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

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Vanadium haloperoxidases and the bacterial class A nonspecific acid phosphatases have a conserved active site. It is shown that vanadate-substituted recombinant acid phosphatase from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* ser. *typhimurium* (PhoN-Se) in the presence of H\(_2\)O\(_2\) are able to oxidize bromide to hypobromous acid. Vanadate is essential for this activity. The kinetic parameters for the artificial bromoperoxidases have been determined. The \(K_m\) value for H\(_2\)O\(_2\) is about the same as that for the vanadium chloroperoxidase, but the turnover frequency is low, and clearly the active site of acid phosphatases is not optimized for haloperoxidase activity. Like the native vanadium bromoperoxidase, the vanadate-substituted PhoN-Sf and PhoN-Se catalyse the enantioselective sulfoxidation of thioanisole.

*Keywords:* acid phosphatase; brominating activity; enantioselective sulfoxidation; vanadium bromoperoxidase; vanadium chloroperoxidase.

The enzymes are named after the most electronegative halide ion they are able to oxidize, therefore chloroperoxidase (EC 1.11.1.7) oxidation of a halide by hydrogen peroxide to the corresponding hypohalous acids according to:

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\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 oxidize, therefore chloroperoxidase oxidizes CF\(_4\), Br\(^-\), I\(^-\) and bromoperoxidase oxidizes Br\(^-\) and I\(^-\). This class of enzymes binds vanadate (HVO\(_4\)\(^2-\)) as a prosthetic group [1,2]. It is possible to prepare an apo form of these enzymes which is re-activated by vanadate. This re-activation is competitively inhibited by structural analogues of vanadate (tetrahedral compounds) such as phosphate and molybdate [3,4]. The crystal structures [5–7] of vanadium chloroperoxidase 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 nonprotein oxygens. The resulting structure shown for the chloroperoxidase (Fig. 1A) is that of a trigonal bipyramid with three nonprotein oxygens in the equatorial plane 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 vanadate-binding amino acids were shown to be conserved in two bromoperoxidases from seaweed and several acid phosphatases among the large group of soluble bacterial nonspecific class A acid phosphatases [5,7–12]. Examples are the nonspecific acid phosphatase from *Shigella flexneri* (PhoN-Sf) and the enzyme from *Salmonella enterica* ser. *typhimurium* (PhoN-Se) [13,14]. On the basis of 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]. Figure 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 apochloroperoxidase has some phosphatase activity, although the turnover with \(p\)-nitrophenol phosphate as a substrate is only 1.7 min\(^{-1}\), which is about 10 000 times slower than that of various acid phosphatases. However, the \(K_m\) for the substrate is less than 50 \(\mu\)M [8,16], which is of the same order of magnitude as various acid phosphatases. These data show that the active site of chloroperoxidase has a good affinity for the substrate but is not optimized for phosphatase activity. On the basis of the similarity of the active sites and the fact that the phosphatase activity of phosphatases is inhibited by vanadate [17,18], we expect that vanadate-substituted phosphatase has haloperoxidase activity. Indeed,
as shown here, recombinant PhoN-Sf and PhoN-Se substituted with vanadate also catalyzed the oxidation of bromide and the enantioselective oxidation of thioanisole [19,20].

MATERIALS AND METHODS

Materials

All standard recombinant DNA procedures were performed as described by Sambrook et al. [21].

The host strains Escherichia coli TOP10 (Invitrogen) and BL21(DE3) (Novagen) were used in subcloning and expression experiments. S. enterica ser. typhimurium strain SB3507 was used as a DNA source for phoN-Se gene cloning. Bacteria were routinely grown at 37 °C in Luria–Bertani medium containing 100 μg/mL ampicillin when required (LA medium). Plasmid pKU102 harbouring the Sh. flexneri phoN locus was a gift from Dr K. Uchiya [13]. Expression vectors pET3a (Novagen) and pBAD/gIIIA (Invitrogen) were used to clone the phoN gene from Sh. flexneri and S. typhimurium, respectively. pBAD/gIIIA holds the gene III signal sequence for secretion of the recombinant protein into the periplasmic space.

Expression and purification of recombinant PhoN-Se

S. enterica ser. typhimurium phoN gene was cloned in the pBAD/gIIIA expression plasmid as follows. The mature sequence (i.e. phoN gene without the 5' end coding for the secretion signal) was PCR amplified from S. enterica chromosomal DNA using the forward primer 5'-ACCA TGGGAATATACATCAGCGAAGA-3' and the reverse primer 5'-CGCAAGCTTTTACCTTCCAGTAATT-3' (the NcoI and HindIII sites, respectively, are underlined). The PCR was performed using the Expand™ High fidelity PCR System (Roche) with the following conditions: 1 μg chromosomal DNA, 1 μM each primer, 200 μM each dNTP, 1.5 mM MgCl₂, 2.6 U high-fidelity polymerase mix in a final volume of 100 μL. A ‘hot start’ of 2 min at 94 °C was followed by 30 cycles of denaturation (15 s at 94 °C), annealing (30 s at 55 °C) and extension (1 min at 72 °C) using a programmable heating block (Eppendorf Mastercycler 5330). The PCR product was restricted with NcoI and HindIII and cloned into the corresponding sites of pBAD/gIIA, in-frame with the gene III signal sequence. The resulting clone was confirmed by DNA sequencing using an Applied Biosystems 373A DNA Sequencer.

Escherichia coli TOP10 carrying the recombinant plasmid was grown at 37 °C in LA medium until the absorbance of the culture suspension reached an A₆₀₀ 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 h. 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) to A₆₀₀ = 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 A₆₀₀ = 5 and incubated on ice for 10 min. The secreted PhoN-Se was obtained in the supernatant (osmotic shock fluid) after centrifuging for 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 an 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).

Expression and purification of recombinant PhoN-Sf

Sh. flexneri phoN was cloned under control of the T7 promoter in pET3a as described below. It 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

Fig. 1. Structure of the active site of (A) vanadium chloroperoxidase from C. inaequalis (PDB ID: 1IDQ) and (B) the acid phosphatase from E. blattae (PDB ID: 1D2T). The phosphatase cocrystallized with sulfate. The figure was prepared using Swiss PDB viewer.
expression was induced with 0.4 mm isopropyl isothio-ß-D-galactoside for 5–7 h at 37 °C.

Soluble PhoN-Sf was released from E. coli by breaking the cells in a French press (5.17–5.24 MPA). 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 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/HCl (pH 7.5) and 1 mm EDTA which has no effect on the phosphatase activity.

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

**Enzymatic assay of phosphatase activity**

The phosphatase activity was measured by hydrolysis of 10 mm p-nitrophenyl phosphate 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 p-nitrophenol was measured at 410 nm (absorption coefficient 16.6 mm M⁻¹cm⁻¹).

**Enzymatic assay of bromoperoxidase activity**

*Assay of PhoN-Sf brominating activity.* 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 Br⁻. This assay is convenient because large color changes are observed which can easily be detected visually [22]. As 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 preincubated with 100 μM vanadate in 100 mm Tris/HCl (pH 7.5) for at least 30 min. The brominating activity of recombinant PhoN-Sf (final concentration 0.5 μM) 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 Br⁻ and 2 mm H₂O₂. The assay mixture also contained 100 μM vanadate.

*Enantioselective sulfoxidation of organic sulfide*

The enantioselective sulfoxidation by the recombinant phosphatases was demonstrated using thioanisole as a substrate [20]. Thioanisole at a concentration of 2 mm was incubated with 2 mm H₂O₂, 100 μM vanadate and 100 nm 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, H₂O₂ remaining in the reaction mixture was quenched with Na₂SO₃. The enantiomeric products were extracted with dichloromethane, evaporated to 20 μL, and dissolved in 1 mL hexane/propan-2-ol (4:1, v/v). A 20-μL sample was used for HPLC analysis on a Diacel chiral OD column (0.46 × 25 cm) equipped with a Pharmacia LKB-HPLC pump 2248 and an LKB Bromma 2140 rapid spectral detector. The column was eluted with hexane/propan-2-ol (4:1, v/v) at a flow rate of 0.5 mL/min⁻¹. 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 (JMBS developments) was used for HPLC data acquisition and evaluation.

**RESULTS AND DISCUSSION**

**Expression of recombinant acid phosphatases in E. coli**

The similarity in the active-site structures of vanadium haloperoxidases and class A bacterial acid phosphatases was first suggested by sequence alignments [8–10]. Indeed, the comparison of the crystal structures of E. blattae acid phosphatase and C. inaequalis vanadium chloroperoxidase (Fig. 1) confirms this structural similarity [15]. Unfortunately, the structure of the acid phosphatase complexed to vanadate is not available, only that of a sulfate and a molybdate complex [15]. The similarity prompted us to investigate whether class A bacterial acid phosphatases with vanadate bound to the active site could also function as vanadium haloperoxidases. S. enterica ser. typhimurium [25] and Sh. flexneri acid phosphatases, which 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 conservation of three separate domains. 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 [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 TOP10, which harbours the expression vector for PhoN-Se, nor BL21(DE3), which harbours the expression vector for PhoN-Sf, showed...
relevant levels of acid phosphatase activity. On induction, the specific activity of acid phosphatase in both strains was about 40 U mg⁻¹.

During purification, the acid phosphatase activity always cochromatographed with a protein of about 30 kDa, in agreement with the molecular mass of each phosphatase. The final preparations with a yield of 1–2 mg Pho-N-Sf per L of culture medium were judged to be at least 90% pure 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 matrix-assisted laser desorption ionization time-of-flight MS (not shown).

In the case of Pho-N-Se, 10–15 mg enzyme, with a specific activity of 140 U mg⁻¹, was obtained from 1 L of 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.

**Haloperoxidase activity of vanadate-substituted acid phosphatases**

The brominating activity of recombinant Pho-N-Sf and Pho-N-Se was tested in a phenol red assay. After overnight incubation of 1 μM Pho-N-Sf and Pho-N-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 Pho-N, bromination of the dye was not detected. This means that the reaction is catalysed by the vanadate-substituted Pho-N-Sf and Pho-N-Se. Binding of vanadate to the active site of Pho-N-Sf is confirmed by the observation that vanadate inhibits the phosphatase activity of Pho-N-Sf with a *K*ₐ of ≈ 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 binding was strikingly similar to that in the vanadium chloroperoxidase from *C. inaequalis* [10]. Therefore, it is likely that vanadate binds to the active site of Pho-N and causes the peroxidase-like activity.

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

As described in Materials and methods, buffers containing citrate or phosphate are not suitable for measuring brominating activity of Pho-N, therefore sodium acetate was used in the assay to determine the pH optimum. Figure 3 shows that the maximal brominating activity is observed at pH 4.6 and pH 4.2 for Pho-N-Sf and Pho-N-Se, respectively. Owing to the restricted choice of buffers, experiments were carried out over a limited pH range. Sodium acetate was used in the pH range 4.2–5.4 and pH 3.8–6.0 for Pho-N-Sf and Pho-N-Se, respectively. This makes it difficult to evaluate the *K*ₐ value of the group involved in the bromination activity of these phosphatases. As only a limited amount of enzyme was available, the determination of the optimum pH of Pho-N-Sf was based on a single substrate concentration (200 mM KBr and 2 mM H₂O₂). For Pho-N-Se it was possible to measure *K*ₐ and *V* at each pH value. Figure 3B shows the pH-dependence of *V*. The data suggest that a group with a *K*ₐ of ≈ 4.3 is involved in the bromination reaction. The *K*ₐ for bromide was also pH-dependent and increases with increasing pH (not shown).

A steady-state kinetic study of the brominating activity of vanadate-substituted Pho-N-Sf and Pho-N-Se was carried out. For Pho-N-Sf, a *K*ₐ of ≈ 350 mM was obtained for bromide (Fig. 4A), and for Pho-N-Se a *K*ₐ of ≈ 160 mM (Fig. 4C). The maximal turnover for the brominating activity of vanadate-substituted Pho-N-Sf is 3.4 min⁻¹ (0.13 U mg⁻¹), which is considerably slower than the values
concentrations of H2O2. The data points are means of triplicate measurements.

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 a function of the substrate concentration. PhoN-Sf was preincubated for 1 h 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 Br–. (B) PhoN-Sf in 300 mM Br– and variable concentrations of H2O2. (C) PhoN-Se in 2 mM H2O2 and variable concentrations of Br–. (D) PhoN-Se in 300 mM Br– and variable concentrations of H2O2. The data points are means of triplicate measurements.

of 120–180 U·mg⁻¹ observed for vanadium haloperoxidases [26,27]. However, the turnover for the brominating activity of the acid phosphatases is of the same order of magnitude as the phosphatase activity of apo-chloroperoxidase (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 for the brominating activity of vanadate-substituted PhoN-Se was 33 min⁻¹ (1.23 U·mg⁻¹), which was about 10-fold higher than that for PhoN-Sf and the phosphatase activity of apo-chloroperoxidase. Although PhoN-Se has higher brominating activity than PhoN-Sf, the K_m for H2O2 was approximately 400 μM (Fig. 4D). The specificity constant (k_cat/K_m), which can be calculated from these data, are 0.16 M⁻¹·s⁻¹ and 2 M⁻¹·s⁻¹ for bromide oxidation by PhoN-Sf and PhoN-Se, respectively. If one compares these values with the specificity constant for bromide oxidation [28] by the bromoperoxidase from A. nodosum (1.8 × 10⁵ M⁻¹·s⁻¹), it is clear that the vanadate-substituted acid phosphatases are poor catalysts in bromide oxidation.

As several vanadium haloperoxidases are able to catalyse the enantioselective sulfidation of thioanisole [19,20], we investigated whether the PhoN-Sf and PhoN-Se catalysed this reaction. Indeed, when 0.5 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 into the R enantiomer of the sulfoxide, with an enantiomeric excess (e.e.) of 57% (results not shown). Owing to the limited amount of enzyme 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 into the sulfoxide was noted in the absence of vanadate, but a racemic mixture resulted (not shown). Also when vanadate was incubated with thioanisole and H2O2, a minor amount of a racemic mixture resulted. It is clear that vanadate is essential for the enantioselective sulfoxidation activity of 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 an enantioselective conversion was also observed (e.e. 24%). However, the conversion was much slower than when vanadate was present. As further incubation of PhoN-Sf, the sulﬁde and H2O2 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 that was not completely removed by dialysis against 1 mM EDTA. Recently, it has been reported that vanadate-incorporated phytase [29], an unrelated phosphatase that mediates the hydrolysis of phosphate esters, also catalyses the enantioselective sulfoxidation of prochiral sulﬁdes with H2O2 to the S-sulfoxides. However, brominating activity, was not detected.

The kinetic data obtained previously [8,16] showed that, despite the similarity in the structure of the active sites of the vanadium haloperoxidases and the acid phosphatases (see Fig. 1), apo-chloroperoxidase is not optimized for the phosphatase activity, and vice versa the vanadate-substituted phosphatases show only moderate peroxidase activity. This means that other residues further away from 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 of which factors determine whether a vanadium haloperoxidase is a bromoperoxidase or a chloroperoxidase [7,16] have also been equivocal. Despite the fact that structural data and kinetic details are available for these enzymes, and even site-directed mutagenesis studies have been carried out [29], the nature of these factors is not clear.

Our findings have important implications. There have been many attempts to construct enzyme mimics or create synthetic enzymes using knowledge of the active-site structure of enzymes. In general, these mimics are comparatively poor catalysts. Our study clearly shows that despite the similarity in active-site structure, the activities of these enzymes differ widely. As pointed out above, these differences are probably due to amino-acid residues outside the active site, which appear to be very important in catalysis and determining specificity. This suggests that construction of an artificial enzyme with similar activity to the original on the basis of its active site is going to be more difficult than expected.

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REFERENCES


