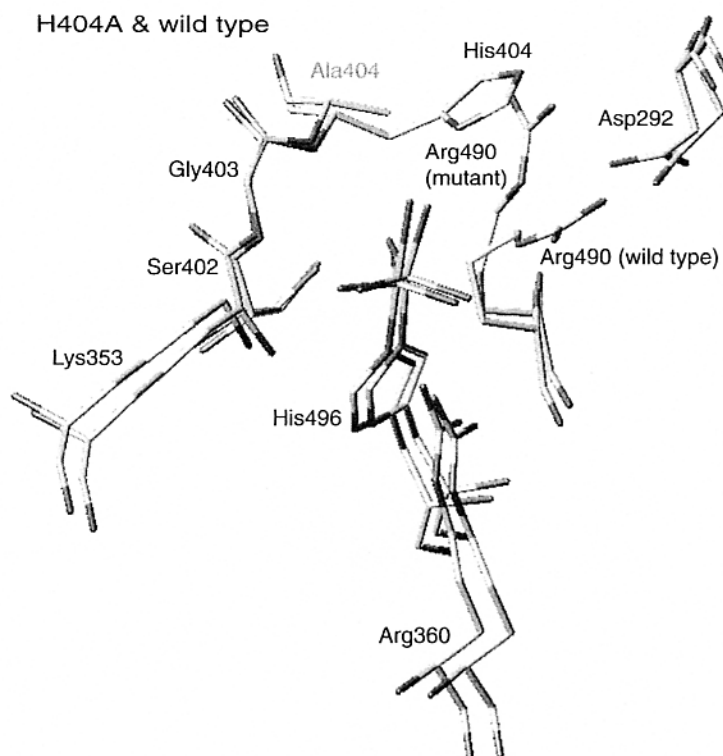


FIG. 10. Overlay of X-ray coordinates of the vanadium site of wild-type V-ClPO and the H404A mutant.

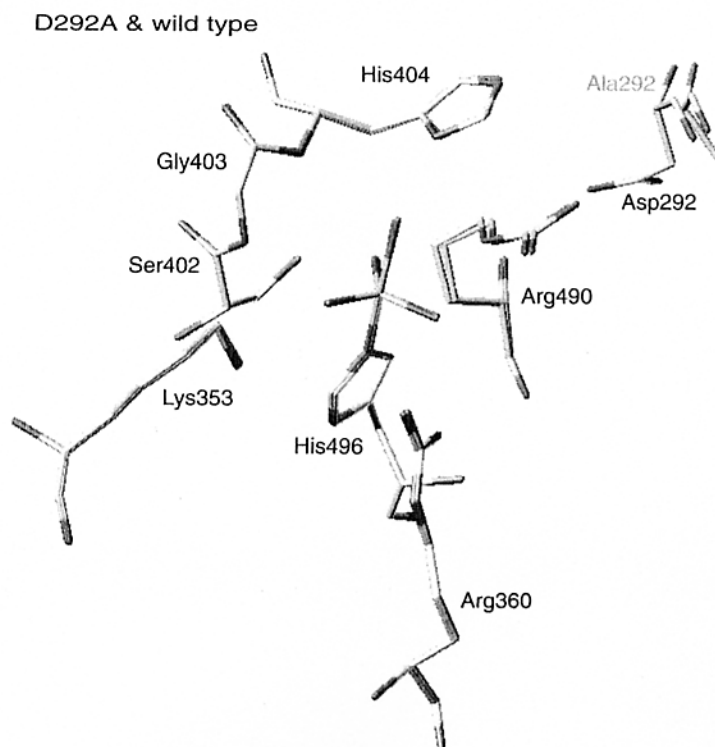


FIG. 11. Overlay of X-ray coordinates of the vanadium site of wild-type V-ClPO and the D292A mutant.

R360A & wild type

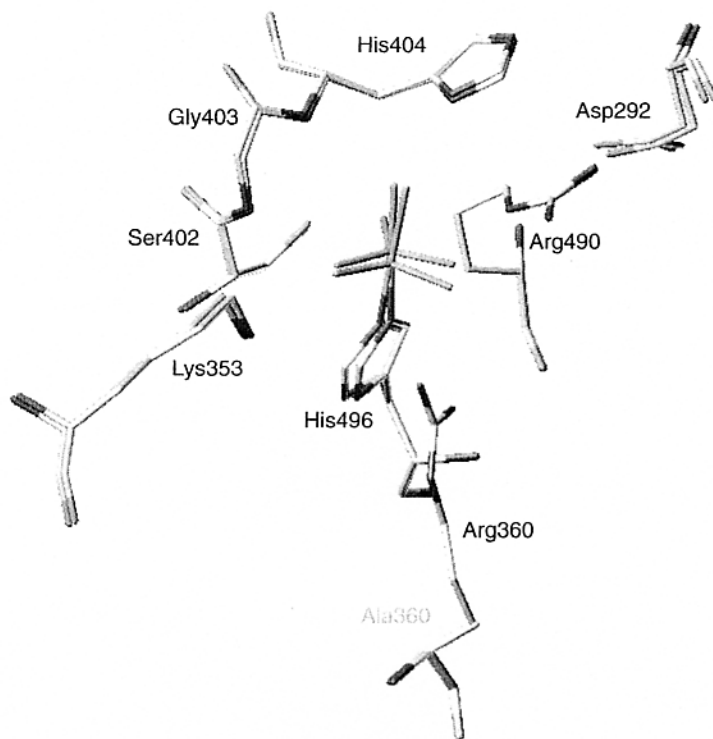


FIG. 12. Overlay of X-ray coordinates of the vanadium site of wild-type V-ClPO and the R360A mutant.

2.2. Vanadium Bromoperoxidase

2.2.1. Occurrence and Putative Biological Role

Vanadium bromoperoxidase (V-BrPO) has been isolated from all the major classes of marine algae, including chlorophyta (green algae), phaeophyta (brown algae), and rhodophyta (red algae). As described above, Fe-heme-containing bromoperoxidases have also been isolated from these classes of algae, as well as other organisms (e.g., certain marine worms [26,27]); however, the vanadium haloperoxidase seems to be more prevalent. In addition to haloperoxidase enzymes, the production of halogenated natural products is also widespread in marine organisms [52,53]. These compounds range from halogenated indoles (Fig. 13), terpenes (Fig. 13), acetogenins, phenols, etc., to volatile halogenated hydrocarbons (e.g., bromoform, chloroform, bromomethane, etc.), which are produced on a very large scale [54]. Often the halogenated compounds isolated from the marine organisms have important biological activities, such as antimicrobial properties, or feeding deterrent properties, suggesting that they play a defensive role in the marine organism. In many cases the halogenated compounds also are of pharmacological interest due to their antimicrobial, antifungal, antiviral, and anti-inflammatory activities.

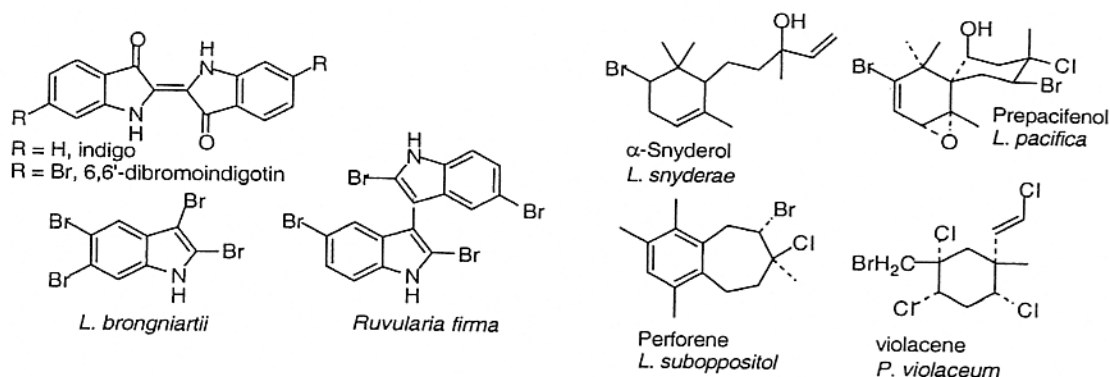


FIG. 13. Examples of halogenated indole and terpene marine natural products. (Adapted from [52,53].)

2.2.2. Molecular Structure: Overall Protein Structure and the Vanadium Site

The vanadium bromoperoxidases are all acidic proteins, with similar amino acid composition, molecular weight, charge (pI 4–5) and vanadium content. Analysis of amino acid sequence similarities of V-BrPO (*Ascophyllum nodosum*) shows a 89% similarity to V-BrPO from the brown alga *Fucus distichus* [55], and a 40.9% and 42% homology to the two bromoperoxidases produced by the red alga *C. pilulifera* [56]. The X-ray crystal structure of native V-BrPO from the brown alga *A. nodosum* has recently been reported at 2.0 Å resolution [57]. The final model reports V-BrPO as a homodimeric protein with approximate dimensions of 90 Å × 77 Å × 75 Å. The secondary structure of the monomer unit is predominately α -helical with a few short β strands. Each monomer has three intramolecular disulfide bridges, as well as two intermolecular disulfide bridges, resulting in the covalently linked homodimer. In addition to intermolecular disulfide connections, four salt bridges and numerous side-chain/side-chain, side-chain/main-chain interactions stabilize the dimer interface. Each monomer of V-BrPO has been calculated to contribute more than 46% of the monomer surface to contacts in the dimer interface. The extensive surface contacts and the compact helical structure of the homodimer provides a structural explanation for the observed thermostability and chemical stability of V-BrPO. The molecular weight of V-BrPO is 120,400 Da, which agrees well with the predicted monomer molecular weight of 65,000 Da from reducing SDS-PAGE gels [58]. Previously the molecular weight of the homodimer predicted by SDS-PAGE was reported as 95,000–100,000 Da [59,60].

The core of the V-BrPO homodimer is built up of two 4-helix bundles [57]. The active site of the enzyme is part of the core structure, with the vanadate cofactor bound at the end of a 15-Å-deep substrate funnel. Both monomers of the homodimer contribute residues to the substrate funnel surface. The funnel entrance has a diameter of approximately 12 Å and narrows to approximately 8 Å near the anion binding site. The funnel surface is composed of both hydrophilic and hydrophobic residues,

with the hydrophobic residues dominating the surface region around the vanadate binding site.

The vanadium atom at the active center of V-BrPO resides in a trigonal bipyramidal coordination geometry similar to the vanadium(V) site in V-ClPO [57]. Vanadium(V) in V-BrPO is coordinated by four oxygen atoms and $N^{\epsilon 2}$ of His-486. The negatively charged cofactor is neutralized by several hydrogen bond interactions from side chain residues at the active site. The protein residues (Ser-416, Gly-417, His-418, Lys-341, Arg-349 $N^{\eta 1}$, $N^{\eta 2}$, Arg-480 $N^{\eta 2}$) act as proton donors to the oxyanion and constitute the central part of the rigid vanadate binding site. The active site of V-BrPO contains an additional histidine residue not present in V-ClPO, His-411, which is not directly coordinated to the vanadate cofactor. His-411 is within hydrogen bonding distance to one of the vanadate oxygen atoms and may participate as a proton donor/acceptor during the enzymatic reaction.

The X-ray structure of the peroxo derivative of V-BrPO has not been reported yet. Curiously, when crystals of V-BrPO were soaked in millimolar concentrations of H_2O_2 , i.e., conditions similar to the preparation of the peroxo derivative of V-ClPO, the electron density did not change [57].

3. VANADIUM ENZYMES OF UNKNOWN STRUCTURE: VANADIUM NITROGENASE

3.1. Occurrence and Biological Significance

The reduction of dinitrogen to ammonia is accomplished by the two-component nitrogenase enzyme system, consisting of a nitrogenase enzyme harboring one of the Mo-Fe, V-Fe, or Fe-only iron-sulfur cofactors and a reductase enzyme (also called the iron protein, or the dinitrogenase-reductase), which supplies electrons to the nitrogenase protein. Vanadium nitrogenase has been purified from several species within the Azotobacteriaceae family, including *Azotobacter vinelandii* [61–63], *A. chroococcum* [64], and *A. paspali* [65]. A vanadium-dependent nitrogenase has also been found in the cyanobacterium *Anabaena variabilis* [66].

Reports of the effect of vanadium on the dinitrogen-dependent growth of the soil bacterium *A. vinelandii* first appeared in the 1930s. Later reports suggested that a vanadium nitrogenase could be obtained by growth on vanadium salts in the absence of molybdenum [67]; however, it was not until 1986, using the mutant strain of *A. vinelandii* lacking the structural genes for the Mo-nitrogenase, that molecular nuances of the alternative nitrogenase were realized [68]. In 1987, vanadium was found to stimulate the growth of strains lacking structural genes encoding Mo-nitrogenase (*nifHDK*) [64]. Vanadium nitrogenase was subsequently isolated from this mutant strain of *A. chroococcum* [64] and the related strain of *A. vinelandii* [62,63]. In 1988, the third nitrogenase that lacks molybdenum and vanadium was isolated [69]. It is now known that each of these nitrogenases is genetically distinct. The alternative nitrogen fixation pathway allows each of these bacteria to survive in an environment

deficient in molybdenum or vanadium. In addition, the vanadium enzyme has been shown to have higher activity at temperatures below 30°C, providing a selective advantage for organisms at cold temperatures [70].

3.2. Structural Considerations and Reactivity

All of the nitrogenase systems are composed of the Mo-, V-, or Fe-only nitrogenase and a reductase, which acts as an electron donor. The nitrogenase reductases of Mo- and V-nitrogenases have 95% homology at the amino acid level [71]. Both are 63-kDa homodimers with a single 4Fe-4S cluster at the interface of the two subunits.

The vanadium- and molybdenum-containing dinitrogenases have different quaternary structures but similar metal sites. Mo-nitrogenase is an $\alpha_2\beta_2$ tetramer with a molecular weight of 230 kDa. This protein undergoes activation from the apo- to the holoenzyme only in the presence of the reductase, which acts as a chaperone-insertase [72]. By contrast, V-dinitrogenase is an $\alpha_2\beta_2\gamma_2$ hexamer. The γ subunit plays a role in processing of the holoenzyme [73].

The metal clusters of these two nitrogenases appear to be very similar, although the structure of the vanadium-iron cofactor has not been explicitly elucidated yet. Both enzymes have two pairs of metal sites, an M center in each a subunit and two P clusters on the interface of the α and β subunits [74]. The M centers are MFe_7S_9 (with M being V or Mo [Fig. 14]). In the $MoFe_7S_9$ cofactor, the octahedral molybdenum is coordinated to the imidazole nitrogen of histidine, two homocitrate oxygen atoms, and three cofactor sulfur atoms. The structure of the VFe_7S_9 cofactor is believed to be very similar to the $MoFe_7S_9$ cofactor based on magnetic circular dichroism [75], EXAFS [76,77], and Mössbauer [78] results. The P clusters of the two nitrogenases are Fe_8S_8 sulfide-bridged double cubanes and are spectroscopically identical (Fig. 14).

All of the nitrogenase systems reduce dinitrogen to ammonia. Other substrates can also be reduced, including acetylene, ethene, and protons, etc. The relative ability of each nitrogenase system to effect reduction of these other substrates distinguishes the V-, Mo-, and Fe-only nitrogenases from each other. The overall reaction for the Mo-nitrogenase [Equation (3)] shows that about 25% of the electron flux goes into the reduction of protons forming dihydrogen, whereas 40–50% of the electron flux in V-nitrogenase goes into proton reduction [Equation (4)], making dinitrogen reduction less efficient by this alternative nitrogenase.



Despite differences in the stoichiometry of substrate reduction between V-nitrogenase and Mo-dinitrogenase, both nitrogenases have similar K_m values for N_2 .

The mechanism of N_2 reduction involves a series of reduction steps in which nitrogenase-reductase docks to the Mo- or V-nitrogenase, transferring electrons one at a time in an MgATP-dependent reaction. The only reduced dinitrogen product in

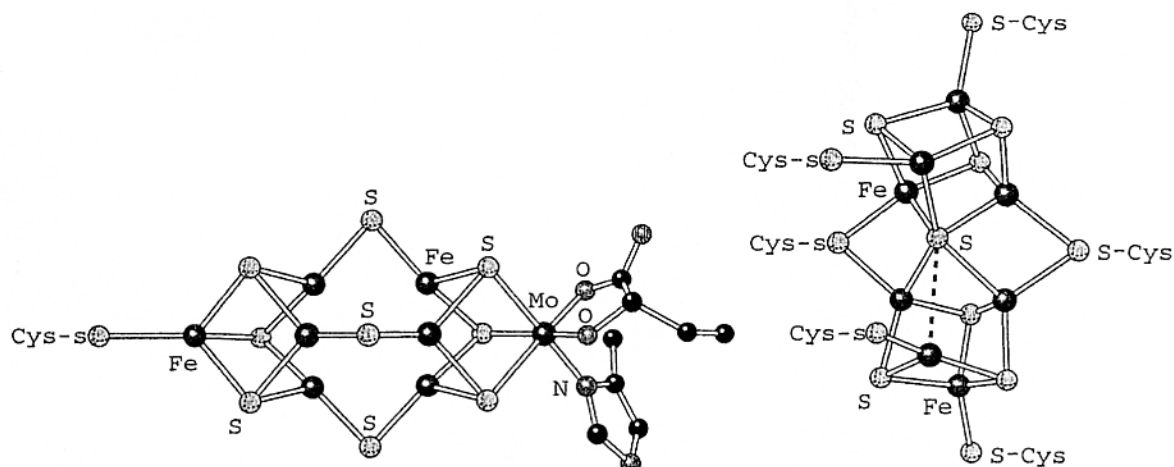


FIG. 14. Structures of the M and P clusters of nitrogenase. (Adapted from [90,91].)

the Mo-dinitrogenase enzyme is ammonia, although hydrazine, N_2H_4 , is observed in the V-nitrogenase system along with ammonia [79]. The proposed mechanism for Mo-nitrogenase involves a series of enzyme-bound reduced dinitrogen species that are not released from the enzyme. All nitrogenases can also reduce acetylene to ethylene. The vanadium enzyme can also form ethane as a minor product of this reaction while the molybdenum enzyme cannot. Ethane production has been suggested as a test for non-molybdenum nitrogenases [80].

4. STRUCTURE-FUNCTION RELATIONSHIPS

4.1. Vanadium Haloperoxidase Expression Systems

In the last 5 years there has been a rapid increase in what is known about the structure and function of haloperoxidases. The wealth of information obtained from X-ray crystal structures and the applications toward mechanism and reactivity are the result of successful cDNA sequence determinations and protein expression systems.

The complete cDNA sequence and the derived amino acid sequence for vanadium chloroperoxidase has been determined [81]. Native V-ClPO from *C. inaequalis* can be isolated from the growth media of this fungus, classifying the enzyme as a secretion protein. However, when the cDNA clone for the enzyme was isolated no putative leader peptide could be assigned, which is normally observed in precursors of secreted proteins. The isolated cDNA of V-ClPO was initially expressed in a bacterial expression system but afforded low yields for the enzyme.

Recently, V-ClPO has been successfully overexpressed in a yeast recombinant expression system [50]. The heterologous expression of V-ClPO in *Saccharomyces cerevisiae* afforded protein yields of 100 mg/L of yeast culture for apo-rV-ClPO.

Experiments were performed to determine if rV-CIPO would be secreted into the growth media for yeast with or without being fused to the yeast mating type α factor, which directs secretion of proteins to the media. Surprisingly, no stable expression was observed when the α factor was fused in frame with the gene for V-CIPO. Furthermore, when the α factor was omitted the protein was not secreted into the media but was found within the cytoplasm of the yeast in high yields. The isolation of large amounts of pure rV-CIPO obtained with the yeast expression system has enabled the creation of site-directed mutants of the enzyme and further structural characterization.

In addition to cloning and expression of vanadium chloroperoxidase, vanadium bromoperoxidase from the red macroalga *Corallina pilulifera* has been cloned and expressed. Cloning of V-BrPO from *C. pilulifera* produced two separate clones; *bpo1* and *bpo2*, where the two clones show 90% homology to one another [56]. The isolated cDNA for *bpo1* was initially expressed in a bacterial expression system. The protein was successfully expressed in the bacterial system, but overexpression of the enzyme was not achieved. The recombinant bromoperoxidase (rV-BrPO1) that was isolated from the bacteria was in the apo form, and bromoperoxidase activity could be observed upon incubation with vanadium. In addition, N-terminal sequencing of rV-BrPO1 was found to wholly agree with the sequence predicted by cDNA nucleotide sequence from *bpo1* with the exception of the N-terminal methionine residue. The N-terminal amino acid of V-BrPO isolated from the alga was masked, but this was not found to be the case for the recombinant enzyme isolated from the bacteria.

The isolation of cDNA clones and the overexpression of recombinant protein from various vanadium-dependent haloperoxidases have allowed the detailed structural characterization of native and mutant forms of the enzyme. In addition, the use of recombinant protein has aided the continued exploration of mechanisms of reactivity between vanadium-dependent chloroperoxidases and bromoperoxidases.

4.2. Comparative Aspects of the Vanadium Sites in V-BrPO and V-CIPO

The X-ray structures of V-BrPO from *A. nodosum* [57] and V-CIPO from *C. inaequalis* [45,46], as well as apo-V-CIPO [47], its metal-substituted derivatives [47], and the site-directed mutants of V-CIPO [49] are remarkably similar, but with certain notable differences. All of the structures reveal strong structural similarities at the vanadium site, which is not surprising given that all of the residues that participate in vanadate binding are conserved (Fig. 15). The protein provides a rigid oxyanion binding site, which is stabilized by hydrogen bonding interactions between these conserved residues and the oxyanion. In addition, both active sites for V-BrPO and V-CIPO are found at the core of a four-helix bundle structural motif in the enzymes. However, the overall amino acid sequence similarity of the structurally aligned residues is surprisingly low, at only 21.5%. As described above, the quaternary structure of V-

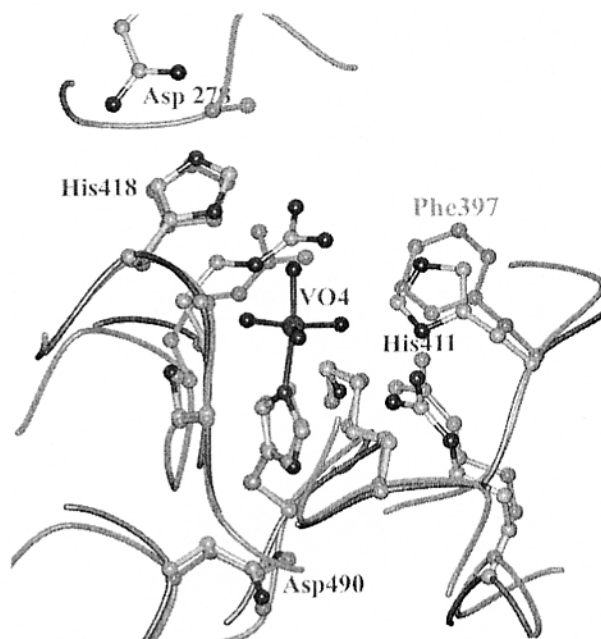


FIG. 15. Overlay of the X-ray coordinates of V-ClPO (*Curvularia inaequalis*) and V-BrPO (*Ascophyllum nodosum*). (Reproduced with permission from [57].) See Figure 6.15 in the color insert.

ClPO (*C. inaequalis*), a monomer, and V-BrPO (*A. nodosum*), a dimer, do differ, as well as their active site channels, undoubtedly as a result of differences in the primary structure.

In both enzymes the vanadium binding site motif is P[S/A]YPSGHAT, and the vanadate is directly coordinated to a histidine in the axial position [45–49]. The structures of V-BrPO and V-ClPO also render the proposed catalytic histidine as axially aligned with the vanadate cofactor, demonstrating again the highly conserved anion binding site. Equatorial ligands of the vanadate cofactor for V-BrPO and V-ClPO also appear to be conserved, although there are different residue-residue interactions and ligand stabilization that occur between the two haloperoxidases. Amino acid alignments of V-ClPO and V-BrPO for the vanadate binding site show that V-BrPO has an additional histidine residue that may be present in the vanadate binding site. Structural alignment of V-BrPO and V-ClPO show the additional histidine aligns with a phenylalanine in V-ClPO. The crystal structure of V-BrPO indicates that the extra histidine has no direct interaction with vanadate oxygen. It has been proposed that the residue might possibly participate as a proton/donor acceptor during the enzymatic reaction. The role of the additional histidine found in V-BrPO will need to be explored further. In spite of the overall low sequence similarity between V-BrPO and V-ClPO, it appears that the residues involved in cofactor binding are highly conserved, which may indicate a common genetic origin.

4.3. Mechanistic Considerations of the Catalytic Cycle

The first step in the catalytic cycle of V-BrPO and V-ClPO is coordination of hydrogen peroxide to vanadium(V) (Fig. 16, step 1). As discussed above, in the resting state, vanadium(V) is bound in a trigonal bipyramidal geometry ligated by a single protein side chain: His-496 in V-ClPO (*C. inaequalis*) or His-486 in V-BrPO (*A. nodosum*). The three equatorial oxygen ligands and the axial hydroxide ligand are hydrogen-bonded to multiple protein side chains or the protein backbone. The acid/base histidine (His-404 in V-ClPO (*C. inaequalis*) or His-418 for V-BrPO (*A. nodosum*)), which is present in the active site channel, must be deprotonated for H_2O_2 to bind [82]. Upon coordination of peroxide, the oxoperoxovanadium(V) intermediate is poised to oxidize the halide (Fig. 16, step 2). However, aqueous solutions of oxoperoxovanadium(V) or oxodiperoxovanadium(V) do not oxidize bromide or chloride [7]. Thus, it is significant that in the X-ray structure of V-ClPO Lys-353 was found to be in hydrogen-bonding contact with the bound peroxide, which could further activate it for oxidation of bromide or chloride. While the structure of the peroxo intermediate of V-BrPO has not been reported, one might anticipate that Lys or His residues could participate in similar hydrogen-bonding interactions. Step 2 in Fig. 16 seems to depend on the type of haloperoxidase; thus, the nature of the oxidized halogen intermediate (see Fig. 2) may vary between enzymes as well as the nature of the organic substrate to be halogenated in the case of algal V-BrPOs. In V-BrPO appropriate organic substrates can bind to the enzyme, blocking the release of an oxidized halogen intermediate [39,40], whereas the function of V-ClPO (*C. inaequalis*) is proposed to be in production of HOCl in the absence of organic substrate. Thus, the oxidized halogen intermediates could differ between V-ClPO and V-BrPO. In step 3 (Fig. 16), the oxidized halogen intermediate can either halogenate the organic substrate, RH, or oxidize a second equivalent of H_2O_2 .

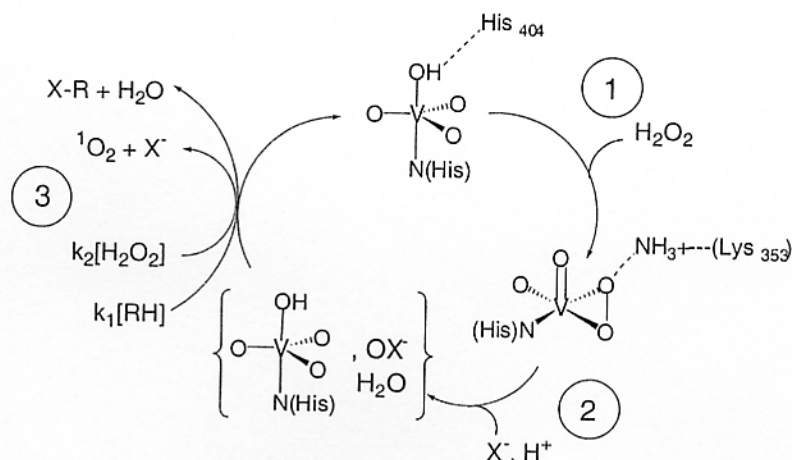


FIG. 16. Proposed catalytic cycle of vanadium haloperoxidase.

What does the reactivity of the site-directed mutants tell us about the mechanism of V-ClPO? All of the changes (i.e., H404A, D292A, H496A, R360A, K353A, R490A) effectively abolish chloroperoxidase activity, although one mutant, R360A, retains 6% of the chlorinating activity of recombinant V-ClPO, which is the highest activity of these mutants [49,50]. The lack of activity of H496A establishes the essentiality of the liganded histidine for reactivity [49]. Little has been reported about the activity of the H404A mutant except that it lacks chlorinating activity (and there was no mention of the brominating activity) [49]. Some of these mutants can, however, catalyze the oxidation of bromide by hydrogen peroxide, including K353A, R360A, and R490A [50]. All of these residues are involved in hydrogen-bonding interactions with the equatorial oxygen atoms ligated to vanadium(V) in the resting state. While the bromoperoxidase activity appears to be substantial, the specificity constant, $k_{\text{cat}}/K_{\text{m}}$, is always one to three orders of magnitude less than rV-ClPO over the pH range investigated, pH 4.2–7.0 [50]. The $k_{\text{cat}}/K_{\text{m}}$ value for the K353A mutant is the worst of the mutants, indicating the importance of this residue in the activation of vanadate-bound peroxide. Clearly, further studies of these mutants and others will be essential to our understanding of the mechanism of the vanadium haloperoxidases.

5. PERSPECTIVES

The active sites of V-BrPO (*A. nodosum*) and V-ClPO (*C. inaequalis*) display sequence homology with a family of acid phosphatases [83–85]. This includes both soluble and membrane-bound isoforms of phosphatidic acid phosphatase (PAP), enzymes crucial in mammalian signal transduction. Glucose-6-phosphatase, which is the enzyme affected in von Gierke disease, also has a homologous domain. The PAP family all have a perfectly conserved motif (GSYPSGHT), that is similar to the vanadate binding region of the haloperoxidases, P[S/A]YPSGHAT. The histidine covalently bound to the vanadate cofactor (residue 496 in V-ClPO) is conserved in each of the PAP family of enzymes. In addition, the haloperoxidase residues that form hydrogen bonds to the equatorial oxygens of the vanadate are also well conserved among phosphatidic acid phosphatases.

It is known that vanadate is a competitive inhibitor of phosphatases. It has also been found that remetallation of apohaloperoxidases is inhibited by phosphate. This information suggests that acid phosphatases and vanadium haloperoxidases may have similar three-dimensional structures at the active site as well as similar amino acid sequences. The similarity was confirmed when apo-V-ClPO was shown to have phosphatase activity [83]. Recombinant apochloroperoxidase hydrolyzes *p*-nitrophenyl phosphate, a commonly used phosphatase substrate. The K_{m} was found to be 51 μM , which compares well with those for acid phosphatases (100–200 μM). However, the maximal turnover for apo-ClPO was only 1.7 min^{-1} , which is much slower than the reported values of acid phosphatases (i.e., 10^2 – 10^3 s^{-1}).

In addition to a haloperoxidase exhibiting phosphatase activity, a vanadate-incorporated phytase has been prepared that has peroxidase activity [86,87]. Phytase isolated from the fungus *Aspergillus ficuum* is a phosphatase in its native state (i.e., without a bound metal ion). The vanadate-phytase derivative catalyzes the oxidation of sulfides by hydrogen peroxide. When the substrate is thioanisole, the (S)-sulfoxide enantiomer is the predominant product, formed with an enantiomeric excess (ee) of 56% and with a turnover frequency of 11 min^{-1} . Vanadium bromoperoxidases from *A. nodosum* [88] and *C. pilulifera* [89], on the other hand, can also catalyze the enantioselective oxidation of sulfides to sulfoxides. These reactions occur slowly, with turnover frequencies of 1 min^{-1} . V-BrPO from *A. nodosum* reacts with aromatic sulfides to form the (R)-sulfoxide in about 80% ee [88].

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ABBREVIATIONS

ee	enantiomeric excess
EXAFS	extended X-ray absorption fine structure
MCD	monochlorodimedone (= 2-chloro-5,5-dimethyl-1,3-dimedone)
NHE	normal hydrogen electrode
PAP	phosphatidic acid phosphatase
rV-CIPO	recombinant vanadium chloroperoxidase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
V-BrPO	vanadium bromoperoxidase
V-CIPO	vanadium chloroperoxidase
V-HPO	vanadium haloperoxidase

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