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Primary structure and characterization of the vanadium chloroperoxidase from the fungus *Curvularia inaequalis*

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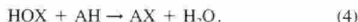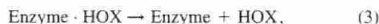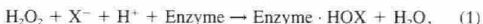[2] E. C. Slater Institute, Department of Biochemistry, Meibergdreef 15, Amsterdam, The Netherlands
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Using reverse transcription of messenger RNA followed by amplification using the polymerase chain reaction, three overlapping cDNA fragments encompassing the encoding sequence of the vanadium chloroperoxidase from the fungus *Curvularia inaequalis* were isolated and sequenced. The sequence was confirmed by DNA sequence analysis of genomic DNA. The deduced amino acid sequence predicts a protein of 609 residues with a mass of 67 488 Da. Competitive reverse-transcription polymerase chain reaction analysis indicates that vanadium chloroperoxidase expression takes place in the secondary-growth phase initiated by nutrient depletion. Southern-blot analysis of genomic DNA indicates that there is only a single gene encoding the vanadium chloroperoxidase and that no isoenzymes are present. The N-terminal amino acid residue was blocked and could not be determined by amino acid sequencing, although evidence is presented showing that the N-terminal region starts very close to the first encoded methionine residue. Although the vanadium chloroperoxidase is secreted, it was not possible to assign a leader peptide. The protein contains two putative N-glycosylation sites but experiments indicate that the protein is non-glycosylated. Two cysteine residues are present in the protein both as free thiols; no disulfide bridging was found. Metal analysis revealed that iron, copper, and calcium do not constitute part of the protein. Zinc was found at a ratio of 0.3 ± 0.04 mol/mol protein. Boiling and subsequent SDS/PAGE of the protein sample showed a typical degradation pattern of the enzyme. Amino acid sequence analysis of the resulting peptides showed that the cleavage took place at Asp-Pro bonds of which six are located throughout the protein. No sequence similarity with other known peroxidases was found except for one small region, sharing limited similarity with bacterial haloperoxidases and other α/β-hydrolase-fold enzymes. In the case of the bacterial bromoperoxidases from this group, a methionine located in this region was suggested to have a role in catalysis. Methionine, however, was not involved in the catalysis of the vanadium chloroperoxidase.

Keywords. Vanadium chloroperoxidase; *Curvularia inaequalis*; cDNA; primary structure.

Haloperoxidases have been isolated from a broad range of organisms. These enzymes catalyze the oxidation of halides (X^-) in the presence of hydrogen peroxide to form hypohalous acids (HOX), which in turn can react with a nucleophilic acceptor [AH; Eqn (1), Eqn (3) and Eqn (4)] if present, to form a halogenated compound (AX) [1]. Specific halogenation [Eqn (1) and Eqn (2)] may, however, occur via a halogenating enzyme intermediate [2].

$$H_2O_2 + X^- + H^+ + Enzyme \rightarrow Enzyme \cdot HOX + H_2O, \quad (1)$$

$$Enzyme \cdot HOX + AH \rightarrow Enzyme + AX + H_2O, \quad (2)$$

$$Enzyme \cdot HOX \rightarrow Enzyme + HOX, \quad (3)$$

$$HOX + AH \rightarrow AX + H_2O. \quad (4)$$

The haloperoxidases are named according to the oxidation of the most electrophilic halide they are able to catalyze, e.g. bromoperoxidases are able to oxidize iodide and bromide while chloroperoxidases are also able to oxidize chloride. Many naturally occurring halogenated compounds have been isolated and, as far as determined, they are involved in a variety of metabolic and physiological functions [1-4]. Haloperoxidases are probably involved in the formation of many of these organohalogens.

Like other peroxidases, haloperoxidases may contain haem as a prosthetic group, e.g. chloroperoxidase from *Caldariomyces fumago* [5]. However, a large group of iodoperoxidases and bromoperoxidases have been discovered containing 1 mol vanadium/mol enzyme, which was shown to be essential for peroxidase activity. These vanadium-containing haloperoxidases were isolated mainly from seaweeds [6-8], although a vanadium bromoperoxidase was also discovered in the terrestrial lichen *Xantheria parietina* [9]. EPR studies [10] and K-edge X-ray studies [11, 12] on vanadium bromoperoxidase from the brown seaweed *Ascophyllum nodosum* showed that the oxidation state

of the metal was vanadium(V). Apparently, the redox state of the vanadate does not change during catalytic turnover. A model has been proposed in which the vanadium(V) coordinates the hydrogen peroxide. The resulting enzyme-peroxo intermediate is able to oxidize bromide to hypobromous acid [12, 13].

Recently, it was shown that certain terrestrial fungi also produce and excrete vanadium chloroperoxidases [14]. The extracellular vanadium chloroperoxidases produced by these fungi may be partly responsible for the formation of naturally occurring organohalogens, which have been found in the terrestrial environment [15]. It has been suggested that the fungal vanadium chloroperoxidases function in degradation and/or penetration of plant tissues (unpublished results), but the precise function of these haloperoxidases has yet to be established.

Two vanadium chloroperoxidases from the fungi *C. inaequalis* and *Drechslera biseptata*, have been biochemically characterized in our laboratory in detail [14, 16, 17]. These enzymes were found to share some interesting features. Besides the ability to form free hypochlorous acid, they display remarkable thermostability and resistance towards denaturation by organic solvents and detergents [17]. Furthermore, in contrast to haem chloroperoxidase from *C. fumago* [5], they show a considerable tolerance to the hypohalous acid product [18]. Finally, when grown in liquid cultures in the absence of vanadate, the fungal vanadium haloperoxidases are secreted into the medium as an apoprotein, which can be easily isolated. This inactive apoform can be reconstituted to full activity when vanadate is added [14].

Although vanadium haloperoxidases constitute a large group of enzymes, little is known about their structure and function. In this study, we describe the cDNA sequence determination of a vanadium chloroperoxidase from the fungus *C. inaequalis* in order to elucidate the primary structure and to initiate a preliminary study of the gene expression. The sequence is compared with other protein sequences to study if any structural relationship exists.

EXPERIMENTAL PROCEDURES

Organisms. The fungi *C. inaequalis* and *D. biseptata* were obtained from the Central Bureau voor Schimmelcultures (CBS strain nos 102.42 and 371.72, respectively; CBS, Baarn, The Netherlands). *Escherichia coli* strain HB101 was used as a host for pUC18/19 derivatives.

Isolation of the chloroperoxidase and protein sequencing. The chloroperoxidase was isolated and purified from liquid cultures of *C. inaequalis* as described previously [14], except that after DEAE chromatography two additional purification steps were performed using an FPLC system (Pharmacia LKB). First a phenyl-Sepharose CL-4B hydrophobic interaction column was used to bind the enzyme in the presence of 2 M NaCl in 50 mM Tris/HCl, pH 8.3, followed by elution with one column volume of 0.1 M NaCl in 50 mM Tris/HCl, pH 8.3. For the final purification, a Mono-Q HR 5/5 anion-exchange column was used to bind the enzyme, followed by elution with a gradient of 0−0.5 M NaCl in 20 mM piperazine/HCl, pH 5.4. Subsequent concentration of the enzyme was carried out using rotation evaporation, followed by dialysis against 50 mM Tris/SO$_4$, pH 8.

The purified chloroperoxidase was enzymatically digested with the proteases *Staphylococcus aureus* V8 and trypsin or chemically cleaved with CNBr [19]. The resulting peptides were separated using SDS/PAGE according to Laemmli [20] or on a Tricine gel according to Schägger and Von Jagow [21] and subsequently blot-transferred to poly(vinylidene difluoride) membranes (Immobilon-P, Millipore) using Caps transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10%

methanol, pH 11] as described by Matsudaira [22]. After electrophoretic elution, the membrane was rinsed for 5 min with deionized water, stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol for 5 min, and destained in 50% methanol, 10% acetic acid, for 10 min at room temperature. The membrane was finally rinsed in deionized water for 10 min and air dried. Peptide bands were submitted to automatic Edman sequencing on a Porton LF 3000 protein sequencer (Beckman Instruments).

Enzyme activity assays. Chloroperoxidase activity was assayed in filtered culture media. Samples were concentrated using Centricon-30 concentrators (Amicon). The chloroperoxidase activity was assayed by measuring the chlorination of 50 μM 1,1-dimethyl-4-chloro-3,5-cyclohexanedione ($\varepsilon = 20.2$ mM · cm^{-1} at 290 nm) [5] using a Varian Cary-17 spectrophotometer. The activity assays were carried out in 0.1 M sodium acetate, pH 5.5, 5 mM potassium chloride, and 1 mM hydrogen peroxide. Qualitative enzyme activity assays were performed using a phenol red assay as described by De Boer et al. [23]. Chloroperoxidase activity on SDS/PAGE gels [20] was detected by soaking the gel in 1 mM *o*-dianisidine, 100 mM potassium bromide, and 100 mM potassium phosphate, pH 6.5, and H$_2$O$_2$ to a concentration of 1 mM. The SDS present during electrophoresis does not interfere with chloroperoxidase activity.

Analyses for metal content. Metal analyses of vanadium chloroperoxidase samples were performed on a Hitachi 180-80 Zeeman polarized atomic absorption spectrophotometer using a Hitachi pyrolysis graphite cuvette.

Determination of protein disulphide bridges. Protein disulphide bridges and free thiols were determined using a method described by Thannhauser et al. [24]. For further identification of putative disulphide bridges in the chloroperoxidase, SDS/PAGE using a 10% gel according to Laemmli [20] was performed. The chloroperoxidase sample mixtures were boiled with or without 2-mercaptoethanol (5%) for 5 min before applying the samples to the gel. After gel electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue R-250 in 25% methanol, 10% acetic acid, for 1 h and destained in 25% methanol, 10% acetic acid. Differences in the running pattern indicated the presence of disulphide bridges within the protein.

Assay for glycosylation of the enzyme. To determine the presence of N-linked sugars in the protein, the purified vanadium chloroperoxidase was treated with *N*-glycanase enzyme (Sanbio). Changes in mobility of the *N*-glycanase-treated enzyme when compared to the untreated enzyme were tested using SDS/PAGE according to Laemmli [20]. The presence of glycan residues was further tested using a sensitive antibody-based dioxygen glycan detection kit (Boehringer Mannheim).

Isolation of mRNA, cDNA synthesis, cloning and analysis. For the isolation of RNA, spores of *C. inaequalis* were inoculated directly into a fermentation medium containing 4 g yeast extract and 2 ml microelement solution/l [14]. After several days of growth, the mycelia were harvested by filtration and lyophilized. The lyophilized *C. inaequalis* mycelia were ground under liquid nitrogen. RNA was extracted by adding an RNA extraction buffer (42 mM sodium citrate, pH 7, 0.83% *N*-laurylsarcosine, 50 mM 2-mercaptoethanol, 1% Triton X-100 and 4 M guanidine isothiocyanate), and incubating for 1 h at room temperature. 0.1 vol. 2 M sodium acetate, pH 4, and 1 vol. phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) was added and the mixture placed on ice for 15 min. After centrifugation for 10 min at $10000 \times g$, 4°C, the aqueous phase was collected, 1 vol. absolute ethanol was added and the mixture was incubated for 1 h at −20°C followed by brief centrifugation at $10000 \times g$. The pellet was resuspended in an appropriate volume of RNA extraction buffer and fractionated by ultracentrifugation in a gradient of cesium chloride [25]. The RNA pellet was carefully washed
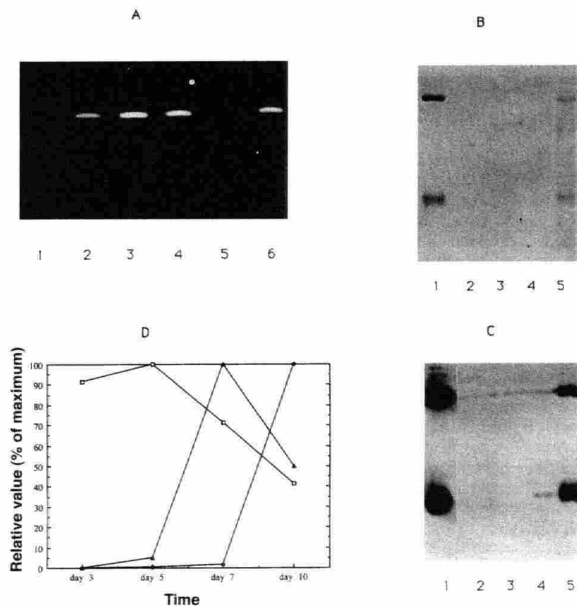
Fig. 1. Growth-stage-specific expression of the vanadium chloroperoxidase by *C. inaequalis*. Cultures were grown for the indicated time periods. (A) RT-PCR result using 5 µg isolated total RNA. Lanes 1−4, days 3, 5, 7, and 10, respectively; lane 5, empty; lane 6, control PCR reaction using the same primers on a cloned cDNA gene fragment. (B) SDS/PAGE gel stained with Coomassie brilliant blue. Lane 1, control (purified vanadium chloroperoxidase); lanes 2−5, concentrated culture medium of days 3, 5, 7, and 10, respectively. (C) Similar SDS/PAGE gel as shown in B after staining for chloroperoxidase activity with *o*-dianisidine in the presence of bromide (see also Experimental Procedures section). (D) Total dry mass of the fungus in the cultures (□), enzyme activity (1,1-dimethyl-4-chloro-3,5-cyclohexanedione chlorination) (◇) and competitive RT-PCR results (△) (using 5 µg isolated RNA per culture) on days 3, 5, 7, and 10. The results are in each case relative to the highest value observed.

and stored in a 75% ethanol solution at $-70\,°C$. For mRNA isolation the RNA was precipitated and resuspended in ribonuclease-free water after which the mRNA was extracted using the poly(A) tract mRNA isolation kit (Promega).

Four 20-residue degenerate oligonucleotides were designed based on the amino acid sequences of chloroperoxidase peptides and used as primers in polymerase chain reactions with first-strand cDNA from *C. inaequalis* as a template. First-strand cDNA synthesis, used in polymerase chain reactions, was carried out on isolated mRNA from *C. inaequalis* using the Pharmacia first-strand cDNA synthesis kit (Pharmacia). Polymerase chain reactions were performed using a thermocycler (Eppendorf mastercycler 5330) and *Taq* polymerase (Promega). For optimal amplification of the chloroperoxidase-encoding cDNA using the degenerate primers, the polymerase chain reaction (RT-PCR) was performed at $46\,°C$ for 30 cycles. The two resulting specific fragments, 71-bp and 62-bp long, were ligated into a pUC18 vector, cloned and sequenced from both strands using the *Taq* Dyedeoxy Terminator Cycle sequencing kit (Applied Biosystems) on a model 373A sequencer (Applied Biosystems). Based on the DNA sequence results, new primers were designed and used for polymerase chain reactions in RT-PCR to obtain the complete encoded region (see also the Results section), or as primers in DNA sequencing for complete double-stranded sequencing of the resulting clones.

To obtain the 5′ region of the mRNA encoding the chloroperoxidase the 5′-Amplifinder RACE kit (Clontech) was used.

The gene-specific primers used in this method were based on DNA sequence data obtained as described above.

Quantification of chloroperoxidase mRNA levels. To obtain semi-quantitative information of chloroperoxidase-encoding mRNA levels in the fungal cells at different stages of growth, competitive RT-PCR [26] was used. Total RNA was isolated from the cells of interest, 5 µg of which was used for reverse transcription as described above. Competitive RT-PCR was carried out using 1 µl of a 1:100 dilution of the first-strand DNA mixture as a template and two gene-specific oligonucleotides as primers for the amplification reaction. An internal control PCR fragment of known size and concentration, containing the same primer template sequences, was added to the PCR mixtures in different concentrations. The amount of this competitor DNA yielding an approximate equimolar amount of product as the target cDNA was taken as a measure for the amount of target mRNA.

Isolation of genomic DNA and analysis. *C. inaequalis* genomic DNA was isolated from lyophilized mycelia ground under liquid nitrogen and extracted with an appropriate amount of extraction buffer (200 mM Tris/HCl, pH 8.5, 25 mM EDTA, 250 mM NaCl, 1% SDS and 0.2 mg · ml^{-1} proteinase K). After incubation overnight at room temperature, 0.7 vol. phenol and 0.3 vol. chloroform were added and mixed vigorously. The tubes were centrifuged at $10000 \times g$ and the aqueous layer was transferred to a clean tube. The genomic DNA was precipitated with 2 vol. absolute ethanol. After centrifugation for 5 min at $5000 \times g$

Table 1. Amino acid sequence data. N-terminal amino acid sequence of five peptides from the chloroperoxidase of *C. inaequalis*. The peptide fragments were obtained by cleavage with trypsin, V8 protease, or CNBr and subsequently separated with PAGE, blotted onto poly(vinylidene difluoride) membranes and analysed using an amino acid sequencer. Sequences used to design degenerated primers for amplification of DNA (see text) are underlined. The numbers of the first and last amino acid residue of each peptide indicate the position in the derived protein sequence (see also Fig. 2).

No.	Amino acid sequence	Cleaved/digested with
1	12 IPDEPEEYN 19	trypsin
2	109 MLSSLYMKPVEQPNPNPGANISDNAYAQLGLVLDRSVLEA 149	CNBr
3	183 GYHPTPGRYKFDDEP 198	V8 protease
4	251 SNADETAEYDDAVRVAIAMGGAQALNSTK 280	trypsin
5	450 DLRQ(S)YDPTAPIEDQPGIVRT 471	trypsin

the pellet was resuspended in 2 ml 10 mM Tris/HCl, pH 8.0, 1 mM EDTA buffer and treated with RNase (Boehringer Mannheim) as recommended by the manufacturer. The genomic DNA-containing solution was extracted with phenol/chloroform/isoamyl alcohol (25:25:1, by vol.) and, after ethanol precipitation, finally dissolved in a suitable volume of 10 mM Tris/HCl, pH 8, 1 mM EDTA.

For Southern-blot analysis of the genomic DNA, the DNA was digested with several combinations of restriction enzymes and after agarose gel electrophoresis blotted onto a nitrocellulose membrane [25]. Hybridization was carried out using a radiolabelled gene-specific fragment, which was made by primer extension using primers 1 and 4 (Fig. 2) and first-strand cDNA as a template. The resulting fragment was labelled by random priming using a-^{32}P-labelled dATP [25]. Based on this result, a minilibrary was made using genomic DNA digested with *Pst*I, which was inserted in the vector pUC18. The library was screened with the same probe as described for the Southern blot. A positive clone was isolated and sequenced from both strands to confirm the cDNA sequence results.

RESULTS

Regulation of chloroperoxidase gene expression. As already shown by Van Schijndel et al. [14], the chloroperoxidase enzyme could only be detected in liquid cultures after several days of growth. Furthermore, we found that the production of the enzyme was retarded for several days when rich growth media were used. To investigate this finding, the expression of the chloroperoxidase-encoding gene in relation to the growth stage of the fungus was studied. Cells were grown in separate cultures and the chloroperoxidase activity in the medium and the chloroperoxidase mRNA content in the fungal cells were monitored after 3, 5, 7, and 10 days of growth. The results are depicted in Fig. 1. At day 3, no chloroperoxidase mRNA could be detected, while a maximum chloroperoxidase-encoding mRNA yield was obtained after 7 days of growth. The increase in chloroperoxidase-encoding mRNA coincides with a decrease in total dry mass of the fungal culture. Chloroperoxidase activity was not detectable after 3 days and was still very low at day 7. At day 10, however, the chloroperoxidase activity increased 60 times compared to day 7. The increase in activity correlates with the amount of the vanadium chloroperoxidase as observed by SDS/PAGE according to Laemmli [20].

Determination of peptide sequences for *C. inaequalis* chloroperoxidase and designing DNA probes. FPLC purification of the chloroperoxidase protein showed a single peak after the final purification step using a Mono-Q anion-exchange column. Isoenzymes for the chloroperoxidase were not found. Sequencing of the purified chloroperoxidase protein revealed that the protein

was N-terminally blocked. After cleavage of the protein with trypsin, V8 protease, or CNBr, five peptide fragments were partially sequenced. The results of the amino acid sequence determination are summarized in Table 1.

Nucleotide sequence of the cDNA. Based on the amino acid sequences of the peptides, four degenerate oligonucleotides were designed (see also Table 1). Using first-strand cDNA as a template and the degenerate primers, the regions encoding peptides nos 2 and 4 (Table 1) were amplified. The small amplification products were cloned into a pUC18 vector and the sequence was determined. Based on these sequence results, two specific primers, 1 and 4 (Fig. 2), were designed and used to amplify the intervening region with RT-PCR. The specific fragment was cloned into pUC18 and sequenced. Two primers, 4 and 5 (Fig. 2), based on the 5' region of this 500-bp fragment, were used as gene-specific primers in the 5'-Amplifinder RACE protocol to obtain the 5' encoding region of the mRNA. After RT-PCR the resulting fragments of about 750 bp were cloned into pUC18 and sequenced. To obtain the 3' coding region of the mRNA, primer 3 (Fig. 2) and the T_{18} Not primer for first-strand cDNA synthesis were used in RT-PCR. The resulting fragment of about 1400 bp was cloned into the pUC18 vector and subsequently sequenced. The three overlapping cDNA clones were all sequenced at least three times from both strands using the cloned PCR fragments derived from three independent amplification reactions in order to exclude possible sequence mistakes due to the incorporation of incorrect bases during the amplification reaction. If necessary, internal sequencing primers were designed and used to obtain a complete double-stranded sequence.

The complete cDNA sequence and its derived amino acid sequence is presented in Fig. 2. The protein sequence is depicted starting with the first methionine residue. The cDNA sequence comprises 2091 bp plus a poly(A) tail and contains an open reading frame that encodes for 609 amino acids. The sequence AATACA, 58 bp upstream of the poly(A) tail, is very similar to the eukaryotic polyadenylation signal AATAAA [27].

To investigate if there are multiple genes encoding for isoenzymes of the chloroperoxidase, a Southern-blot experiment was carried out with differently digested genomic DNA (Fig. 3). The blot was hybridized using a radiolabelled 500-bp PCR fragment, obtained with primers 1 and 4 (Fig. 2) and cDNA as template, as a probe. The result (Fig. 3) shows that only one specific DNA fragment reacts with the chloroperoxidase gene-specific probe, confirming the absence of isoenzymes. Using the results obtained with this Southern blot, a *Pst*I fragment, containing the complete vanadium chloroperoxidase gene, was isolated and the encoding DNA sequence was determined on both strands to confirm the cDNA sequence results (Fig. 2).

Translation of the full open reading frame gives a protein of 609 amino acid residues with a calculated molecular mass of

gttggc**tataaa**gcctgtgagct

                    ──────►
ttctcgttctatatcagaatcacaattcacttgctcagtctactcactacttacaatcacacacattatcacattcacg

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   1 ATG GGG TCC GTT ACA CCC ATC CCA CTC CCT AAG ATC GAT GAA CCC GAA GAG TAC AAC ACC
   1 Met Gly Ser Val Thr Pro Ile Pro Leu Pro Lys Ile Asp Glu Pro Glu Glu Tyr Asn Thr

  61 AAC TAC ATA CTA TTC TGG AAC CAT GTC GGT TTG GAA CTC AAC CGC GTA ACT CAT CAC ACT GTT
  21 Asn Tyr Ile Leu Phe Trp Asn His Val Gly Leu Glu Leu Asn Arg Val Thr His Thr Val

 121 GGA GGC CCC CTG AGC GGA CCA CCT CTC TCT GCC AGG GCT CTG GGT ATG CTG CAC TTG GCT
  41 Gly Gly Pro Leu Ser Gly Pro Pro Leu Ser Ala Arg Ala Leu Gly Met Leu His Leu Ala

 181 ATT CAC GAC GCC TAC TTT TCT ATC TGC CCT CCG ACC GAC TTC ACC ACC TTC CTC TCA CCT
  61 Ile His Asp Ala Tyr Phe Ser Ile Cys Pro Pro Thr Asp Phe Thr Thr Phe Leu Ser Pro

 241 GAT ACT GAG AAT GCC GCG TAC CGT CTA CCT AGC CCT AAT GGT GCA AAT GAT GCT CGC CAA
  81 Asp Thr Glu Asn Ala Ala Tyr Arg Leu Pro Ser Pro Asn Gly Ala Asn Asp Ala Arg Gln

 301 GCA GTC GCT GGA GCT GCC CTC AAG ATG CTG TCT TCA CTG TAC ATG AAG CCC GTC GAG CAG
 101 Ala Val Ala Gly Ala Ala Leu Lys Met Leu Ser Ser Leu Tyr Met Lys Pro Val Glu Gln

 361 CCT AAC CCT AAC CCC GGC GCC AAC ATC TCC GAC AAC GCT TAT GCT CAG GTT GGC TTG GTT
 121 Pro Asn Pro Asn Pro Gly Ala Asn Ile Ser Asp Asn Ala Tyr Ala Gln Leu Gly Leu Val
     2<───────────────────>1 ***

 421 CTC GAC CGA TCA GTT CTG GAG GCA CCT GGT GGC GTG GAC CGA GGA TCA GCC AGT TTC ATG
 141 Leu Asp Arg Ser Val Leu Glu Ala Pro Gly Gly Val Asp Arg Gly Ser Ala Ser Phe Met

 481 TTT GGT GAG GAT GTA GCC GAT GTC TTC TTC GCA CTC CTC AAC GAT CCT CGA GGT GCT TCG
 161 Phe Gly Glu Asp Val Ala Asp Val Phe Phe Ala Leu Leu Asn Asp Pro Arg Gly Ala Ser
                                                                      5<──────────╌│╌─────────

 541 CAG GAG GGC TAC CAC CCT ACA CCC GGC CGC TAT AAA TTT GAC GAT GAA CCT ACT CAC CCT
 181 Gln Glu Gly Tyr His Pro Thr Pro Gly Arg Tyr Lys Phe Asp Asp Glu Pro Thr His Pro

 601 GTC GTC CTC ATT CCA GTA GAC CCC AAC ACC CCT AAT GGT CCC AAG ATG CCT TTC CGT CAG
 201 Val Val Leu Ile Pro Val Asp Pro Asn Asn Pro Asn Gly Pro Lys Met Pro Phe Arg Gln
                                                                                    †

 661 TAC CAC GCC CCA TTC TAC TAC GGC AAG ACC ACG AAG CGT TTT GCT ACG CAG AGC GAG CAC TTC
 221 Tyr His Ala Pro Phe Tyr Tyr Gly Lys Thr Thr Lys Arg Phe Ala Thr Gln Ser Glu His Phe

 721 CTG GCC GAC CCA CCG GGC CTG CGT TCT AAT GCG GAC GAG ACC GCG GAG TAT GAC GAC GTC
 241 Leu Ala Asp Pro Pro Gly Leu Arg Ser Asn Ala Asp Glu Thr Ala Glu Tyr Asp Asp Ala
                                                                              †

 781 GTC CGC GTC GCT ATC GCC ATG GGT GGT GCT CAG GCT CTC AAC TCC ACC AAG CGT AGC CTA
 261 Val Arg Val Ala Ile Ala Met Gly Gly Ala Gln Ala Leu Asn Ser Thr Lys Arg Ser Pro
     4<───────────────────────>3 ***

 841 TGG CAG ACA GCA CAG GGC CTA TAC TGG GCC TAC GAT GGG TCA AAC CTC ATT GGC ACA CCA
 281 Trp Gln Thr Ala Gln Gly Leu Tyr Trp Ala Tyr Asp Gly Ser Asn Leu Ile Gly Thr Pro

 901 CCT CGC TTT TAC AAC CAG ATC GTA CGT CGC ATC GCA GTT ACG TAC AAG AAG GAA GAG GAC
 301 Pro Arg Phe Tyr Asn Gln Ile Val Arg Arg Ile Ala Val Thr Tyr Lys Lys Glu Glu Asp

 961 CTT GCC AAC AGC GAA GTC AAC AAT GCG GAT TTC GCC CGC CTC TTC GCC CTC GTC GAC GTC
 321 Leu Ala Asn Ser Glu Val Asn Asn Ala Asp Phe Ala Arg Leu Phe Ala Leu Val Asp Val

1021 GCT TGC ACA GAC GCT GGT ATC TTT TCC TGG AAG GAG AAA TGG GAG TTC GAA TTC TGG CGC
 341 Ala Cys Thr Asp Ala Gly Ile Phe Ser Trp Lys Glu Lys Trp Glu Phe Glu Phe Trp Arg

1081 CCA CTA TCT GGT GTG CGA GAC GAC GGC CGT CGA GAC CAT GGA GAT CCT TTC TGG CTC ACT
 361 Pro Leu Ser Gly Val Arg Asp Asp Gly Arg Arg Asp His Gly Asp Pro Phe Trp Leu Thr
                                                                          †

1141 CTC GGT GCC CCA GCT ACT AAC ACC AAC GAC ATT CCA TTC AAG CCT CCT TTC CCA GCT TAC
 381 Leu Gly Ala Pro Ala Thr Asn Thr Asn Asp Ile Pro Phe Lys Pro Pro Phe Pro Ala Tyr

1201 CCA TCT GGT CAC GCG ACC TTT GGC GGT GCC GTG TTC CAA ATG GTG CGT CGA TAC TAC AAC
 401 Pro Ser Gly His Ala Thr Phe Gly Gly Ala Val Phe Gln Met Val Arg Arg Tyr Tyr Asn

1261 GGC CGC CTA GGT ACA TGG AAG GAC GAC GAA CCC GAC AAC ATT GCC ATC GAT ATG ATG ATC
 421 Gly Arg Leu Gly Thr Trp Lys Asp Asp Glu Pro Asp Asn Ile Ala Ile Asp Met Met Ile

1321 TCG GAG GAG CTC AAC GGC GTG AAC CGC GAC CTA CGC CAG CCT TAT GAC CCC ACG GCC CCA
 441 Ser Glu Glu Leu Asn Gly Val Asn Arg Asp Leu Arg Gln Pro Tyr Asp Pro Thr Ala Pro

1381 ATC GAA GAC CAA CCC GGT ATC GTG CGC ACC CGC ATT GTT CGC GAC TTC GAC TCG GCC TGG
 461 Ile Glu Asp Gln Pro Gly Ile Val Arg Thr Arg Ile Val Arg Asp Phe Asp Ser Ala Trp

1441 GAA CTC ATG TTC GAA AAC GCC ATT TCG CGC ATC TTC CTC GGT GTC CAC TGG CGT TTC GAT
 481 Glu Leu Met Phe Glu Asn Ala Ile Ser Arg Ile Phe Leu Gly Val His Trp Arg Phe Asp

1501 GCC GCC GCC GCC CGC GAC ATT CTC ATT CCC ACG ACG ACA AAG GAC GTC TAC GCT GTC GAC
 501 Ala Ala Ala Ala Arg Asp Ile Leu Ile Pro Thr Thr Thr Lys Asp Val Tyr Ala Val Asp

1561 AAC AAT GGC GCC ACC GTG TTC CAG AAC GTA GAG GAC ATT AGG TAC ACA ACC AGG GGT ACG
 521 Asn Asn Gly Ala Thr Val Phe Gln Asn Val Glu Asp Ile Arg Tyr Thr Thr Arg Gly Thr

1621 CGT GAG GAC CCC GAG GGC CTC TTC CCT ATC GGT GGT GTC CCA CTG GGT ATC GAG ATT GCG
 541 Arg Glu Asp Pro Glu Gly Leu Phe Pro Ile Gly Gly Val Pro Leu Gly Ile Glu Ile Ala

1681 GAT GAG ATT TTT AAT AAT GGA CTT AAG CCT ACG CCC CTG GAG ATC CAG CCT ATG CCG CAG
 561 Asp Glu Ile Phe Asn Asn Gly Leu Lys Pro Thr Pro Pro Glu Ile Gln Pro Met Pro Gln

1741 GAG ACA CCG GTG CAG AAG CCG GTG GGA CAG CAG CCG GTT AAG GGC ATG TGG GAG GAA GAG
 581 Glu Thr Pro Val Gln Lys Pro Val Gly Gln Gln Pro Val Lys Gly Met Trp Glu Glu Glu

1801 CAG GCG CCG GTA GTC AAG GAG GCG CCG TAG atgaggtatcgtgagagtatggggtaggcgtgagtgggga
 601 Gln Ala Pro Val Val Lys Glu Ala Pro AMB
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cgtcttggagtaaggggctgatgcggaagtttagtttgattggtacgggtctggtttagggcgtgcgctcgatactctgcgggttaa
                                                                                    ──
tacacctatttcgatattagataagaatgctcagatacctagaatgtgatgatcagctgtct

Fig. 2. Nucleotide sequence for the vanadium chloroperoxidase cDNA from *C. inaequalis*. The transcription initiation site is indicated by a solid arrow (→). The sequence given in italics was only obtained from the sequence analyses of genomic DNA while the rest of the sequence was obtained from sequence analyses of cDNA. The complete 3′ region up to base pair number 180 and the 5′ region from base pair number 1141 to the *pvu*II site (cagctg) located at the end of the transcribed region, were further confirmed by double-stranded sequencing of a genomic DNA clone containing the gene encoding the vanadium chloroperoxidase. A typical TATAAA box upstream of the transcription initiation site is underlined. The amino acid sequence of the predicted translation product is shown below the nucleotide sequence. The vanadium chloroperoxidase coding region is flanked by a 75-bp 5′ noncoding region and a 186-bp [excluding the poly(A) tail] 3′ noncoding region. No leader peptide can be assigned to the extracellular enzyme. Potential N-glycosylation sites at positions 128 and 274 are marked (***). A putative polyadenylation signal is overlined. Primers used for amplification reactions are indicated (⊶). Vertical crosses (†) indicate the position of an Asp-Pro bond (see text).

1 2 3 4 5 6

Fig. 3. Southern-blot analysis of genomic DNA from *C. inaequalis* probed with a RT-PCR fragment for the vanadium chloroperoxidase. The genomic DNA from *C. inaequalis* was digested with different restriction enzymes and subsequently separated by electophoresis in a 0.5% agarose gel, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labelled probe as described in the text. Lanes 1−6 contain genomic DNA digested with *Eco*RI, *Bam*HI, *Eco*RI/*Bam*HI, *Xba*I, *Pst*I and *Xba*I/*Pst*I, respectively.

67 488 Da, which is in good agreement with the experimentally determined molecular mass of about 67 kDa, as found by SDS/PAGE according to Laemmli [20]. Although the protein was extracellular, no putative leader peptide, normally present in precursors of extracellular proteins [28, 29] can be assigned. In order to ascertain whether the first encoded methionine residue was present in the mature protein, the protein was treated with CNBr in order to remove this putative N-terminal methionine residue. The resulting peptide mixture was submitted to amino acid sequence analysis. No amino acid sequence in the N-terminal region could be detected indicating that the mature protein lacks a N-terminal methionine residue. A tryptic digestion of the chloroperoxidase yielded a sequenced amino peptide corresponding to residues 12−19 of the encoded protein (Table 1).

Liu et al. [18] reported that the enzyme is tetrameric. This is in agreement with our results presented in Fig. 1, which show two active bands on SDS/PAGE gels, one in the high-molecular-mass range, being a multimeric form of the vanadium chloroperoxidase, and one with a lower molecular mass, representing a monomer. The multimeric form is apparently stable as it is not affected by the presence of SDS (Fig. 1).

It was previously suggested that the vanadium bromoperoxidase from the seaweed *A. nodosum* that the thermostability and chemostability of the enzyme are (at least partly) due to the presence of eight disulphide bridges [30]. Therefore, it was investigated if the remarkable stability of the vanadium chloroperoxidase could be explained by the presence of disulphide bridges. Determination of thiols in the protein by the method of Thannhauser et al. [24] revealed the presence of two reactive thiols. As the deduced amino acid sequence shows the presence of only two cysteine residues (Fig. 2), these results indicate that no disulphide bridges are present in the mature protein. Treatment of the protein with 2-mercaptoethanol did not affect the mobility of the protein when compared to the non-treated sample, which is in agreement with the finding that the protein lacks disulphide bridges.

An intriguing phenomenon occurred when the vanadium chloroperoxidase was submitted to denaturing SDS/PAGE. Minor bands appeared in the gel, which increased in intensity upon prolonged boiling of the sample (data not shown). Subsequent amino acid sequencing of the resulting peptides showed that the protein is cleaved at Asp-Pro sequences. The vanadium chloroperoxidase contains six of these Asp-Pro sequences (Fig. 2). Interestingly, a similar cleavage pattern was observed for vanadium chloroperoxidase isolated from the fungus *D. biseptata* (unpublished results), after submitting the sample to denaturing SDS/PAGE. This finding supports the suggestion that the fungal vanadium chloroperoxidases are homologous.

Metal content of the vanadium chloroperoxidase. As reported previously, the vanadium content is 1 mol vanadium/mol chloroperoxidase [14]. According to Liu et al. [18], the enzyme contained 0.7 mol iron and 2.2 mol zinc as deduced from X-ray fluorescence. However, the role of these metals in enzyme activity was not established. In our experiments, zinc and/or iron addition had no effect on the enzyme activity. We determined the amount of zinc, iron, and also calcium and copper, using purified vanadium chloroperoxidase. The measurements were carried out on four different batches of isolated chloroperoxidase. After dialysis for 24 h in 25 mM Tris/SO$_4$, pH 8.5, the samples were passed through a Sephadex G-25 or Chelex-100 column to remove all residual traces of metal ions. After both procedures, vanadium was found in a ratio of 1 ± 0.1 mol/mol enzyme, as expected. Zinc was found in a ratio of 0.3 ± 0.04/ mol protein. Addition of 2 mM 1,10-phenanthroline or 10 mM EDTA to the dialysis buffer did not affect the amount of zinc present in the protein, indicating that the zinc is strongly bound. Iron and copper were found at ratios less then 0.2 and 0.02 mol/ mol enzyme, respectively. Although calcium was found in a ratio of about 1 mol/mol enzyme after passing it through a G-25 column, this ratio decreased to less then 0.2 after passing the sample through a Chelex column.

Glycosylation of the vanadium chloroperoxidase. After treatment of the vanadium chloroperoxidase with *N*-glycanase, the mobility of the treated enzyme was not altered when compared with the untreated enzyme. This strongly indicates that no N-linked sugars are present in the purified protein, although two putative N-glycosylation sites are present in the deduced primary structure (Fig. 2). A general glycan detection kit was also used to test if the protein was glycosylated. No reaction was observed indicating that the protein is not glycosylated.

Possible role of methionine in the halogenation catalysis. Comparison of the primary structure of the vanadium chloroperoxidase from *C. inaequalis* with bacterial non-haem haloperoxidases from *Pseudomonas pyrrocinia* [31], *Streptomyces aureofaciens* [32, 33], and *Streptomyces lividans* [34] showed that there is one small region region sharing amino acid sequence similarity (Fig. 4). Since these bacterial haloperoxidases did not contain metals, amino acid residues were proposed to be involved in catalysis of the halogenation reaction [35]. For the bromoperoxidases of *S. aureofaciens* (BPO-A1 and BPO-A2), it was shown that incubation of the enzymes in the presence of cyanide, bromide, and hydrogen peroxide resulted in cleavage of the enzymes after a specific methionine due to the formation of CNBr. Therefore, Haag et al. [35] proposed that this methionine residue is involved in the catalytic activity of the bromoperoxidases. This methionine residue is located in the region showing similarity with the vanadium chloroperoxidase from *C. inaequalis* (Fig. 4). To study if a similar cyanide-dependent cleavage

CPO	*C. inaequalis*	D A V R U A I A M G G A Q A L N S T K R S P	290
CPO	*S. lividans*	G A V H I G H S T G G G E V A R Y V A R A E	111
BPOA1	*S. aureofaciens*	D V T L V A H S M G G G E L A R Y V G R H G	109
BPOA2	*S. aureofaciens*	D A V L V G F S M G T G E V A R Y V S S Y G	108
CPO	*P. pyrrocinia*	N A V H I G H S T G G G E V A R Y V A N D G	111
BphD	*P.* sp. KKS102	K A H L V G N S M G G A G A L H F A L E Y P	126
DmpD	*P.* sp. CF600	Q A D L V G N S F G G G I A L A L A I R H P	121
		* * * * * * * * * + *	

Fig. 4. Sequence comparison of vanadium chloroperoxidase from *C. inaequalis* with bacterial non-haem haloperoxidases and two other α/β-hydrolase-fold enzymes. The figure shows the alignment of one small region of the vanadium chloroperoxidase sequence sharing similarity with six α/β-hydrolase-fold enzymes from *S. lividans* chloroperoxidase [34], *S. aureofaciens* bromoperoxidases A1 and A2 [32, 33, 36], *P. pyrrocinia* chloroperoxidase [31], *Pseudomonas* sp. strain KKS102 BphD (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase) [37], and *Pseudomonas* CF600 DmpD (2-hydroxymuconate-semialdehyde hydrolase) [38]. Boxed regions indicate identical residues. Where five or more sequences, including the CPO from *C. inaequalis*, contain the same residue an asterisk (*) appears below the residue; a plus symbol (+) denotes three or four sequences, including the CPO from *C. inaequalis* with the same residue.

occurs in the fungal vanadium chloroperoxidase, the enzyme was incubated with cyanide, bromide, and hydrogen peroxide, as described for the bacterial haloperoxidases [35], and subjected to SDS/PAGE. No cleavage was observed suggesting that this methionine residue is not involved in the catalytic activity of the fungal vanadium chloroperoxidase. This region also has sequence similarity with other α/β-hydrolase-fold enzymes, a group with an increasing number of members to which the bacterial non-haem haloperoxidases now belong [36], though the potentially functional nucleophile is not conserved in the chloroperoxidase sequence. The significance of this similarity, in other than a purely structural sense is not clear, though it is interesting to note that the other aligned hydrolase sequences (Fig. 4) are involved in a dehalogenation pathway [37−39].

DISCUSSION

The results described in this study show that the vanadium chloroperoxidase of *C. inaequalis* is not a normal secretion protein. A signal sequence to guide a precursor into the endoplasmic reticulum, as found for many eukaryotic extracellular proteins, is lacking. The finding that the vanadium enzyme might be secreted via an alternative route is supported by the observation that the vanadium enzyme is not N-glycosylated and does not contain disulphide bridges. These post-translational modifications normally take place in the endoplasmic reticulum. It is not clear how the vanadium enzyme is secreted by the fungus, but it would be interesting to study the mechanism by which the enzyme is crossing the membrane.

Although the N-terminal amino acid residue could not be determined by amino acid sequencing, the results described in this study strongly suggest that the mature vanadium chloroperoxidase does not contain methionine as a N-terminal amino acid residue but the first amino acid should be in close vicinity to the first encoded methionine residue. X-ray crystal structure analysis of the vanadium chloroperoxidase, also indicates that the first amino acid residue of the mature protein is in close vicinity to the first encoded methionine residue (Messerschmidt, A., unpublished results).

Another interesting observation is that the vanadium chloroperoxidase from *C. inaequalis* is secreted in an apoform when grown in the absence of vanadate [14]. This apoenzyme can be easily activated by the addition of vanadate. Apparently these enzymes do not require an additional enzyme system for the incorporation of the vanadate, but are able to obtain their prosthetic group directly from the environment.

A computer database (EMBL) was screened using the Blast program for the presence of proteins that share amino acid sequence similarity to the vanadium chloroperoxidase from *C. inaequalis*. No proteins having sequence similarity were found in this search. Several fungi belonging to the hyphomycetes are able to produce an apparently similar vanadium chloroperoxidase (unpublished results). It therefore appears that the vanadium chloroperoxidases constitute a new family of peroxidases present in the terrestrial environment. Protein fingerprinting and immunoblotting experiments revealed that the fungal vanadium chloroperoxidases from *C. inaequalis* and *D. biseptata* are very similar to each other (unpublished results). However, no similarity was found with peptide sequences of the vanadium bromoperoxidase from the seaweed *A. nodosum* [40]. Therefore, it is presently unclear whether the fungal haloperoxidases are structurally related to the haloperoxidases from marine sources. EPR spectra from both fungal vanadium chloroperoxidases and the vanadium bromoperoxidase from *A. nodosum* [14], however, are very similar, indicating that the environment of the vanadate is identical in these haloperoxidases.

It was demonstrated for three bacterial non-haem haloperoxidases that methionine residues are involved in the halogenation reaction [35]. It has been suggested for these enzymes that the reaction mechanism involves the oxidation of methionine to methionine sulphoxide, which subsequently reacts with halide ions [35]. Our results indicate that in the vanadium chloroperoxidase from *C. inaequalis*, a methionine is not involved in catalysis. Several experiments on the vanadium bromoperoxidase from the seaweed *A. nodosum* indicate that the vanadium in this enzyme is not involved in a redox reaction but serves to bind the hydrogen peroxide and to activate it to allow reaction with bromide and subsequent formation of HOBr [12, 13]. The vanadium in the fungal vanadium chloroperoxidase probably has a similar function in the halogenation reaction and on the basis of steady-state kinetics a peroxo intermediate has been postulated [17].

Although the fungal vanadium chloroperoxidases were found to be very thermostable and chemostable, no disulphide bridges are present in the protein. Secondary structure prediction, using the Garnier prediction method (PC-Gene), indicate that the protein mainly consists of α-helix structures (40−50%), which could be involved in stabilization of the vanadium enzyme. The metal analysis of the vanadium chloroperoxidase shows that iron, copper, and calcium do not constitute part of the enzyme. The amount of zinc is also apparently too low to constitute part of the monomer of the vanadium chloroperoxidase monomer and the determined zinc content of about 0.3 mol/mol enzyme could be due to a high background of zinc that is hard to remove.

There are six Asp-Pro bonds, which are known to be weak peptide bonds [41], found in the protein, which easily break upon boiling of the protein sample. This is especially striking as the protein was found to be very stable at temperatures up to 80°C or in the presence of different organic solutions [17]. The Asp-Pro sequences might play an essential role in folding of the protein possibly providing sharp turns in the tertiary structure.

The expression studies described in this study clearly show that the vanadium enzyme is produced in the secondary growth phase, the so-called idiophase. The expression is regulated at the level of transcription and is apparently dependent on nutrient limitations. Previously it has been suggested that the fungal vanadium chloroperoxidases might be involved in degradation and/or penetration of plant tissues (unpublished results). This would be in agreement with the expression of the *C. inaequalis* vanadium chloroperoxidase in the secondary growth phase as a result

of nutrient depletion. The mechanism by which the vanadium enzyme is involved in plant tissue disruption is unclear. It is conceivable that the hypochlorous acid formed by the enzyme directly oxidizes the polymeric structures present in the plant cell wall in order to solubilize these rather tough structures before penetration. It has been suggested for cellulolytic and lignolytic micro-organisms that a small oxidizing agent, e.g. hydrogen peroxide, is necessary to initiate the solubilization of the polymeric backbone of the plant tissues [42, 43], before cellulases and/or ligninases take over the breakdown of these polymeric structures.

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