Biochimica et Biophysica Acta, 1161 (1993) 249–256

BBAPRO 34388

The chloroperoxidase from the fungus *Curvularia inaequalis*; a novel vanadium enzyme

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(Received 18 May 1992)

Key words: Fungus; Chloroperoxidase; Vanadium; (*C. inaequalis*)

The presence of vanadium-containing bromoperoxidases in various types of seaweed is well-documented. We now report that the terrestrial fungus *Curvularia inaequalis* excretes a novel chloroperoxidase which also contains vanadium as a prosthetic group. The chloroperoxidase is excreted in the medium as the only protein and is, therefore, almost purely obtained. Atomic absorption spectroscopy measurements showed that the chloroperoxidase contained vanadium, which was essential for enzymatic activity, in a stoichiometry of 1 mol vanadium per mol of enzyme. When the fungus was grown in media containing low concentrations of vanadate (VO_4^{3-}) or when vanadate was absent, the enzyme was excreted in an apoform. Addition of vanadate to the apoenzyme purified from the medium, dialyzed holo-enzyme or growth medium led to incorporation of the metal and to a subsequent increase in specific activity from 0.7 to about 7.5 units/mg. The reduced enzyme showed an axially symmetric EPR spectrum ($g_o = 1.971$, $A_o = 91.7 \cdot 10^{-4}$ cm^{-1}) with 16 hyperfine lines that is essentially the same as the EPR spectrum of the vanadium-containing bromoperoxidase of the seaweed *Ascophyllum nodosum*. This demonstrates that the active sites in the two enzymes are very similar. The chlorinating and brominating activities of the chloroperoxidase from *C. inaequalis* were also studied and compared to those of the vanadium bromoperoxidase from *A. nodosum*. The chlorinating reaction catalyzed by the chloroperoxidase had a pH optimum around 5.5 and the K_m for Cl^- was small (0.25 mM at pH 4.5), but the logarithm of its value increased linearly with increasing pH. At high bromide concentrations, the pH optima of chloroperoxidase and bromoperoxidase in the brominating reaction were about the same (5.5). However, at low bromide concentrations the pH optimum of the chloroperoxidase was at higher pH values than that of the bromoperoxidase.

Introduction

Haloperoxidases form a class of enzymes which are able to oxidize halides (Cl^-, Br^-, I^-) in the presence of hydrogen peroxide to the corresponding hypohalous acids according to:

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

If a convenient nucleophilic acceptor is present, a reaction will occur with HOX and a halogenated compound will be formed.

Haloperoxidases have been isolated from various organisms: mammals [1], marine animals [2], plants [3], algae [4], a lichen [5], fungi [6] and bacteria [7,8]. It is generally accepted that haloperoxidases are the enzymes responsible for the formation of halogenated compounds, although other enzyme systems may be involved [9]. The presence in nature of halogenated antibiotics, hormones and halogenated gases is probably due to the activity of these enzymes. For a long time it was believed that the only enzymes catalyzing halogenation were heme-containing peroxidases [6,10,11], but recently it has become clear that marine algae, lichens and microorganisms contain non-heme haloperoxidases [5,12–16]. One of the properties of the prosthetic group in vanadium bromoperoxidases is that it can be removed at low pH (rendering the enzyme inactive) by dialysis against 0.1 M citrate-phosphate buffer containing EDTA. Incubation of the enzyme with vanadate at neutral pH results in recovery of the brominating activity. This observation suggests that the active site in native bromoperoxidase consists of a vanadate type of structure and that the valence state of the metal in the native enzyme is 5+ ($3d^0$). This structure is in line with results of spectroscopic techniques [17] with which vanadium has been identified as the prosthetic group involved in the generation of HOBr. Upon reduction a vanadyl type of EPR spec-

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trum is observed for bromoperoxidases with EPR parameters which are very similar [18,19]. In recent years, there has been considerable interest in the application of these enzymes, e.g., in the use of these enzymes in industrial halogenation reactions. However, the potential uses of some peroxidases have been hampered by instability and costs of isolation of these enzymes [20].

During the course of screening for haloperoxidases, with a high tolerance with respect to basic pH and temperature, it was found that fungi belonging to the group of dematiaceous hyphomycetes produced haloperoxidase activity [21]. These fungi were, in fact, isolated from plant material and soils collected in the Death Valley. This desert contains one of the world's largest salt pans and is very hot and dry. Shortly after this report Liu et al. [22] reported the presence and isolation of a non-heme chloroperoxidase from the fungus *Curvularia inaequalis*, one of the dematiaceous hyphomycetes, which was surprisingly stable towards oxidative attack. According to Liu et al. [22], the enzyme contained 0.7 mol of iron and 2.2 mol of zinc per mol of enzyme as measured by X-ray fluorescence. The authors, however, did not determine whether these metals were essential for enzymic activity and the nature of the active site in this enzyme was not established. We now report that the chloroperoxidase, that is excreted in the medium of the terrestrial fungus *Curvularia inaequalis*, is also a vanadium-containing enzyme and that it shows similarities with the bromoperoxidases from various seaweeds.

Materials and Methods

Our studies were carried out with *Curvularia inaequalis* which was obtained from the Centraal Bureau voor Schimmelcultures (CBS, Baarn, Netherlands) strain No. 102.42. In the first, so-called germination, phase a piece of fungal material was aseptically brought into a 250-ml flask containing 50 ml of a sterile germination medium. This germination medium consisted of 15 g of glucose, 3 g of yeast extract, 1 ml microelement solution (0.8 g of KH_2PO_4, 0.64 g of $CuSO_4 \cdot 5H_2O$, 0.11 g of $FeSO_4 \cdot 7H_2O$, 0.8 g of $MnCl_2 \cdot 4H_2O$, 0.15 g of $ZnSO_4 \cdot 7H_2O$ in 1 liter of distilled water), 100 μg of thiamin and 5 μg of biotin in 1 liter of millipore water. The flask was shaken for 5 days at 23°C. The second phase of growth was performed in 3-l flasks containing 1 liter of a sterile fermentation medium. This fermentation medium consisted of 5 g of tryptone, 3 g of malt extract, 1 g of glucose and 3 g of yeast extract in 1 liter of millipore water. The 50-ml germination medium with the grown fungal material was transferred to fermentation medium and the flasks were shaken for 14 days at 23°C.

After growing the fungus for 14 days in the fermentation medium, the fungal material was separated from the medium by centrifugation ($4000 \times g$) and the medium was bound batch-wise to DEAE-Sephacel (50 ml of DEAE-Sephacel per liter medium). The ion-exchange material was poured into a column which was washed extensively with 0.05 M Tris-HCl (pH 8.3) and 0.2 M NaCl, and the enzyme was eluted with 0.05 M Tris-HCl (pH 8.3) and 0.6 M NaCl. The active fractions were pooled and concentrated using Amicon Centricon 30 microconcentrators. The yield of enzyme was 10 mg per liter of fermentation medium. After concentration, the samples were filtered over active coal to remove some brown colored material. In the experiments in which the bromination reaction was studied, the enzyme was dialyzed extensively against water to remove chloride. Bromoperoxidase from *A. nodosum* was prepared according to Wever et al. [23] as modified by de Boer et al. [13].

The enzymatic activity of the chloroperoxidase was determined spectrophotometrically on a Varian Cary-17 spectrophotometer by measuring the bromination or chlorination of monochlorodimedone (ϵ = 20.2 $mM^{-1}cm^{-1}$ at 290 nm) [4]. The chlorinating activity was measured in a medium containing 1 mM H_2O_2, 0.1 M sodium acetate buffer (pH 5.0), 50 μM monochlorodimedone and 5 mM potassium chloride. 1 unit of chloroperoxidase is defined as 1 μmol of monochlorodimedone chlorinated per min under these conditions. The brominating activity of the chloroperoxidase and bromoperoxidase was measured in a medium containing 1 mM H_2O_2, 0.1 M potassium phosphate (pH 6.5), 50 μM monochlorodimedone and 100 mM potassium bromide. Conditions as mentioned above were used unless specified otherwise. For the inactivation/reactivation experiments, the enzyme was dialyzed against 0.1 M citrate buffer (pH 3.8) with 1 mM EDTA and thereafter against 0.2 M Tris-HCl (pH 8.3). During this treatment, the enzyme partially loses its prosthetic group. Activity was restored by adding vanadate (VO_4^{3-}), after which chlorinating activity was measured as mentioned above. To assess the effect of vanadate on the amount of chloroperoxidase excreted and its specific activity, several batches of *C. inaequalis* were grown in media containing different amounts of vanadate (0–10 μM). The enzyme was purified from the fermentations as described above and the amount of vanadium in the enzyme preparations was determined.

Vanadium was determined with the standard addition method using a Hitachi 180–80 Zeeman polarized Atomic Absorption spectrophotometer equipped with a Hitachi pyrolysis graphite cuvet. EPR measurements were performed on a Varian E-9 EPR spectrometer coupled to an Apple II computer. The instrument was operating at X-band frequency. Temperature, magnetic field and microwave frequency were measured as described by Albracht [24].

The protein content of the samples thus obtained was determined by the method of Bradford [25] using a protein reagent from Bio-Rad with bovine serum albumine as a standard. SDS-PAGE was carried out with 8% gels as described by Laemmli [26]. Standard proteins (Pharmacia, Uppsala, Sweden) used for molecular mass determination ranged from 14.4 kDa to 94 kDa and protein staining was performed with Coomassie brilliant blue R-250. Bromoperoxidase activity was detected on gels by soaking them in a solution of 1 mM *o*-dianisidine, 100 mM potassium bromide and 100 mM potassium phosphate (pH 6.5), after which H_2O_2 was added to a concentration of 1 mM. All chemicals used were of analytical grade. Sodium acetate used for buffers was from Merck, Darmstadt, Germany and contained 0.001% chloride, dipotassium phosphate and monopotassium phosphate were from Merck, Darmstadt, Germany and contained 0.003% and 0.0005% chloride, respectively. Water was filtered and deionised by leading it through an Elgastad B12H (Elga group) and a Milli-Q (Millipore) water-purification system.

Results

The purified enzyme was tested both for activity and for purity by SDS-PAGE. The results are depicted in Fig. 1.

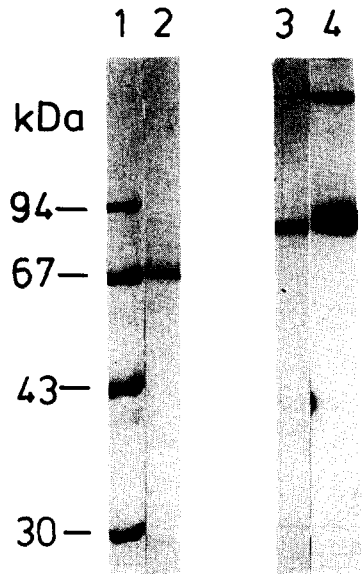

Fig. 1. Gel electrophoresis of purified chloroperoxidase. Lane 1, LMW marker (14400–94000), boiled for 5 min in 20% glycerol, 0.1 M Tris-HCl (pH 6.8), 0.001% Bromophenol blue and 1% SDS, Coomassie staining; lane 2, 10 μg chloroperoxidase boiled for 5 min in 20% glycerol, 0.1 M Tris-HCl (pH 6.8), 0.001% Bromophenol blue, 5% β-mercaptoethanol and 1% SDS, Coomassie staining; lane 3, 10 μg chloroperoxidase in 20% glycerol, 0.1 M Tris-HCl (pH 6.8), 0.001% Bromophenol blue and 1% SDS, Coomassie staining; lane 4, Chloroperoxidase from *C. inaequalis*, *o*-dianisidine activity staining, non-denaturing conditions as in lane 3.

After denaturation by boiling the enzyme for 5 min in a medium containing 5% β-mercaptoethanol and 1% SDS, a single band at 67 kDa was observed. The molecular mass of this band is the same as reported by Liu et al. [22]. Under denaturing conditions, SDS-PAGE yields for all vanadium bromoperoxidases a single subunit with a molecular mass of also about 65 kDa [27]. We assume therefore that the chloroperoxidase from *C. inaequalis* consists of one single subunit (67 kDa). Under these denaturing conditions all enzymic activity was lost. When the enzyme was not boiled in SDS, it remained active and two bands were observed which stained both for activity and protein. The band with the high molecular mass probably represents an aggregated form of the enzyme.

Little is known about the steady-state kinetics of the chlorination catalyzed by chloroperoxidase except for the limited study by Liu et al. [22]. Fig. 2a and 2b show the activity of the chloroperoxidase as a function of pH at various Cl^- and Br^- concentrations. As in most peroxidases, the enzyme showed a pH-optimum in both reactions. As can be seen, the pH optima for bromination and chlorination catalyzed by the chloroperoxidase from *C. inaequalis* were about equal (pH 5.5). The enzyme, however, was inhibited at low pH values by excess bromide but not by excess chloride. The concentration of chloride slightly affected the position of the pH optimum in the chlorination. Comparison of Fig. 2a with 2b also shows that bromide is oxidized by the chloroperoxidase at a slightly higher rate than chloride (Cl^- turnover = 12 s^{-1}, Br^- turnover = 41 s^{-1}). The low activity detected at pH 4–5 when no chloride was present may be due to traces of chloride in the buffer. However, another peroxidative reaction, the origin of which is unknown, may also occur. For comparison, the pH-dependence of the bromoperoxidase from *A. nodosum* is also given (Fig. 2c). It is clear that the pH optimum for bromination by the chloroperoxidase from *C. inaequalis* (pH 5.2) is higher than the pH optimum for bromination by the bromoperoxidase from *A. nodosum* when measured at a Br^- concentration of 10 mM. Fig. 2a also shows that at high pH the chloride concentrations used were not high enough to saturate the enzyme completely. Therefore, the K_m for chloride was determined as a function of pH. Fig. 3 shows that, in line with Fig. 2a, the logarithm of the K_m increased linearly with increasing pH from 0.25 mM at pH 4.5 to 116 mM at pH 8. The high affinity for chloride at low pH probably explains why the enzyme is able to use traces of chloride in the buffer.

In our initial attempts to isolate the enzyme, highly variable yields in terms of activity were observed in some batches of the fermentation medium. Further, when the turnover of the purified chloroperoxidase in the monochlorodimedone assay was compared with the turnover found by Liu et al. [22] for the same enzyme

($240\ s^{-1}$), it was found that our enzyme preparations had a much lower activity ($1\ s^{-1}$). Since it is known that vanadium bromoperoxidases in the apoform can be activated by vanadate [12,13] and that vanadium bromoperoxidases when isolated from seaweed contain in general less than stoichiometric amounts of vanadium, we assumed that *C. inaequalis* excreted chloroperoxidase as an apo-enzyme. To investigate this, batches of *C. inaequalis* were grown in media containing different amounts of vanadium (0–10 μM final concentration) added as sodium orthovanadate and the enzymic activity was measured both in these media as

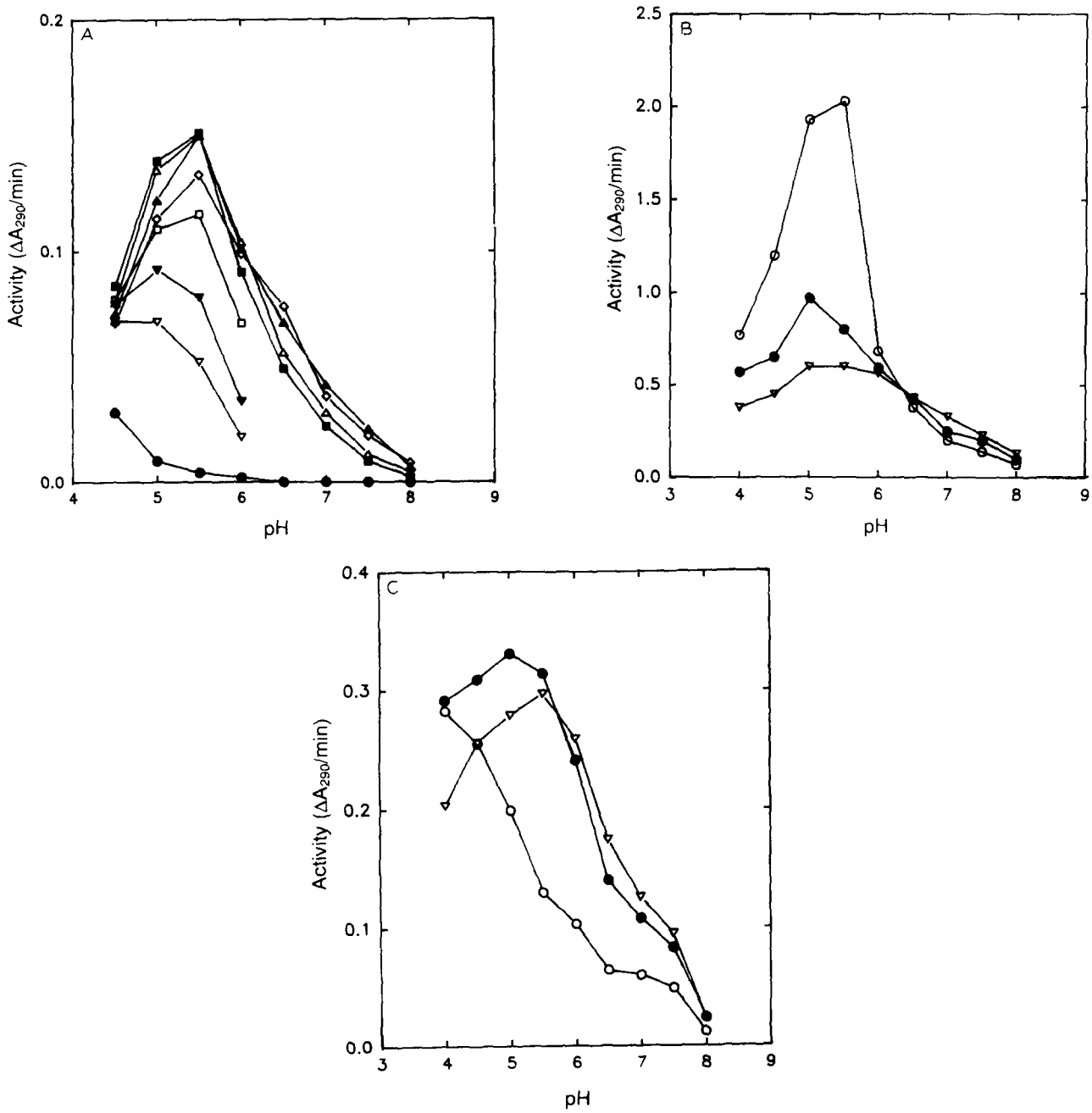

Fig. 2. (a), chlorinating activity of chloroperoxidase at different pH and Cl^- concentrations as measured by the monochlorodimedone assay (conditions as in Materials and Methods, except for halide concentration and pH). (●——●), 0 mM; (▽——▽), 1 mM; (▼——▼), 2 mM; (□——□), 5 mM; (■——■), 20 mM; (△——△), 40 mM; (▲——▲), 80 mM; (◇——◇), 100 mM. Chloroperoxidase concentration in the assay; 0.69 μg/ml. (b), brominating activity of chloroperoxidase at different pH and Br^- concentrations as measured by the monochlorodimedone assay (conditions as in Materials and Methods, except for halide concentration and pH). (○——○), 10 mM; (●——●), 50 mM; (▽——▽), 200 mM. Chloroperoxidase concentration in the assay; 2.7 μg/ml. (c), brominating activity of bromoperoxidase of *A. nodosum* at different pH and Br^- concentrations as measured by the monochlorodimedon assay (conditions as in Materials and Methods, except for halide concentration and pH). (○——○), 10 mM; (●——●), 50 mM; (▽——▽), 200 mM. Bromoperoxidase concentration in the assay; 0.12 μg/ml.

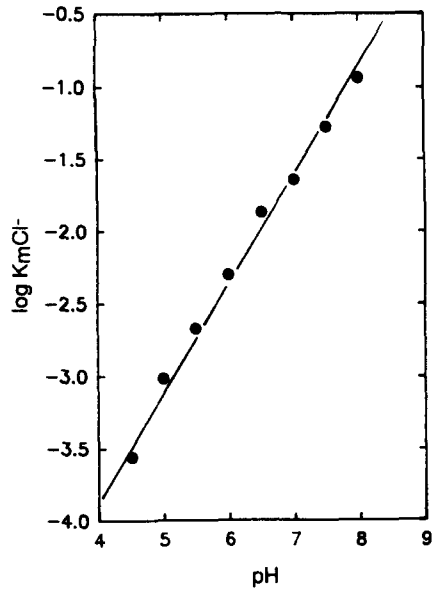

Fig. 3. Log K_{m,Cl^-} for chloroperoxidase from *C. inaequalis* at different pH values. For experimental conditions, see Materials and Methods.

in the enzyme purifications. It was observed that, when 10 μM vanadate was present, the enzymic activity increased a factor of 10 compared to media to which no vanadate was added. Further, enzyme prepared from media to which no or low concentrations of vanadate were added contained less than stoichiometric amounts of vanadium (results not shown) assuming a molecular mass of 67 kDa, as suggested by SDS-PAGE.

It was also tested whether the enzyme purified from media containing vanadate could be inactivated al low pH as is observed for the vanadium bromoperoxidases and, therefore, purified chloroperoxidase was inactivated at low pH by treatment with citrate buffer (pH 3.8) containing 1 mM EDTA to remove all vanadium (see Materials and Methods). After inactivation and dialysis against 0.1 M Tris-HCl (pH 8.3), the specific activity of the enzyme decreased from 5.6 U/mg to 2.3 U/mg in the inactivated enzyme. It was not possible to inactivate the enzyme completely using this procedure. Lowering of the pH in the inactivation buffer from 3.8 to 3.2 resulted in denaturation of the protein and loss of activity. Fig. 4 shows that the enzyme was reactivated completely after incubation of the apo-protein in vanadate solutions for 24 h and suggested that vanadate was essential for the activity of the chloroperoxidase from *C. inaequalis* as had already been suggested by the observation that an inactive apo-enzyme was excreted into the medium when no vanadate was present. To establish that there is a relationship between specific activity and vanadium content a reactivation experiment was performed with apo-enzyme, which was obtained by growing the fungus in medium without

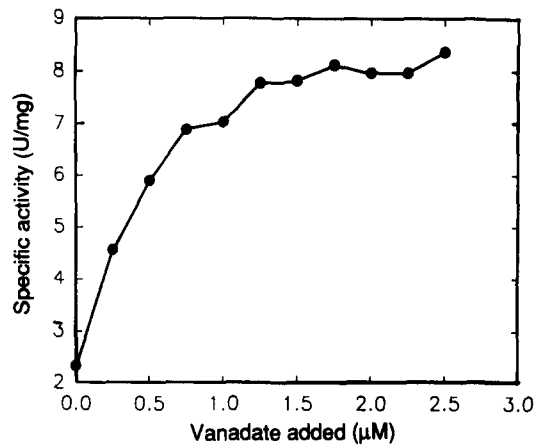

Fig. 4. Reconstitution of apo-chloroperoxidase from *C. inaequalis* by vanadate. The reconstitution was carried out in 50 mM Tris-HCl (pH 8.3) by addition of various amounts of sodium orthovanadate; enzyme concentration, 2.5 μM. After 24 h incubation, the specific activity was determined. The apo-enzyme was prepared as described in Materials and Methods.

vanadate and isolation of the apo-enzyme. The results of this vanadate titration experiment are depicted in Fig. 5. The apo-enzyme obtained in this way had a much lower specific activity of 0.8 U/mg and a vanadium content of less than 0.1 mol per mol of enzyme. A linear relationship was obtained between specific activity and the concentration of vanadium incorporated in the enzyme. Maximal specific activity (7.5 U/mg) is reached when 1.0 mol of vanadium per mol

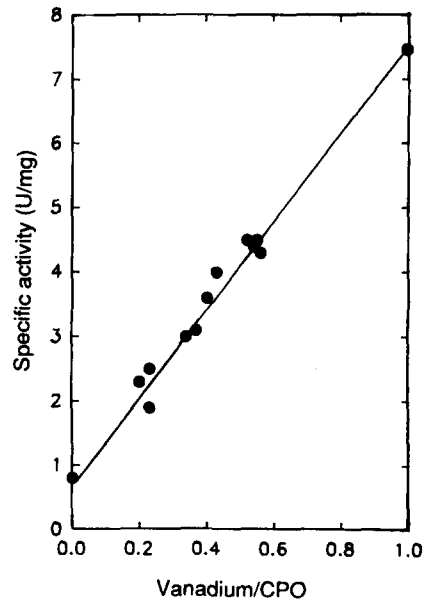

Fig. 5. Relationship between specific activity of the chloroperoxidase and its vanadium content. Different amounts of vanadate were added to apochloroperoxidase purified from cultures grown without vanadate. The protein samples were incubated for 24 h in 50 mM Tris-HCl (pH 8.3) and sodium orthovanadate. After incubation, the samples were centrifuged through a Chelex-100 column to remove free or aspecifically bound vanadium.

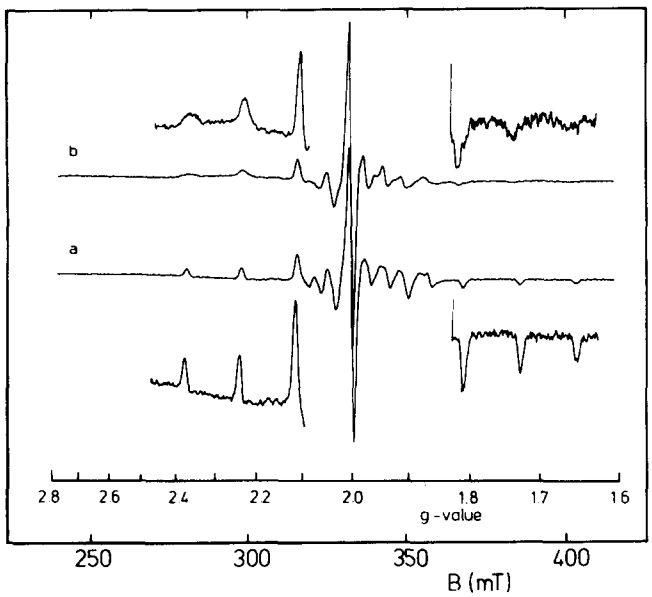

Fig. 6. EPR spectra of bromoperoxidase from *A. nodosum* and chloroperoxidase from *C. inaequalis* after reduction with sodium dithionite. Trace a, bromoperoxidase from *A. nodosum* (5 mg/ml); Trace b, chloroperoxidase from *C. inaequalis* (3 mg/ml). Instrument settings: Microwave power, 24 dB; microwave frequency, 9323.66 MHz; Modulation amplitude, 1 mT; temperature, 90 K. The hyperfine lines were recorded at a 10-times higher gain.

of enzyme is incorporated. Thus, titration of apo-chloroperoxidase obtained by incubation at low pH or apo-chloroperoxidase obtained by growing the fungus in media without vanadate give the same result. The addition of vanadate after 2 weeks of growth to media to which no additional vanadate was added led to a rise in activity within one day, also showing that vanadate had been incorporated in an already excreted apo-enzyme in the medium.

The evidence that vanadium was present in the isolated chloroperoxidase was confirmed by electron paramagnetic resonance. In the enzyme as isolated no EPR signals were detectable. However, when the chloroperoxidase was reduced with a small excess of sodium dithionite, a typical vanadyl type of spectrum is observed (Fig. 6, trace b). For comparison the spectrum of the vanadium containing bromoperoxidase from *A. nodosum* is also shown (Fig. 6, trace a). It is obvious that the spectra of bromoperoxidase from *A. nodosum* and chloroperoxidase from *C. inaequalis* are very similar. The linewidth of the hyperfine lines in chloroperoxidase is, however, larger. For instance, the linewidth of the parallel $m_{\|} = -5/2$ hyperfine line of reduced bromoperoxidase was 2.25 mT, whereas that in chloroperoxidase was 3.75 mT. The spectral parameters are $g_o = 1.971$ and $A_o = 91.7 \cdot 10^{-4}$ cm^{-1} where g_o is derived from the relation $g_o = g_{\|}/3 + 2 \cdot g_{\perp}/3$ and the hyperfine coupling constant $A_o = A_{\|}/3 + 2 \cdot A_{\perp}/3$. This result confirms that the chloroperoxidase from *C. inaequalis* is also a vanadium enzyme. Furthermore, it suggests that the ligands for the prosthetic group and the way the protein is wrapped around the prosthetic group are very similar for the chloroperoxidase from the fungus *C. inaequalis* and the bromoperoxidase from the seaweed *A. nodosum*.

Discussion

In this study, we have presented data on the nature of the prosthetic group of the chloroperoxidase from *C. inaequalis* and some of its properties. SDS-PAGE and staining for both protein and enzymatic activity showed that the isolated enzyme was pure. Boiling in the presence of SDS and β-mercaptoethanol yielded a single band with a mass of around 67 kDa, which corresponds to that seen in all vanadium bromoperoxidases [27,28].

When the activity of fully-reconstituted chloroperoxidase preparations in our studies was compared to results obtained by Liu et al. [22] a much lower activity in the chlorination of monochlorodimedone was observed (12 vs. 240 s^{-1}). This discrepancy remains unexplained. The yield of enzyme in terms of enzymatic activity was also highly variable, which suggested that an apo-enzyme was secreted into the medium. Therefore, vanadate was added to the fermentation medium and, indeed, this led to the production of more enzyme activity in the medium. The purified enzyme from such cultures had a much higher specific activity than that from cultures to which no vanadate was added. The specific activity in the chlorination (7.5 U/mg) however, was still lower than reported for various fully reactivated vanadium bromoperoxidases, such as the bromoperoxidases from *Fucus distichus* (1500 U/mg) [29], *L. saccharina* (615 U/mg [18]) and *A. nodosum* I (126 U/mg) [30]. The low enzymic activity of preparations isolated from media to which no vanadate was added is probably due to traces of vanadium in the growth media. From vanadate titration experiments with enzyme from *C. inaequalis* grown in media without vanadate, it was found that full activity was restored at a stoichiometry of 1 mol vanadate per mol of the apo-enzyme. Further, a linear relationship was found between specific activity and vanadium content of the preparation and our observations show clearly that vanadate is essential for the enzymatic activity of the chloroperoxidase from *C. inaequalis*. Whether other metal ions, like iron and zinc, which have been shown to be present in the chloroperoxidase isolated by Liu et al. [22], have a role in catalysis is not clear yet. It should be noted, however, that zinc is often found as a contaminant in proteins and is difficult to remove.

When the purified enzyme was reduced with sodium dithionite, an EPR signal was detected that was typical of a vanadyl species and with EPR parameters compa-

rable to those of the vanadium bromoperoxidases from *A. nodosum* and other seaweed species. From this observation one may conclude that the native chloroperoxidase also contains vanadium in the 5 + oxidation state [13] which is in line with the observation that the apo-enzyme was activated by orthovanadate (VO_4^{3-}) in which vanadium is in its highest oxidation state. That the EPR parameters are so similar indicates that the structure and the way in which the vanadyl group is ligated into the protein are very similar in both chloroperoxidases and bromoperoxidases. This is extremely interesting; in some way the protein part and surrounding amino acids have been tuned and modified via evolution to increase the oxidizing ability of the prosthetic group of the enzyme, which allows the enzyme to oxidize chloride. In this respect, it is of great interest that it has been shown that the bromoperoxidase from *A. nodosum* also catalyzes chlorination reactions [31] with a specific activity that is about 15-times lower than the value reported here for the chloroperoxidase from *C. inaequalis*. However, in the case of bromoperoxidase, very high concentrations of chloride (up to 1.5 M) had to be used, whereas the chloroperoxidase was able to use much lower concentrations of chloride.

The pH optima in the bromination and chlorination reactions catalyzed by the chloroperoxidase are almost equal (pH 5.5). Soedjak and Butler [31] also report that the pH optimum for chloroperoxidase activity by bromoperoxidase is at pH 5.0, which is the same for bromoperoxidase activity. In this respect these enzymes seem to differ from heme-containing haloperoxidases. For instance for myeloperoxidase, the pH optimum for chlorinating activity is about 0.5 pH unit lower than that for brominating activity [32].

The logarithm of the K_m for Cl^- is linearly dependent on the pH, the slope of the line being about 0.7. This suggests that during the catalytic cycle protons have to be taken up simultaneously with chloride or alternatively a group has to be protonated to allow binding of chloride. A more detailed kinetic analysis at higher and lower pH is required to distinguish between the two possibilities. This behavior is in clear contrast to that which was observed in the oxidation of bromide by bromoperoxidase [33]. It is striking that excess chloride did not inhibit the chlorinating activity of chloroperoxidase, whereas excess bromide did inhibit the brominating activity as was also observed for the vanadium bromoperoxidase from *A. nodosum*. These differences in kinetic behaviour may reflect changes in the catalytic mechanism and/or the presence of other amino-acid residues involved in the catalytic cycle. Detailed analysis of similarities and differences between the two enzymes in their kinetic properties may well lead to important clues concerning the catalytic action. However, definite conclusions still have to await knowledge of the tertiary structure of these interesting enzymes.

Acknowledgements

The support by Unilever Research, Vlaardingen and Gist-Brocades/IBIS, Delft/Rijswijk, Netherlands, is gratefully acknowledged. This research is supported by the Netherlands Foundation for Chemical Research (SON) and was made possible by financial support from the Netherlands Technology Foundation (STW) and from the Netherlands Organization for Scientific Research (NWO). The authors further would like to thank Drs. L. de Boer and W.R. de Boer (Gist Brocades) for their advise in the initial stages of this investigation, Drs. J. Haverkamp and G.S.P. Groot for stimulating discussions and A.M.Ph. de Jong and E.C. Duin for their help with the EPR experiments.

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