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Author(s)	J.W.P.M. van Schijndel, P. Barnett, J. Roelse, E.G.M. Vollenbroek, R. Wever
Faculty	FNWI: Swammerdam Institute for Life Sciences (SILS)
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The stability and steady-state kinetics of vanadium chloroperoxidase from the fungus *Curvularia inaequalis*

Johannes W. P. M. VAN SCHIJNDEL, Philip BARNETT, Jeanet ROELSE, Esther G. M. VOLLENBROEK and Ronald WEVER
E. C. Slater Institute, University of Amsterdam, Amsterdam, The Netherlands

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In this article we report on the steady-state kinetics of the chlorination and the stability of the vanadium chloroperoxidase from the fungus *Curvularia inaequalis*. The data show that the kinetics of this enzyme resemble that of the vanadium bromoperoxidase from the seaweed *Ascophyllum nodosum*. At low pH, chloride inhibited the enzyme, but the inhibition was of a dual nature. At pH 4.1 a mixed type of inhibition by chloride with respect to hydrogen peroxide was observed whereas at pH 3.1 the nature of the inhibition became competitive. The log K_m for hydrogen peroxide decreased linearly with pH with a slope of −1 in the pH range 3–5. A reaction mechanism is presented to explain the observed data.

We also showed that this class of enzymes is inhibited by nitrate. At pH 5.5, nitrate inhibits the chlorination reaction competetively with respect to chloride (K_i = 2 mM) and uncompetetively with respect to hydrogen peroxide. Furthermore, we showed that the enzyme produces HOCl as a reaction product. The enzyme exhibited a high thermostability (t_m of 90°C) and displayed high stability in organic solvents (solutions of 40% methanol, ethanol or 2-propanol) and moderate stability in the presence of the chaotropic agent guanidine/HCl ($G_{1/2}$, the concentration of guanidine/HCl at which the enzyme activity was half the original activity was 3.7 M).

After the discovery of the first non-haem (vanadium) bromoperoxidase from the alga *Ascophyllum nodosum* a whole range of other vanadium-dependent bromoperoxidases was found in many other different seaweeds such as *Laminaria saccharina*, *Ceramium rubrum*, *Fucus distichus*, *Macrocystis pyrifera* and also in a terrestrial lichen *Xantoria parietina* [1–6]. The bromoperoxidase from *A. nodosum* has now been extensively studied by a variety of kinetic and biophysical methods. These studies have revealed the nature of the prosthetic group and the reaction mechanism [7].

K-edge X-ray studies show that the prosthetic group of vanadium bromoperoxidase has features similar to vanadate [8]. Both the vanadium K-edge X-ray study and EPR studies [9] showed that the valence state of the vanadium in the enzyme is vanadium(V) and that vanadium(IV) is not involved in catalytic turnover. This finding confirms the model in which vanadium(V) in the native enzyme serves to bind hydrogen peroxide and bromide [9].

Detailed kinetic studies of the bromination reaction catalysed by the enzyme showed that it does not react with H_2O_2 when groups on the enzyme are singly or doubly protonated and, in addition, that at low pH, bromide is a competitive inhibitor with respect to H_2O_2. In the reaction scheme that was derived for this enzyme, hydrogen peroxide reacts first with the enzyme to form a hydrogen-peroxide-enzyme complex after which bromide and a proton react with the complex to form an enzyme-bound HOBr complex which decays to yield enzyme and HOBr [7].

The role of the bromoperoxidases in the seaweeds has not yet been established, but it is very likely that the enzyme is involved in the synthesis of bromometabolites such as CH_2Br_2 and $CHBr_3$ by the seaweed [10–12]. Another halometabolite that has been shown to be produced by the seaweed *A. nodosum* is $CHBr_2Cl$. As shown by Soedjak and Butler [13], the bromoperoxidase has a low chloroperoxidase activity though with a low affinity for chloride. It is conceivable that bromoperoxidase is involved in the biosynthesis of this chlorinated metabolite.

In an earlier study we reported on the discovery of the first vanadium-dependent chloroperoxidase, isolated from the growth medium of the terrestrial fungus *Curvularia inaequalis* [14]. In this report we give a more detailed analysis of the steady-state kinetics of the chlorination reaction of the chloroperoxidase and in addition we present the reaction mechanism and stability properties of the enzyme from *C. inaequalis*. Finally, we compare the results with those obtained from the related vanadium bromoperoxidase from *A. nodosum*.

MATERIALS AND METHODS

Our studies were carried out with the fungus *C. inaequalis* which was obtained from the Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands, strain no. 102.42.). Growth of the fungus and isolation of the enzyme were performed according to [14] except that after the DEAE column chromatography, hydrophobic-interaction chromatography was also carried out using a 20-ml Phenyl Sephar-

Correspondence to R. Wever, E. C. Slater Institute, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands
Fax: +3120 5255124.
Enzyme. Peroxidases (EC 1.11.1.–).

ose CL 4B hydrophobic-interaction column (Pharmacia). The enzyme was eluted with a gradient from 2 M to 0 M NaCl in 50 mM Tris/HCl, pH 8.3.

The enzymic activity of the chloroperoxidase was determined spectrophotometrically on a Varian Cary-17 spectrophotometer by measuring the chlorination of monochlorodimedone (ε = 20.2 mM^{-1} cm^{-1} at 290 nm) to dichlorodimedone (ε = 0.1 mM^{-1} cm^{-1} at 290 nm) [15]. In the temperature stability experiments chloroperoxidase was incubated for selected time intervals in 50 mM Tris/HCl, pH 8.3, and at various temperatures, after which activity was measured. To test stability in guanidine/HCl the enzyme was incubated for 5 min in 50 mM Tris/HCl, pH 8.3, containing up to 5 M guanidine/HCl, activity being measured with the monochlorodimedone assay.

The formation of free HOCl was measured by incubating chloroperoxidase (64 nM) in 100 mM potassium phosphate, pH 4.5, 200 µM H_2O_2 and 1 mM Cl^- in an Amicon concentration cell equipped with a PM 30 filter. By applying pressure on the cell after the addition of chloroperoxidase to the reaction mixture the reagents and products were pushed through the filter which retained the enzyme. HOCl in the effluent was measured by collecting aliquots of the filtrate in tubes, which had been previously weighed and contained a 50 µM monochlorodimedone solution in 100 mM sodium acetate, pH 5.0. By determining the masses of the tubes before and after the addition of the aliquot and from measurement of absorbance a corrected absorbance decrease could be calculated. From the latter it was possible to determine the concentration of HOCl in the filtrate.

HOCl formation was also measured spectrophotometrically using monochlorodimedone as a scavenger. The enzyme (27 nM) was added to 2.5-ml quartz cuvettes containing the same reagents as in the Amicon experiment. At selected time intervals after starting the reaction by addition of the enzyme, monochlorodimedone was added to the cuvettes to a final concentration of 45 µM. Directly after addition of the scavenger, the absorbance decrease was monitored on a Cary 17 spectrophotometer. It was also possible to measure HOCl formation directly in a quartz cuvette containing 20 mM chloride and 5 mM hydrogen peroxide in 0.1 M sodium citrate buffer, pH 3.8. After addition of enzyme, absorbance spectra from 200 nm to 350 nm were recorded, using a Hewlett Packard 8452 A diode array spectrophotometer.

All chemicals used were of analytical grade. Water was filtered and deionised by passing it through an Elgastad B12H (Elga group) and a Milli-Q (Millipore) water-purification system.

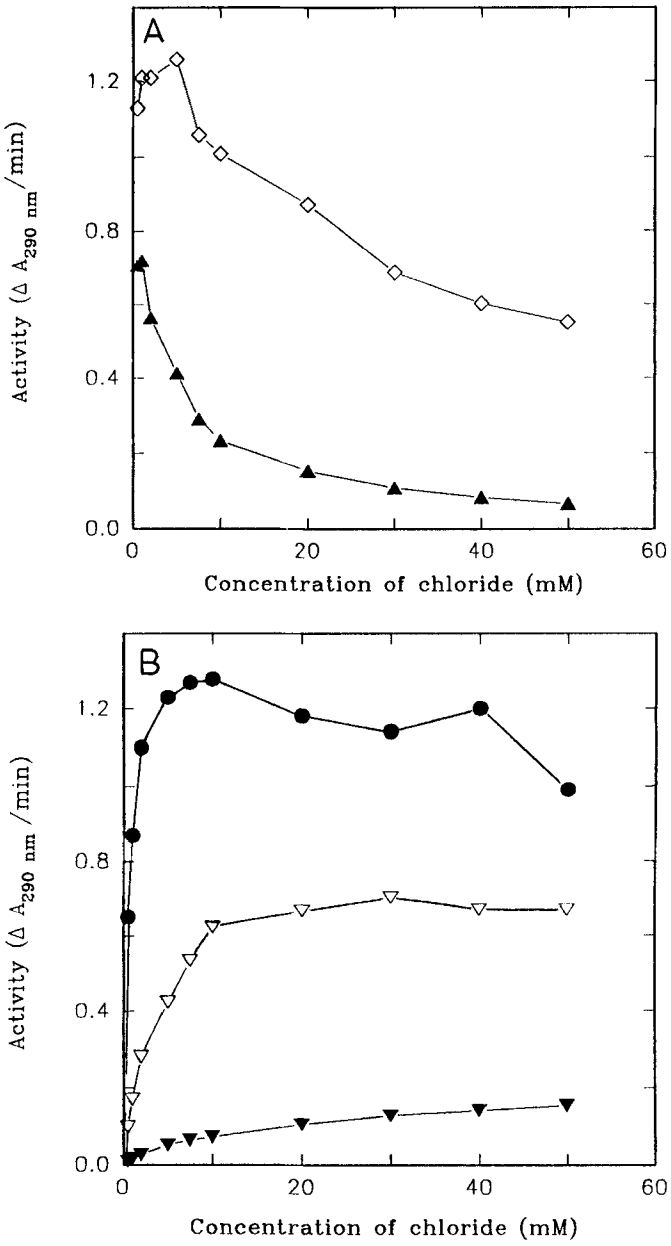

Fig. 1. Activity versus chloride concentration at various pH values for vanadium chloroperoxidase from *C. inaequalis*. Assay conditions were 0.1 M sodium citrate, 1 mM H_2O_2, 50 µM monochlorodimedone and 60 nM chloroperoxidase. (A), (▲), pH 3.0; (ε), pH 4.0; (B), (●), pH 5.0; (▽), pH 6.0; (▼), pH 7.0. The data points are the means of two measurements.

RESULTS

In Fig. 1 the activity versus chloride concentration is depicted at five different pH values with a constant hydrogen peroxide concentration. The resultant curves change from a chloride-inhibited reaction at pH 3.0 and pH 4.0 to a more Michaelis-Menten type of curve at pH values of 5.0, 6.0 and 7.0, showing that chloride acts as a substrate for the reaction but can also inhibit the enzyme. Similar curves were obtained with other concentrations of hydrogen peroxide (data not shown).

The inhibition of the enzyme by chloride at low pH values was studied in more detail (Fig. 2). At pH 3.1 the Lineweaver-Burk plot is typical for a competitive type of inhibition by chloride with respect to hydrogen peroxide (Fig. 2A). The slopes of the lines in this reciprocal plot are affected by increasing the inhibitor concentration, but the V of the reaction is independent of the concentration of chloride. The K_i for chloride at pH 3.1, calculated from a Dixon plot [16] (data not shown), was found to be 6 mM. To determine whether the inhibition by chloride is due to an ionic strength effect on the activity of the enzyme, the chlorination reaction was also studied in the presence of 0.2 M sodium sulphate. No effect of ionic strength on the enzyme kinetics was observed. The type of inhibition depends upon the pH. A complex pattern of inhibition was observed at pH 3.8 (data not shown) but the lines at pH 4.1 (Fig. 2B) show a typical mixed type of inhibition. Both the value of V and the slopes

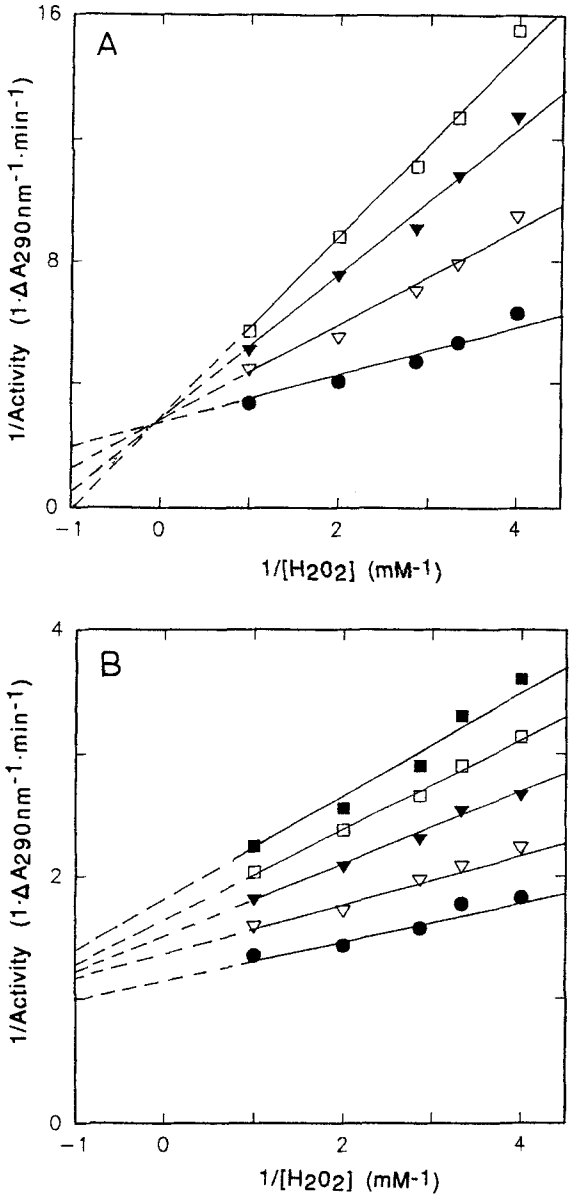

Fig. 2. Inhibition by chloride of the chloroperoxidase from *C. inaequalis*. Assay conditions were 0.1 M sodium citrate, 50 μM monochlorodimedone and 25 nM chloroperoxidase. Double-reciprocal plots of activity as a function of hydrogen peroxide concentration at different chloride concentrations and pH values. (A) pH 3.1, (●), 10 mM chloride; (▽) 20 mM chloride; (▼), 30 mM chloride; (□), 40 mM chloride. (B) pH 4.1, (●), 20 mM chloride; (▽), 40 mM chloride; (▼), 60 mM chloride; (□), 80 mM chloride; (■), 100 mM chloride. The data points are the means of three measurements.

of the lines are affected by chloride, but the lines intersect at a common point in the upper-left quadrant. The inhibition constant, calculated from a Dixon plot, was found to be 70 mM. At neutral pH the enzyme obeys Michaelis-Menten kinetics.

When the V, which at pH 4.1 cannot be derived directly from the Lineweaver-Burk plot, but has to be calculated, was plotted as a function of pH (Fig. 3) a curve was obtained with a maximum value at about pH 4 indicating the presence of three ionization states in the rate-determining step. Fig. 3 also reveals the pH dependence of the K_m for hydrogen peroxide which was obtained from Lineweaver-Burk plots at different

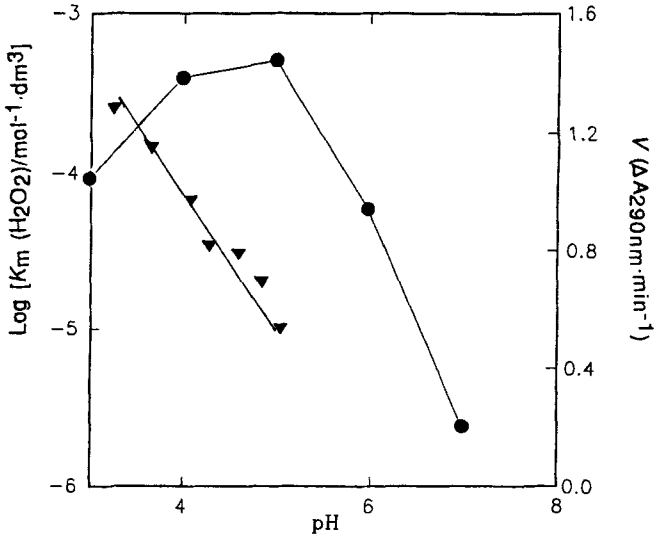

Fig. 3. Logarithm of the K_m for hydrogen peroxide and V as a function of pH for vanadium chloroperoxidase from *C. inaequalis*. The K_m for hydrogen peroxide (▼) was measured at a fixed chloride concentration of 10 mM and the V (●) was obtained at saturating and non-inhibitory concentrations of substrates. Concentration of chloroperoxidase 52 nM.

pH values. Log K_m for H_2O_2 was found to be linearly dependent on pH with a slope of -1. At pH 5 the values of K_m for hydrogen peroxide and the absorbance changes in the measurements became too small to obtain accurate data. Extrapolating the straight line to neutral pH gives a Michaelis constant for hydrogen peroxide of chloroperoxidase of the order of 1 μM.

Inhibitors of vanadium haloperoxidases have not yet been reported, despite the fact that nitrate is known to affect the kinetics of the bromination reaction catalysed by the vanadium bromoperoxidase from the lichen *Xantoria parietina* [17]. We therefore investigated if this inhibition by nitrate was also found for the vanadium chloroperoxidase (Fig. 4). At pH 5.5, nitrate inhibits the enzyme in a competitive way with respect to chloride and in an uncompetitive way with respect to hydrogen peroxide. The inhibition constant (K_i) for nitrate, calculated from a Dixon plot (data not shown), is about 2 mM.

The vanadium bromoperoxidase from *A. nodosum* produces oxidized bromine species upon incubation with substrates [7]. Since this is a topic of much current interest [18], the role of chloroperoxidase in the formation of oxidized chlorine species was investigated in greater detail. As described in Materials and Methods the formation of an oxidized chlorine species was measured in three different experimental systems. The results of the experiment, in which the enzyme was withheld by a filter and the filtrate was tested for the presence of an oxidized chlorine species, suggested that the enzyme produced a product that was able to chlorinate monochlorodimedone. Thus, in the concentration cell a chlorinating product is formed which is able to pass through the filter. Calculations from the maximum absorbance changes of the amount of monochlorodimedone that has reacted show that about 50 μM of the chlorinating species was detectable which corresponds to about 0.25 mol of hydrogen peroxide originally present.

In the experiment to assess formation of an oxidized chlorine species in a cuvette, as described in Materials and

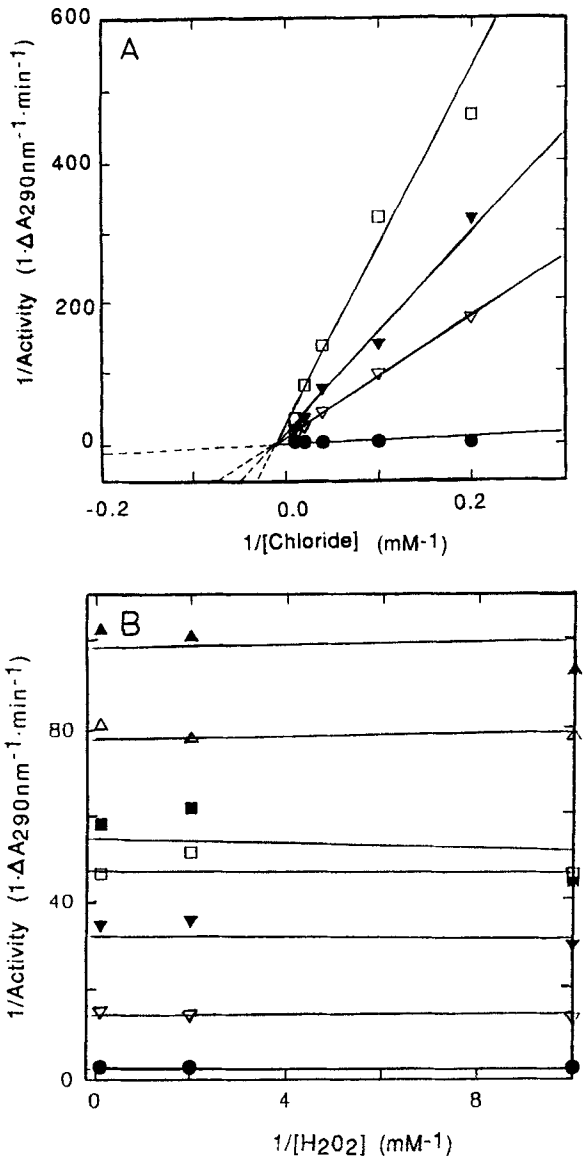

Fig. 4. Inhibition by nitrate of the chloroperoxidase of *C. inaequalis*. Double-reciprocal plots of activity as a function of chloride and hydrogen peroxide concentration at pH 5.5 and at different nitrate concentrations. Assay conditions were 0.1 M sodium citrate, 50 μM monochlorodimedone and 15.5 nM chloroperoxidase. (A) Inhibition of nitrate with respect to chloride. Hydrogen peroxide at 1 mM; (●), no nitrate; (▽), 5 mM nitrate; (▼), 10 mM nitrate; (□), 20 mM nitrate. (B) Inhibition of nitrate with respect to hydrogen peroxide. Chloride at 20 mM; (●), no nitrate; (▽), 2 mM nitrate; (▼), 5 mM nitrate; (□), 7.5 mM nitrate; (■), 10 mM nitrate; (△), 15 mM nitrate; (▲), 20 mM nitrate.

Methods, the immediate decrease in absorbance was monitored after addition of monochlorodimedone to a cuvette in which the reaction was already started (Fig. 5) by addition of chloroperoxidase. Theoretically, the absorbance would instantaneously decrease from 0.9, corresponding to 45 μM monochlorodimedone, to an absorbance corresponding to the amount of HOCl generated in the time interval between addition of the enzyme and addition of monochlorodimedone. Such an immediate absorbance decrease was observed, and this shows that an oxidized chlorine species is accumulating in the cuvette which reacts directly with the monochlorodimedone when added. In Fig. 5 the initial absorbance

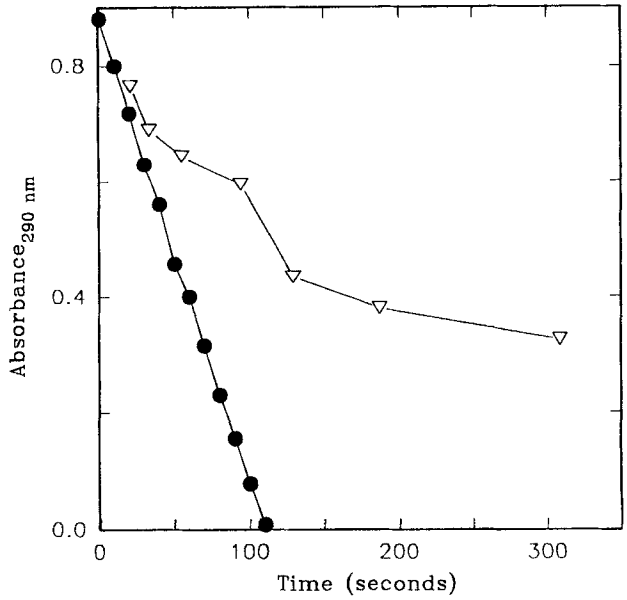

Fig. 5. Generation of oxidized chlorine species by the chloroperoxidase from *C. inaequalis*. Experimental conditions as described in Materials and Methods. (●), time-course of the absorbance decrease of monochlorodimedone upon addition of chloroperoxidase. (▽), absorbance measured directly after addition of monochlorodimedone at selected time intervals after starting the enzymic reaction.

decrease is plotted as a function of the time elapsed after starting the enzymic reaction and before addition of monochlorodimedone to the cuvette. For comparison the time-course of the absorbance observed when monochlorodimedone was present before addition of the enzyme is also shown. At short time intervals after starting the reaction, the absorbance decrease corresponds to the value calculated. At longer time intervals, however, the measured absorbance decrease is less than expected, showing that the product of the enzymic reaction has reacted in a side reaction. Clearly an oxidized chlorine species is generated by the enzyme reaching a concentration of about 25 μM after 200 s.

Anbar and Dostrovsky [19] reported that HOCl has an absorbance spectrum in aqueous solutions with peaks at 245 nm and 315 nm. Therefore, it was possible to spectrophotometrically directly measure the formation of the oxidized chlorine species. Fig. 6 shows the spectral changes occurring when chloroperoxidase was added to hydrogen peroxide and chloride. From this figure it can be seen that absorbance bands develop at 245 nm and 315 nm in time. The absorbance spectrum corresponds to that reported by Anbar and Dostrovsky and to that of free HOCl in aqueous buffer (Fig. 6) indicating that the product (the oxidized chlorine species) of the enzymic reaction is HOCl.

Vanadium bromoperoxidases have been reported [25] to be stable enzymes and the chloroperoxidase shows similar stability features. When the chloroperoxidase was incubated in 40% methanol, ethanol or 2-propanol and aliquots of the incubation mixture were taken and tested for activity the chloroperoxidase activity was hardly affected by incubation even after three weeks of incubation (data not shown). There is, however, a decrease of about 20% in activity after one day of incubation, but this was also observed in the absence of alcohol.

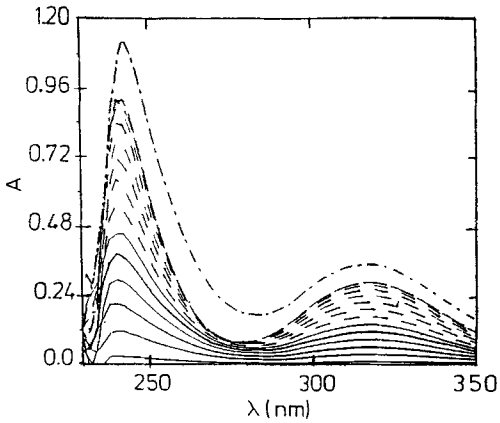

Fig. 6. Direct spectroscopic measurement of HOCl. (——) and (– –); HOCl formation by the chloroperoxidase from *C. inaequalis*, conditions as described in Materials and Methods. 1 μM chloroperoxidase in 0.1 M sodium citrate, pH 3.8. Time between scans, 30 s, (–·–), commercially available HOCl (4.5 mM) in 0.1 M sodium citrate, pH 3.8.

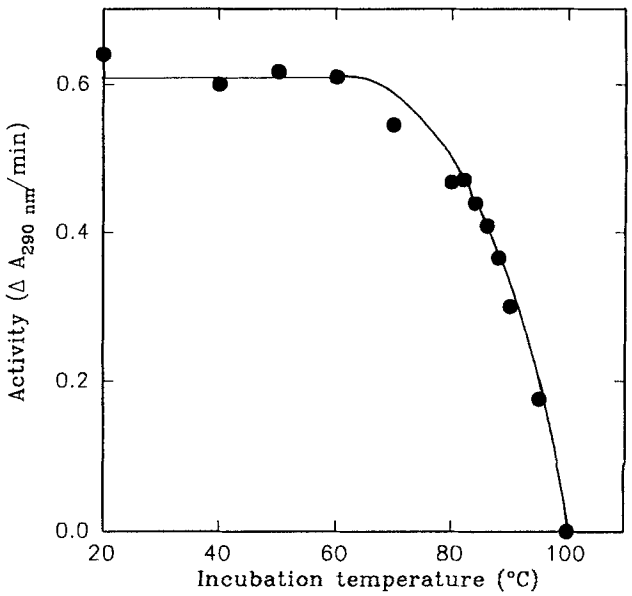

Fig. 7. Stability of the chloroperoxidase from *C. inaequalis* at different temperatures. Samples of chloroperoxidase (0.25 mg/ml) in 50 mM Tris/H_2SO_4, pH 8.3, were incubated for 5 min at the given temperatures in an Eppendorf 5436 thermomixer after which enzymic activity of 25-μl aliquots was measured as described in Materials and Methods.

The stability of the chloroperoxidase was also tested in the presence of the chaotropic agent guanidine/HCl (data not shown). The decrease in activity starts at a concentration of about 2 M of guanidine/HCl and the enzyme was completely inactivated at a concentration of 5 M guanidine/HCl. The $G_{1/2}$, the concentration of guanidine/HCl at which the activity of the enzyme is half the original activity, is 3.7 M.

The thermal stability of the chloroperoxidase was also investigated. Incubation of the enzyme at 80°C resulted in an initial decrease in activity of about 20% after which the enzyme remains stable for 6.5 h (data not shown). The t_m (midpoint temperature) of chloroperoxidase was calculated from an experiment, in which the enzyme was incubated for 5 min at a given temperature after which the activity was determined. The t_m found was 90°C (Fig. 7). Prolonged incubation (15 min) of the enzyme did not affect the t_m. It is obvious from these results that chloroperoxidase is a remarkably stable enzyme.

DISCUSSION

In this study the steady-state kinetics of the chlorination reaction and stability of the vanadium chloroperoxidase from the fungus *C. inaequalis* were investigated. At low pH (3.0–4.0), high chloride concentrations inhibited the enzyme, whereas at higher pH values the activity showed a Michaelis-Menten type of behaviour as a function of chloride concentration. Log K_m for hydrogen peroxide showed a linear dependence with respect to pH in the range 3–5 and V showed a maximum in the pH range 3–7. These observations, together with results from earlier studies [14], which showed that the K_m for chloride is also pH dependent, have been used to develop a scheme for the mechanism of the vanadium chloroperoxidase from *C. inaequalis* (Fig. 8).

From the double-reciprocal plots at pH 6 and 7 of 1/activity versus 1/chloride concentration at different hydrogen peroxide concentrations and 1/activity versus 1/hydrogen peroxide concentration at various chloride concentrations (data not shown) it can be concluded that in this pH range the enzyme mechanism is of a ping-pong type. In the uninhibited reaction cycle of the enzyme, the enzyme first forms a complex with hydrogen peroxide which is converted into an activated peroxo-intermediate E* after which chloride and a proton react and a enzyme-HOCl complex is formed which decays rapidly to enzyme and HOCl. That protons are needed in the reaction can be deduced from the dependence of log K_m for chloride on pH [14]. This conclusion was also derived from earlier studies on the vanadium bromoperoxidase from *A. nodosum* [7].

Protons appear to play a dual role in the reaction of chloroperoxidase. On the one hand they are required for the reaction, but on the other hand at low pH a protonated species is formed that reacts with chloride to inhibit catalytic activity. The inhibition by chloride at pH 3.1 is of a competitive nature with respect to hydrogen peroxide, which in the mechanism is represented by the occurrence of the inhibitory $E \cdot H_2^{2+}Cl^-$ species. This species originates from the reaction of the double-protonated form of the enzyme, $E \cdot H_2^{2+}$, with chloride.

The inhibition at pH 4.1, however, is of a mixed type. This effect is explained in the mechanism by the occurrence of inhibitory $E \cdot H^+ \cdot Cl^-$ and $E \cdot H^+ \cdot H_2O_2 \cdot Cl^-$ complexes, which originate from the binding of chloride to the $E \cdot H^+$ and $E \cdot H^+ \cdot H_2O_2$ species and which should differ from the productive complex yielding HOCl.

Similar effects of the substrates on the enzyme kinetics were observed for the vanadium bromoperoxidase from the seaweed *A. nodosum* [7] where bromide was reported to have a competitive inhibitory effect in the pH range of 4.5–6 and no inhibition was observed at lower than pH 4.5 and at higher than pH 6. In contrast, Soedjak and Butler [20, 21] reported that the inhibition by bromide of the bromoperoxidase from *A. nodosum* was non-competitive with respect to hydrogen peroxide. It is conceivable that the inhibition of the bromoperoxidase by bromide is also of a dual nature as was observed in our measurements of the chloride inhibition for the chloroperoxidase from *C. inaequalis* and that these conflicting observations may be due to measurements made at different pH values.

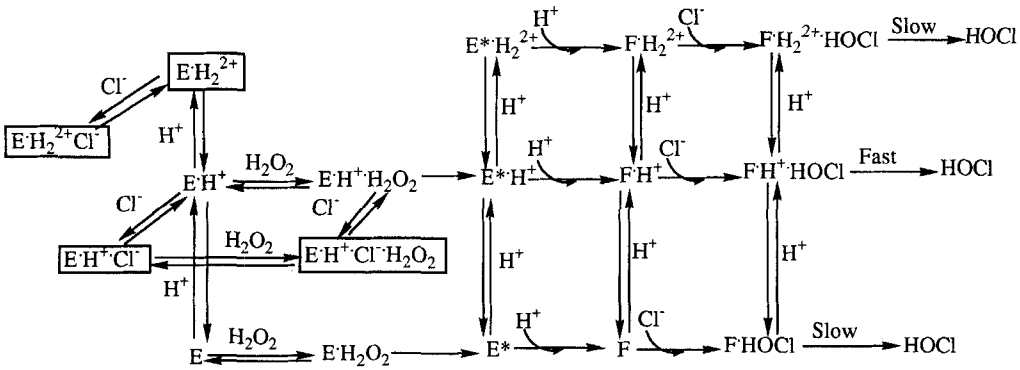

***Fig. 8.* Proposed reaction scheme for the vanadium chloroperoxidase from the fungus *C. inaequalis* based on steady-state kinetic results.** The enzyme species in boxes represent the inhibited forms. E* may represent an activated vanadium-peroxocomplex. The reaction scheme only depicts those protonated states which according to our steady-state study are present. More ionization states may occur.

De Boer and Wever [7] stated that bromide does not appreciably react with doubly protonated or deprotonated forms of the native bromoperoxidase. For the chloroperoxidase from *C. inaequalis* we similarly conclude that chloride reacts with a protonated form of the enzyme and not with the deprotonated form. As reported previously [14] the log K_m for chloride for this chloroperoxidase is linearly dependent on pH with a slope of 1. This corresponds in the mechanism to the step from E* to E·HOCl where a protonated enzyme-peroxo intermediate should be formed before chloride is able to bind. Similarly, the linear dependency of the log K_m for hydrogen peroxide on pH for the chloroperoxidase with a slope of −1 in the pH 3−5 range suggests that a protonable group, with a pK_a larger than 5, is involved in the binding of hydrogen peroxide. When the group is protonated, an inhibitory $E \cdot H_2^{2+}$ species is formed (Fig. 8) and hydrogen peroxide is unable to bind.

The bromoperoxidase from *A. nodosum* also showed a linear dependence of K_m for hydrogen peroxide at low pH but at neutral pH a deviation was found. This was explained by the presence of an ionizable group with a pK_a of 5.7 and it was speculated that the group in bromoperoxidase was a histidine residue [7]. In agreement with this, we have recently obtained evidence, on the basis of chemical modification of histidines using diethylpyrocarbonate, that in the chloroperoxidase from *C. inaequalis* at least three histidines are present in close vicinity of the vanadate prosthetic group and may be possibly involved in the binding of the metal [22].

Under saturating but non-inhibitory substrate concentrations the *V* showed a maximal rate at about pH 4.0 (Fig. 3). Fig. 2 in [14] also shows a bell-shaped pH optimum curve in the rate of the chlorination reaction at saturating chloride concentrations. These observations are consistent with three ionization states in the rate-determining step to yield the product. These correspond in the mechanism (Fig. 8) to the decay of $F \cdot H_2^{2+}$, $F \cdot H^+$ and F to enzyme and HOCl.

The bromination reaction catalysed by the vanadium bromoperoxidase from the lichen *X. parietina* [17] is affected by nitrate. The nature of the inhibition of this enzyme was not fully understood. Inhibition of the vanadium enzyme from *C. inaequalis* by nitrate with respect to chloride and hydrogen peroxide is less complicated. Nitrate inhibition at pH 5.5 showed that the enzyme is competitively inhibited (K_i of 2 mM) with respect to chloride and uncompetitively with respect to hydrogen peroxide.

In the study of the formation by the enzyme of a free oxidized chlorine species, three different methods were used which lead to the conclusion that the enzyme produces HOCl, indicating that the presence of a stable enzyme-HOCl complex is unlikely. In the filtrate of the ultrafiltration cell only 50 μM oxidized chlorine species could be detected whereas 200 μM hydrogen peroxide was originally present. That only a part of the oxidizing chlorine species is detectable is probably due to side reactions of the free oxidizing chlorine species. A rapid reaction of HOCl with hydrogen peroxide yielding singlet oxygen as described by Allen [23] may occur, but the possibility of a reaction of HOCl with, for instance, traces of contaminants in the buffer or on the surface of the Amicon cell or the filter cannot be excluded. Furthermore, the pressure-induced flow through the cell after addition of the chloroperoxidase will limit the amount of hydrogen peroxide that is available for reaction, resulting in a lower amount of oxidized chlorine species formed than expected. Similarly such side reactions may occur also in the experiment in which monochlorodimedone was added after the onset of the enzymic reaction, since the decrease in absorbance was not as large as the calculated value.

The spectrum of the species generated by the enzyme obtained by us is the same as that of HOCl and our results show that hypochlorous acid is the enzymic product. The enzyme also remains catalytically active at very high concentrations of HOCl. From Fig. 6 it can be calculated, using a molar absorption coefficient of 60 mM^{-1} cm^{-1} [19], that a concentration as high as 4 mM of HOCl is generated without loss of enzymic activity. Liu et al. [24] also showed high resistance of the enzyme towards HOCl up to a concentration of 2.5 mM, but above this concentration the enzyme loses its function. In the direct spectroscopic experiment a much higher concentration of HOCl is found than in the other experiments, designed to detect the presence of free HOCl, this is probably due to the lower pH value used in the reaction. HOCl will, in particular at higher pH, rapidly react with hydrogen peroxide [23].

We have found that the vanadium chloroperoxidase from *C. inaequalis* is very stable in mixtures of organic solvents with water. Incubation with the chaotropic agent guanidine/HCl reveals a $G_{1/2}$ of 3.7 M, somewhat lower than that of 4.7 M reported for the vanadium bromoperoxidase from *A. nodosum* [25]. The observation that incubation of the enzyme at 80 °C causes an initial loss of activity of about 20%, but prolonged incubation does not decrease the activity further, was also found for the vanadium bromoperoxidase from the lichen *X. parietina* [5]. The latter enzyme also showed initial

loss of activity upon incubation at 70 °C, but no further effect was observed upon prolonging the incubation time.

Tombs [26] has presented data for a series of proteins on the relation between physical and chemical stability. Bromoperoxidase from *A. nodosum* is, according to these data, extremely chemically stable and moderately thermally stable. However, the chloroperoxidase from *C. inaequalis* is moderately chemically stable, but has high thermal stability that approaches that observed for enzymes from thermophilic organisms. The vanadium chloroperoxidase is much more stable than its haem-containing counterpart and the stability enhances the application of the enzyme in potential commercial uses [27, 28].

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