Vanadium haloperoxidases

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Handbook of Metalloproteins

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Vanadium haloperoxidases

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FUNCTIONAL CLASS

Enzyme; vanadate dependent peroxidases; EC 1.11.1.7; donor:hydrogen peroxide oxidoreductase.

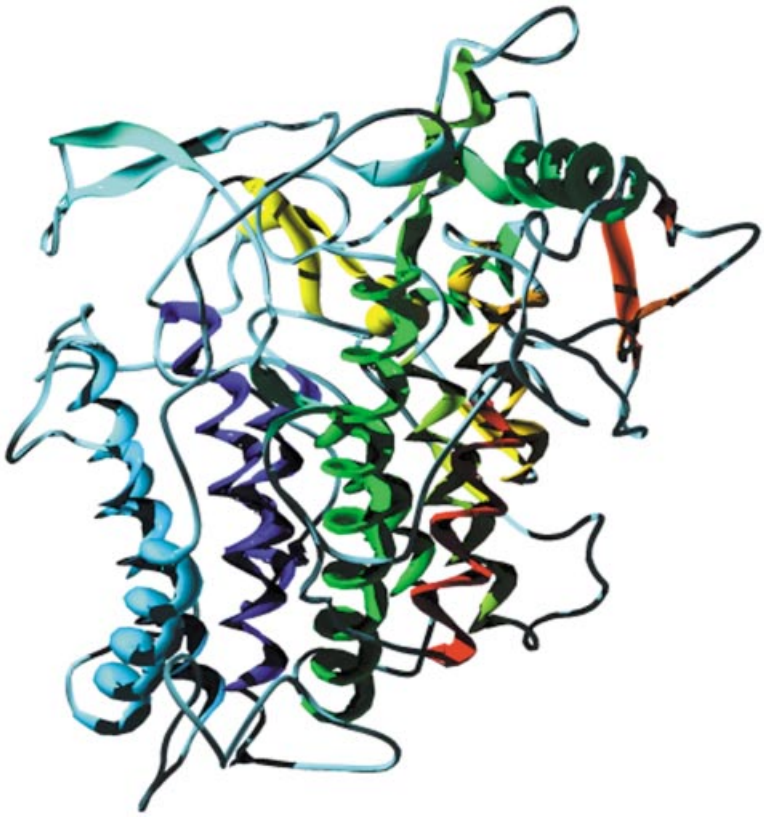
The vanadate dependent haloperoxidases are enzymes that catalyse the two-electron oxidation of a halide by hydrogen peroxide to the corresponding hypohalous acids according to Equation (1):

$$H_2O_2 + X^- + H^+ \rightarrow H_2O + HOX \qquad (1)$$

HOX reacts with a broad range of organic substrates to form a diversity of halogenated compounds. Haloperoxidases capable to catalyse the oxidation of chloride, bromide and iodide in the presence of H_2O_2 are designated as chloroperoxidases (CPOs), when they are able to oxide bromide and iodide, as bromoperoxidases (BPOs) and enzymes oxidising only iodide are characterised as iodoperoxidases. The distinction, however, is sometimes arbitrary.

OCCURRENCE

Vanadium bromoperoxidases (VBPOs) are present[1] in a great variety of brown and red seaweed samples (Great Barrier Reef in Australia, the Japanese Sea, the East Pacific and the North Atlantic Ocean). Vanadium iodoperoxidases have also been identified in some brown seaweeds but they have hardly been studied.[2,3] Most of the haloperoxidases are found on the thallus surface of these plants, although in the brown seaweed *Ascophyllum nodosum* a major part of the activity is found[4] in the so-called fruiting receptacles. Several species in the group of the dematiaceous hyphomycetes (fungi) produce haloperoxidases.[5] A number of these haloperoxidases have been identified[6] as being vanadium chloroperoxidases (VCPOs). These include the



3D Structure 3D Ribbon-type presentation of the CPO molecule. Prepared with Swiss PDB Viewer and POVRAY (3.1). PDB code: 1V NC.

enzyme from the species *Curvularia inaequaelis Drechslera biseptata*, *D. subpapendorfii*, *Embellisia didymospora* and *Ulocladium chartarum*.

BIOLOGICAL FUNCTION

Vanadium bromoperoxidases

Seaweeds release various volatile halogenated compounds particularly bromoform, dibromomethane and dibromochloromethane[7] and there is strong evidence that the BPOs are involved in the biosynthesis of these compounds. In some seaweeds, BPOs are located on the surface of the thallus and the seaweeds are able to release HOBr in seawater.[4] HOBr will rapidly react with organic matter that in seawater consists mainly of fulvic and humic acids, to give rise to brominated compounds that upon decay lead to formation of bromoform and dibromomethane. The formation of HOBr and brominated compounds by these algae appears to be part of a defence system of these seaweeds since HOBr is a bactericidal agent and also some of the halogenated compounds that are produced by seaweeds, have antimicrobial activity. The enzyme may prevent fouling of the seaweed by micro-organisms or may act as an antifeeding system.[8]

Vanadium chloroperoxidases

The VCPOs are produced by dematiaceous hyphomycetes. Some of these hyphomycetes are pathogenic towards plants and/or are saprophytes[9] and there may be a relation between the function of the enzyme and pathogenicity of these fungi. The enzyme forms HOCl, which is a strong bactericidal and oxidising agent known to degrade lignine.[10] It has been shown[11] that the enzyme is found both in the growth medium and on the surface of the fungal hyphen. The suggestion has been made[11] that the CPO is used by the fungus as an attack mechanism to oxidise the lignocellulose in the cell walls of the plant in order to facilitate penetration of the fungal hyphen into the host. It is likely that the HOCl which is generated by fungi also reacts specifically with organic compounds in plants resulting in the formation of organohalogens. This process may explain the presence of chlorinated compounds[12,13] in some soils.

AMINO ACID SEQUENCE INFORMATION

CPOs from dematiaceous hyphomycetes

- *Curvularia inaequalis*, 609 amino acid residues based on cDNA and confirmed on genomic DNA.[14]
- *Drechslera biseptata*, partial sequence based on genomic DNA.[15]
- *Embellisia didymospora*, 614 amino acids, based on genomic DNA.[16]

Bromoperoxidases from seaweed

- *Ascophyllum nodosum*, 1111 amino acids based on cDNA, peptide sequences and electron density interpretation.[17]
- *Corallina pilulifera*, 598 amino acid residues based on cDNA and partial peptide sequences.[18]
- *Fucus distichus*, 676 amino acid residues based on cDNA.[19]

PROTEIN PRODUCTION, PURIFICATION AND MOLECULAR CHARACTERIZATION

The vanadium iodo- and bromoperoxidases have been isolated from various families of red and brown seaweed but isolation from green seaweed has not yet been reported. For the enzyme from the brown seaweed *A. nodosum* that has been studied in detail, two different isolation procedures have been reported. The first procedure[20,21] consists of extraction of the chopped seaweed with Tris-buffer, removal of the alginate from the extract by addition of calcium ions, ammonium sulphate precipitation of the enzyme and ethanol extraction of the precipitate, resulting in a two-phase system. The alcohol phase is collected and the enzyme is further purified by ion-exchange chromatography on DEAE-Sephacel and Mono-Q. The other procedure[22] consists of an extraction by an aqueous polymer (PEG1550) two-phase system from freeze dried and powdered material followed by hydrophobic interaction chromatography. The procedure of the isolation of the enzyme from the red seaweed *C. pilulifera* consists again of extraction of the chopped seaweed with buffer preferably Tris-buffer, ammonium sulfate fractionation followed by ion-exchange chromatography or gel filtration.[23,24] For the enzyme from *C. pilulifera* an expression system has been reported.[18]

Under strong denaturating conditions (boiling of the samples in the presence of SDS) all these enzymes show bands with a molecular mass ranging from 58 to 70 kDa.[25] Some of these enzymes are very resistant towards denaturation and exhibit activity even in the presence of 1% SDS.[21] Only when boiled in the presence of the detergent the enzymes seem to unfold completely. For the enzyme from *A. nodosum*, assuming a molecular mass of 97 kDa, the presence of eight sulphydryl groups per protein molecule were reported.[26] This mass was obtained from the sedimentation coefficient and equilibrium centrifugation. The very high equilibrium value shows that this enzyme is a very compact molecule. On basis of binding to Concanavalin A some of these BPOs may be

glycosylated the linked carbohydrate moieties have not been identified. Anyway glycosylation appears to be limited.

Vanadium chloroperoxidases

It is possible to purify a CPO from the growth media of the fungus *C. inaequalis*.[27] Since the enzyme is mainly produced in the iodophase when nutrients become limiting, the procedure consists of growing the fungus for a period of 10 days, centrifugation, collection of the media and batch-wise addition of buffered DEAE Sephacel to bind the enzyme. After stirring overnight, the DEAE is loaded on to a column. The column is washed and the enzyme is eluted. As final purification steps, a hydrophobic interaction column and a Mono-Q ion-exchange column are used. For the enzyme from the fungus *Embellisia didymospora*, a slightly different procedure[16] was used since a large part of the enzyme remains bound to the mycelia. After the growth period 0.05% SDS was added to the culture which was incubated at room temperature with gentle agitation for 30 min. The medium was then filtered to remove the mycelium and the enzyme was further purified from the medium essentially as above. A procedure has been developed [28] to produce a recombinant CPO in yeast and the X-ray structure of this recombinant enzyme has been reported.[29]

When the VCPO is submitted to SDS/PAGE under denaturing conditions a band is found at approximately 67 kDa which is consistent with the calculated molecular mass. Prolonged incubation at boiling temperature results in the appearance of specific bands with lower mass that are due to cleavage of the main peptide chain at the six Asp–Pro linkages.[14]

METAL CONTENT AND COFACTOR

To determine the amount of vanadium present in these enzymes AA spectrophotometry is normally used.[20] Alternatively, it is possible to obtain accurate data on the vanadium content by double integration of the EPR signal of a reduced sample and comparison with a suitable standard.

Vanadium haloperoxidases commonly loose their enzymatic activity in phosphate containing buffers at low pH. Also during purification procedures the peroxidases slowly inactivate in phosphate buffers. However, in the absence of phosphate-containing buffers the enzymes retain their activity for months.[30] It is possible to obtain an inactive apo-enzyme by dialysis at low pH in a phosphate buffer containing EDTA.[31] Incubation of the apo-enzyme at neutral pH with orthovanadate (VO_4^{3-}, which is the vanadium(V) oxy-anion) results in recovery of the enzymatic activity as discovered originally by Vilter[32] for the bromoperoxidase of *A. nodosum*. The affinity of the apohaloperoxidases for vanadate is quite high. Values of the Kd of 35–55 nM have been reported[26] for the bromoperoxidase from *A. nodosum* which are close to the concentration of vanadate in sea water which is about 50 nM.[33] This suggests that, *in vivo*, no additional enzyme system is required for incorporation of vanadate in the apo-enzyme. This high affinity for vanadate decreases sharply at lower pH values.[26]

The affinity of the apoCPO for vanadate is somewhat less (140 nM) and also decreases at lower pH values.[34] The apoform of the enzyme, which is secreted by the fungus can easily be activated upon addition of vanadate (VO_4^{3-}) to the growth medium.[5] Apparently, these enzymes also do not require an additional enzyme system for the incorporation of vanadate. Also the recombinant CPO inactively produced by yeast[28] is easily reactivated by the addition of vanadate. In this respect, it should be noted that in most soils very high concentrations (100 ppm) of vanadium are present[35] and the amount of vanadium in soils does not appear to be a limiting factor in the conversion of the apo- to holo conversion of the VCPO once produced by the fungi. In addition, as shown[36] recently, the affinity of the apo-enzyme for vanadate increases at least 200-fold in the presence of H_2O_2.

ACTIVITY TEST

The vanadium peroxidases differ from the heme-containing peroxidases in their specificity for halides. The vanadium enzymes do not oxidize classical organic electron donors such as guaiacol, *o*-dianiside and benzidine.[37] Thus, assay systems are used that detect the formation of HOCl, HOBr or HOI. Routinely, the halogenating activity of these enzymes is determined by measuring the bromination or chlorination of the cyclic diketone monochlorodimedon (extinction coefficient of 20.2 $mM^{-1}\,cm^{-1}$ at 290 nm) to the dihalogenated compound (extinction coefficient of 0.2 $mM^{-1}\,cm^{-1}$ at 290 nm).[21] At more alkaline pH values and in particular at high concentrations of H_2O_2 a competing reaction (Equation (2)) between H_2O_2 and HOBr occurs.[38] This results in singlet oxygen formation and an apparent decrease in the rate of bromination or chlorination of monochlorodimedon.

$$H_2O_2 + HOX \rightarrow O_2 + H_2O + HX \qquad (2)$$

It is also possible to measure enzyme activity by monitoring the rate of dioxygen formation directly using an oxygen electrode.[39] At high pH, this is a more accurate measure of bromoperoxidase activity. A convenient method to assay the haloperoxidase activity qualitatively is following the bromination of phenol red to bromophenol blue,[30] this results in a marked colour change taking place which allows screening of haloperoxidase activity of large

numbers of samples by simple visual inspection. The turnover value of the VBPO varies considerably among the various seaweeds species and ranges from 130 s^{-1} for the bromoperoxidase from *A. nodosum*[40] to 1900 s^{-1} for the bromoperoxidase from the seaweed *Macrocystis pyrifera*.[41] The CPO from the fungus *C. inaequalis* has a turnover value for chloride oxidation of 22 s^{-1} and 250 s^{-1} for bromide oxidation.[16,28] The turnover values of the enzyme from *E. didymospora*[16] are 10 times lower for chloride and four times lower for bromide.

SPECTROSCOPY

The vanadium haloperoxidases exhibit no bands in the visible region of the optical spectrum. However, in the near UV spectrum of the VBPO of *A. nodosum* a shoulder is present[26] absorbing around 315 nm. This band is absent in the apo-enzyme and is recovered when vanadate is added to the enzyme. The intensity of this band decreases upon addition of H_2O_2 and the decrease is stable for a long period. Upon addition of bromide the original spectrum is restored and native enzyme is recovered. A similar band absorbing at 315 nm has been discovered[36] also in the VCPO from *C. inaequalis*, the intensity of which also decreases upon addition of H_2O_2. As we know now these changes correspond to the formation of a peroxo-intermediate. Identification of this band allowed detailed stopped-flow studies to monitor the binding of hydrogen peroxide to active site and the subsequent reaction of the peroxo-intermediate with halides. The kinetic parameters obtained are in agreement with those obtained from steady-state measurement.

Vanadium(IV) has one unpaired electron which is strongly coupled to the ^{51}V nucleus ($I = 7/2$). This spin–spin interaction gives rise to either 8 or 2 sets of 8 overlapping lines in axial symmetric complexes. By the EPR technique, it is possible to observe relatively low concentrations of the vanadium peroxidases.[40] Unfortunately, when the metal is reduced to the vanadium(IV) state the enzyme is inactive. These EPR studies[40] and also K-edge X-ray studies[42,43] on the VBPO from the brown seaweed *A. nodosum* have shown that the oxidation state of the metal in the native form is vanadium(V) and that its redox state does not change during turnover. A model was proposed[40,44] in which the vanadium(V) co-ordinates and activates hydrogen peroxide after which the halide is able to react with this activated peroxide to yield hypobromous acid. Vanadium may be considered to function as a Lewis acid in which the metal effectively withdraws electron density from the metal in such a way that the halide is able to react with this activated state of the enzyme to yield hypohalous acid. In this mechanism, no redox changes occur in the vanadium metal center. Also ESEEM studies on the reduced VBPO have been carried out. These spectroscopic methods have not only yielded insight in the mechanism but also by these techniques structural data have been obtained. For example, the conclusion was reached[46] that the geometry was five-coordinate. This was based on the EXAFS results obtained[42] earlier applying the bond-valence sum analysis and by comparing the intensity of the pre-edge feature in the EXAFS spectra of the enzyme with that of vanadium model compounds. EPR and ESEEM studies on the reduced enzyme demonstrated[40,45] clearly that water is in the direct coordination sphere of the metal oxide. The EXAFS data[42] showed the presence of multiple scattering effects from outer atoms of a group corresponding to a histidine ligated to vanadium. Also the ESEEM experiments[45] clearly indicated the presence of a histidine residue in the active site. As we know now the active site in the VBPO contains three histidines. The fungal CPO has not yet been studied by these detailed biophysical techniques but since this enzyme shares many properties with the VBPOs, it is likely that it will essentially show the same basic features.

X RAY STRUCTURES

Vanadium CPO crystallization

The enzyme crystallizes easily using ammonium sulphate as a precipitant.[47] However, owing to the inert nature of the enzyme it was initially difficult to prepare a heavy-metal derivative. Fortunately, it was also possible to prepare the apo-enzyme and to crystallize it in the presence of Na_2WO_4. This metal oxide will bind to the site where vanadate is normally bound.[48] Further, it was possible to prepare a mercury derivative by soaking the crystals in mercury acetate. The X-ray structures of the CPO heterologously expressed in *Saccharomyces cerevisiae* have been determined[29] both in the apo- and in the holo-form at 1.66 and 2.11 Å resolution, respectively. Crystallization requires slightly altered conditions and takes 2–3 months.

Overall description of the structure

Originally the CPO was crystallized as an azide derivative[47] at 2.1 Å. Somewhat later the native holo- and apo-enzyme were crystallized with a resolution of 2.03 Å revealing details of the structure and the active site. The enzyme molecule has an overall cylindrical shape with a length of about 80 Å and a diameter of 55 Å. The protein fold is mainly α helical with two four-helix bundles as the main structural motifs. The structure also contains some antiparallel β sheets (Figure 1).

The very compact structure is different from other known protein structures and it appears that the high stability is due to compact packing of the helices, which exhibit a strong stabilising hydrophobic effect. There are

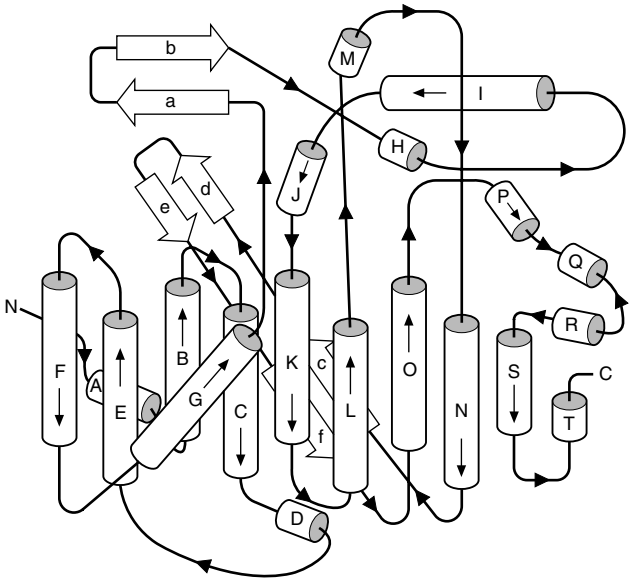


Figure 1 Schematic representation of the structural elements of CPO.[47] All helices are drawn as cylinders; β strands are depicted as arrows.

no disulphide bridges which is in contrast to the VBPO. The vanadium binding centre is located on top of the second four-helix bundle and the residues binding the prosthetic group are located at the C-terminal part of the protein and span a length of approximately 150 residues in the primary structure.

Actives site geometries

The X-ray structure analysis (Figure 2) shows that vanadium is bound as orthovanadate in a trigonal bipyrimidal coordination geometry in which three oxygen

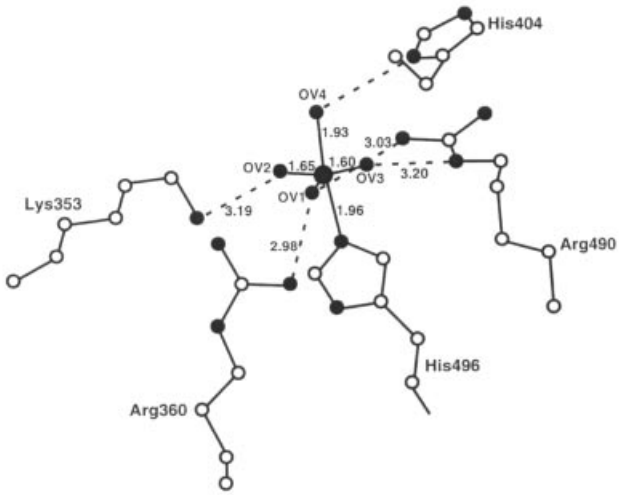


Figure 2 The vanadate binding site in VCPO modified from reference 47 using Swiss PDB Viewer. For clarity residues Ser402 and Gly403 are omitted.

atoms form a plane (bond length about 1.65 Å). The fourth apical oxygen ligand has a bond length of 1.93 Å which is in the range of a vanadium(V) hydroxyl bond and it is hydrogen bonded to nitrogen $N_{\delta 1}$ of His404 found at the apex.

The $N_{\varepsilon 2}$ atom from a histidine (His496) ligates directly to the metal and completes the trigonal bipyrimidal coordination. The negative charge of the vanadate group is compensated by hydrogen bonds to three positively charged residues (Arg360, Arg490, Lys353). Further, the vanadate forms hydrogen bonds with a glycine (Gly403) and a serine residue (Ser402). The Arg490 forms a salt bridge with residue Asp292. The vanadate binding pocket seems to form a very rigid frame stabilising oxyanion binding. The rigidity of this active site matrix is the result of large number of hydrogen bonding interactions involving the side chains and the main chain of residues lining the active site.[29,48,49]

The vanadate binding pocket is at the end of a channel that supplies access and release to the small substrates and products of the reaction. One half of the surface of the channel is mainly hydrophobic with Pro47, Pro221, Trp350, Phe393 and Pro395 as contributing side chains. The other half of the channel is predominantly polar with several carbonyl oxygens and the ion pair Arg490–Asp292. It is interesting to note that the Arg490 is also directly binding the orthovanadate.

Active site mutants

The overall structure and the active site of the recombinant enzyme are very similar to the native enzyme. The structures of the single site mutants to alanine have been determined[28,29] for His404, His496, Arg360 and Asp292. In general, no large changes are observed at the active site for any of these mutants, confirming the view of active site of the CPO as a rigid matrix providing the oxy-anion binding site. The exception is His404Ala, in this mutant the Arg490 moves at the expense of breaking the strong salt bridge with Asp292. The space generated by replacement of the large residues in the other mutants by alanines is usually replaced by water molecules that partially replaces the hydrogen bonding interactions to the vanadate. These mutations have strong effect on catalytic activity as will be discussed later.

Vanadium bromoperoxidases crystallization

Crystals of the holo-enzyme and the apo-enzyme were obtained[17] from ammonium sulphate and diffracted to 2.4 Å, the structure solution was performed by isomorphic replacement using tungstate that as has been shown[48] bind at the same site as vanadate.

Vanadium haloperoxidases

Overall description

The crystallized enzyme is a homodimer[17] containing two vanadate binding sites and consisting of 1111 amino acids with a corresponding molecular mass of 120 kDa. The dimeric enzyme is a compact ellipsoid of approximately 90 Å in length and with a diameter of about 75 Å. The structure of the vanadium BPO monomer is dominated by helical secondary structures (Figure 3). More than 46% of each monomer is involved in the dimer surface. The monomers are linked covalently in the N-terminal region by two disulphide bridges, four well-defined salt-bridges and a large number of hydrogen bonds involving both side chain and main chain residues. The two long helices at the N-terminus in each monomer that contains the disulphide bridges are aligned antiparallel with respect to each other. Additionally, each monomer has three intra-molecular disulphide bridges. Thus each dimer contains eight disulphide bridges in line with previous estimates.[26] The core of the dimer consists of two four-helix bundles and six additional helices.

Active site geometry

Vanadate is bound on the N-terminal side of a four-helix bundle and the metal is the centre of a trigonal bipyramid with three oxygen atoms (bond length about 1.6 Å) in the equatorial plane, one oxygen in the axial position (bond length about 1.8 Å) and a covalent linkage to the $N_{\varepsilon 2}$ of His486. The nitrogen–vanadium bond is 2.11 Å. The negatively charged vanadate group is hydrogen bonded to Lys341, Arg349, Gly416, Ser417, His418 and Arg480. Both hydrophilic and hydrophobic residues cover the entrance channel with hydrophobic residues dominating the lower part near the vanadate-binding site. Interestingly, amino residues from both monomers and vice versa contribute to the substrate access channel of each active centre. This channel clearly differs from the channel in vanadium CPO. For the vanadium BPO enantioselective sulphoxidation has been reported[50] whereas that for the VCPO is not enantioselective. This difference in selectivity may be caused by different structural factors associated with the access channel to the active sites.

Comparison of the overall structure and active site of the vanadium peroxidases

Sequence alignment[17,47] of the vanadium BPO and CPO shows very low sequence similarity except for the sequence identity near the active site. The two overall structures differ considerably although they are mainly α helical and share the vanadate binding four-helix bundle. In contrast,

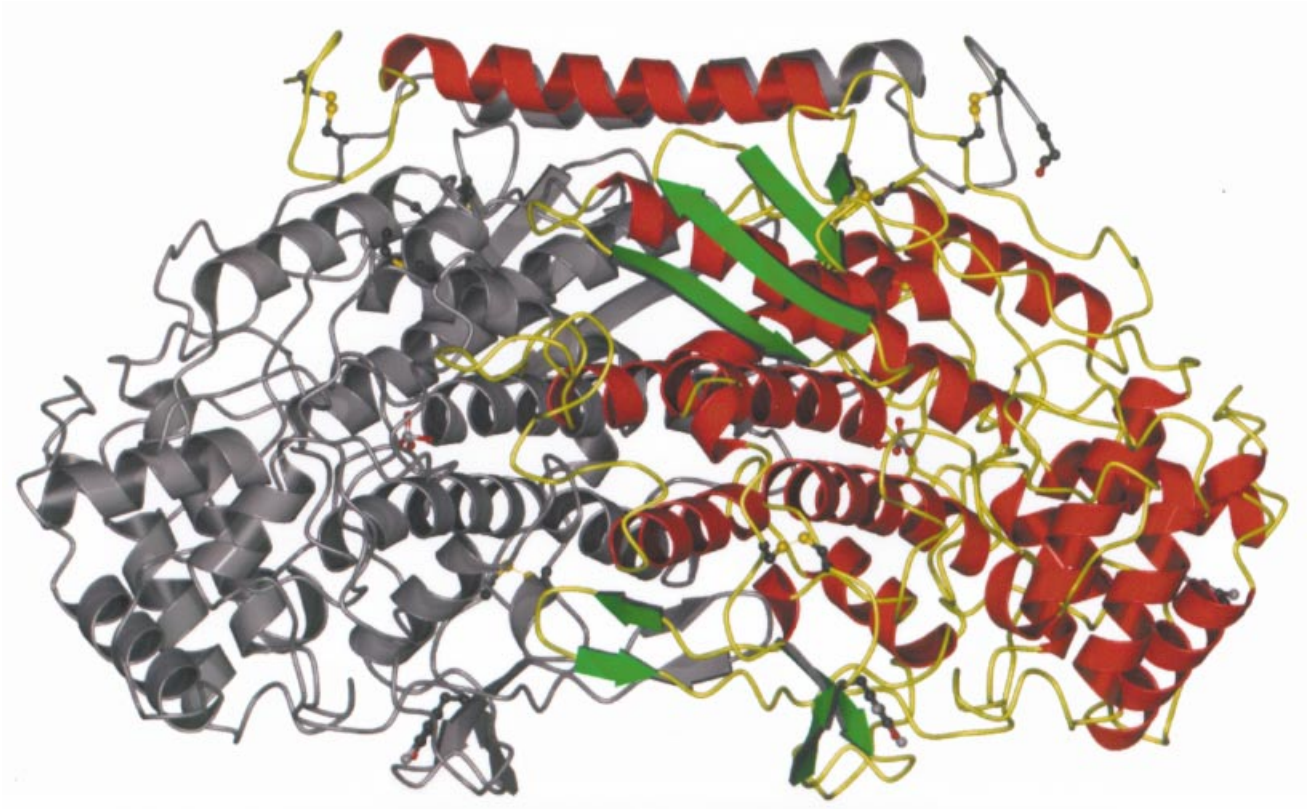


Figure 3 Ribbon-type representation of the vanadium homodimer of VBPO.[17] Monomer A, α helices in red, β strands in green and coil structure in yellow. Monomer B in grey.

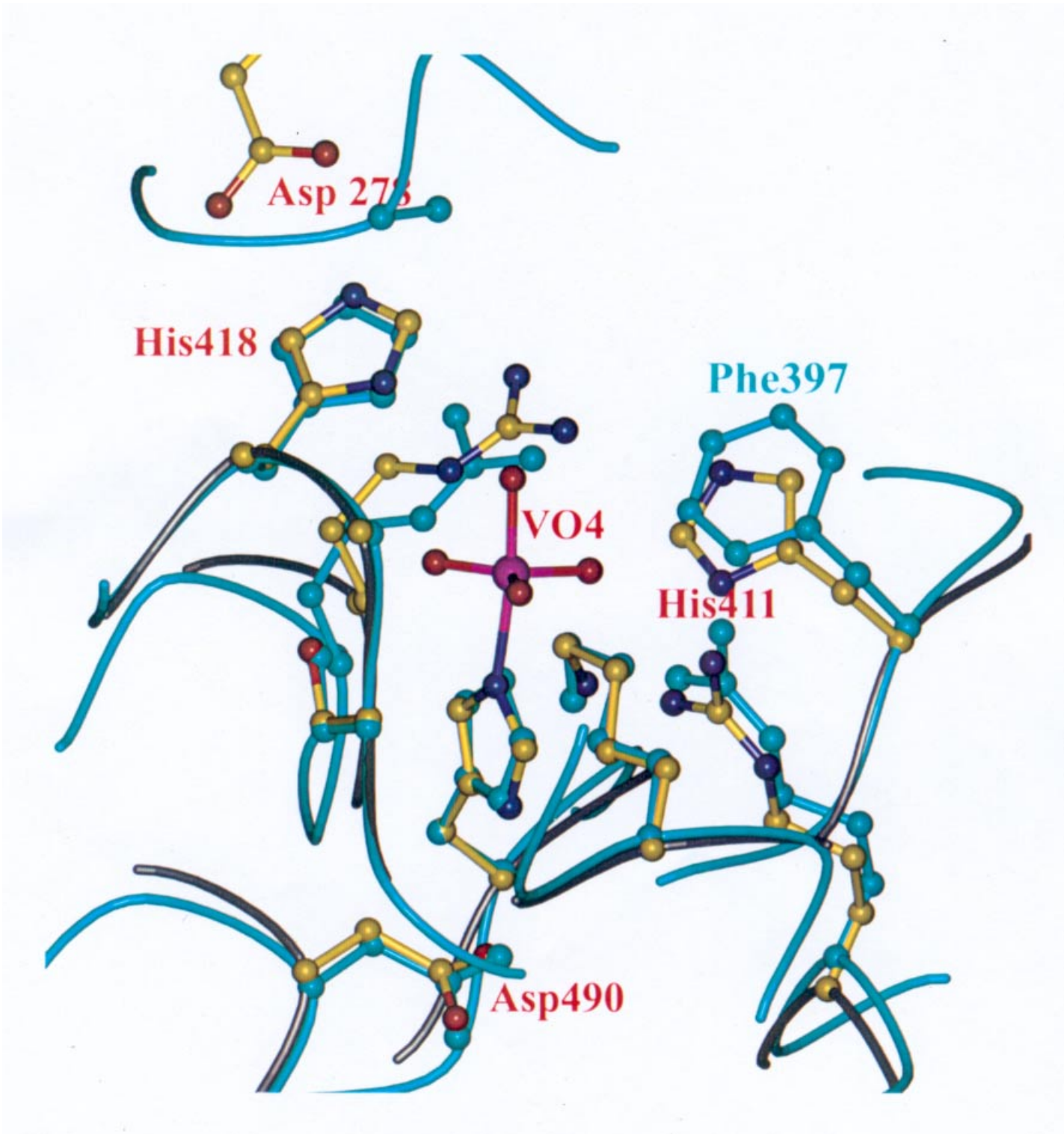


Figure 4 Superposition of VBPO and VCPO structures near the vanadate groups of BPO, based on the secondary structure alignment of both enzymes.[17] VBPO residues in atom colours. VCPO in blue.

the active sites show very high structural similarity and are structurally nearly superimposable (Figure 4).

As predicted[15,47] the residues involved in vanadate binding are conserved. There are, however, differences in the residues that are hydrogen bonded to the active site residues. In CPO Arg490 forms a salt bridge to Asp292 whereas in BPO the corresponding Arg480 has only two hydrogen bonds to the carbonyl oxygen atom of Thr275

and to two water molecules. His404 is hydrogen bonded in CPO to the carbonyl oxygen atoms of Trp289 and Ala290 whereas in BPO the corresponding His418 is strongly hydrogen bonded to the carbonyl of Asp278. Asp278 may be an important factor in determining the pK_a of His418. A major difference between CPO and BPO is that the Phe397 in CPO is replaced by a histidine (His411) in BPO which is hydrogen bonded to a water molecule close to the vanadate group.

HOMOLOGY BETWEEN HALOPEROXIDASES AND SEVERAL FAMILIES OF ACID PHOSPHATASES

Initial database searches[14] did not provide proteins homologous to the vanadium CPO. Only the vanadium BPO from *A. nodosum* showed[47] three consensus stretches of high similarity in the regions providing the metal anion-binding site. In the intervening regions the similarity turned out to be very low. Using these three stretches of high similarity as templates in database searches, it was shown by several groups[15,51–53] that similar stretches are present in a large number of acid phosphatases that were previously considered unrelated and for which no X-ray structures are available yet. It must be noted, however, that these highly similar stretches are interposed by highly variable regions both varying in sequence and in length. Based on the high similarity to the domains providing the metal binding site in vanadium CPO, it was proposed[15,51,52] that the binding pocket for vanadate in the peroxidases is similar to the phosphate-binding site in the aligned phosphatases. This is supported by the observations that phosphate and vanadate are structurally very similar, that many phosphatases are inhibited by vanadate[54,55] and the observation the vanadium haloperoxidases loose their activity in phosphate-containing buffers.[26] These observations led to the question whether apo-CPO can function as a phosphatase which was subsequently shown[15] to be the case. Since the initial database searches the available search algorithms have improved considerably and more than 40 (putative) proteins containing the three domains have been identified.[56] Glucose-6-phosphatase was also identified as carrying the proposed active site domains and studies[57,58] on mutants of this enzyme revealed that the amino acid residues Arg83, His119 and His 1176 are indispensable for glucose-6-phosphatase activity. These residue correspond to the active site residues Arg360, His404 and His496 in the vanadium CPO. Since a histidine residue is binding the vanadate in the peroxidases and as suggested also the phosphoryl moiety in the acid phosphatases, this family of related enzyme has been called the histidine phosphatases/peroxidase (HPP) superfamily.[59] In this superfamily there is a bias towards membrane proteins. So far only two families have been identified[56] that are soluble proteins; these are the vanadium haloperoxidases and the Class A non-specific acid phosphatases present in bacteria. Proteins from the HPP superfamily have very different physiological functions and are found from bacteria to humans. A number of groups[58–62] working on these phosphatases have modelled the active sites and topology of these enzymes using the active site and co-ordinates of the VCPO. Since phosphate and phosphate-metabolising enzymes entered evolution at a very early stage it seems likely that vanadate was coined by nature more recently to become the prosthetic group in the vanadium-containing haloperoxidases and thus these enzymes have evolved from the phosphatases.

FUNCTIONAL ASPECTS

Steady-state kinetics

The steady-state kinetics of the bromoperoxidases have been studied in great detail.[39,41,63] The results show that these enzymes oxidize bromide in the presence of hydrogen peroxide through a so-called 'bi–bi ping-pong' mechanism. Hydrogen peroxide binds first to the vanadium metal oxide forming an activated peroxo-intermediate, which facilitates the attack of the halide to yield hypobromous acid, this can further react with either an organic compound or an additional equivalent of hydrogen peroxide to form a halogenated compound or singlet molecular oxygen, respectively. The kinetic mechanisms for the bromoperoxidases from various sources are all very similar and slight reversible inhibition of BPO by high concentrations of H_2O_2 (20–400 mM) with stronger inhibition at higher pH and partial irreversible inactivation at low pH has been observed.[64] Bromide has been reported[63,64] to be a competitive inhibitor with respect to H_2O_2 as well as a non-competitive inhibitor. Bromide inhibition is observed only at pH values lower than the pH optimum. For the peroxidases studied, the Michaelis–Menten constant (K_m) for H_2O_2 increases with increasing H^+ concentration. For the bromoperoxidase from *A. nodosum* the K_m for H_2O_2 is 22 μM at pH 8.0 and its value increases to 3.1 mM at pH 4.0. The data indicate that an amino acid residue with a pK_a of 5.7–6.5 is important in the binding of peroxide to these enzymes. A histidine residue has been suggested[63] to be responsible for this phenomenon and these are indeed present in the active site of the enzyme.

The k_{cat} for the enzyme from *A. nodosum* is slightly pH dependent[63] and decreases from 300 s^{-1} at pH 4.0–50 s^{-1} at pH 8.0. In contrast, the K_m for bromide increases from 1.7 mM at pH 4.0 to 18.1 mM at pH 8.0. The specificity constants k_{cat}/K_m at pH 4.0 and 8.0 for Br^- are $1.8 \times 10^5\ M^{-1}\ s^{-1}$ and $2.8 \times 10^3\ M^{-1}\ s^{-1}$, respectively. The specificity constants for H_2O_2 at pH 4.0 and 8.0 are 1.0×10^5 and $2.3 \times 10^6\ M^{-1}\ s^{-1}$, respectively.

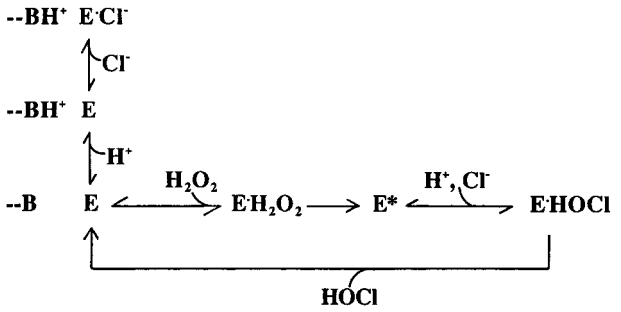


Figure 5 Simplified representation of the reaction of substrates with the vanadium haloperoxidases. For simplicity a number of protonated states have been omitted. The enzyme species E^* presents the activated vanadium peroxo complex. B represents an acid–base group. When this group becomes protonated, hydrogen peroxide is unable to react with the enzyme.[37]

A steady-state kinetic analysis[65] showed that the kinetics of the CPO of *C. inaequalis* resemble those of the VBPOs.[39,63] The CPO exhibits a pH profile similar to that of VBPO although the optimal pH of 5.0 is at a lower pH value than that for the BPOs. At low pH, high chloride concentrations inhibit the enzyme in a competitive way[65] whereas at higher pH values the activity displays a normal Michaelis–Menten type of behaviour. Both the K_m for chloride and the K_m for hydrogen peroxide are a function of pH. The log K_m for chloride increases linearly with pH whereas that for hydrogen peroxide decreases with pH demonstrating[27,65] that protons are involved in the catalytic cycle. These observations have led to the following simplified ping-pong type of mechanism for the haloperoxidases (Figure 5).

In the uninhibited reaction cycle, the enzyme first reacts with peroxide to form a peroxo-intermediate after which a halide ion and a proton react and an enzyme-hypohalous species is formed which decays rapidly to enzyme and free HOX. The linear dependency of the log K_m for hydrogen peroxide on pH suggests that an ionisable group is involved in the binding of hydrogen peroxide. When this group is protonated hydrogen peroxide is unable to bind. This phenomenon and the inhibition by chloride probably cause the decrease in enzymatic activity at low pH. The decrease at higher pH value is due to both a decrease in the V_{max} and the pH dependence of the K_m for halide.[65] These effects account for the skewed curve of the activity versus pH seen for the CPO. It has been speculated[65] that the group responsible for these effects was a histidine residue near the active site which is in agreement with the X-ray structural data now available. The k_{cat} of the chloroperoxidase calculated from the data[27,65] reaches a maximum of 22 s^{-1} at pH 4.0. The K_m values for Cl^- and H_2O_2 at this pH are both 0.1 mM and the specificity constants are both 2.2×10^5 M^{-1} s^{-1} at pH 4.0.

FUNCTIONAL DERIVATIVES

General remarks

Accidentally, the VCPO was originally crystallized in the presence of azide and the X-ray study revealed[47] direct

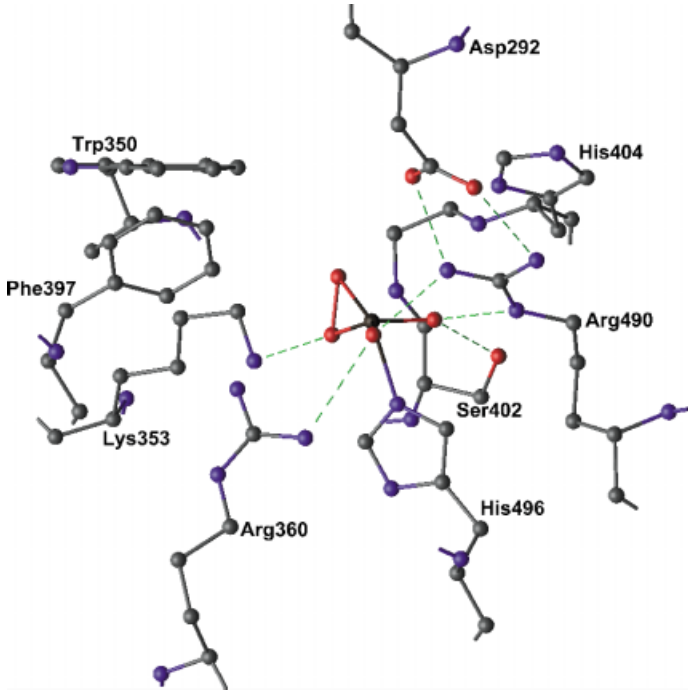


Figure 6 Hydrogen-bonding pattern of the peroxide form of the active site of the VCPO around the vanadate-binding site.[49] All residues proposed to have some role in the catalytic mechanism are included. Prepared with Swiss PDB Viewer and POVRAY (3.1).

coordination of azide to the metal center. The enzyme is also strongly inhibited by azide although mechanistic details are lacking. Steady-state kinetics[63,65] of both CPO and BPO indicated the presence of a vanadium peroxo-intermediate during catalysis and optical spectroscopy data also pointed to such an intermediate. Furthermore, many inorganic vanadium(V) compounds form stable peroxide intermediates.[66] Indeed, the crystal structure of the peroxide intermediate has been obtained[49] confirming the proposed basic catalytic steps in mechanism of the vanadium CPO.

X-ray structure of the peroxide form of VCPO

The X-ray structure of this peroxo-intermediate has been determined at 2.24 Å. Crystals were incubated in mother liquor containing 20 mM H_2O_2 for 2 h, shock frozen in a cryobuffer and kept at 100 K. The different electron density map that was obtained shows that the apical oxygen OV4 has been released and that the peroxide binds side-on in the equatorial plane to the vanadium (Figure 6).

The coordination geometry is that of a distorted tetragonal pyramid with only four oxygen atoms and one nitrogen atom. The apical ligand is oxygen OV3 with a bond length of about 1.6 Å identifying this as a V=O bond. There is a longer VO bond of about 1.93 Å and the two peroxide atoms are at about 1.87 Å. The distance between the two peroxide oxygens OV2 and OV4 is 1.47 Å.

There are changes in the hydrogen-bonding network, the most important being that His404 is no longer hydrogen bonded to any of the vanadate oxygens. Interestingly, one of the peroxide oxygens is now hydrogen bonded to Lys353 which may have important implications for the catalytic mechanism.

SITE DIRECTED MUTAGENESIS AND CATALYTIC MECHANISM

Figure 5 shows already a simplified mechanism based on the steady-state kinetic analysis. Basically, there are two steps: formation of the peroxide intermediate and reaction of the halide with this peroxide intermediate. Site directed mutagenesis studies[28,29,36] have been performed that shed some light on the role of the highly conserved active site residues in catalysis.

When the vanadate-binding residue His496 is changed into an alanine, the mutant enzyme loses the ability to bind vanadate covalently, resulting in inactive enzyme. Analysis of the crystal structure revealed that the metal ion is still present in the active site of the mutant. However, the covalent bond is lost, and vanadate is found in a tetrahedral conformation hydrogen bonded to the active site residues. Apparently, the covalent bond to the histidine and the penta-coordination are essential for catalytic activity.

On the basis of analogy to heme-peroxidases His404 was originally suggested[47] to play a role as acid–base catalyst in the binding of hydrogen peroxide by accepting a proton of the peroxide. More recently, it was proposed[28,29] that this residue has a more indirect role in activating the apical hydroxide to deprotonate the peroxide. The His4040Ala mutant has been studied and the mutant has lost its chlorinating activity. However, the X-ray structure of this mutant shows that there is a change in conformation with an effect on the active site making direct conclusions about the role of this residue difficult.

The effects of the mutations of the equatorial residues Arg490, Arg360 are less dramatic and although mutant R360 has residual CPO activity, the basic feature of each of the mutants is that it has changed into a bromoperoxidase. This is evident when the specificity constants of the mutants are compared[28] with those of the VBPO from *A. nodosum*. The specificity constants for Br^- have a maximal value around pH 4 and are 1.8×10^5, 1.5×10^6 and $5.5 \times 10^5\ M^{-1}\,s^{-1}$ for *A. nodosum*, Arg360Ala and Arg490Ala, respectively. The specificity constants for H_2O_2 are maximal around pH 7 and are 2.8×10^6, 7.7×10^5 and $1.8 \times 10^5\ M^{-1}\,s^{-1}$ for *A. nodosum*, Arg360Ala and Arg490Ala, respectively. Mutation of Lys353 into an alanine has a much stronger effect on the catalytic efficiency. The specificity constant of this mutant for Br^- at pH 4 is only $5.3 \times 10^3\ M^{-1}\,s^{-1}$ and for H_2O_2 has decreased to $2.9 \times 10^4\ M^{-1}\,s^{-1}$ at pH 7.

Figure 7 shows the catalytic mechanism for the VCPO proposed by Hemrika *et al.*[28] accounting for the consequences of the mutations and which is a combination and extension of previous schemes.

The native enzyme is shown in panel 7A and panel 7B–C describe the formation of the peroxide intermediate in which His404 activates the apical hydroxide to deprotonate the peroxide and leading to formation of the side-on bound peroxide. The vanadium atom acts as a Lewis acid withdrawing electron density from the bound peroxide. The two arginine residues Arg490 and Arg360 further assist electron withdrawal and Lys353 by forming a hydrogen bond to one of the peroxide oxygens causes polarisation of the bound peroxide. Model complex studies[67,68] have suggested that the protonation of the bound peroxide may also occur, thus increasing its reactivity. The next step in catalysis is nucleophilic attack by the halide (Panel D) breaking the oxygen–oxygen bond and formation of the nucleophilic OX-group. This group will take up a proton to form HOCl (panel E) which leaves the co-ordination sphere. This model explains why mutations of Arg360 and Arg490 have less drastic effects than that of mutation of Lys353. Arg360 only forms a hydrogen bond to the oxygen (OV1) of the vanadate and Arg490 forms hydrogen bonds to both (OV1 and OV2). This may explain the relatively mild effect of the Arg360

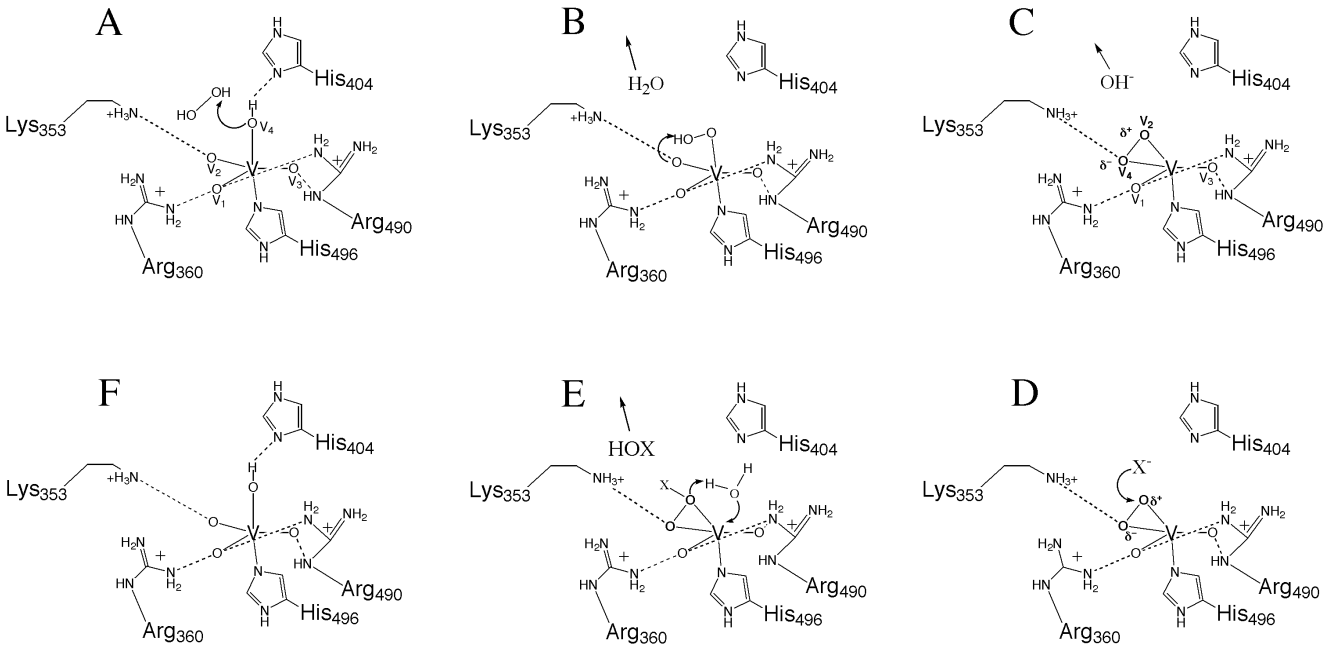


Figure 7 Minimal reaction scheme for vanadium CPO catalysis.[28]

mutation compared to that of the Arg490 mutation. When Asp292 which forms a strong salt bride with Arg490 is mutated into an alanine the enzymes also loses its chlorinating activity[29] highlighting the importance of this residue. It is obvious that there is an intricate balance of charges and protonation that tunes the reactivity of the active site in the VCPO. Apparently, any disturbance will convert the CPO into a BPO. This raises the important question as to which factors determine whether an enzyme is a bromo- or CPO. A comparison of the active site structures of the two enzymes (Figure 4) shows that they are essentially identical with only one difference. Phe397 in vanadium CPO is substituted by His411 in BPO and which is within hydrogen-bonding distance of a modelled peroxo-intermediate.[17] It is conceivable that this His411 decreases the reactivity of the enzyme by preventing protonation of the bound peroxide and thus decreasing its reactivity.

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