Isolation, Characterization, and Primary Structure of the Vanadium Chloroperoxidase from the Fungus *Embellisia didymospora*

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Here we describe the isolation, purification, and basic kinetic parameters of a vanadium type chloroperoxidase from the hyphomycete fungus *Embellisia didymospora*. The enzyme proved to possess similar high substrate affinities, a $K_m$ of 5 μM for a bromide, 1.2 mM for a chloride, and 60 μM for a hydrogen peroxide, as those of the vanadium chloroperoxidase from *Curvularia inaequalis*, although with lower turnover rates for both $\text{Cl}^-$ and $\text{Br}^-$ . Substrate bromide was also found to inhibit the enzyme, a feature subsequently also noted for the chloroperoxidase from *C. inaequalis*. The gene encoding this enzyme was identified using DNA Southern blotting techniques and subsequently isolated and sequenced. A comparison is made between this vanadium chloroperoxidase and that of the fungus *C. inaequalis* both kinetically and at the sequence level. At the primary structural level the two chloroperoxidases share 68% identity, with conservation of all active site residues.

First isolated in 1984 (1), the vanadium haloperoxidases represent a group of peroxidases that possess a single bound vanadate ion as a prosthetic group (2–4). These enzymes are defined by their ability to oxidize a suitably electrophilic halide species (X) (in the case of chloroperoxidase, $\text{Cl}^-$, $\text{Br}^-$ , and $\Gamma^-$ ) to its corresponding hypohalous acid (HOX) in the presence of hydrogen peroxide (Equation 1) (5, 6).

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

If a suitable nucleophilic acceptor (AH) is present, the hypohalous acid species may further react to give rise to a halogenated species (AX) (Equation 2). The presence in nature of such enzymes could go some way to explaining the formation of at least some of the wide diversity of halogenated compounds that can be isolated from the environment (7–10).

Historically, the first isolated and characterized vanadium haloperoxidases were the marine vanadium bromoperoxidases (5, 11–13) typified by the enzyme from the seaweed *Ascophyllum nodosum*. However, such enzymes are not restricted solely to the aquatic environment. As a result of a fungal survey carried out by Hunter-Cevera and Sotos (14) several species of fungi isolated from plant material were identified with the ability to produce haloperoxidases. Subsequently, a non-heme chloroperoxidase was isolated and partially purified from one of these fungal species, namely *Curvularia inaequalis* (15), which, several years later, was shown to be a novel vanadium containing enzyme (3).

EPR studies (3–5) have already shown that the environments around the orthovanadate when bound at the active site of both the terrestrial chloroperoxidase and the marine bromoperoxidase were very similar and since both enzymes possessed similar thermo- and chemostability profiles and high substrate affinities it was presumed that the structure of these enzymes would be very similar (3). However, comparison of the sequence of the gene encoding the fungal vanadium chloroperoxidase from *C. inaequalis* (16) and the related marine vanadium bromoperoxidase from *A. nodosum* has shown that these enzymes are very dissimilar at the primary structural level, sharing homology only in small regions of the active site. In fact, a recent sequence study has shown that certain groups of phosphatases also share this homology of active site residues, and the results revealed that the bromoperoxidase shares more homology with the phosphatases than with the fungal chloroperoxidase (17). With the recent publication of the X-ray structure of the fungal vanadium chloroperoxidase (2), it will be interesting to see if this relationship still holds at the tertiary structural level, since it is known that proteins can possess different primary structures and yet still possess a similar protein fold (18).

We have reported that vanadium chloroperoxidases are present in many other species of terrestrial fungi (19), many of which, including *C. inaequalis*, are phytopathogens and speculated that in such a case the function of the vanadium chloroperoxidase is to assist in fungal penetration of host cells (20). Here we report the isolation, partial characterization, and gene sequencing of the vanadium chloroperoxidase from the fungus *Embellisia didymospora*, a species first isolated from the South Adriatic sea (21). We compare some of the basic kinetic parameters of the chloroperoxidase to those of the vanadium chloroperoxidase from *C. inaequalis* and also compare their primary structures.

EXPERIMENTAL PROCEDURES

Organisms—The fungus, *E. didymospora*, used in this study was obtained from the Central Bureau voor Schimmelcultures (CBS strain 766.79; CBS, Baarn, The Netherlands). *Escherichia coli* strain DH5a was used as host for the vector pUC 18 derivatives.

Isolation of the Vanadium Chloroperoxidase—*E. didymospora* was grown in 3-liter flasks containing 1 liter of liquid culture medium composed of 5 g/liter yeast extract (Difco), 1 g/liter NH$_4$Cl, 1 g/liter glucose, 2 ml/liter mineral elements (4.5 mM K$_2$HPO$_4$, 2.5 mM CuSO$_4$·5H$_2$O, 2.75 mM FeSO$_4$·7H$_2$O, 4 mM MnCl$_2$·4H$_2$O, 5 mM ZnSO$_4$·7H$_2$O) in 1 liter of deionized water. Growth of inoculated cultures was carried out at 25 °C on an orbital shaker (Gallenkamp) set at 100 rpm for 10 days. After the growth period, 5 ml of a 10% SDS solution was added to the culture which was then incubated at room temperature.
temperature with gentle agitation for a further 30 min. The medium was then filtered to remove the mycelia. Approximately 50 ml of buffer (50 mM TrisSO4, pH 8.0) was added to DEAE (Amersham Pharmacia Biotech) was added to a total collected medium volume of 3 liters and left overnight gently stirring on a magnetic stirrer at 10 °C. The DEAE was loaded onto a 3-cm diameter column and subsequently washed with 10 column volumes of 0.1 M NaCl in 50 mM TrisSO4, pH 8.0. Column elution was carried out using 0.25 M NaCl in 50 mM TrisSO4, pH 8.0, and vanadium chloroperoxidase (vCPO)active fractions, assessed using a phenol red qualitative assay (22), were pooled, and NaCl was added to a final concentration of 2 M. A 5-ml prepacked, buffer washed (2 M NaCl in 50 mM TrisSO4, pH 8.0), phenyl-Sepharose Cl-4B (Amersham Pharmacia Biotech) column was used to bind the vCPO. This was then washed with 10 column volumes of 0.6 M NaCl in 50 mM TrisSO4 and eluted with 50 mM NaCl in 50 mM TrisSO4, pH 8.0. vCPO active fractions were pooled, dialyzed overnight against 50 mM TrisSO4, pH 8.0. As a final purification step, a Mono-Q HR 5/5 anion exchange column was used to bind the enzyme followed by elution with a 0–1.0 M NaCl gradient in 50 mM TrisSO4, pH 8.0. Concentration of samples was carried out using either lyophilization or rotation evaporation.

**SDS-Polyacrylamide Gel Electrophoresis Analysis of vCPO—** SDS-polyacrylamide gel electrophoresis was carried out using 7% gels according to Laemmli (23) under either denaturing conditions (boiling of sample for 5 min in the presence of SDS and β-mercaptoethanol). Denatured samples were observed using Coomassie Brilliant Blue R250.

**Protein Sequencing—** Purified vCPO was digested with trypsin and separated using a 10% Tricine gel according to Schagger and Von Jagow (24) and subsequently electroblotted to polyvinylidene difluoride membrane (Immobilon-P, Millipore) using 1× Tris-borate-EDTA transfer buffer (25). After blotting, the membrane was soaked for 5 min in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and then destained in 50% methanol. Peptide bands were submitted to automatic Edman sequencing on an Applied Biosystems 494 protein sequencer.

**Enzyme Activity Assays—** vCPO activity measurements were carried out in 50 mM citrate buffer at the pH value in the presence of 1 mM hydrogen peroxide and either by following the chlorination/bromination of monochlorodimedone (e = 20.2 m M cm−1 at 290 nm) to dichlorodimedone (e = 0.1 m M cm−1 at 290 nm) (26) using Popspec software on an Atari 1040ST computer coupled to a single beam Zeiss spectrophotometer via the Poptronics 4 channel A/D converter. Protein sample concentrations to derive specific activity values were determined following the method of Bradford (27). For the determination of pH optimum an overlapping range of 50 mM acetate and citrate buffers was used. Thermal stability studies were carried out by incubating samples of enzyme in 50 mM TrisSO4, pH 8.0, in an Eppendorf Mastercycler 5330, at the appropriate temperature for 5 min. After this time samples were cooled to room temperature, briefly centrifuged, and then assayed for activity.

**DNA Isolation, Cloning, and Analysis—** The isolation of DNA from *E. didymospora* was carried out as described previously for the isolation of DNA from *C. inaequalis* (16). 15 µg of DNA were used for restriction enzyme digestion. After separation on a 0.8% agarose (Sigma) gel, DNA was denatured in situ and Southern transferred to a nitrocellulose membrane (25). A vCPO gene-specific polymerase chain reaction fragment encompassing 700 bp of the portion encoding for the vCPO-active site was created using cDNA from *C. inaequalis* (16), the primer sequences used for this were P1 (forward) 5′-CAGGCTCTCTAACCTCACC-3′ and P2 (reverse) 5′-GTCCTTTGTCGTCGTGGG-3′. This fragment was then used as a template for Klenow fragment (Boehringer Mannheim) random-primer labeling using [α-32P]dATP (25). Hybridization of this probe to the Southern blotted DNA was carried out overnight at 55°C in 6× SSC (25), 0.2% SDS, 5× Denhardt’s solution (25), and 5 µg of sheared salmon sperm DNA (Sigma). Based on this result a 1.8-kb HindIII-sized fragment was isolated from a subsequent genomic DNA digest and used to create a mini-library in the vector pUC 18. A positive clone was isolated and the DNA sequenced (T7 Sequencing kit, Amersham Pharmacia Biotech). This clone was subsequently used to create a second, *E. didymospora*, vCPO-specific probe to isolate a genomic DNA BamHI/SalI restriction fragment, for cloning and sequencing, encompassing the 5′ portion of the vCPO coding sequence.

1 The abbreviations used are: vCPO, vanadium-containing peroxidase; EvCPO, vanadium-containing peroxidase from *E. didymospora*; CvCPO, vanadium-containing peroxidase from *C. inaequalis*; Tricine, N/2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; bp, base pair(s); kb, kilobase pair(s).

**RESULTS**

**SDS-Polyacrylamide Gel Electrophoresis Analysis of Purified E. didymospora Vanadium Chloroperoxidase—** Based upon a previous investigation by Vollenbroek et al. (19), showing that like *C. inaequalis* (3) several other hyphomycete species also possessed a vanadium-type chloroperoxidase similar in immunoreactivity to the vanadium chloroperoxidase (CvCPO) from *C. inaequalis*, the vanadium chloroperoxidase (EvCPO) from the fungus *E. didymospora* (21) was chosen for further characterization. *E. didymospora* was grown in a fixed-medium liquid shake culture for 10 days, after which time vanadium chloroperoxidase activity could be detected in the medium. Fig. 1 shows the SDS-polyacrylamide gel electrophoresis results of denatured purified EvCPO. Similar to CvCPO, EvCPO under these conditions exhibits a single band of approximately 67 kDa. A common feature of this group of enzymes is their high stability, usually making it possible to also run non-denatured samples under SDS-polyacrylamide gel electrophoresis conditions and then detect the presence of vanadium chloroperoxidase in the gel using the peroxidase activity stain o-dianisidine in the presence of peroxide and bromide (3, 5, 16). Under these conditions EvCPO typically gave rise to a single lightly stained peroxidase band, which failed to leave the stacking layer gel (results not shown). A similar band under these conditions has also been described and accounted for in *CvCPO* (16, 20) as being a multimeric structure of undefined size, although this form was able to enter the separating gel and exhibited a reproducible mobility.

**Kinetic Parameters of Bromination and Chlorination—** By spectrophotometrically following the bromination and or chlorination of monochlorodimedone at 290 nm, it was possible to establish a few basic kinetic parameters for EvCPO. As with *CvCPO*, which was used as an activity reference during these experiments, EvCPO displayed a typical peak pH optimum for chlorination at around pH 5.2 (Fig. 2A), 5.5 being the reported pH optimum for CvCPO (3) though results from these experiments indicated this value to be somewhat high, a value closer to 5.0 for CvCPO is probably more accurate. A similar attempt to characterize the pH profile for bromination by EvCPO proved more complex. Fig. 2B shows the effect of different concentrations of bromide on the activity of EvCPO at pH 4, 5, and 6. It is evident from this that at pH 4 and 5 EvCPO experiences dramatic substrate inhibition at levels of bromide.
exceeding 500 μM. Using bromide concentrations of less than 500 μM and a range of pH values it was possible to derive $K_m$ constants of 5 μM for bromide and 60 μM for hydrogen peroxide from double reciprocal plots (Fig. 2C). Also using double reciprocal plots the $K_m$ for chloride was calculated to be 1.2 mM (results not shown), while the $K_m$ for hydrogen peroxide using chloride as the second substrate gave the same value as was found with bromide.

The high substrate affinity and yet substrate inhibition for bromide was also noted for the vanadium bromoperoxidase from the lichen Xantoria parietina, the first documented terrestrial vanadium haloperoxidase (28). Here a $K_m$ of 28 μM for bromide was observed and reported as being the highest substrate affinity ever recorded for such an enzyme. Similarly, although the $K_m$ for bromide for CvCPO has never actually been reported, results obtained during these experiments indicated that the $K_m$ for bromide is also low (9 μM) and that substrate inhibition is also experienced as low as 500 μM (results not shown). This inhibition by bromide was noted by Van Schijndel et al. (6), but was apparent only in the lower pH ranges (pH 3–4). A detailed steady state kinetic analysis of the marine vanadium bromoperoxidase from A. nodosum by De Boer and Wever (29) showed that bromide was also able to inhibit the marine bromoperoxidase, but only when in the presence of in excess of 200 mM Br$^-$ and when the enzyme was in its single protonated state. At high pH little or no inhibition was observed.

Examination of the turnover of EvCPO derived from $V_{\text{max}}$ calculations show that, catalytically, EvCPO is less active than CvCPO. Calculated turnover values for Cl$^-$ and Br$^-$ were 2 s$^{-1}$ and 60 s$^{-1}$, respectively, for EvCPO, and from this study values were found of 22.5 s$^{-1}$ and 201 s$^{-1}$, respectively for CvCPO. These values are higher than those previously reported by Van Schijndel et al. (3).

A common feature associated with most vanadium-type haloperoxidases is their high thermostability (6, 11, 30), although with exceptions for example the vanadium bromoperoxidase from Ceramium rubrum (12). CvCPO is known to still be active at temperatures exceeding 80 °C, although activity begins to drop after this point (6). Fig. 2C shows the results of a similar thermostability experiment carried out with EvCPO. Small volume samples of enzyme were incubated for 5 min at the selected temperature and then assayed for the remaining activity. After incubation at 60 °C for 5 min the enzyme is still fully active; however, at 70 °C the enzyme is almost completely inactivated. Another interesting feature that can be seen in Fig. 2C is the apparent increase in activity from room temperature up to 45 °C. Prolonged incubation at 45 °C was not found to affect activity further (results not shown).

**Determination of Peptide and Primary Sequence**—Purified EvCPO was digested with trypsin protease and separated on a Schägger and Von Jagow (24) type polyacrylamide gel. After electroblotting to polyvinylidene difluoride membrane, a suitable fragment was cut from the blot and subjected to direct Edmann sequencing. The determined sequence of this peptide was DLSNPYDP(L)(R)PITDQPGIVRT. This fragment was found to closely resemble a similar peptide sequence from CvCPO, DLRQPYDPTAPIEDQPGIVRT (16). Subsequently, a
DNA fragment was made using polymerase chain reaction extension of two primers, based upon CvCPO gene sequence as template, which encompassed all residues involved in the active site of CvCPO (2) and included the new peptide sequence. The polymerase chain reaction synthesized fragment was then used as a template to create a random labeled probe for hybridization with Southern blotted, restriction digested, genomic DNA from *E. didymospora*. Fig. 3 shows the hybridization results using the CvCPO-based probe and identifies a 1.8-kb sized positively hybridizing HindIII fragment. Upon the basis of this result, a 1.0–2.5-kb HindIII mini-library was created in pUC 18.

Successful screening of this *E. didymospora* DNA mini-library with the random labeled probe used in the previous Southern blot, gave rise to two clones, both of which gave similar sequencings results. A comparison of this sequence with that obtained for the vCPO from *C. inaequalis* (16) and the protein sequence obtained from peptide sequencing of EvCPO revealed that the sequence coded for approximately 80% of the vanadium chloroperoxidase, with the remaining 20% missing from the 5'-coding region. Upon the basis of these results and other Southern blotting results using various combinations of restriction digested genomic DNA (not shown), a further Southern blot hybridization was carried out using SalI/BamHI digested DNA, which was predicted to contain the CvCPO 5'-coding region. Using a randomly labeled probe based upon the newly sequenced EvCPO-encoding DNA, a 3.5-kb positive reacting fragment was identified, and after mini-library screening several positive clones were isolated. The sequence obtained from these clones perfectly overlapped the previous sequence and the results are presented in Fig. 4.

The complete DNA sequence and its derived amino acid sequence is presented in Fig. 4A. The DNA sequence comprises a single 1839-bp open reading frame together with a portion of both the 3'- and 5'-noncoding regions. The open reading frame translates to 614 amino acids with a derived molecular mass of 67,547 Da as compared with the 609-amino acid open reading frame and 67,488 predicted *M* of the vanadium chloroperoxidase gene from *C. inaequalis* (CvCPO) (16). Upon comparison with CvCPO sequence, a start methionine was easily located and is the only methionine coded for in this region. Upstream of this at −75 bp, a putative TATA box could be identified, a feature also identified in the promoter region of CvCPO (20). A putative translation start point is overlined with an arrow, based on a prediction made using the MacVector DNA analysis software. Similarly, a predicted polyadenylation signal AATACA closely resembling that of the eukaryotic polyadenylation signal AATAAA (31) and that of CvCPO (16) was also identified.

Fig. 4A also shows the positions of the HindIII and SalI sites which were used in isolation of the gene from genomic DNA digests. Upon the basis of several Southern blot hybridization experiments, a 5-kb restriction map could be drawn up which is presented in Fig. 4B, showing the relative position of the CvCPO coding sequence in respect to the restriction sites.

**Sequence Comparison**—Primary sequence alignment of EvCPO with CvCPO, Fig. 5, reveals approximately 65% identity between these two fungal chloroperoxidases. As expected, all residues involved in the binding of the vanadium at the active site, marked with filled circles, and their surrounding residues in the sequence, are conserved. Surprisingly, perhaps, is that many of the substitutions of amino acids which have occurred are of a nonconservative nature for example the substitution of a noncharged hydrophobic residues for charged ones, for example EvCPO contains 81 positively charged residues whereas CvCPO possess 65. Such substitutions are particularly evident in the C'-terminal region. Above the sequence in Fig. 5, the relative position of secondary structural elements, based upon the crystal structure of CvCPO (2), have been sketched, helices being represented by cylinders and β-sheets by open arrows. From this, it is clear that the majority of significant sequence changes occur between helices in random coil structural elements, with the exception of helix χ. Helices b, c, e, f, k, l, n, and o, which contribute to the two 4-helical bundle motives (2) can be seen to be well conserved in sequence, particularly helix b that is 100% conserved in the sequence. From Fig. 5B, a schematic representation of the x-ray crystal structure of CvCPO, helix χ can be seen to occupy an external cross-lying position and as such may permit sequence divergence without significant structural change.

From the alignment it can be seen that there are three regions which possess a particularly high degree of sequence variation. These can be located at the N terminus, the C terminus, and between helices m and n, although in this respect the partial sequence obtained for the vanadium chloroperoxidase from the fungus *Drechslera biseptata*, also depicted in Fig. 5A, remains highly similar to CvCPO. This region m to n, varying in both sequence and length, is perhaps of particular interest, since it would seem somewhat unusual that such a significant variation should occur between active site residues in what one would then presume is an important area of the protein structure, particularly in view of the otherwise high degree of similarity. A recent study carried out by our laboratory into sequence similarities in the active sites of both vanadium haloperoxidases and certain groups of phosphatases also highlighted this area of particular sequence dissimilarity, varying not only between the haloperoxidases and phosphatases, but also between the phosphatase groups themselves (17).

The EvCPO sequence, Fig. 4, can also been seen to possess 3 cysteine residues, none of which, as depicted in Fig. 5, align with those from CvCPO. However, on the basis of positioning on the secondary structural model in Fig. 5B, it can be presumed that like CvCPO (16) EvCPO does not possess sulfide bridging cysteines.

**DISCUSSION**

In the characterization of any one system, it is obviously very useful to compare and contrast similar systems, both kinetically and structurally providing not only possible functional insights, but a good reference point for molecular analysis techniques such as mutational studies. Although now well characterized kinetically (3, 6) structurally (2, 16), and even functionally (20), work upon the fungal vanadium chloroperoxidase remains restricted to one system.

A recent study carried out by our laboratory revealed that the vanadium-type chloroperoxidase appears to be quite widespread, occurring in several different species of hyphomycete fungi (19). Using a probe based upon the active site of CvCPO, it was possible to identify, isolate, and sequence a vanadium
chloroperoxidase-encoding gene from the hyphomycete fungus *E. didymospora*. Primary structural alignment of this sequence with that of CvCPO (16) reveals a sequence identity between the two of approximately 68%. Typically, based upon secondary structural elements of CvCPO, the majority of amino acid substitutions appear to occur in coil and turn parts of the structure, leaving helices generally free from alteration, with the exception of an external cross-lying helix. These helical structures, particularly the two 4-helical bundles, have been proposed to be the major structural elements of the protein, pro-
A strong stabilizing hydrophobic effect (2), and as such are probably responsible for the high thermostability seen for CvCPO. However, a thermal inactivation study carried out on EvCPO reveals that it is inactivated at lower temperatures than those observed for CvCPO. Although without studying the exact processes involved in unfolding it is impossible to state which parts of the protein are responsible for this effect, it is quite possible that the changes which have occurred in the primary structure, particularly those associated with helical amino acid substitutions, are sufficiently large so as to destabilize this tight hydrophobic nature of the vanadium chloroperoxidase fold and as such reduce the stability of the protein. It may therefore be of interest to study the effect of helical sequence substitution of CvCPO via directed mutagenesis and study their effect on the stability of CvCPO.

Another interesting fact this thermal study revealed is the higher activity EvCPO appears to have after being incubated at temperatures up to 45 °C. One explanation for this could be the presence of multimer structures, which also apparently prevented gel analysis under native conditions. Such structures have also been shown to occur in CvCPO (16), although not to the extent as observed for EvCPO. Mild heating of the enzyme could therefore result in the break up of such structures, then permitting better substrate accessibility.

The presence in the upstream noncoding region of the EvCPO gene of a TATA box indicates that this gene, like that of CvCPO, is probably well regulated (20). Similarly, if it proves that EvCPO is also secreted, then a similar mechanism of export probably exists between the two fungi, since both CvCPO and EvCPO lack a typically defined signal sequence normally associated with secreted eukaryotic proteins (32, 33). In a previous study of CvCPO, it was suggested that the C-terminal portion of the protein may play a role in secretion. Comparison of the C termini of CvCPO and EvCPO reveals the particularly heavily charged nature of EvCPO. However, both proteins, based upon secondary structure predictions, possess putative C-terminal amphiphilic helices, a feature noted for certain metalloproteases secreted by enterobacteria via a C-terminal signal sequence (34).

During the accumulation of kinetic data on EvCPO, it be-
came clear that some of the results obtained for the reference enzyme, namely CvCPO, differed from those previously reported (3). A turnover value for Cl\(^-\) was found of 22.5 s\(^{-1}\) and 201 s\(^{-1}\) for Br\(^-\), contrasting to the previously published values of 12–17 s\(^{-1}\) and 40 s\(^{-1}\) for chlorination and bromination, respectively. This difference is probably due to improved techniques employed in purification of this enzyme (16). Previous samples of seemingly pure enzyme were often heavily discolored. This impurity is now removed by the additional Mono-Q ion exchange step resulting in the higher activity measurement. It can thus be concluded that the values reported here for turnover and \(K_m\) constants are more representative of the pure enzyme system.

Purified EvCPO, although possessing similar substrate affinities at pH 5.0 for both Cl\(^-\) and Br\(^-\), with respect to those found for CvCPO, has actual turnover values which are 10 times lower for Cl\(^-\) and approximately 4 times lower for bromide. It is quite interesting to note at this point that the bromoperoxidase from the terrestrial lichen, a symbiosis between an alga and a fungus, Xantoria parietina (28) shares very similar kinetic parameters for bromination; \(K_m\) for Br\(^-\) of 28 \(\mu\)M and a turnover of approximately 85 s\(^{-1}\). Interestingly, the \(K_m\) for peroxide in both X. parietina enzyme and EvCPO are at least 60 times higher compared with that of CvCPO. However, chlorination by the X. parietina enzyme was not investigated and therefore it would probably be of interest to further investigate this enzyme and attempt to gain structural information.

Since the primary structures of CvCPO and EvCPO proved to be very similar, it was possible to model the structure of EvCPO using CvCPO as a backbone template. The resulting model (not shown), as expected, showed a high degree of overall similarity to that of the x-ray structure of CvCPO. Attempts are now under way to crystallize the vanadium chloroperoxidase from E. didymospora for x-ray crystal analysis, the results of which may hopefully shed more light on the role and importance of some of the residues in or near the active site in catalysis of the vanadium peroxidases.

REFERENCES

1. Vilter, H. (1984) *Phytochemistry* **23**, 1387–1390
2. Messerschmidt, A., and Wever, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 393–396
3. Van Schijndel, J. W. P. M., Vollenbroek, E. G. M., and Wever, R. (1993) *Biochim. Biophys. Acta* **1161**, 249–256
4. De Boer, E., Van Kooyk, Y., Tromp, M. G. M., Plat, H., and Wever, R. (1986) *Biochim. Biophys. Acta* **869**, 48–53
5. De Boer, E., Tromp, M. G. M., Plat, H., Krenn, G. E., and Wever, R. (1986) *Biochim. Biophys. Acta* **872**, 104–115
6. Van Schijndel, J. W. P. M., Barnett, P., Roelse, J., Vollenbroek, E. G. M., and Wever, R. (1994) *Environ. Sci. Technol.* **28**, 391–409
7. Vollenbroek, E. G. M., Van Schijndel, J. W. P. M., Barnett, P., Roelse, J., Vollenbroek, E. G. M., and Wever, R. (1994) *Environ. Sci. Technol.* **28**, 377–385
8. Wever, R., Tromp, M. G. M., Van Schijndel, J. W. P. M., Vollenbroek, E. G. M., Olsen, R. L., and Fogelqvist, E. (1991) in *Biogeochemistry of Global Change* (Oremland, R., ed), pp. 811–825, Chapman & Hall, San Francisco
9. Gribble, G. W. (1994) *Environ. Sci. Technol.* **28**, 310A–319A
10. Van Pee, K.-H. (1996) *Annu. Rev. Microbiol.* **50**, 375–399
11. Wever, R., Plat, H., and De Boer, E. (1985) *Biochim. Biophys. Acta* **830**, 181–186
12. Krenn, B. E., Plat, H., and Wever, R. (1987) *Biochim. Biophys. Acta* **912**, 287–291
13. Krenn, B. E., Isumi, Y., Yamada, H., and Wever, R. (1989) *Biochim. Biophys. Acta* **996**, 63–68
14. Hunter-Cevera, J. C., and Sotos, L. S. (1986) *Microb. Ecol.* **12**, 121–127
15. Liu, T. N. E., M’Timkulu, T., Geigert, T., Wolf, B., Neidleman, S. L., Silva, D., and Hunter-Cevera, J. C. (1987) *Biochem. Biophys. Res. Commun.* **142**, 329–333
16. Simons, L. H., Barnett, P., Vollenbroek, E. G. M., Dekker, H. L., Muijser, A. O., Messerschmidt, A., and Wever, R. (1995) *Eur. J. Biochem.* **229**, 567–574
17. Henrika, W., Renarie, R., Dekker, H. L., Barnett, P., and Wever, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2145–2149
18. Thornton, J. M., Jones, D. T., MacArthur, M. W., Orengo, C. M., and Swindells, M. B. (1996) in *Protein Folding* (Ohlson, C. M., and Fersht, A. R., eds), pp. 71–79, Cambridge University Press, Cambridge, UK
19. Vollenbroek, E. G. M., Simons, L. H., Van Schijndel, J. W. P. M., Barnett, P., Balzar, M., Dekker, H. L., Van Der Linden, C., and Wever, R. (1995) *Biochem. Soc. Trans.* **23**, 267–271
20. Barnett, P., Kruidbosch, D. L. K., Henrika, W., Dekker, H. L., and Wever, R. (1997) *Biochim. Biophys. Acta* **1332**, 73–84
21. Muntanjola-Cvetkovic, M. (1976) *Mycolagia* **68**, 47–51
22. De Boer, E., Plat, H., Tromp, M. G. M., Wever, R., Franssen, M. C. R., Van Der Plas, H. C., Meijer, E. M., and Schoemaker, H. E. (1987) *Biotechnol. Bioeng.* **30**, 607–610
23. Laemmli, U. K. (1970) *Nature* **227**, 680–685
24. Schagger, H., and Von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
25. Sammock, J., Pritzf, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Hewson, W. D., and Hager, L. P. (1980) *J. Phycol.* **16**, 340–345
27. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
28. Plat, H., Krenn, B. E., and Wever, R. (1987) *Biochem. J.* **248**, 277–279
29. de Boer, E., and Wever, R. (1988) *J. Biol. Chem.* **263**, 12326–12332
30. Sherlock, D. J., Harry, T., Smith, A. J., and Rogers, L. J. (1993) *Phytochemistry* **30**, 263–269
31. Proudfoot, N. J., and Brownlee, G. G. (1976) *Nature* **263**, 211–214
32. Von Heijne, G. (1985) *J. Mol. Biol.* **184**, 99–105
33. Perlman, D., and Halvorsen, H. O. (1983) *J. Mol. Biol.* **167**, 391–409
34. Ghigo, J., and Wandersman, C. (1992) *Mol. Gen. Genet.* **236**, 135–144