From vanadium haloperoxidases to acid phospatases

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Class A acid phosphatases from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* (PhoN-Se), which share the conserved active site with vanadium haloperoxidases and glucose 6-phosphatases, show brominating activity when substituted with vanadate.

**Bromoperoxidase activity of vanadate-substituted acid phosphatases from *Shigella flexneri* and *Salmonella enterica* ser. *typhimurium***

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Haloperoxidase Activity of Phosphatases

Bromoperoxidase activity of vanadate-substituted acid phosphatases from Shigella flexneri and Salmonella enterica ser. typhimurium

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Abstract

Vanadium haloperoxidases and the bacterial class A non-specific acid phosphatases share a conserved active site. It is shown for the first time that vanadate-substituted recombinant acid phosphatase from Shigella flexneri (PhoN-SF) and Salmonella enterica ser. typhimurium (PhoN-Se) in the presence of H2O2 are able to oxidise bromide to hypobromous acid. Vanadate is essential for this activity. The kinetic parameters for the artificial bromoperoxidases have been determined. The Km value for H2O2 is about the same as that for the vanadium bromoperoxidases from the seaweed Ascophyllum nodosum. However the Km value for Br- is about ten to twenty times higher and the turnover values of about 3.4 min-1 and 33 mm-1 for PhoN-SF and PhoN-Se, respectively, are much slower than that of the native bromoperoxidase. Thus, despite the striking similarity in the active site structures of the vanadium haloperoxidases and the acid phosphatase, the turnover frequency is low and clearly the active site of acid phosphatases is not optimised for haloperoxidase activity. As the native vanadium bromoperoxidase, the vanadate-substituted PhoN-SF and PhoN-Se catalyse the enantioselective sulfoxidation of thioanisole.

Keywords: Vanadium bromoperoxidase; Vanadium chloroperoxidase; Acid phosphatase; Brominating activity; Enantioselective sulfoxidation

1. Introduction

Vanadium haloperoxidases are enzymes that catalyse the oxidation of a halide by hydrogen peroxide to the corresponding hypohalous acids according to:

\[ \text{H}_2\text{O}_2 + \text{H}^+ + \text{X}^- \rightarrow \text{H}_2\text{O} + \text{HOX} \]

The enzymes are named after the most electronegative halide ion they are able to oxidise; therefore chloroperoxidase (CPO) oxidises Cl-, Br-, I- and bromoperoxidase (BPO) oxidises Br- and I-. This class of enzymes binds vanadate (HVO2-) as a prosthetic group [1,2]. It is possible to prepare an apo-form of these enzymes which is reactivated by vanadate. This reactivation is competitively inhibited by structural analogues of vanadate (tetrahedral compounds) such as phosphate and molybdate [3,4]. The crystal structures [5-7] of vanadium chloro- and bromoperoxidase from fungus Curvularia inaequalis and the seaweed Ascophyllum nodosum show that vanadate in these enzymes is covalently attached to a histidine residue while five residues donate hydrogen bonds to the non-protein oxygens. The resulting structure shown for the chloroperoxidase (Fig. 1A) is that of a trigonal bipyramid with three non-protein oxygens in the equatorial plane and which are hydrogen bonded to Arg360, Arg490, Lys353, Ser402, and Gly403. The fourth oxygen (hydroxide group) at the apical position is hydrogen bonded to His404. The nitrogen atom from a histidine residue (His496) is at the other apical position. The above mentioned vanadate-binding amino acids were shown to be conserved in two bromoperoxidases from seaweed and several acid phosphatases among others the large group of soluble bacterial non-specific Class A acid phosphatases [5, 7, 8-12]. Examples are the non-specific acid phosphatase, PhoN from Shigella flexneri (PhoN-SF) and the enzyme from Salmonella enterica ser. typhimurium (PhoN-Se) [13,14]. Based on sequence similarity it has been proposed [8-12] that the architecture of the active site in the two classes of enzymes is very similar. Recently the X-ray structure of a novel acid phosphatase from Escherichia blattae was determined [15]. Fig. 1B shows the active site structure of this acid phosphatase. The similarity of the residues involved in binding oxyanions is remarkable. Sulfate co-crystallises with the acid phosphatase and its binding site (Fig. 1B) is comparable to that of vanadate in the chloroperoxidase (Fig. 1A) confirming that these families are indeed evolutionary related and share the same ancestor [8]. Hemrika et al [8] showed that apo-CPO has some phosphatase activity though the turnover with para nitrophenyl phosphate (p-NPP) as a substrate is only 1.7 min-1 which is about 10,000 times slower that of various acid phosphatases. However, the Km for the substrate is less than 50 uM [8,16] which is of the same order of magnitude as various acid phosphatases. These data show that the active site of CPO has a good affinity for the substrate but is not optimised for phosphatase activity. On basis of the similarity of the active sites and the fact that the phosphatase activity of phosphatases is inhibited by vanadate [17,18], we
Fig. 1 Structure of the active site of vanadium chloroperoxidase from C. inaequalis (PDB ID: 1IDQ) and that of the acid phosphatase from E. blallae (PDB ID: 1D2T) The phosphatase co-crystallized with sulfate. The figure was prepared using Swiss PDB viewer.

expect that vanadate-substituted phosphatase has haloperoxidase activity. Indeed, as shown here, the recombinant acid phosphatases from Shigella flexneri (PhoN-Sf) and Salmonella enterica ser. typhimurium (PhoN-Se) substituted with vanadate also catalyzed the oxidation of bromide and the enantioselective oxidation of thioanisole [19,20].

2. Materials and methods

2.1 Materials

All standard recombinant DNA procedures were performed as described in Sambrook et al. (1989) [21]. The host strains Escherichia coli TOP10 (Invitrogen) and BL21(DE3) (Novagen) were used in subcloning and expression experiments. Salmonella enterica ser. typhimurium strain SB3507 was used as a DNA source for phoN-Se gene cloning. Bacteria were routinely grown at 37°C in LB medium containing 100 µg/ml ampicillin when required (LA medium). Plasmid pKU102 harbouring Shigella flexneri phoN locus was a gift from Dr. K. Uchiya [13]. Expression vectors pET3a (Novagen) and pBAD/gIII A (Invitrogen) were used for cloning the phoN gene from S. flexneri and S. typhimurium, respectively. pBAD/gIII A holds the gene III signal sequence for secretion of the recombinant protein into the periplasmic space.

2.2 Expression and purification of recombinant PhoN- Se

S. enterica ser. typhimurium phoN gene was cloned in the pBAD/gIII A expression plasmid as follows. The mature sequence (i.e. phoN gene without the 5’ end coding for the secretion signal) was PCR amplified from Salmonella enterica chromosomal DNA using the forward primer 5’-

ACCATGGAATATACATCAGCAGAA-3’ and the reverse primer 5’-CGCAAGCTTTCACCTTTCAGTAATT-3’ (the NeoI and HindIII sites, respectively, are underlined). The PCR was performed using the Expand™ High fidelity PCR System (Roche) with the following conditions: 1µg of chromosomal DNA, 1µM of each primer, 200 µM of each dNTP, 1.5 mM MgCl2, 2.6 U high fidelity polymerase mix in a final volume of 100µl. A "hot start" of 2 min at 94°C was followed by 30 cycles of denaturation (15 sec at 94°C), annealing (30 sec at 55.5°C) and extension (1 min at 72°C) using a programmable heating block (Eppendorf Mastercycler 5330). The PCR product was restricted with NeoI and HindIII and cloned into the corresponding sites of pBAD/gIII A, in frame with the gene III signal sequence. The resulting clone was confirmed by DNA sequencing using an Applied Biosystems 373A DNA Sequencer.

E. coli TOP10 carrying the recombinant plasmid was grown at 37°C in LA medium until the absorbance of the culture suspension reached an OD600 of 0.4-0.6. The expression of recombinant PhoN-Se was induced by adding 0.02% L-arabinose and the growth was continued at 37°C for 4 hours. The bacterial cells were harvested by centrifugation and secreted PhoN-Se was released from E. coli periplasmic space by osmotic shock. The cell pellet was resuspended in osmotic shock solution 1 (20 mM Tris-HCl pH 8, 2.5 mM EDTA, 20% sucrose) to an OD600 of 5 and incubated on ice for 10 min. After centrifugation for 1 min at 4°C, the cell pellet was resuspended in osmotic shock solution 2 (20 mM Tris-HCl pH 8, 2.5 mM EDTA) to an OD600 of 5 and incubated on ice for 10 min. The secreted PhoN-Se was obtained in the supernatant (osmotic shock fluid) after centrifuging 10 min at 4°C. The osmotic shock fluid was dialysed overnight at 4°C against 20 mM sodium acetate buffer (pH 6.0). The solution was passed through a 0.45 µM filter (Millipore) and then applied to
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a SP Sepharose Fast Flow ion exchange column (Pharmacia Biotech). The recombinant protein was eluted with a linear gradient of NaCl (0-0.3 M) in 20 mM sodium acetate buffer (pH 6.0).

2.3 Expression and purification of recombinant PhoN-Sf

S. flexneri phoN was cloned under control of the T7 promoter in pET3a as described below. S. flexneri phoN was generated by PCR using pKU102 as a template and suitable primers that allowed cloning of phoN between NdeI and HindIII sites of pET3a. The construct was transformed into the T7 polymerase expressing strain BL21(DE3). PhoN-Sf expression was induced with 0.4 mM IPTG for 5-7 hours at 37°C.

Soluble PhoN-Sf was released from E. coli by breaking the cells in a French press (750-760 psi). The soluble fraction was applied to a BioCAD ion exchange column (Perseptive Biosystems) and the enzyme was eluted with a gradient of NaCl (0-1 M) in 30 mM Tris-HCl buffer (pH 7.5). The active fractions were pooled and applied to a Sephacryl 200HR column (Pharmacia). Elution was done with 30 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl and 10% glycerol.

The purity of the preparations was checked on SDS-PAGE gels stained with Coomassie Brilliant Blue R-250. To remove possible contaminating metal ions, the purified phosphatases were eventually dialysed against 100 mM Tris-Cl (pH 7.5) and 1 mM EDTA which has no influence on the phosphatase activity.

The protein concentration was determined by using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

2.4 Enzymatic assay of phosphatase activity

The phosphatase activity was measured by hydrolysis of 10 mM para-nitrophenyl phosphate (p-NPP) as a substrate in 100 mM MES (pH 6.0). The reaction mixtures were quenched with 0.5 M NaOH to change the pH to 12 and the production of para-nitrophenol phenol (p-NP) was measured at 410 nm (extinction coefficient 16.6 mM⁻¹·cm⁻¹).

2.5 Enzymatic assay of bromoperoxidase activity

PhoN-Sf brominating activity assay

The brominating activity of the recombinant phosphatases was measured qualitatively by the bromination of 40 μM phenol red in 100 mM citrate buffer (pH 5.0) containing 2 mM H₂O₂ and 100 mM Br₂.

This assay is convenient since large colour changes are observed which can easily be detected visually [22]. Since phosphate ions inhibit the brominating activity of PhoN-Sf, it is likely that phosphate binds at the active site of the enzyme and prevents binding of the vanadate. Therefore phosphate should be absent in the assay. To

induce the brominating activity of PhoN-Sf the recombinant PhoN-Sf was pre-incubated with 100 μM vanadate in 100 mM Tris-Cl (pH 7.5) for at least 30 minutes. Brominating activity of a final concentration of 0.5 μM recombinant PhoN-Sf was quantitatively measured by monitoring the bromination of 50 μM monochlorodimethane (MCD) at 290 nm (extinction coefficient 20.2 mM⁻¹·cm⁻¹) in 100 mM sodium acetate buffer (pH 4.6) containing 200 mM Br₂ and 2 mM H₂O₂ on a Cary 50 [23]. The kinetic parameters were determined using the EnzymeKinetics program from Trinity Software.

PhoN-Se brominating activity assay

Brominating activity of PhoN-Se was measured by phenol red assay as mentioned above but using sodium acetate (pH 4.6) instead of citrate. It is well known [24] that vanadate interacts with most buffers normally used. Therefore the vanadate-induced brominating activity of PhoN was measured in two different buffers. Since PhoN-Se brominating activity was absent in citrate buffer and since it is likely that citrate forms a complex with vanadate inhibiting its incorporation in the active site of PhoN, sodium acetate was used as a buffer. Brominating activity of PhoN-Se was quantitatively measured by monitoring the bromination of 50 μM MCD at 290 nm in 100 mM sodium acetate buffer (pH 4.2) containing 300 mM Br₂, 2 mM H₂O₂. The assay mixture also contained 100 μM vanadate.

2.6 Enantioselective sulfoxidation of organic sulfide

The enantioselective sulfoxidation by the recombinant phosphatases was demonstrated using thioanisole as a substrate [20]. 2 mM of thioanisole was incubated with 2 mM H₂O₂, 100 μM vanadate and 100 mM enzyme in 100 mM acetate buffer (pH 5.0) at 25 °C in 1.7 ml sealed glass vials to prevent evaporation of the substrate. After overnight incubation, remaining H₂O₂ in the reaction mixture was quenched with Na₂S₂O₃. The enantiomeric products were extracted with CH₂Cl₂, evaporated to 20 μl and dissolved in 1ml hexane/2-propanol (4:1). 20 μl of sample was used for HPLC analysis on a Diacel chiral OD column (0.46 x 25 cm) equipped with a Pharmacia LKB-HPLC pump 2248 and a LKB Bromma 2140 rapid spectral detector. The column was eluted with hexane/2-propanol (4:1) at a flow rate of 0.5 ml/min. The retention times for the R and S isomer were 14 and 17 min, respectively. The HPLC effluent was monitored at 254 nm. The Borwin software program (JMS developments) was used for HPLC data acquisition and evaluation.
3. Results and Discussion

3.1 Expression of recombinant acid phosphatases in E. coli

The similarity in the active site structures of vanadium haloperoxidases and Class A bacterial acid phosphatases has been first suggested by sequence alignments [8-10]. Indeed, the comparison of the crystal structures of Escherichia blattae acid phosphatase and Curvularia inaequalis vanadium chloroperoxidase (Fig. 1) confirms this structural similarity [15]. Unfortunately, the structure of the acid phosphatase complexed to vanadate is not available, but only that of a sulfate and a molybdate complex [15]. The similarity prompted us to investigate whether Class A bacterial acid phosphatases when vanadate is bound to the active site of these enzymes could also function as vanadium haloperoxidases. S. enterica ser. typhimurium [25] and S. flexneri acid phosphatases, that show respectively 40% and 80% homologies with E. blattae acid phosphatase, were chosen for this study. A sequence alignment (not shown) of vanadium chloroperoxidase with these enzymes points to three separate domains that are conserved. Domain 1 contains Lys353 and Arg360; Domain 2, Ser402, Gly403, His404 and Domain 3, Arg490 and His496. This shows clearly that the binding pocket for vanadate in the peroxidases is very similar to the phosphate-binding site in phosphatases. However, the overall similarity between vanadium chloroperoxidase and these phosphatases is very low (see also Ref. 8) and the domains are connected by regions that are highly variable. Both phosphatases were expressed as recombinant proteins in E. coli, as described in Materials and Methods. No acid phosphatase activity was detected in E. coli host strains TOP10 or BL21(DE3). In the absence of inducer, neither TOP 10 harboring the expression vector for PhoN-Se, nor BL21(DE3) harboring the expression vector for PhoN-Sf, showed relevant levels of acid phosphatase activity. Upon induction, the specific activity of acid phosphatase in both strains was about 40 U/mg.

During purification, the acid phosphatase activity always co-chromatographed with a protein of about 30 kDa, a size in agreement with the molecular mass of each phosphatase. The final preparations with a yield of 1-2 mg of Pho-Sf per liter culture medium were judged at least 90% pure as determined by SDS-PAGE. There is a minor band present with a slightly lower molecular mass. However, this band originates from proteolytic degradation of the native phosphatase according to a mass analysis of its tryptic peptides by MALDI-TOF (not shown). In the case of PhoN-Se, 10 to 15 mg of enzyme, with a specific activity of 140 U/mg, were obtained from 1 liter culture, indicating a high level of expression in E. coli. Moreover, the purification procedure was greatly simplified by targeting the phosphatase to E. coli periplasmic space.

3.2 Haloperoxidase activity of vanadate-substituted acid phosphatases

The brominating activity of recombinant non-specific acid phosphatases from Shigella flexneri (PhoN-Sf) and Salmonella enterica ser. typhimurium (PhoN-Se) was tested in a phenol red assay. After overnight incubation of 1 μM PhoN-Sf and PhoN-Se, respectively, in the presence of 100 μM vanadate, phenol red was clearly brominated to bromophenol blue by both phosphatases. In the absence of vanadate or PhoN, bromination of the dye was not detected. This means that the reaction is catalysed by the vanadate-substituted PhoN-Sf and PhoN-Se. That vanadate binds to the active site of the PhoN-Sf is confirmed by the observation that vanadate inhibits the phosphatase activity of PhoN-Sf with a K_1 about 70 nM at pH 6.0 (results not shown). Many other phosphatases are inhibited by vanadate [17,18], which is homologous in structure to phosphate. Although it has no sequence similarity to the bacterial acid phosphatases, the crystal structure of the vanadate-substituted rat acid phosphatase shows clearly that vanadate bound in a striking similar way as in the vanadium

![Fig. 2 Panel A. Dependence of the bromoperoxidase activity of PhoN-Sf (200 nM) on the vanadate concentration. 20 μM PhoN-Sf was pre-incubated for 1 hour with various concentrations of vanadate and the activity was measured in the MCD assay (pH 4.6). Panel B. Dependence of the bromoperoxidase activity of PhoN-Se (1 μM) on the vanadate concentration by the MCD assay (pH 4.2).]

![Fig. 3 Panel A. pH dependence of the brominating activity of 200 nM PhoN-Sf. 20 μM PhoN-Sf was pre-incubated for 1.5 hour with 100 μM vanadate in 100 mM Tris-Cl (pH 7.5) and the activity was measured in the MCD assay. Panel B. pH dependence of the brominating activity of 1 μM PhoN-Se. K_m and V for KBr at each pH were collected. The activity measurements were carried in triplicate using the MCD assay containing 100 μM vanadate.]

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chloroperoxidase from *C. inaequalis* [10]. Therefore, it is likely that vanadate binds to the active site of PhoN, and causes the peroxidase-like activity.

Further quantitative kinetic studies were carried out using the MCD assay. Fig.2A shows that about 10 μM vanadate is necessary to obtain full activity of 500 nM PhoN-Sf. From a Hill plot (not shown) it was possible to obtain a *K*~m~ of about 1 μM at pH 4.6. In the presence of 100 μM vanadate it takes approximately 20 minutes to fully induce the brominating activity of PhoN-Sf (result not shown). Therefore, at least 30 minutes preincubation with 100 μM vanadate was carried out with PhoN-Sf as mentioned in Materials and Methods. Fig.2B shows that about 20 μM vanadate is necessary to activate 1 μM PhoN-Se, and a *K*~m~ of about 2 μM at pH 4.2 was obtained. PhoN-Se reaches full peroxidase activity within 2 minutes when 100 μM vanadate is present (result not shown). In case of PhoN-Se preincubation was not necessary, therefore 100 μM vanadate was added to the MCD assay mixture for further experiments.

As mentioned in Materials and Methods, citrate and phosphate containing buffers are not suitable for brominating activity of PhoN and sodium acetate was used in the assay to determine the pH optimum. Fig.3 shows that the maximal brominating activity is observed at pH 4.6 and 4.2 for PhoN-Sf and PhoN-Se, respectively. Due to the restrictions in choice of buffers, experiments were carried out in a limited pH range. Sodium acetate was used in the pH range 4.2 to 5.4 and pH 3.8 to 6.0 for PhoN-Sf and PhoN-Se, respectively. This makes it difficult to evaluate the *pK*~a~ value of the group involved in the bromination activity of these phosphatases. Since there was limited amount of enzyme available, optimum pH determination of PhoN-Sf was based on single substrate concentration (200 mM KBr and 2 mM H₂O₂). For PhoN-Se it was possible to measure *K*~m~ and *F* at each pH value. Fig.3B shows the pH dependence of the *F*. The data suggest that a group with a *pK*~a~ of about 4.3 is involved in the bromination reaction. The *K*~m~ for bromide was also pH dependent and increases with increasing pH (not shown).

A steady-state kinetic study of vanadate-substituted PhoN-Sf and PhoN-Se brominating activity was carried out. For PhoN-Sf a *K*~m~ value for bromide was obtained of about 350 mM (Fig.4A) and for PhoN-Se a *K*~m~ of about 160 mM (Fig.4C). The maximal turnover value of the brominating activity of vanadate-substituted PhoN-Sf is 3.4 min⁻¹ (0.13 U/mg) which is considerably slower than the values of 120-180 U/mg observed for vanadium haloperoxidases [26,27]. However, the turnover in the brominating activity of the acid phosphatases is of the same order of magnitude as the phosphatase activity of apo-CPO (1.7 min⁻¹) [8]. The *K*~m~ for H₂O₂ was also determined and a value of 15 μM was obtained with a maximal turnover of 2.7 min⁻¹ (Fig.4B). Surprisingly, the maximal turnover value of the brominating activity of vanadate-substituted PhoN-Se was 33 min⁻¹ (1.23 U/mg) which was about 10 times higher than that of

Fig. 4 Bromoperoxidase activity of vanadate-substituted PhoN-Sf (0.2 μM) at pH 4.6 and PhoN-Se (1 μM) at pH 4.2 as function of the substrate concentrations. PhoN-Sf was preincubated for 1 hour in 100 mM Tris-HCl (pH 7.5) with 100 μM vanadate and the activity measured in the MCD assay. A, PhoN-Sf in 2 mM H₂O₂ and variable concentrations of Br₂. B, PhoN-Se in 300 mM Br₂ and variable concentrations of H₂O₂. C, PhoN-Se in 2 mM H₂O₂ and variable concentrations of Br₂. D, PhoN-Se in 300 mM Br₂ and variable concentrations of H₂O₂. The data points are the means of triplicate measurements.

PhoN-Sf and the phosphatase activity of apo-CPO. Although PhoN-Se has higher brominating activity than PhoN-Sf, the *K*~m~ value for H₂O₂ was about 400 μM (Fig. 4D). The specificity constants (*k*ₐᵣₑᵣ/K*ₚₚᵣ*) that can be calculated from these data are for bromide oxidation by PhoN-Sf and PhoN-Se 0.16 M⁻¹ s⁻¹ and 2 M⁻¹ s⁻¹, respectively. If one compares these values with the specificity constant for bromide oxidation [28] by the bromoperoxidase from *Ascophyllum nodosum* (1.8 10⁻² M⁻¹ s⁻¹) it is clear that the vanadate substituted acid phosphatases are poor catalysts in bromide oxidation. Since several vanadium haloperoxidases are able to catalyse the enantioselective sulfoxidation of thioanisole [19,20], we investigated whether the PhoN-Sf and PhoN-Se catalysed this reaction. Indeed, when 0.500 μM PhoN-Sf was incubated overnight with 2 mM thioanisole and 2 mM H₂O₂ in 100 mM acetate (pH 5.0) in the presence of 100 μM vanadate, the thioanisole was partially converted to the R-enantiomer of the sulfoxide with an enantiomeric excess (e.e.) of 57 % (results not shown). Due to the limited amount of enzymes available further studies were carried out at a relatively low enzyme concentration of 0.1 μM. At the lower concentration of PhoN-Sf (0.1 μM) the e.e. decreased to 39 %. This has been noted before and is due to an increased contribution of the direct reaction between the sulfide and H₂O₂ leading to a racemic mixture [20]. Some conversion to the sulfoxide was noted in the absence of vanadate but a racemic mixture resulted (not shown). Also when vanadate was incubated with
thioanisole and H$_2$O$_2$, a minor amount of a racemic mixture resulted. It is clear that vanadate is essential for the enantioselective sulfoxidation activity of the PhoN-Sf. PhoN-Se also catalyzes the sulfoxidation of thioanisole but in this case the S-enantiomer was produced with a selectivity of 36%. Surprisingly, in the absence of vanadate also an enantioselective conversion was observed (e.e. 24%). However, the conversion was much slower than when vanadate was present. Further addition of the PhoN-Sf, the sulfide and H$_2$O$_2$ with 1 mM EDTA resulted in a lower e.e. The sulfoxidation observed in the absence of vanadate may be due to metal contamination in the preparation which was not completely removed by dialysis against 1 mM EDTA. Recently, it has been reported that also vanadate-incorporated phytase [29], a non-related phosphatase that mediates the hydrolysis of phosphate esters, catalyses the enantioselective sulfoxidation of prochiral sulfides with H$_2$O$_2$ to the S-sulfoxides. Brominating activity, however, was not detected. The kinetic data obtained earlier [8,16] already showed that despite the great similarity in the architecture of the active sites of the vanadium haloperoxidases and the acid phosphatases (see Fig.11) that apo CPO is not optimised for the phosphatase activity. Vice versa the vanadate-substituted phosphatases show only moderate peroxidase activity. This means that other residues farther away of the active site and probably near or at the entrance to the active site play a very important role in tuning the activity and specificity of these enzymes. Identification of these residues even with a full knowledge of the crystal structure and sequence is difficult if possible at all. Studies carried out to which factors determine whether a vanadium haloperoxidase is a bromo- or chloroperoxidase [7,16] have also been elusive. Despite the fact that for these enzymes structural data and kinetic details are available and even site-directed mutagenesis studies have been carried out [29] there is no clear answer as to the nature of these factors.

Our findings have also important implications. There are many attempts to build enzyme mimics or create synthetic enzymes based on knowledge of active site structure of enzymes. In general these mimics are poor catalysts compared to the natural enzymes. Our study clearly shows that despite the good similarity in active site structure the activity of these enzymes differ widely. As pointed out these differences are probably due to amino acid residues outside the active site and these are apparently very important in catalysis and determining specificity. Considering this it is obvious that the construction of artificial enzymes based on an active site of an enzyme and with activities similar to enzymes is much more difficult than anticipated.

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