Heterologous Expression of the Vanadium-containing Chloroperoxidase from Curvularia inaequalis in Saccharomyces cerevisiae and Site-directed Mutagenesis of the Active Site Residues His$^{496}$, Lys$^{353}$, Arg$^{360}$, and Arg$^{490}$*  

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The vanadium-containing chloroperoxidase from the fungus Curvularia inaequalis is heterologously expressed to high levels in the yeast Saccharomyces cerevisiae. Characterization of the recombinant enzyme reveals that this behaves very similar to the native chloroperoxidase. Site-directed mutagenesis is performed on four highly conserved active site residues to examine their role in catalysis. When the vanadate-binding residue His$^{496}$ is changed into an alanine, the mutant enzyme loses the ability to bind vanadate covalently resulting in an inactive enzyme. The negative charges on the vanadate oxygens are compensated by hydrogen bonds with the residues Arg$^{360}$, Arg$^{490}$, and Lys$^{353}$. When these residues are changed into alanines the mutant enzymes lose the ability to effectively oxidize chloride but can still function as bromoperoxidases. A general mechanism for haloperoxidase catalysis is proposed that also correlates the kinetic properties of the mutants with the charge and the hydrogen-bonding network in the vanadate-binding site.

Haloperoxidases are enzymes catalyzing the two-electron oxidation of a halide (X$^-$) to the corresponding hypohalous acid (HOX) according to Equation 1.

$$\text{HO}_2^- + \text{H}^+ + \text{X} \rightarrow \text{H}_2\text{O} + \text{HOX} \quad \text{(Eq. 1)}$$

This hypohalous acid may further react with a broad range of nucleophilic acceptors to form a diversity of halogenated compounds. The haloperoxidases are named after the most electrophilic halide they are able to oxidize, implying that a chloroperoxidase can oxidize chloride, bromide, and iodide, whereas a bromoperoxidase can oxidize bromide and iodide.

The vanadium haloperoxidases (VHPO)$^1$ form one of the three groups of haloperoxidases that are currently known.

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1 The abbreviations used are: VHPO, vanadium haloperoxidase; VCPO, vanadium-containing chloroperoxidase; rVCPO, recombinant enzyme VCPO; VIBO, vanadium bromoperoxidase; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis.

These enzymes were first discovered in marine macroalgae (seaweeds) (1–3) but are also shown to be present in fungi (4) and in a lichen (5). The most studied fungal vanadium haloperoxidase is the vanadium chloroperoxidase (VCPO) from the plant pathogenic fungus Curvularia inaequalis (6–13). This enzyme is found associated with the fungal hyphae (13) but can also be isolated from the growth medium of the fungus. When vanadate is absent from the growth medium of the fungus, the enzyme is present in its inactive apo form. This apoenzyme can easily be activated by the addition of vanadate (6) indicating that auxiliary protein factors are not essential for incorporation of the cofactor.

The gene encoding the C. inaequalis VCPO has been cloned and sequenced (10). The gene codes for a protein of 609 amino acids with a calculated molecular mass of 67,488 Da. Determination of the crystal structure of this enzyme (11) revealed a molecule with an overall cylindrical shape measuring about 80 × 50 Å. The secondary structure is mainly α-helical with two four-helix bundles as main structural motifs of the secondary structure. The active site of the enzyme is located on top of the second four-helix bundle. The vanadium ion, which is present in its highest oxidation state (5+) as hydrogen vanadate (HVO$_2^-$), is coordinated to the protein in a trigonal bipyramidal fashion with three oxygen atoms in the equatorial plane (Fig. 1). A covalent bond is formed at one apical position with atom N$^{66}$ of His$^{496}$, and a hydroxide group is present at the other apical position where it forms a hydrogen bond with N$^{31}$ of His$^{496}$ (14). The negative charges of the equatorial vanadate oxygens are compensated by hydrogen bonds to surrounding hydrophilic or positively charged protein groups (Lys$^{353}$, Arg$^{360}$, Ser$^{492}$, Gly$^{495}$, and Arg$^{490}$).

Analysis of the peroxide form of the enzyme reveals a distorted tetragonal coordination geometry (14). The apical hydroxide group has been released, and His$^{496}$ is no longer hydrogen-bonded to any oxygen function of the vanadate. The peroxide binds side-on in the equatorial plane. One of the peroxide oxygens is hydrogen-bonded to Lys$^{353}$ and to the amide nitrogen of Gly$^{495}$ a residue that also makes a hydrogen bond to the other oxygen of the peroxide. Arg$^{360}$ and Arg$^{490}$ remain hydrogen-bonded to the same oxygens as in the native structure.

The active site residues are conserved in three short domains in other VHPOs and most interestingly in several families of acid phosphatases that were considered unrelated (11, 15, 16). Therefore we proposed that these enzymes have structurally very similar active sites. The homologous acid phosphatases contain important enzymes such glucose-6-phosphatase (17–
19), an enzyme that plays a key role in gluconeogenesis and glucose homeostasis (20), and the phosphatidic acid phosphatases (21–23), which are enzymes thought to be involved in signal transduction.

Although spectroscopic and kinetic studies have revealed many details about the metal-binding site and the reaction mechanisms of both the VPPOs and the VCPOs, the precise role of the active site residues has remained elusive. In trying to gain information about such roles we have created heterologous expression systems in which the C. inaequalis VCPO gene is expressed in Escherichia coli (24) or in the yeast Saccharomyces cerevisiae. Although the bacterial expression system was shown to produce recombinant enzyme (rVCPO) that could be activated by adding vanadate, the amount of enzyme that could be isolated from this system was very low, hampering in-depth analysis of the recombinant enzyme (24).

Here we describe a yeast recombinant expression system that enables us to isolate large amounts of recombinant (apo) enzyme. Determination of the kinetic parameters of this recombinant enzyme shows that these are essentially identical to those of the native enzyme. This S. cerevisiae expression system enables us to create site-directed mutants for rVCPO.

Binding and coordination of the vanadate cofactor in the VCPO active site are expected to be essential for enzymic activity. As a first approach in the analysis of the roles of the active site residues in catalysis, we have examined the effects of mutating the basic residues His496, Lys353, Arg360, and Arg490 into alanines. We show that His496 is essential for VCPO catalysis and that the K353A, R360A, and R490A mutations basically convert the mutant enzymes into VPPOs. Finally the (gradual) analysis and that the K353A, R360A, and R490A mutations basically convert the mutant enzymes into VPPOs. Finally the (gradual)

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C. inaequalis VCPO was performed according to Ref. 25. Transformants were selected in C. inaequalis VCPO wild type or the mutant and that the K353A, R360A, and R490A mutations basically convert the mutant enzymes into VPPOs. Finally the (gradual)

mistic representation of the DNA fragments and the plasmids used to construct the recombinant expression vector.

The 0.8-kb EcoRI-BamHI fragment containing the inducible S. cerevisiae Gal1 promoter (26, 27) was cloned into BamHI-EcoRI-digested Yeplac195 (a multi-copy yeast shuttle vector) (28) giving plasmid pTNT2. The EcoRI site of pTNT2 was removed by digestion with EcoRI, filling the ends with Klenow polymerase and religation, giving pTNT12. The C. inaequalis VCPO gene was present in pUC18 as a 2.5-kb PstI-EcoRI genomic DNA fragment containing the 1.1-kb 5'-half of the VCPO gene or as a 0.8-kb EcoRI-PvuII genomic DNA fragment containing the 3'-half of the gene, respectively (10).

A BamHI restriction site was created, at position –44 with respect to the VCPO start codon, by polymerase chain reaction using Tac polymerase (Promega) and as primers the M13/pUC 22-mer reverse sequence primer and primer WH1-Bam, 5'-GAGGAGATGACTACTACTATTATCACC-3'. After polymerase chain reaction amplification this fragment was isolated and digested with BamHI and EcoRI. The BamHI site present in pUC18 containing the 3'-end of the VCPO gene was destroyed by BamHI digestion, filling of the ends, and religation, giving pTNT13. Digestion with EcoRI and XhoI liberated the 3' VCPO fragment. A three-point ligation was used to ligate the 5' BamHI-VCPO-EcoRI fragment and the 3' EcoRI-VCPO-XhoI fragment into pTNT12 digested with BamHI and XhoI. This resulted in pTNT14, the inducible yeast expression vector. Plasmid pTNT14 was transformed to yeast, and transformants were checked for VHPO activity using the o-dianisidine or the phenol red assay.

Isolation of rVCPO—Yeast cells shown to express rVCPO were inoculated into starter cultures containing 0.67% yeast nitrogen base without amino acids (YNB-WO Difco), 2% (w/v) glucose, and 20 μg/ml (w/v) uracil and grown until the end of the log phase. Starter cultures were diluted 1:10 in 1% (w/v) yeast extract (Difco), 1% casein hydrolysate (Difco), and 1% (w/v) glucose and grown until the end of the log phase. The cultures were induced by the addition of galactose to a final concentration of 4% (w/v) and allowed to grow for another 2 days.

The yeast cells were harvested by centrifugation (5 min at 3000 × g) and resuspended to 1 g/ml in 50 mM Tris/HCl, pH 8.1. An equal volume of acid-washed glass beads (425–600 μm, Sigma) was added, and the yeast cells were broken using an ice water-cooled bead beater (Biospec) for 5 × 2 min. The suspension was collected, and the glass beads were washed with 2 volumes of 50 mM Tris/HCl, pH 8.1. After centrifugation (15 min at 5000 × g) an equal volume of isopropyl alcohol was added to the supernatant to precipitate nucleic acids. After centrifugation (20 min at 13,000 × g), the clear supernatant was applied to a DEAE-Sephaloc column (Amersham Pharmacia Biotech) (0.2 ml slurry/ml supernatant) equilibrated with 50 mM Tris/HCl, pH 8.1. After washing of the column with 2 volumes of 50 mM Tris/HCl, pH 8.1, and 2 volumes of 0.1 M NaCl in 50 mM Tris/HCl, pH 8.1, the enzyme was eluted with 0.6 M NaCl in 50 mM Tris/HCl, pH 8.1. Upon dialysis against 20 mM piperazine HCl, pH 5.4, a final purification step was performed using a Poros 20 HQ anion exchange column (Perspective Biosystems). The enzyme was eluted with a linear gradient of 0.1 to 1.0 M NaCl in 20 mM piperazine HCl, pH 5.4. Finally the pure apoenzyme was dialyzed against 50 mM Tris-Cl, pH 8.0, or against 100 μM orthovandate in 50 mM Tris cacitate, pH 8.1, followed by 50 mM Tris acetate, pH 8.1, to obtain the reconstituted holoenzyme.

Production of the Mutant Enzymes—rVCPO mutants were produced using either the Altered Sites II (Promega) or the Quickchange Site-directed Mutagenesis Kit (Stratagene). For the H496A and R490A...
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RESULTS

Expression of the C. inaequalis VCPO in S. cerevisiae—Since the VCPO of C. inaequalis and other hyphomycetes can be isolated from the growth media of these fungi (4), these enzymes are considered to be secretion proteins. Analysis of the predicted amino acid sequence and of the crystal structure of the C. inaequalis VCPO, however, did not show the cleavable N-terminal signal sequence that usually directs eukaryotic secretion proteins to the endoplasmic reticulum (10). This feature, together with the absence of post-translational modification steps like the formation of disulfide bridges and N-linked glycosylation, suggested the presence of an alternative secretion route for the fungal VCPOs (10). To investigate whether such an alternative route would also be taken when the C. inaequalis enzyme was expressed in S. cerevisiae, we decided to clone the VCPO gene into our yeast expression vector without fusing it to a yeast signal sequence, such as that of the yeast mating type α-factor (32).

Yeast colonies transformed with the pTNT14 expression vector (Fig. 2), as indicated by their Ura⁺ phenotype, were streaked on plates containing rich growth medium supplied with either glucose or galactose. After 2 days of growth, cells from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were separately re-separated from the glucose and the galactose plates were 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pression of the rVCPO could be obtained with this system. This feature was not further investigated since using the internal expression system under optimized growth and isolation procedures (as described under “Experimental Procedures”), we are now able to isolate approximately 100 mg of pure (apo) rVCPO from 1 liter of yeast culture.

Characterization of rVCPO—Apo-rVCPO as isolated from the yeast expression system can, like the native protein as isolated from C. inaequalis, easily be activated by incubating the apoenzyme with 100 μM orthovanadate in 50 mM Tris/SO₄, pH 8.1. Furthermore, addition of 100 μM orthovanadate to enzyme activity assay mixtures results in a very rapid activation of the apoenzyme. Fig. 3 shows the results of an SDS-PAGE experiments in which 3-μg samples of purified VCPO were run under either denaturing (Fig. 3A) or native (Fig. 3B) conditions. Fig. 3A shows that the native VCPO (lane 1) and the rVCPO (lane 2) both run as a single 67-kDa band. It was shown (10) that VCPO remains active in SDS-PAGE gels when β-mercaptoethanol is omitted from the sample buffer, and the samples are not boiled prior to loading. Fig. 3B, lanes 1 and 2, shows that, although the monomeric form of the enzymes is also visible, under these conditions both the native and the recombinant CPO run mainly as higher molecular weight aggregates that can be stained for activity.

EPR spectra were taken to characterize further the rVCPO. After reduction of the sample with dithionite, the typical vanadyl spectrum, as already observed for the native VCPO (6) and several VBPOs (33, 34), was obtained (results not shown). Determination of the rate of chloride oxidation by rVCPO as a function of pH by spectrophotometrically following the chlorination of monochlorodimedon revealed an optimum around pH 5 and a maximal value of 40 μM s⁻¹. Furthermore, at this optimal pH the Kₘ values for H₂O₂ and Cl⁻ were determined to be 0.5 and 0.9 mM, respectively. The specificity constant calculated from our data for chloride and H₂O₂ are 2.9×10⁴ M⁻¹ s⁻¹ and 6.5×10⁴ M⁻¹ s⁻¹, respectively. These kinetic parameters are very similar to those reported for the native VCPO (kcat of 23 s⁻¹, Kₘ H₂O₂ 10 μM and Kₘ Cl⁻ 0.9 mM) (6, 8).

The x-ray crystal structure of the apo-rVCPO and its reconstituted holo form have been determined very recently (35). It is reported that the overall structure of the rVCPO is nearly identical to that of the native VCPO, which holds particularly for the vanadate-binding site (35). These results confirm that the recombinant enzyme is very similar to the native one. We therefore decided to use this rVCPO as the wild type control in the experiments describing the parameters of the mutant enzymes.

Kinetic Properties of rVCPO and the Mutants—Fig. 3, A and B, lanes 3–6, shows the purified mutant enzymes run on SDS-PAGE and stained with Coomassie Brilliant Blue for protein identification (Fig. 3A) or with o-dianisidine to determine activity (Fig. 3B). It can be seen in Fig. 3A that all mutant proteins run similarly to the native VCPO and the rVCPO. Fig. 3B, lanes 3–5, shows that mutants R490A, R360A, and K353A, respectively, retain (bromo) peroxidase activity with that of mutant K353A being very little (lane 5). Mutant H496A (Fig. 3B, lane 6) shows no activity at all. However, when the activity stained gel is subsequently incubated with Coomassie Brilliant Blue, also in lane 6 a protein band is visible running at the same height as those in lanes 1–5 of Fig. 3B (results not shown).

The results obtained with the non-denaturing SDS-PAGE analysis indicate that the overall structure of the mutant proteins is similar to that of the native VCPO and the rVCPO. This is confirmed by the crystal structures of the mutants that have very recently been analyzed (35). From the mutants studied here, x-ray crystal structures are obtained for mutants H496A and R360A. The crystal structure analysis indicates that the vanadate-binding pocket forms a very rigid frame, stabilizing oxyanion binding. Furthermore, the empty spaces left by the replacement of the large side chains by an alanine are usually occupied by new solvent molecules, which partially replace the hydrogen bonding interactions to the vanadate (35).

A kinetic analysis of the rVCPO and the mutant enzymes was started to evaluate importance of these active site residues in catalysis. It is conceivable that the mutations also affect the affinity of the apoenzyme for the cofactor. This was not investigated, but to compensate for such potential effects the kinetic experiments were performed in the presence of excess (100 μM) orthovanadate.

When the activity of the rVCPO and the mutants was measured with chloride as a substrate it was found that, as expected, mutant H496A was inactive, but also mutants R490A and K353A showed very low chloroperoxidase activity. When the rate of the chlorination reaction was measured as a function of chloride concentration for mutants R490A and K353A, Vmax conditions were not reached even in the presence of 1 mM Cl⁻. Thus we were not able to determine the Kₘ for chloride for these mutants. Furthermore, activity remained less than 1.5% of that of the rVCPO. R360A is the only mutant showing appreciable chloroperoxidase activity although with a much higher Kₘ for Cl⁻ (19 mM) and a reaction rate at saturating chloride concentrations which is only 14% that of the rVCPO.

Mutants R490A and K353A have lost the ability to oxidize chloride, but lanes 4 and 5 of Fig. 3 indicate that these mutants are still able to oxidize bromide; therefore, we decided to determine bromoperoxidase activity in order to characterize further the changes in the kinetic properties of the mutants. It has recently been shown (36) that the native VCPO has a very high affinity for bromide (Kₘ 9 μM at pH 5) and that strong substrate inhibition by bromide already occurs at concentrations higher than 0.5 mM. In order to establish the dependence of enzyme activity on pH we therefore measured monochlorodimedon bromination at several pH values at low concentrations of the substrates H₂O₂ and Br⁻ (0.5 and 0.1 mM, respec-
R490A and R360A are even higher than that of the rVCPO as higher values, and strikingly, the specific activities of mutants the rVCPO activity is strongly inhibited, but the reaction rates still lower than those from the rVCPO.

As anticipated from the results depicted in Figs. 3 and 4, mutant R360A is least affected in its kinetic properties. The optimal value of $k_{\text{cat}}$ (298–333 s$^{-1}$) of the bromoperoxidase reaction of this mutant is shifted approximately 2 pH units to higher pH when compared with that of the rVCPO. However, the optimal value of $k_{\text{cat}}$ of the mutant is of similar magnitude as that of the rVCPO at pH 4.2 (230–253 s$^{-1}$). Mutant R490A also shows a shift in the optimum of the $k_{\text{cat}}$ value although apparently to a less alkaline pH as the R360A mutant. Table I shows that the $K_m$ for H$_2$O$_2$ of the R490A mutant below pH 5.5 has increased to 11.6 mM. Thus part of the sharp decline in the activity of this mutant at low pH as depicted in Fig. 4B results from the use of 40 mM H$_2$O$_2$. Strikingly, $k_{\text{cat}}$ of mutant R490A is also similar to that of the rVCPO when both are compared under optimal conditions.

From the $k_{\text{cat}}$ values it could be concluded that mutants R360A and R490A are better enzymes at high pH values than rVCPO. However, to make such a comparison the specificity constant ($k_{\text{cat}}/K_m$) needs also to be taken into account. Inspection of the specificity constants of rVCPO and the mutants in Table I reveals that the mutants are severely affected in catalytic activity. This is evident when we compare rVCPO to the mutant R360A which is least affected. Even at pH 4.2, where the specificity constant of R360A for Br$^-$ has its optimum, and at pH 7 where this constant is high for H$_2$O$_2$, these values are still lower than those from the rVCPO.

The kinetic parameters are most impaired in the case of mutant K353A (Table I), and no clear pH optimum is found in the activity of this mutant. $k_{\text{cat}}$ of this mutant is only 14% that of rVCPO when both are compared under optimal conditions.

**DISCUSSION**

Steady-state kinetic analysis of VHPoOs has indicated that these enzymes work according to a substrate-inhibited ping
KINETIC PARAMETERS FOR ONE SUBSTRATE (H₂O₂ OR Br⁻) MAXIMAL AROUND pH 7 AND ARE 2.8 TIMES THOSE OF THE VBPO OF C. INAEQUALIS. THE SPECIFICITY CONSTANTS OF THE MUTANTS ARE COMPARED WITH THOSE OF THE VBPO OF ASCOPELUM Y. NODOSUM (1). THE SPECIFICITY CONSTANTS FOR Br⁻ HAVE A MAXIMAL VALUE AROUND pH 4 AND ARE 1.8 × 10⁵, 1.5 × 10⁶, AND 5.5 × 10⁵ M⁻¹ s⁻¹ FOR A. NODOSUM, R360A, AND R490A, RESPECTIVELY. THE SPECIFICITY CONSTANTS FOR H₂O₂ ARE MAXIMAL AROUND pH 7 AND ARE 2.8 × 10⁷, 7.7 × 10⁸, AND 1.8 × 10⁹ M⁻¹ s⁻¹ FOR A. NODOSUM, R360A, AND R490A, RESPECTIVELY.

IT IS OBVIOUS THAT MUTATION R360A HAS A RELATIVELY MILD EFFECT AS COMPARED WITH R490A OR K353A, WHICH STRONGLY AFFECT THE CATALYTIC EFFICIENCY. WE WILL DESCRIBE BELOW HOW THE CONSEQUENCES OF THE MUTATIONS CAN BE EXPLAINED WHEN THE CHARGE AND THE HYDROGEN-BONDING NETWORK IN THE VCPO ACTIVE SITE ARE TAKEN INTO ACCOUNT.

THE NATIVE ENZYME IS SHOWN IN FIG. 5A, THE NATIVE ENZYMES DESCRIBED IN A–C ARE IDENTICAL TO THOSE PROPOSED BY MESSERSCHMIDT ET AL. (14) AND LEAD TO THE SIDE ON BOUND PEROXIDE AS OBSERVED IN THE STRUCTURE OF THE PEROXIDE INTERMEDIATE (14).

HALIDE BINDING IS THE NEXT STEP IN CATALYSIS. BINDING CAN OCCUR DIRECTLY AT THE VANADATE PRIOR TO ITS OXIDATION (14) OR THE HALIDE CAN PERFORM A NUCLEOPHILIC ATTACK ON ONE OF THE OXgens OF THE BOUND PEROXIDE. BASED ON MECHANISTIC STUDIES WITH MODEL COMPOUNDS (41) AND THE ASSUMPTION THAT DIRECT BINDING OF THE HALIDE WILL INCREASE ITS NUCLEOPHILICITY, WE FAVOR THE LATTER POSSIBILITY.

THE BASIC RESIDUES LYS⁵⁵³, ARG⁶⁰⁶, AND ARG⁴⁹⁰ COMPENSATE FOR THE NEGATIVE CHARGES ON THE VANADATE OXgens BY FORMING HYDROGEN BONDS WITH THESE ATOMS. CHANGING ANY OF THESE RESIDUES INTO AN ALANINE WILL INCREASE THE OVERALL NEGATIVE CHARGE IN THE ACTIVE SITE, WHICH IS EXPECTED TO INFLUENCE CATALYSIS. ACCORDINGLY, WE HAVE SHOWN THAT MUTANTS K353A AND R490A HAVE LOST THE ABILITY TO OXIDIZE CHLORIDE WHILE ALSO MUTANT R360A IS SEVERELY IMPAIRED IN CHLORIDE OXIDATION. HOWEVER, THESE THREE MUTANTS ARE STILL ABLE TO OXIDIZE THE LESS ELECTRONEGATIVE BROMIDE AND HAVE THERFORE INCREASED THEIR CHLORINATING ACTIVITY.}

**TABLE I**

| Enzyme            | pH   | Km (μM) | Vmax (μM s⁻¹) | Km (μM) | Vmax (μM s⁻¹) | Km (μM) | Vmax (μM s⁻¹) | Km (μM) | Vmax (μM s⁻¹) |
|-------------------|------|---------|---------------|---------|---------------|---------|---------------|---------|---------------|
| rVCPO H₂O₂        | 4.2  | 90      | 250           | 4.2     | 90            | 250     | 4.2           | 90      | 250           |
| Br⁻                | 5.2  | 5.5     | 105           | 5.2     | 5.5           | 105     | 5.2           | 5.5     | 105           |
| Br⁻                | 6.3  | 10.5    | 200           | 6.3     | 10.5          | 200     | 6.3           | 10.5    | 200           |
| Br⁻                | 7.0  | 15.0    | 300           | 7.0     | 15.0          | 300     | 7.0           | 15.0    | 300           |

Kinetic parameters were determined in sodium citrate/citric acid buffers adjusted to the desired pH and in the presence of 100 μM vanadate. Kinetic parameters for one substrate (H₂O₂ or Br⁻) were determined in the presence of a concentration of 5–10 times the determined Kₘ for the other substrate (Br⁻ or H₂O₂) and were calculated using Enzyme Kinetics (version 1.4 Macintosh Trinity software) on the basis of direct linear fits using at least eight substrate concentrations varying from 0.2 to 5 times the Kₘ for each mutant under each condition measured.

It is obvious that mutation R360A has a relatively mild effect as compared with R490A or K353A, which strongly affect the catalytic efficiency. We will describe below how the consequences of the mutations can be explained when the charge and the hydrogen-bonding network in the VCPO active site are taken into account.

The native enzyme is shown in Fig. 5A, the kinetic events described in A–C are identical to those proposed by Messerschmidt et al. (14) and lead to the side on bound peroxide as observed in the structure of the peroxide intermediate (14).

Halide binding is the next step in catalysis. Binding can occur directly at the vanadate prior to its oxidation (14) or the halide can perform a nucleophilic attack on one of the oxgens of the bound peroxide. Based on mechanistic studies with model compounds (41) and the assumption that direct binding of the halide will decrease its nucleophilicity, we favor the latter possibility.

The basic residues Lys⁵⁵³, Arg⁶⁰⁶, and Arg⁴⁹⁰ compensate for the negative charges on the vanadate oxgens by forming hydrogen bonds with these atoms. Changing any of these residues into an alanine will increase the overall negative charge in the active site, which is expected to influence catalysis. Accordingly, we have shown that mutants K353A and R490A have lost the ability to oxidize chloride while also mutant R360A is severely impaired in chloride oxidation. However, these three mutants are still able to oxidize the less electronegative bromide and have thus become bromoperoxidases by definition.

It can be rationalized that the positively charged residues in the active site (together with vanadate as a Lewis acid) decrease the electron density of the bound peroxide thereby activating this substrate. The effects of the mutations in the equatorial positions are most severe for mutant K353A. The Kₘ values for H₂O₂ and Br⁻ are largely increased, and also kₗcat is only 15% of the rVCPO when both are compared at their optimal pH values.

In the crystal structure of the peroxide form of the enzyme (14), it can be seen that Lys⁵⁵³ is the only positively charged residue that is directly linked to the bound peroxide, via a strong hydrogen bond with peroxide oxygen Ov4. This can explain the importance of this residue in catalysis since in addition to the above-mentioned general electron-withdrawing effect, this hydrogen bond (see Fig. 5, C and D) may also polarize the bound peroxide. This will make the peroxide oxygen Ov2 more susceptible toward a nucleophilic attack by the halide (Fig. 5D) as already suggested by Hamstra et al. (42).

Binding of the halide to the partially positive peroxide oxygen breaks the peroxide bond, and the nucleophilic OX− group is formed (Fig. 5D). The OX− group will take up a proton from an incoming water, possibly activated by His⁴⁰⁴ (Fig. 5E), and leave the coordination sphere as hypohalous acid. The formed hypohalous acid, resulting from the deprotonation of the water molecule, can simultaneously take the empty coordination site on...
the vanadium, and the native structure is formed back again (Fig. 5F).

Arg360 and Arg490 do not form hydrogen bonds with any of the oxygens of the bound peroxide, and as such, the effects of the Arg360 or Arg490 to Ala mutations are expected to be less drastic than those of the Lys353 to Ala mutation. The increased negative charge in the active site, however, will weaken the activation of the bound peroxide. Since Arg360 donates one hydrogen bond only (to OV1) and Arg490 donates one to OV1 and one to OV3, the effects observed in the R360A mutant are expected to be milder than those observed in the R490A mutant. This is confirmed by the analysis of these mutants.

The decreased activation of this bound peroxide may also result in the 100–1000-fold increase in the $K_m$ values for bromide as observed for the mutants. In this respect it is noteworthy that the R360A mutant, for which the $K_m$ values for bromide are affected the least, still has residual chlorinating activity. It has been suggested (40) that the activated oxygen OV2 (Fig. C and D) is protonated, thus creating a full positive charge on this atom. If one assumes that such protonation occurs in VCPO while the activated oxygen is only partially positive in the VBPOs and the mutants, one can explain both the high oxidizing ability and the relatively low value of the $K_m$ for chloride in VCPO (6), whereas the $K_m$ for bromide in VBPO and the mutants is only slightly affected when the pH is varied (1, 39).

The observed pH shift in the optimal value of $k_{cat}$ (Table I) upon mutation of Arg360 and Arg490 may be the consequence of the decrease in positive charge in the active site, which might affect the $pK_a$ of the residues governing the pH dependence. However, residues that are involved in the decrease of $k_{cat}$ as a function of pH have not been identified, and further analysis has to answer this question.

The results of the kinetic analysis of the site-directed mutants point to the importance of the activation of the bound peroxide prior to oxidation of the halide, and very recently we indicated why this activation may be less in the VBPOs as compared with the VCPOs (43). Alignment of the active site domains 1, 2, and 3 of the VCPOs, the currently known VBPOs, and the homologous acid phosphatases suggested that the most important difference between the VCPOs and the acid phosphatases on one hand and the VBPOs on the other hand might be the number of residues in domain 1 bridging the lysine and arginine, which are the counterparts of the vanadate bridging Lys363 and Arg490 of C. inaequalis. Only in the VBPOs are there seven instead of six bridging residues. This extra residue will result in a slightly different geometry of the active site of the VBPOs. This in turn may result in a less strong hydrogen bond between the lysine and the bound peroxide and therefore in a less positively charged and less activated peroxide that is unsusceptible to a nucleophilic attack by chloride at physiological concentrations.

This study has shown that His406 is essential for activity of the VCPO, and it is expected that the corresponding histidine is of equal importance for catalysis of the VBPOs and the homologous acid phosphatases, as was recently shown to be the case in glucose-6-phosphatase (44). Analysis of the mutants R490A, R360A, and K353A has revealed the importance of these side chains in compensating the negative charges of the vanadate oxygens. Furthermore, the importance of the hydrogen bond between Lys353 and the bound peroxide was shown. The mutations in the vanadate coordinating side chains may also affect the stability of the mutant enzyme for the cofactor, and we are currently performing experiments to investigate this. These results, together with the results of the analysis of site-directed mutants for His404 and Asp292 of which the crystal structures were recently determined (35), will hopefully deepen our understanding of the catalytic mechanism of the VCPO and the homologous enzymes.

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Fig. 5. Minimal reaction scheme for VCPO catalysis. (See text for details.)
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