Peroxidase and Phosphatase Activity of Active-site Mutants of Vanadium Chloroperoxidase from the Fungus *Curvularia inaequalis*

**IMPLICATIONS FOR THE CATALYTIC MECHANISMS**

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Mutation studies were performed on active-site residues of vanadium chloroperoxidase from the fungus *Curvularia inaequalis*, an enzyme which exhibits both haloperoxidase and phosphatase activity and is related to glucose-6-phosphatase. The effects of mutation to alanine on haloperoxidase activity were studied for the proposed catalytic residue His-404 and for residue Asp-292, which is located close to the vanadate cofactor. The mutants were severely impaired in their ability to oxidize chlorite but still oxidized bromide, although they inactivate during turnover. The effects on the optical absorption spectrum of vanadium chloroperoxidase indicate that mutant H404A has a reduced affinity for the cofactor, whereas this affinity is unchanged in mutant D292A. The effect on the phosphatase activity of the apoenzyme was investigated for six mutants of putative catalytic residues. Effects of mutation of His-496, Arg-490, Arg-360, Lys-353, and His-404 to alanine are in line with their proposed roles in nucleophilic attack, transition-state stabilization, and leaving-group protonation. Asp-292 is excluded as the group that protonates the leaving group. A model based on the mutagenesis studies is presented and may serve as a template for glucose-6-phosphatase and other related phosphatases. Hydrolysis of a phospho-histidine intermediate is the rate-determining step in the phosphatase activity of apochloroperoxidase, as shown by burst kinetics.

Vanadium haloperoxidases are enzymes that catalyze the oxidation of halides to their corresponding hypohalous acids at the expense of hydrogen peroxide.

\[ \text{H}_2\text{O}_2 + \text{H}^+ + \text{X} \rightarrow \text{H}_2\text{O} + \text{HOX} \]  
(Eq. 1)

Haloperoxidases are named after the most electronegative halide they are able to oxidize; thus, vanadium chloroperoxidase (VCPO)\(^1\) from *Curvularia inaequalis* oxidizes Cl\(^-\), Br\(^-\), and I\(^-\).

As illustrated in Fig. 1, the cofactor vanadate (HVO\(_5^{2-}\)), which is present in this enzyme, is bound covalently to His-496, resulting in a trigonal bipyramidal structure with 3 vanadate oxygens in the equatorial plane (1, 2). The Ne2 nitrogen of His-496 and the remaining vanadate oxygen occupy the 2 apical positions. The negative charge on the equatorial oxygens is compensated by H-bonds to 3 positively charged residues (Arg-490, Arg-360, and Lys-353). The apical oxygen has been assigned as a hydroxide group, and it is hydrogen-bonded to His-404. This residue is proposed to activate the hydroxide in the deprotonation of \(\text{H}_2\text{O}_2\) before binding of the peroxide to the vanadium center (2). A second residue that is a candidate to play a role as acid-base group in catalysis is Asp-292 (3), which is also in the vicinity of the apical hydroxide and forms a salt bridge with Arg-490 (not shown in Fig. 1).

Hydrogen peroxide is activated via binding to the vanadium center, after which the halide is oxidized. During this process the vanadium remains in the V\((5+)\) state. Thus, it has been proposed that the vanadate acts as a Lewis acid, withdrawing electron density from the bound peroxide (2, 4, 5). On the basis of mutagenesis studies it has been proposed that this withdrawal of electron density is enhanced by the positively charged residues Arg-490, Arg-360, and Lys-353, which are in direct contact with the vanadate cofactor (6). In the crystal structure of the peroxo form of the enzyme it can be seen that Lys-353 makes direct contact with one of the oxygens of the bound peroxide, and it has been suggested that this residue further activates the peroxide via charge separation on the peroxide oxygens. Recently, a scheme was published that describes in detail a proposal for the catalytic mechanism of vanadium haloperoxidases (6). It was shown that the binding of vanadate, the binding of hydrogen peroxide, and the oxidation of halides can be monitored optically at 300–330 nm. As a consequence, individual steps in the reaction mechanism can be measured directly via stopped-flow techniques (27), and such studies indicate that the oxidation of Cl\(^-\) by the peroxo intermediate occurs in at least two steps. Furthermore, it was shown that mutation of the vanadate binding residues His-496, Arg-360, Arg-490, and Lys-353 has a clear effect on the optical band.

Three domains that are far apart in the primary structure form the active site of VCPO. These domains are also found in several classes of phosphatases (7–9). It has also been demonstrated that the apo form of CPO exhibits phosphatase activity, although at a low rate compared with the phosphatases and to its peroxidase activity (7). Therefore, it was suggested that the active sites of VCPO and the aligned phosphatases are similar. The structural basis for this similarity is the resemblance of phosphate and vanadate and, perhaps more importantly, the resemblance of the trigonal bipyramidal structure of bound vanadate in the native VCPO and the proposed transition state in the phosphatases. This is in line with the fact that these phosphatases are strongly inhibited by vanadate (10). On the
basis of this transition state analogy, it was suggested that in apoCPO His-496 acts as a nucleophile, whereas several residues stabilize the pentacoordinated transition state, and His-404 plays a role in leaving group protonation. (7) Glucose-6-phosphatase (G6Pase), the enzyme involved in von Gierke’s disease, is one of the phosphatases predicted to have an active site similar to that of VCPO (7). For this enzyme, a phosphohistidine enzyme intermediate has been isolated (11, 12), and based on mutation studies, His-119 was predicted to act as the nucleophile in catalysis (13). However, based on the active-site structure of VCPO, we have predicted that His-176 of G6Pase is the nucleophile in catalysis, whereas His-119 is predicted to act as an acid base group (7). This also led to a newly predicted nine-transmembrane helix topology for G6Pase, which was subsequently confirmed by experimental evidence (14–16).

In the present report, the haloperoxidase activity of mutants H404A and D292A is described. It is shown that the effects on activity are in agreement with the effects on the optical spectra, and the implications for the peroxidase mechanism are discussed. Also, the phosphatase activity of the apo forms of H496A, R490A, R360A, K353A, D292A, and H404A is compared with that of apo-recombinant CPO (rCPO). On the basis of the results presented in this paper, in combination with crystal structure data of VCPO mutants, and clues from the peroxidase activity of the holo forms of these mutants, a reaction mechanism for the haloperoxidase activity is presented and discussed. Furthermore, the presteady state kinetics of the hydrolysis of p-nitrophenyl phosphate (p-NPP) has been investigated, and we have obtained evidence as to which step in the proposed reaction mechanism is rate-limiting.

**EXPERIMENTAL PROCEDURES**

**Production and Isolation of rCPO and Mutant Enzymes**—The production and isolation of rCPO, H496A, R490A, R360A, and K353A is described in detail elsewhere (6). Mutants D292A and H404A are made directly in plasmid TNT14 (6) according to the Quikchange protocol (Stratagene) using primer 1- (D292A)5’-CTGGGCCCTAGCCGGG-TCAAAACCTC-3’, the complementary primer 2-(D292A)5’-GAGGGTTTG-ACCCGGCTAGGCCCAAG-3’, 3-(H404A)5’-ACCCATCTGGTGCCGC- GACCTTTGG-3’, and the complementary primer 4-(H404A)5’-CTAGG- GCCTCGCCGGACCGATGTG-3’. After checking for the desired mutation by manual sequencing using the T7 sequencing kit (Amersham Pharmacia Biotech), the mutated pTNT14 derivatives were transformed to yeast, and mutant enzymes were isolated as described before (6). rCPO is produced as apoenzyme by the yeast expression system.

The relative amount of apoenzyme was determined by comparison of the activity of the preparations in the absence and presence of 100 μM vanadate in an activity assay and was larger than 99% for rCPO.

**Enzyme Activity Assays**—Haloperoxidase activity was measured qualitatively by the chlorination or bromination of 40 μM phenol red in the pH range 4–8 (100 mM citrate for pH 4–7 and 100 mM Tris-citric acid for pH 8) using a substrate concentration range of 100 μM–100 mM for both H2O2 and halide in the presence of 100 μM vanadate. Haloperoxidase activity was measured quantitatively by measuring the chlorination or bromination of monochlorodimedone (Δε = 20.1 mM–1 cm–1 at 290 nm) using a Union-Giken RA-401 stopped-flow spectrophotometer thermostated at 20 °C. Steady-state phosphatase activity of the mutants was measured by following the hydrolysis of p-NPP using 200 nM enzyme. After incubation with this substrate at different pH values (100 mM citrate for pH 4–7 and 100 mM Tris-citric acid for pH 8), reaction mixtures were quenched with NaOH, and production of para-nitrophenol was measured at 410 nm using an extinction coefficient of 16.6 mM–1 cm–1. Presteady state phosphatase activity was monitored continuously at 410 nm using an extinction coefficient of 15.4 mM–1 cm–1 at pH 8.3, assuming a pKa of 7.15 for p-NPP. Steady-state phosphatase activity of the various mutants was measured on a single-beam Zeiss spectrophotometer, and phosphatase burst kinetics was measured on a Hewlett Packard 8452A diode array spectrophotometer.

**Miscellaneous**—Protein concentrations were determined using the Bradford assay (17), and the H2O2 concentration was determined using a molar extinction coefficient of 43.6 mM–1 cm–1 at 240 nm. H2O2 stocks were prepared daily. Enzyme solutions were concentrated using the Centricon-30 system (Amicon).

**RESULTS**

**Peroxidase Activity and UV Absorbance Spectra of Mutant H404A**—The chlorinating activity of mutant H404A was investigated in the pH range 4–8; no activity was observed. However, at a bromide concentration of 100 mM clear bromoperoxidase activity was found with a pH optimum around pH 7 (not shown). However, quantitative kinetic analysis of this mutant is difficult due to partial inactivation of the enzyme, as can be seen in Fig. 2. After an initial burst of about 25 μM HOBr formation, which corresponds to about 100 turnovers, a much slower activity is observed. The initial rate of 25 s–1 is about 20% of the brominating activity of recombinant VCPO, which remains constant in time (not shown). The slower phase has a turnover of 1 s–1. The extent of the initial burst is proportional to the enzyme concentration (not shown), and therefore it is difficult to observe and to quantify the burst at low enzyme concentrations. The observed inactivation of H404A during the first fast phase requires all components of turnover, since preincubations with individual components did not inactivate the enzyme. To test whether the inactivation is reversible, the assay mixture was centrifuged in a Centricon-30 after a turn-
over experiment, and the enzyme solution was subjected to 3 rounds of dilution and concentration with 20 volumes of buffer to remove all turnover components. The recovered enzyme was tested in a new assay mixture containing vanadate, and the initial burst was not observed, indicating that the inactivation is irreversible. The remaining activity corresponds to 75% of the slow phase, showing that only a minor percentage of the mutant enzyme is lost during dilution and concentration. For the heme-containing enzyme myeloperoxidase, direct oxidation of the HOBr-scavenger monochlorodimedone is observed (18). Direct oxidation of monochlorodimedone has not been observed for the wild type vanadium enzyme, but mutations could in principle create this activity and related artifacts. However, no activity is found at the chlorinating activity (not shown). Additional H2O2 did not induce a further decrease, indicating complete formation of the peroxo form of H404A. Interestingly, the peroxo species is formed on a timescale of seconds (not shown), whereas this process occurs on a millisecond timescale for rCPO (27). The addition of 1 mM Cl− to the peroxo form of H404A did not affect its spectrum. In contrast, in rCPO the original spectrum is restored immediately upon the addition of 1 mM Cl− at optimal pH. The addition of H2O2, and 10 mM Br−. Trace c, the reaction was started by the addition of enzyme; trace b, the reaction was started by H2O2 and Br− after preincubation of the mutant at pH 4.9 for 10 min; trace e, the reaction was started by Br− after preincubation of the mutant for 10 min at pH 4.0 in the presence of H2O2.

![FIG. 3. UV absorbance of bound vanadate in H404A and the effect of H2O2 at pH 8.3. Panel A, spectrum a, 200 μM apoH404A; spectrum b, mixture of holo- and apoenzyme formed after addition of 200 μM of vanadate; spectrum c, the effect of addition of 200 μM of H2O2. Panel B, a, titration of 200 μM apoH404A with 0–800 μM vanadate; b, absorbance of 0–200 μM free vanadate. The line shown is a fit to the data points for a simple dissociation equilibrium.](image)

![FIG. 4. Inactivation of D292A during turnover and upon preincubation at pH 4.0. Conditions after mixing: 78 nM holo-D292A, 100 mM citrate, pH 4.0, 50 μM monochlorodimedone, 100 μM vanadate, 3 mM H2O2, and 10 mM Br−. Trace a, the reaction was started by the addition of enzyme; trace b, the reaction was started by H2O2 and Br− after preincubation of the mutant at pH 4.9 for 10 min; trace c, the reaction was started by Br− after preincubation of the mutant for 10 min at pH 4.0 in the presence of H2O2.](image)

As in recombinant CPO (27), the intensity of the band of holo H404A decreases upon the addition of an equimolar amount of H2O2 (Fig. 3A, spectrum c). The resulting peroxo species is stable for at least 30 min. Additional H2O2 did not induce a further decrease, indicating complete formation of the peroxo form of H404A. Interestingly, the peroxo species is formed on a timescale of seconds (not shown), whereas this process occurs on a millisecond timescale for rCPO (27). The addition of 1 mM Cl− to the peroxo form of H404A did not affect its spectrum. In contrast, in rCPO the original spectrum is restored immediately upon the addition of 1 mM Cl− at optimal pH. The addition of H2O2, and 10 mM Br−. Trace c, the reaction was started by the addition of enzyme; trace b, the reaction was started by H2O2 and Br− after preincubation of the mutant for 10 min at pH 4.0 in the presence of H2O2.

Peroxidase Activity and UV Absorbance Spectra of D292A—The chlorinating activity of D292A was investigated in the pH range 4–8. Under standard assay conditions at pH 5.0 (1 mM H2O2, 5 mM Cl−), chlorinating activity is very low and corresponds to about 1% that of the wild type. A slightly higher activity is found at pH 4 (2%), and qualitative screening for brominating activity revealed an optimum value at low pH similar to that seen for the chlorinating activity (not shown). However, as for the H404A mutant, quantitative analysis is complicated by inactivation, as shown in Fig. 4. Trace a shows that this mutant is inactivated rapidly. After 25 s, the mutant has completely lost its activity (not shown). About 33 μM HOBr
is formed, corresponding to about 420 turnovers. The amount of HOBr formed is proportional to the enzyme concentration (not shown), and the initial rate (475 s$^{-1}$) is somewhat higher than that reported for the wild type (6). The inactivation of D292A may be caused by loss of cofactor at low pH. Indeed, as can be seen from trace b, the mutant is inactivated by preincubation at pH 4.0. After washing the preincubated enzyme 3 times with 20 equivalents of 100 mM Tris acetate buffer, pH 8.3, however, the activity is restored (not shown). This indicates that this inactivation is reversible, in contrast to that observed during turnover (trace a). Furthermore, trace c shows that preincubation of the mutant at pH 4.0 in the presence of H$_2$O$_2$ does not result in inactivation, confirming that the inactivation observed in trace a is not due to the low pH. As in trace a, inactivation only occurs during turnover.

The crystal structure of mutant D292A indicates that the mutant is still able to bind vanadate (19). Upon the addition of vanadate to apoD292A, a band is formed in the near UV with a peak at 315 nm, as shown in Fig. 5A. This spectrum is identical to that of rCPO. Fig. 5B shows the titration of 120 µM apoD292A with vanadate. A sharp point of intersection at 128 µM is observed, indicating stoichiometric binding of vanadate.

The absorption increase at vanadate concentrations higher than 128 µM (Fig. 5B) is due to that of free vanadate at 316 nm. From Fig. 5B, an extinction coefficient $\Delta \varepsilon$ (holo-apo) at 316 nm of 2.8 m$^{-1}$ cm$^{-1}$ was determined that is identical to that of rCPO (27). The extinction coefficients and estimated dissociation constants for vanadate for the various mutants and recombinant CPO are summarized in Table I.

The formation of the peroxo species of this mutant occurs within the mixing time of the instrument (not shown), and no further decrease in intensity is observed upon further titration with H$_2$O$_2$, indicating that binding of H$_2$O$_2$ is both fast and strong in this mutant. Upon the addition of 1 mM chloride to the peroxo form of D292A, the native spectrum is not regenerated, even after an incubation of 30 min, whereas for rCPO this occurs rapidly. Spectra d and e (Fig. 5) show that the native spectrum of D292A is restored after the addition of Br$^-$, in line with the observed brominating activity for this mutant.

At pH 5.0, the optical absorbance band of D292A is still present, but it is shifted to 305–310 nm (not shown), a shift similar to that seen in rCPO (27). Interestingly, the $\Delta \varepsilon$ (holo-apo) at 316 of 2.8 m$^{-1}$ cm$^{-1}$ remains the same, whereas in rCPO, the value decreases upon lowering the pH. After the addition of 1 mM Cl$^-$, the native spectrum is regenerated in 1 min, which is slower than for rCPO but in line with the remaining chlorinating activity of mutant D292A observed at this pH in steady state experiments. The addition of 100 µM Br$^-$ immediately converts the peroxo spectrum back to that of the native enzyme. Attempts were made to study the optical spectrum at pH 4.0, which is the optimum for activity observed in the steady state brominating experiments. At this pH value, however, 100 µM D292A precipitates. rCPO also precipitates at this concentration, indicating that this effect is not due to the mutation.

**Phosphatase Activity of Active-site Mutants of Chloroperoxidase**—To confirm the proposed role of the various residues in the mechanism of the phosphatase reaction catalyzed by apoCPO, mutants of these residues were investigated. Table II shows the phosphatase activity of rCPO and mutant CPOs at various concentrations of the phosphatase substrate p-NPP and different pH values. p-NPP concentrations were chosen around and well above the previously reported $K_m$ value for this substrate (about 50 µM at pH 4.5–8.0 (7)). Mutant H496A was inactive under all substrate concentrations tested, consistent with the proposed role of His-496 as nucleophile in the phosphatase reaction (7). In line with their proposed role in binding phosphate oxygens in the trigonal bipyramidal transition state (7), the single-site mutants of the positively charged residues Arg-490, Arg-360, and Lys-355 exhibit decreased activity. R490A is completely inactive, and a significant decrease in maximal activity of R360A and K353A as compared with rCPO is observed. This is not a consequence of increased $K_m$ values for the substrate, since an increase of the concentration of p-NPP from 1 to 10 mM did not affect the activity. Both His-404 and Asp-292 have been proposed to be involved in the protonation of the leaving group (3, 7). However, for D292A, considerable activity is still found (62%), although the $K_m$ value has increased to the mM range. For H404A, no activity is observed.

**Burst Kinetics of the Phosphatase Activity of Apochloroperoxidase**—The phosphatase activity of apoCPO is low compared with "normal" phosphatases, and reaction times are small. This low activity allows presteady state studies on the second time scale, even at high enzyme concentrations. When p-NPP was mixed with apoCPO, a presteady state burst of para-nitrophenol was observed (Fig. 6). The first equivalent of para-nitrophenol was released rapidly as compared with the steady state release. The steady state release as observed in the linear parts of the traces in Fig. 6 has a rate of about 0.5 min$^{-1}$ and is of similar magnitude to the rate obtained at low enzyme concentrations (Table II). Extrapolation of the steady state rate to...
and enzyme-substrate complex formation is fast ([p-NPP]k1 is large compared with (k1 + k2 + k3)), then

\[ \pi \approx 1/(1 + k2/k3) \]  

where \( \pi \) is the intercept on the product axis, and \( c0 \) is the total enzyme concentration. One can calculate that for the phosphatase activity of apoCPO, \( k2 = 3.4k3 \).

**DISCUSSION**

**Peroxidase Activity and UV Absorbance Spectra of H404A and D292A**—The individual activity traces of D292A and H404A shown in Figs. 2 and 4 show that both residues are required for stability of the enzyme during turnover. Since irreversible inactivation only occurs when all components of turnover are present, the most likely explanation for the observed inactivation is bromination or oxidation of active-site residues by the reaction product. In this context it is of interest to note that irreversible inactivation of VBPO under turnover at low pH in the presence of high concentrations of \( \text{H}_2\text{O}_2 \) has been reported (22). Under these conditions 2-oxohistidine is formed, which is accompanied by the release of vanadium. This may also be an explanation for the irreversible inactivation of H404A and D292A observed in this work. The reversible inactivation of D292A by incubation at pH 4.0 is not, however, due to instability of the protein at this pH, since it can be prevented by the addition of \( \text{H}_2\text{O}_2 \) (Fig. 4c). It is more likely due to a decrease in affinity for vanadate at low pH. In rCPO (27) vanadate binds much more strongly in the presence of \( \text{H}_2\text{O}_2 \), a phenomenon that probably also occurs in D292A; this may explain why \( \text{H}_2\text{O}_2 \) prevents reversible inactivation of D292A. Unfortunately, direct measurement of the affinity for vanadate via optical spectroscopy was not possible at pH 4.0, since the apoprotein precipitated. An alternative explanation for the reversible inactivation of this mutant is that the mutation creates space, allowing the incorporation of a multimeric vanadate form (e.g., a dimer) at the active site. These multimers are formed mainly at low pH values (23), and \( \text{H}_2\text{O}_2 \) prevents their formation. In this respect it is noteworthy that also the R490A mutant described previously (6) is inactivated in an identical fashion at low pH, which is also prevented by the addition of \( \text{H}_2\text{O}_2 \) (not shown).

As shown by activity assays (Figs. 2 and 4) and the effect of substrates on the optical absorption band at 316 nm (Figs. 3 and 5), both mutants show residual brominating activity. Interestingly, both residues are important for the chlorinating activity of this vanadium haloperoxidase. Previously, loss of chlorinating activity was also observed for R490A and K353A, and a clear reduction in chlorinating activity was observed for R360A (6). These data together with the results presented in this paper indicate that the contributions of several interacting residues are responsible for the chlorinating activity exhibited by native VCPO. Details of this interaction are not yet understood, and the mutagenesis studies presented in this paper give no conclusive answers. The large reduction in the activity upon mutation of Asp-292 may not be caused by a direct involvement of this residue in the catalytic mechanism of the enzyme. Although the crystal structure of D292A shows that the conformation of the active site remains almost unchanged, it was shown that the electrostatic potential distribution is clearly influenced (19), which could also cause the observed decrease in activity. Conclusions about the role of His-404 also have to be drawn carefully. The crystal structure of H404A shows that vanadate is still bound, but that removal of the His-404 side chain also causes a change in the position of Arg-490 and Asp-292 (19). However, our results clearly show that H404A is still able to bind \( \text{H}_2\text{O}_2 \) (Fig. 3). On the basis of analogy to heme-peroxidases, His-404 was suggested to play a role as

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**TABLE II**

<table>
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<th>Enzyme</th>
<th>[p-NPP]</th>
<th>pH 4</th>
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<th>pH 6</th>
<th>pH 7</th>
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<td>10</td>
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<td>13</td>
<td>10</td>
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**Fig. 6. Burst kinetics in the phosphatase reaction catalyzed by apochloroperoxidase.** The different activity traces correspond to different enzyme concentrations at a constant substrate concentration (1 mM p-NPP) at pH 8.3 (100 mM Tris acetate).
acid-base catalyst in the binding of $\text{H}_2\text{O}_2$ by deprotonation of the peroxide (1). More recent proposals (2, 6) suggest a more indirect role of His-404 in activating the apical hydroxide to deprotonate the peroxide. In the structure of the peroxo form of the wild type enzyme (2), His-404 makes no contact with the bound peroxide, which may explain why H404A can still bind $\text{H}_2\text{O}_2$. The result that the rate of formation of the peroxo form of H404A is lower than that of rCPO may be explained by the absence of His-404. In conclusion, the results are in line with a model in which His-404 is not absolutely essential for formation of the peroxo structure but may influence the rate at which it is formed. The lower affinity for the vanadate cofactor in this mutant ($K_d = 112 \mu\text{M}$) as compared with wild type can be explained by a combination of the loss of the hydrogen bond between the cofactor and His-404 and the loss of stabilizing interactions with Arg-490, as observed in the crystal structure of this mutant (19).

Very recently, the x-ray structure of VBPO from Ascophyllum nodosum was published (24). The overall amino acid sequence identity to VCPO from C. inaequalis is very low (21.5%). Still, VBPO and VCPO share a four-helix bundle motif, and moreover, the active sites are very similar as predicted previously (1, 3, 7). This raises the important question as to which factors determine whether the enzyme is a bromo- or a chloroperoxidase. All residues involved in binding the cofactor are conserved in both bromo and chloroperoxidases as well as the Lys-353 in VCPO from C. inaequalis, which make direct contact with one of the oxygen atoms of the bound peroxide. The major difference in the active site is the substitution of Phe-397 in VCPO by His-411 in VBPO. It has been suggested (24) that, since the His-411 is within hydrogen-bonding distance of a modeled peroxy vanadate, this residue in VBPO may protonate the peroxide form, altering the reactivity as compared with VCPO. Protonation of the peroxo species was previously also suggested (6) to be a possible explanation for the difference between VCPO and VBPO. However, protonation of the bound peroxide as illustrated in Fig. 7 rather increases the reactivity of the bound peroxide, and this may be a crucial factor. Thus, His-411 in VBPO may in fact decrease the reactivity by deprotonation of the bound peroxide. As model studies suggested, protonation of the bound peroxide at this stage may be very important in tuning the reactivity of the enzyme (25, 26). The following overall picture of the binding and activation of peroxide by these enzymes emerges. 1) The vanadate atom acts as a Lewis acid, withdrawing electron density from the bound peroxide. 2) The two arginine residues (Arg-490 and Arg-360) further assist electron withdrawal. 3) A lysine residue (Lys-353) in VCPO causes charge separation on the peroxide oxygen atoms. 4) One of the peroxide oxygen atoms is protonated in the VCPO. This protonated oxygen atom is more strongly oxidizing, and in this enzyme, nucleophilic attack by chloride occurs. We clearly deal with an intricate balance of charges and protonation of the VCPO, and any disturbance appears to convert the VCPO into a VBPO.

Phosphatase Activity of Chloroperoxidase and Active-site Mutants—Fig. 8 shows a proposal for the reaction mechanism of the phosphatase activity of apoCPO based on the crystal structure of VCPO, the conserved active site residues, and the mutagenesis studies presented in this paper; it is in line with previous proposals (7, 9). The crystal structure of holo-CPO with vanadate at the active site provides the model for the transition state of the phosphatase mechanism (Fig. 1 (VCPO) and Fig. 8, panels B and E). The mechanism consists of three important steps: 1) nucleophilic attack on the phosphorus, 2) stabilization of the trigonal bipyramidal transition state, and 3) leaving group protonation. Our model points to His-496 as the nucleophile in this reaction (panel A), and indeed mutation of this residue results in complete inactivation (Table II). The crystal structure of H496A (19) shows that the position of other residues is not affected by this mutation, excluding other factors that might explain loss of activity. This result confirms previous mutagenesis studies on the corresponding residue in the aligned G6Pase, His-176 (16). On the basis of the prediction that His-176 in G6Pase would be essential for activity (7), seven different substitutions were made, and none of these mutants had detectable G6Pase activity (16). It is clear from our data, which are supported by crystal structures of native and mutant enzymes, that CPO can serve as a template for the membrane-bound G6Pase, for which crystal structural information is not yet available.

The model points to Arg-360, Arg-490, and Lys-353 as residues that stabilize the pentacoordinated transition state (panels B and E). Indeed, R490A, R360A, and K353A are clearly affected in their activity. The effect is most severe in R490A, which is in line with the fact that this residue makes contact to two of the equatorial oxygens in VCPO (2) and, thus, probably to two oxygens in the phosphatase transition state. The conclusion that the optimal environment for the transition state is affected in R490A is supported by the observation that vanadate binding was not observed spectroscopically (27). This mutant did show some peroxidase activity (6), which makes it unlikely that large structural rearrangements occur. However, the presence of peroxidase activity only shows that pervanadate is present at the active site, not necessarily vanadate. It should be recalled that the resulting peroxy structure is very different from the trigonal bipyramidal structure of the native protein, which resembles the phosphatase transition state, and it has been shown that vanadate binds much more strongly to the enzyme in the presence of $\text{H}_2\text{O}_2$ than in the absence of this substrate (27). For K353A it is not known whether vanadate is bound, since no crystal structure is available, and no optical band associated with vanadate binding could be identified (27). This mutant had some peroxidase activity (6), again pointing to correct overall folding. The phosphatase activity of this mutant is also significantly decreased, suggesting that this residue is important for transition state stabilization. We propose that corresponding residues in the aligned phosphatases play a similar role. For R360A, both the crystal structure (19) and
optical spectroscopy data (27) indicate that the cofactor is still bound. However, the phosphatase activity is significantly decreased, demonstrating the importance of this residue in the phosphatase reaction. For the corresponding Arg-83 in G6Pase, nine different substitutions were tested, all of which eliminated G6Pase activity (13). This is in contrast to the residual phosphatase activity still observed for the CPO mutant R360A and shows that comparisons should be made carefully. Still we predict that mutation of Arg-170 and Lys-176 in G6Pase, which correspond to Arg-490 and Lys-353 in VCPO, will strongly decrease G6Pase activity.

Two residues that may act as acid-base group during catalysis (Fig. 8) are His-404 and Asp-292. Since His-404 is conserved in the acid phosphatases, this residue is more likely to play such a role. The aligned His-119 in G6Pase has been shown to be essential for activity, further supporting the idea that His-404 provides the proton needed to liberate the product. The possibility that Asp-292 acts as proton donor also has been put forward (3). Considering the substantial phosphatase activity (62%) for D292A, this seems unlikely. In contrast, the H404A mutant does lack phosphatase activity. However, a large shift in the position of Arg-490 is observed in the crystal structure of the H404A mutant (19), and Arg-490 has also been shown to be essential for phosphatase activity (Table I). Thus, in the range 4.5–8.0, the Km-values are strongly increased, and measurements were done at a much higher p-NPP concentrations. Therefore, the data reported at lower pH are correct.

In conclusion, we have shown that combining kinetics and other data from two different enzymic activities catalyzed by CPO provide a powerful tool to study the mechanisms of these reactions. We are now producing other active-site mutants of VCPO for which the peroxidase and phosphatase activity will be analyzed in combination with crystal structure data. Hopefully, these data will provide further clues about the mechanism of vanadium haloperoxidases and the phosphate mechanism of apo-CPO and related enzymes such as human G6Pase. It has been shown that mutation of several residues of VCPO results in mutants that still exhibit BPO activity. To fully understand the chlorinating activity, the goal is now to do the reverse and create chlorinating activity by mutation of a VBPO.

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REFERENCES

FIG. 8. Mechanistic scheme for the hydrolysis of a phosphate monoester by apochloroperoxidase. R is the para-nitrophenyl group of the artificial phosphatase substrate p-NPP. B stands for base, presumably His-404.
Peroxidase and Phosphatase Activity of Vanadium-CPO Mutants