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Inhibition of vanadium chloroperoxidase from the fungus *Curvularia inaequalis* by hydroxylamine, hydrazine and azide and inactivation by phosphate

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Abstract

The first detailed inhibition study of recombinant vanadium chloroperoxidase (rVCPO) using hydroxylamine, hydrazine and azide has been carried out. Hydroxylamine inhibits rVCPO both competitively and uncompetitively. The competitive inhibition constant K_{ic} and the uncompetitive inhibition constant K_{iu} are 40 and 80 μM, respectively. The kinetic data suggest that rVCPO may form a hydroxylamido complex, hydroxylamine also seems to react with the peroxovanadate complex during turnover. The kinetic data show that the type of inhibition for hydrazine and azide is uncompetitive with the uncompetitive inhibition constant K_{iu} of 350 μM and 50 nM, respectively, showing that in particular azide is a very potent inhibitor of this enzyme. Substitution of vanadate in the active site by phosphate also leads to inactivation of vanadium chloroperoxidase. However, the presence of H_2O_2 clearly prevents the inactivation of the enzyme by phosphate. This shows that pervanadate is bound much more strongly to the enzyme than vanadate.
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1. Introduction

Vanadium haloperoxidases are enzymes that catalyse the oxidation of a halide (X^-) by hydrogen peroxide to the corresponding hypohalous acids according to the following reaction:

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

The enzymes are named after the most electronegative halide ion they are able to oxidize, therefore chloroperoxidase (CPO) oxidizes Cl^-, Br^-, I^- and bromoperoxidase (BPO) oxidizes Br^- and I^-. This class of enzymes binds vanadate (HVO_4^{2-}) as a prosthetic group [1,2], which can be removed by dialysis against 1 mM EDTA in 100 mM citrate/phosphate (pH 3.8) [3]. Apo enzymes are reconstituted readily to the active holo form by addition of vanadate. Phosphate, which is a structural analogue of vanadate, inhibits the reconstitution by vanadate completely [1]. The crystal structures [4–8] of vanadium chloroperoxidase (VCPO) from the fungus *Curvularia inaequalis* and bromoperoxidase (VBPO) from the brown seaweed *Ascophyllum nodosum* and the red algae *Corallina* species show that vanadate in these enzymes is covalently attached to a histidine (i.e. $N^{\varepsilon 2}$ of His^{496} in VCPO) while five residues (i.e. Arg^{360}, Arg^{490}, Lys^{353}, Ser^{402} and Gly^{403} in VCPO) donate hydrogen bonds to the non-protein oxygens. The resulting structure is that of a trigonal bipyramid with three non-protein oxygens in the equatorial plane. The fourth oxygen (hydroxide group) and the nitrogen atom from a histidine residue are at the apical positions. A simplified ping-pong type of mechanism for the haloperoxidases has been reported [9]. The enzyme first reacts with peroxide to form a peroxo-intermediate after which a halide

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and a proton react to form an enzyme-hypohalous acid intermediate, which rapidly decays to enzyme and hypohalous acid.

Incubation in phosphate buffer slowly inactivates VBPO from *A. nodosum* [10], and it was observed that this inactivation was prevented by the presence of H_2O_2. It is known now that these haloperoxidases form a peroxo intermediate upon addition of H_2O_2 [5]. It has been also demonstrated that the affinity of apo CPO for vanadate becomes much higher in the presence of H_2O_2, and this peroxo-intermediate is fairly stable [11].

Despite extensive research on VCPO including steady-state kinetics [2,12,13], crystal structure determination [4,5] and mutagenesis studies [14–16], few inhibition studies on VCPO have been reported. Van Schijndel et al. [13] showed that nitrate inhibited the enzyme in a competitive way with respect to chloride and in an uncompetitive way with respect to hydrogen peroxide with the inhibition constant value K_i of 2 mM. A high concentration of chloride also inhibits the enzyme [13], and at low pH the enzyme is inhibited by 200 μM bromide [2,16]. A preliminary study reported by Messerschmidt and Wever [4], showed that azide inhibited VCPO at very low concentrations (<1 μM). In this report we investigate in detail the inhibition of VCPO by sodium azide, hydroxylamine and hydrazine. We also study the inactivation of VCPO by phosphate and the protective effect of H_2O_2 on this inactivation process.

2. Experimental

2.1. Production and isolation of rCPO

The recombinant CPO (rCPO) was created in the expression vector pTNT14 [14]. The pTNT14 derivatives were transformed to *Saccharomyces cerevisiae* BJ1991 for protein expression. The enzyme was isolated and purified as described by Hemrika et al. [14]. The purity of the preparations was checked on SDS–PAGE gels stained with Coomassie Brilliant Blue R-250, and the protein concentration was determined by using a protein assay kit (Bio-Rad) with BSA as the standard.

2.2. Enzyme activity assays

Quantitative VCPO activity was measured by monitoring the chlorination or bromination of monochlorodimedone (MCD, $\varepsilon = 20.2\ mM^{-1}\ cm^{-1}$ at 290 nm) to dichlorodimedon or monobromo-monochlorodimedon ($\varepsilon = 0.1\ mM^{-1}\ cm^{-1}$ at 290 nm) in 100 mM sodium citrate or sodium acetate (pH 5.0) for bromination or chlorination assay, respectively, and using the appropriate concentrations of the substrates chloride or bromide and H_2O_2. Since rCPO is produced as an apo enzyme by the yeast expression system, incubation with cofactor vanadate is necessary to obtain the holo enzyme. For the inhibition study, 1 μM apo CPO was incubated with 10 μM sodium orthovanadate in 50 mM Tris/acetate (pH 8.3) prior to the activity assay to exclude the effect of vanadate dissociation. To study the inactivation by phosphate 5 μM apo CPO was incubated with an equimolar concentration of 5 μM sodium orthovanadate in 50 mM Tris/acetate (pH 8.3) to minimize the presence of free vanadate in the incubations and assays. In this report, the data points are means of at least triplicate measurements.

2.3. Inhibition of VCPO by sodium azide, hydroxylamine and hydrazine

The activity of rVCPO was measured by MCD assay containing varying concentrations of each inhibitor and H_2O_2 with a fixed concentration of KBr (0.5 mM). Each measurement was carried out by addition of rVCPO to the MCD assay. $(NH_2OH)\cdot H_2SO_4$ and $N_2H_4\cdot H_2O$ were purchased from Sigma (USA) and NaN_3 was purchased from BDH Chemicals (England).

2.4. Inactivation of VCPO by phosphate and protection of inactivation by H_2O_2

rVCPO (50 nM) was incubated in 1 ml of MCD assay mixture (without substrates) containing varying concentration of potassium phosphate buffer (10–1000 μM, pH 5.0) for various time periods in the absence or presence of H_2O_2. The reaction was initiated by addition of 5 mM chloride and 1 mM H_2O_2 when H_2O_2 was absent during preincubation, or by addition of 5 mM chloride when 1 mM of H_2O_2 was present during preincubation.

3. Results and discussion

3.1. Inhibition of rVCPO activity by hydroxylamine, hydrazine and azide

The inhibition of the enzymatic activity of rVCPO using hydroxylamine, hydrazine and sodium azide was investigated. The steady-state rate of MCD bromination in the absence of inhibitors is linear in time. However, in the presence of inhibitors, the rate of MCD bromination decreases during turnover (not shown). Prior to the detailed inhibition studies, the effect of each inhibitor was checked by starting the bromination reaction in different ways. Starting the reaction in the presence of an inhibitor by addition of KBr, H_2O_2 or addition of rVCPO resulted in the same initial brominating activities (not shown). These results indicate that inactivation occurred during turnover. Since the way of starting the reactions did not result in significant differences in ac-

tivity, all reactions were initiated by addition of rVCPO and initial rates were determined. Fig. 1 shows the inhibition of rVCPO by hydroxylamine (pH 5.0). In panel (a), a plot is given of $1/v$ against the inhibitor concentration and in panel (b) the ratio S/v against the inhibitor concentration. These two plots show (see also [17]) that the mechanism of the inhibition is a mix of competitive (panel (a)) and uncompetitive inhibition (panel (b)) with respect to H_2O_2. From Fig. 1(a) the competitive inhibition constant K_{ic} for hydroxylamine was calculated as approximately 40 μM, whereas the uncompetitive inhibition constant K_{iu} was about 80 μM (Fig. 1(b)). A mixed type of inhibition implies that the inhibitor hydroxylamine binds not only to the enzyme–substrate complex but also to the enzyme itself [17]. It is known [5,11] that VCPO first reacts with H_2O_2 to form a peroxovanadate intermediate. According to Tracy and coworkers [18–21], hydroxylamine and hydrogen peroxide are isoelectronic with each other and they react similarly with vanadate. Vanadium (V) hydroxylamido complexes studies have been described of which especially the hydroxylamido vanadate-imidazole complex [22] is of interest. Considering the similarity of this complex with the active site and the peroxovanadate intermediate complex, it is likely that hydroxylamine forms a complex with the enzyme preventing the binding of H_2O_2. Keramidas et al. [22] have reported that the two hydroxylamido groups are coordinated to vanadate side-on with the hydroxylamido nitrogen trans to the imidazole ligand in the equatorial plane and these compounds are homologous to the known side-on biperoxovanadate complexes. However, as shown by the crystal structure of the peroxovanadate form of rVCPO [5], a monoperoxo complex is formed as a first step in catalysis. Therefore, it is likely that in rVCPO a monohydroxylamido vanadate complex is formed. Interestingly, hydroxylamine seems to bind to the enzyme–substrate complex as well, which is the peroxo form of rVCPO, showing uncompetitive inhibition during catalysis (Fig. 1(b)).

The inhibition of rVCPO by hydrazine is illustrated in Fig. 2. The parallel lines in Fig. 2(a) and the intersecting lines in Fig. 2(b) indicate uncompetitive inhibition (catalytic inhibition) with respect to H_2O_2. From Fig. 2(b) the inhibition constant value K_{iu} was calculated to be approximately 350 μM. The uncompetitive inhibition suggests that hydrazine binds to the peroxo form of rVCPO and not to the free rVCPO.

Fig. 3 shows the inhibition of the brominating activity of rVCPO by sodium azide. As is observed with hydrazine, the inhibition type is uncompetitive (Fig. 3(b)). The inhibition constant value K_{iu} is 50 nM. This very small value of the inhibition constant shows that azide is a much stronger inhibitor of VCPO than hydroxylamine or hydrazine. Although the crystal structure of a native VCPO–azide complex is available [4], our inhibition studies suggest that azide presumably preferentially binds to peroxovanadate intermediate rather than to rVCPO itself. Azide has also been reported to be an inhibitor for the VBPO from marine algae [23,24]. The inhibition by azide of this enzyme is weak, requiring 10–100 mM of azide for inhibition and it leads to irreversible inactivation. The difference in inhibitory effect of azide on VCPO and VBPO is surprising since the architecture of the active site of the two enzymes is very similar [5,25]. An explanation may be that in VCPO during catalysis, the vanadium peroxo complex becomes protonated to allow oxidation of chloride to occur

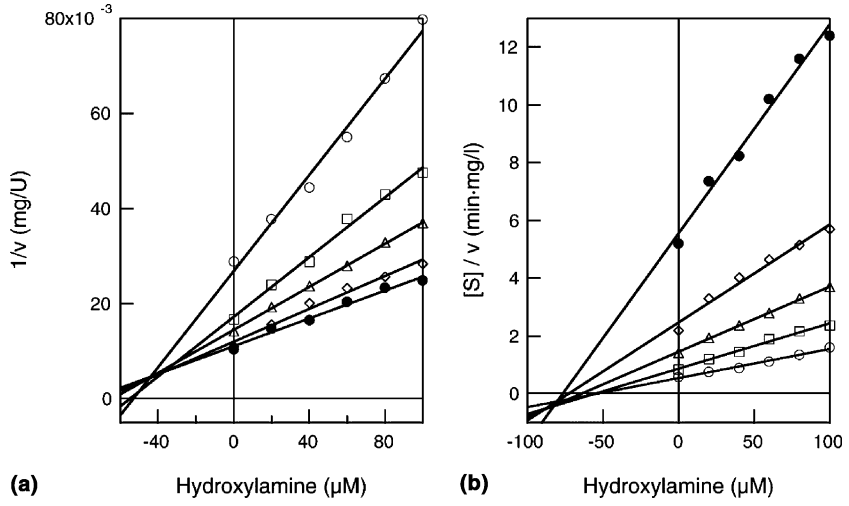


Fig. 1. The inhibitory effect of hydroxylamine on the brominating activity of rVCPO. (a) K_{ic} is obtained from plotting $1/v$ against the hydroxylamine (inhibitor) concentrations at various H_2O_2 concentrations at a fixed concentration of bromide (100 μM). (b) K_{iu} is obtained from plotting [S]/v against the hydroxylamine concentrations at various substrate concentrations with a fixed concentration of bromide (100 μM). The concentrations of H_2O_2 (substrate) were: 20 μM (○), 50 μM (□), 100 μM (△), 200 μM (◇) and 500 μM (●). The combination of intersecting lines in (a) and (b) indicate mixed type of inhibition. Competitive inhibition was seen as in (a) with K_{ic} of 40 μM and uncompetitive inhibition was seen as in (b) with K_{iu} of 80 μM. The assay was carried out in 100 mM citrate (pH 5.0).

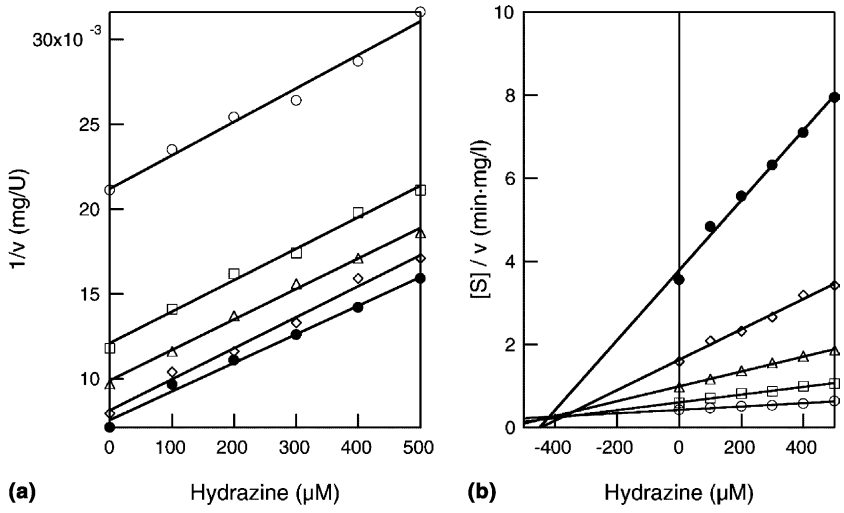


Fig. 2. The inhibitory effect of hydrazine on the brominating activity of rVCPO. (a) Plots of $1/v$ against the hydrazine (inhibitor) concentrations at various H_2O_2 concentrations at a fixed concentration of bromide (100 μM). (b) K_{iu} is obtained from plotting [S]/v against the hydrazine concentrations at various substrate concentrations. The concentrations of H_2O_2 (substrate) were: 20 μM (○), 50 μM (□), 100 μM (△), 200 μM (◇) and 500 μM (●). The parallel lines in (a) and the intersecting lines in (b) indicate uncompetitive inhibition (catalytic inhibition). Uncompetitive inhibition was seen as in (b) with K_{iu} of 350 μM. The assay was carried out in 100 mM citrate (pH 5.0).

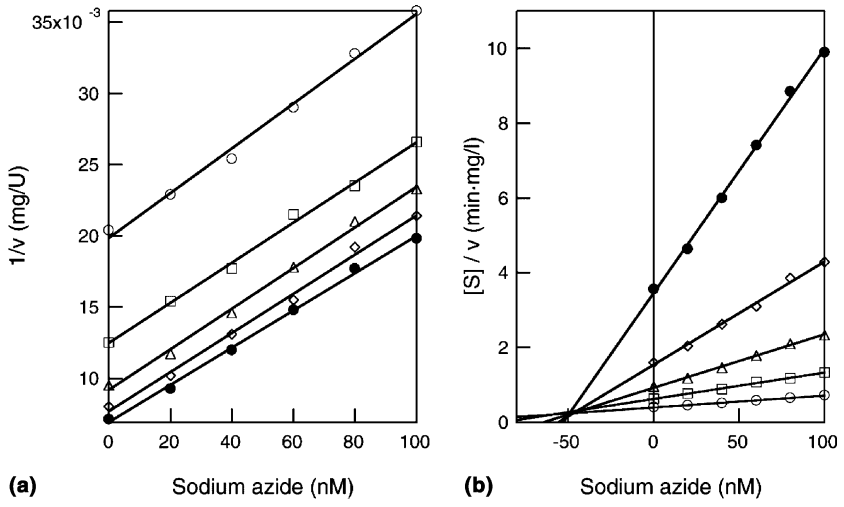
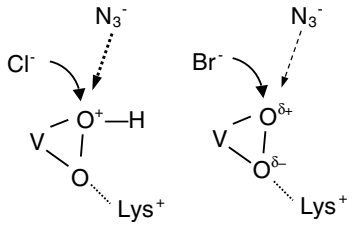


Fig. 3. The inhibitory effect of sodium azide on the brominating activity of rVCPO. (a) Plots of $1/v$ against the sodium azide (inhibitor) concentrations at various H_2O_2 concentrations at a fixed concentration of bromide (100 μM). (b) K_{iu} is obtained from plotting [S]/v against the sodium azide concentrations at various substrate concentrations. The concentrations of H_2O_2 (substrate) were: 20 μM (○), 50 μM (□), 100 μM (△), 200 μM (◇) and 500 μM (●). The parallel lines in (a) and the intersecting lines in (b) indicate uncompetitive inhibition (catalytic inhibition). Uncompetitive inhibition was seen as in (b) with K_{iu} of 50 nM. The assay was carried out in 100 mM citrate (pH 5.0).

[14–16]. This activated protonated state is very susceptible to reaction with the strong nucleophile azide. In contrast in the VBPO, protonation of the bound peroxide is not required to allow oxidation of bromide. The peroxo intermediate will therefore be less electrophilic and for less reactive towards azide. Scheme 1 illustrates the difference in reactivity for azide for VCPO and VBPO, respectively.

As outlined in the presence of these inhibitors the brominating activity decreased in time during turnover and in order to determine whether the inhibition was irreversible, the following experiment was carried out. After an activity measurement the MCD assay mixtures containing 100 nM rVCPO and inhibitor were centrifuged through a Centricon-30. Each sample was washed extensively with 100 mM sodium citrate (pH 5.0) for at least five times by concentration and dilution cycles. The recovered enzyme samples were tested in a new assay mixture. However, activity was not recovered suggesting that inhibition by hydroxylamine, hydrazine and azide is irreversible in line with the inhibition of VBPO by azide [23,24].



Scheme 1. Protonation state of the side-on-bound peroxide in vanadium haloperoxidases that is postulated to be attacked by the incoming halide. (Left) Strongly oxidizing protonated form of the side-on bound peroxide with which azide reacts more effectively. (Right) Less oxidizing unprotonated form of the side-on bound peroxide.

3.2. Inactivation of rVCPO by phosphate and effect of peroxo vanadate intermediate

It has been shown that incubation of VBPO in phosphate buffers leads to a slow inactivation requiring hours of completion [10]. Preincubation of rVCPO with 10–100 mM phosphate resulted in inactivation of the enzyme within 1 min (results not shown). Thus VCPO is inactivated by phosphate much faster than VBPO. Because of the fast inactivation, further inactivation studies were carried out by direct incubation of enzyme in the assay mixture containing phosphate. Fig. 4 shows the effect of phosphate concentration on the activity of the enzyme. It is obvious that a relatively low concentration of phosphate inactivates the enzyme. The K_d value for the inactivation process is approximately 20 μM. The enzyme was only inactivated to about 50% and 50% remained active after 10 min incubation. Higher concentration of phosphate (10 and 100 mM) resulted in similar extent of inactivation after 60 min incubation

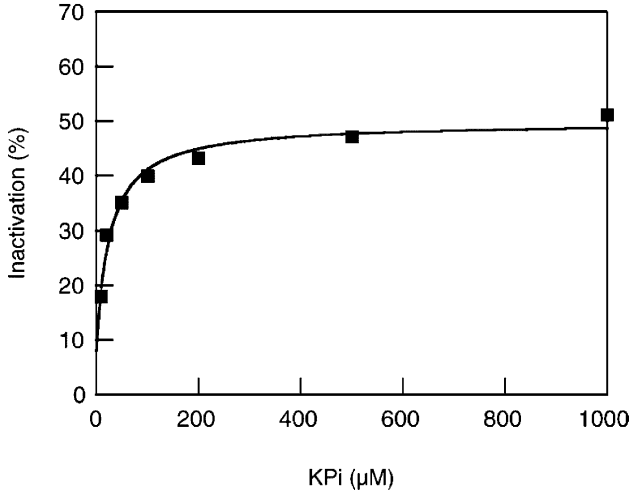


Fig. 4. Inactivation of 50 nM rVCPO as a function of phosphate concentration. rVCPO was preincubated for 10 min with phosphate in the MCD assay and the reaction was initiated by addition of 5 mM NaCl and 1 mM H_2O_2. The experiment was carried out in 100 mM acetate (pH 5.0).

(results not shown). However, the initial inactivation rate with higher phosphate concentration was faster than that of 1 mM phosphate.

Fig. 5(a) illustrates the time course study of rVCPO inactivation by 1 mM phosphate for 1 h in the bromination assay. Upon incubation with 1 mM phosphate, rVCPO was inactivated to 50% of its original activity. The inactivation is probably due to substitution of vanadate in the active site by the competitive analogue phosphate. Preincubation of rVCPO with 1 mM H_2O_2 in MCD assay mixture prevents this inactivation by phosphate completely.

It is well known [5] that H_2O_2 forms a stable peroxo-intermediate and that pervanadate has a very high affinity for the enzyme [11]. The observation that H_2O_2 prevents the inactivation by phosphate suggests that the peroxo-complex is bound much more strongly than vanadate itself. Therefore, the effect of the concentration of H_2O_2 on the inactivation phenomenon was studied. Fig. 5(b) shows that exposure to very low concentrations of H_2O_2 (50 nM) already hampered the inactivation somewhat whereas 500 nM completely prevented the inactivation by 1 mM phosphate. This shows that in the presence of H_2O_2 the affinity of the enzyme for the vanadate cofactor is much higher than in the absence of H_2O_2. Addition of low concentrations of H_2O_2 to the enzyme leads to the formation of the peroxo-intermediate and as Fig. 5 shows that phosphate is unable to inactivate the enzyme. This suggests that this intermediate is bound much more strongly than vanadate to the active site. This has been observed before by Renirie et al. [11]. The active site of VCPO contains a histidine (His^{496}) that is covalently bound to the vanadate [4,5]. In this respect it is of interest that imidazole complexes bind peroxovanadate more strongly than vanadate [26]. The stronger binding of the peroxo-intermediate probably explains why also in BPO the presence of H_2O_2 prevents the inactivation by phosphate [10]. As Fig. 4 illustrates only part of the rVCPO is inactivated by phosphate. This may relate to the presence of more stable peroxo species in the enzyme preparation. However, we have been unable to detect this species experimentally by physical means. The concentration of H_2O_2 to prevent the inactivation by phosphate was found to be very low. This may have a physiological reason. In nature, phosphate is abundant and it may be that a very low amount of H_2O_2 protects VCPO and VBPO against inactivation by phosphate.

In summary, we present here the inhibition and inactivation of rVCPO by nucleophilic compounds, and propose a mechanism for the inhibition. As shown previously [5,9] and illustrated in Scheme 1, hydrogen peroxide binds first to the metal oxide to yield an activated peroxo-intermediate, which is able to react with the halide to produce hypohalous acid. As shown in this paper hydrazine and azide act as uncompetitive

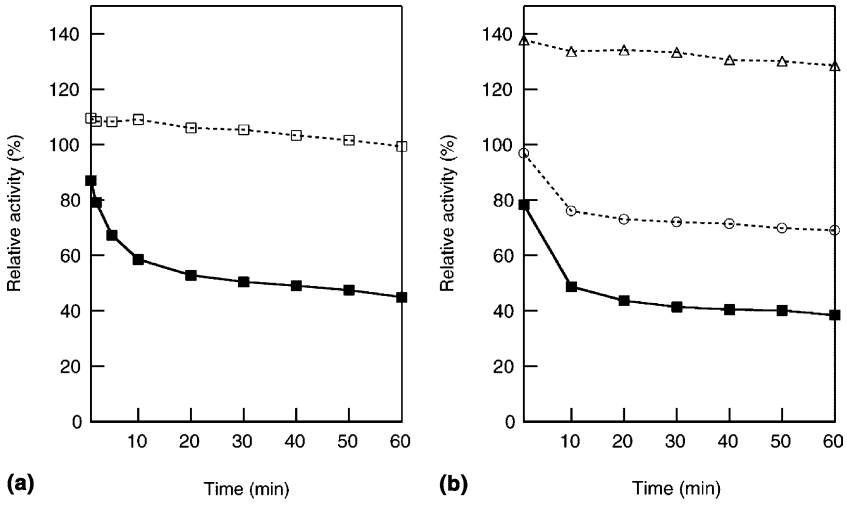


Fig. 5. Phosphate-induced inactivation of rVCPO and prevention by H_2O_2. (a) The effect of H_2O_2 on the phosphate-induced inactivation. (■), 50 nM rVCPO was preincubated with 1 mM phosphate in MCD assay for selected time intervals (0–60 min) and the enzymatic activity was initiated by addition of 5 mM NaCl and 1 mM H_2O_2. (□), 50 nM rVCPO was first preincubated with 1 mM H_2O_2 for 5 min to form the peroxovanadate intermediate. The enzyme was subsequently added to the MCD assay in 100 mM sodium acetate (pH 5.0) containing 1 mM phosphate and after selected time intervals (0–60 min) the enzymatic activity was initiated by addition of 5 mM NaCl. (b) The effect of H_2O_2 concentration on the phosphate-induced inactivation. (■), 2.5 μM rVCPO was incubated for 5 min in 50 mM Tris/SO_4 (pH 8.3). (○), 2.5 μM rVCPO was incubated for 5 min with 2.5 μM H_2O_2 in 50 mM Tris/SO_4 (pH 8.3). (△), 2.5 μM rVCPO was incubated for 5 min with 25 μM H_2O_2 in 50 mM Tris/SO_4 (pH 8.3). After the incubations, samples were taken and diluted 50 times in the MCD assay containing 1 mM phosphate and 100 mM sodium acetate (pH 5.0). This resulted in the final VCPO concentration of 50 nM and H_2O_2 concentrations of 50 and 500 nM, respectively. The enzymatic activity was initiated by addition of 5 mM NaCl and 1 mM H_2O_2. The slightly higher activity in the presence of vanadate and H_2O_2 relates to the fact that in the native preparation a small amount of apo enzyme was present.

inhibitors with respect to hydrogen peroxide confirming that indeed hydrogen peroxide reacts first with the enzyme before the inhibitor reacts with the activated peroxo-intermediate. We also show that the presence of H_2O_2 clearly prevents inactivation of the enzyme by phosphate as pervanadate is bound much more strongly than vanadate to the active site in VCPO.

4. Abbreviations

rVCPO	recombinant vanadium chloroperoxidase (SWISS-PROT primary accession No. P49053)
rCPO	recombinant vanadium chloroperoxidase (apo form)
VBPO	vanadium bromoperoxidase
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Acknowledgements

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