Phosphatase activity of non-heme chloroperoxidase from the bacterium *Serratia marcescens*

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Received 19 July 2002; revised 25 December 2002; accepted 30 December 2002

First published online 14 January 2003

Edited by Vladimir Skulachev

Abstract Haloperoxidases are enzymes capable of formation of carbon–halogen bonds in the presence of hydrogen peroxide and halide ions. A mechanism of halogenation catalyzed by heme- and metal-independent bacterial haloperoxidases differs from other representatives of this group of enzymes. Here we report for the first time that bacterial non-heme haloperoxidases possess a phosphatase activity. Chloroperoxidase from *Serratia marcescens* W 250 purified up to homogeneity is shown to catalyze *p*-nitrophenylphosphate hydrolysis (K_m value, 1.8 ± 0.1 mM at pH 5.7). The reaction is activated by Mg^{2+} and F^-, and is inhibited by WO_4^{2-}, tartrate, acetate and phosphate anions. The irreversible inhibition by phenylmethanesulfonyl fluoride, modificator of serine residue in active site, decreases in the presence of phosphate ions. A mechanism of phosphoesters hydrolysis by non-heme haloperoxidases is proposed.

Key words: Chloroperoxidase; *Serratia marcescens*; *p*-Nitrophenylphosphate

1. Introduction

Haloperoxidases are enzymes capable of incorporating halogen atoms into the molecules of organic substrates under the influence of hydrogen peroxide and halide ions (EC 1.11.1.10). Depending on the type of prosthetic group that allows haloperoxidases to carry out the redox reaction, they can be subdivided into heme- and vanadium-dependent [1]. Some bacterial haloperoxidases have no prosthetic group and the halogenation reaction catalyzed by them follows another mechanism under the influence of acetate ions. In the active site of such enzymes the catalytic triad Asp–His–Ser has a key position [2]. Acetate ions bind covalently with the nucleophilic Ser residue and thus obtained ester is then hydrolyzed by hydrogen peroxide to form peracetic acid. The latter as a strong oxidizing agent is able to oxidize halide ions to the corresponding hypohalous acid which readily incorporates the halogen atom into organic substrates [3] (Scheme 1).

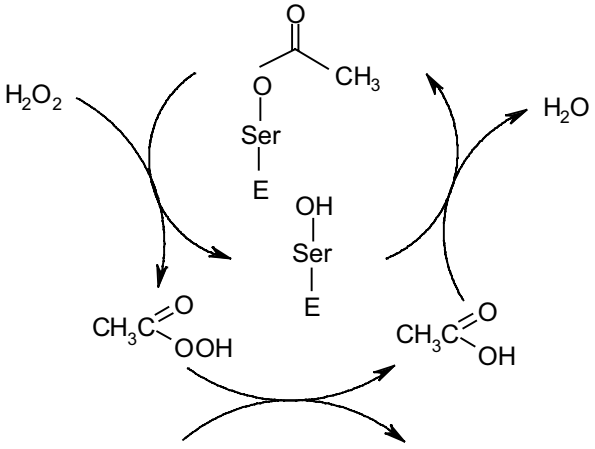
As all haloperoxidases, bacterial heme- and metal-independent representatives of this group of enzymes catalyze other oxidative reactions, for instance, sulfide oxidation [3], and the transformation of aromatic amino into nitro groups [4–6]. Perhaps non-heme haloperoxidases appear to hydrolyze ester bonds due to the high resemblance of their active site structure to that of serine hydrolases [3,7]. Such a broad range of catalyzed reactions in addition to high stability in reaction conditions [8] allows the consideration of these enzymes as very useful with regard to preparative transformations.

Recently a communication about the phosphatase activity of vanadium haloperoxidase and its similarity in the active site to acid phosphatases has been published [9]. Here we report on the exhibition of phosphatase activity by non-heme haloperoxidase from the bacterium *Serratia marcescens* W 250.

2. Materials and methods

2.1. Protein purification

Cells of *S. marcescens* were grown and the crude extract of the



sulfide	→	sulfoxide
olefin	→	bromohydrin
amine	→	nitrocompound
β-diketon	→	bromodiketon
phenol	→	phenol haloderivative
pyrrol	→	pyrrol haloderivative

Scheme 1. Mechanism of the catalysis by non-heme bacterial haloperoxidases.

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Abbreviations: CPO, chloroperoxidase; *p*NPP, *p*-nitrophenylphosphate

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doi:10.1016/S0014-5793(03)00008-5

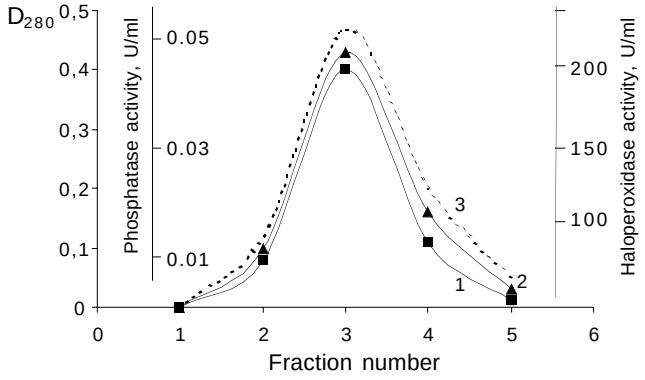


Fig. 1. Affinity chromatography of CPO on Blue Sepharose. 1: Haloperoxidase activity; 2: phosphatase activity; 3: protein.

chloroperoxidase (CPO) was prepared as described previously [8]. The only difference was that the enzyme was extracted with 50 mM potassium phosphate (pH 7.5). Precipitation with ammonium sulfate occurred from a saturation of 45% to 85%. After centrifugation the precipitate was dialyzed against 50 mM potassium phosphate (pH 7.5). Then the enzyme was applied to a 150-ml DEAE–Sephadex (Pharmacia, Biotechnology, Sweden) column which had been previously equilibrated with 50 mM potassium phosphate (pH 7.5, 1.5% glycerol). The elution was performed by linear gradient $0 \rightarrow 1$ M NaCl in four column volumes of the same buffer and the CPO fraction eluted at around 0.12 M NaCl. That fraction was concentrated by ultrafiltration and applied to gel filtration Sephadex G-200 column (2.5×100 cm), equilibrated and eluted with 0.1 M potassium phosphate (pH 6.2). Fractions containing CPO activity were collected, concentrated by ultrafiltration and applied to a column of Blue Sepharose (0.5×10 cm) eluted with 20 mM potassium phosphate (pH 5.7).

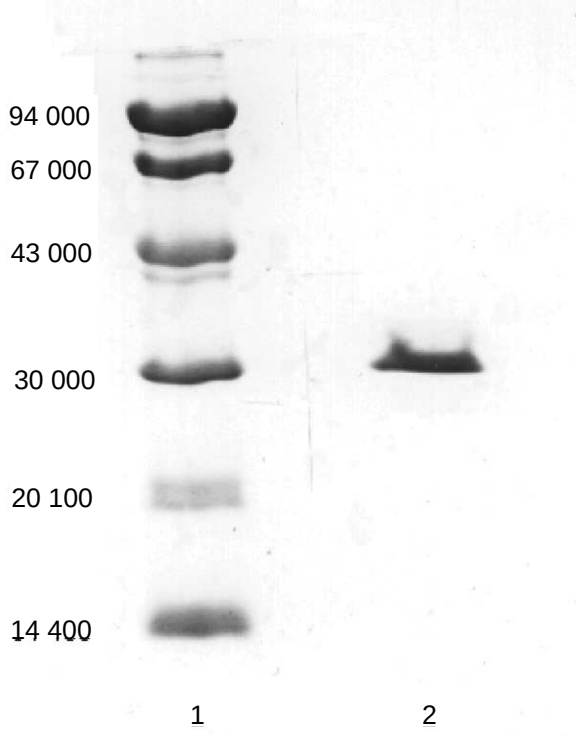


Fig. 2. SDS–PAGE of purified CPO. 1: Molecular weight (Da) markers; 2: CPO. Proteins were stained with Coomassie Brilliant Blue.

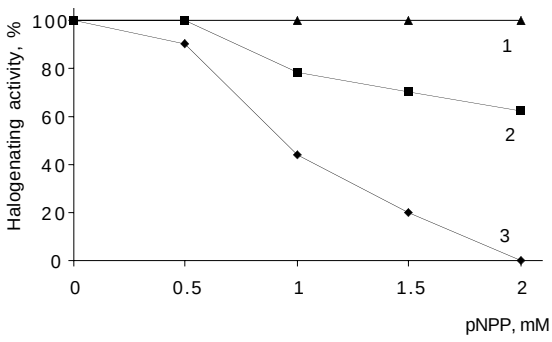


Fig. 3. Effect of *p*NPP on halogenating activity of CPO in the presence of different acetate concentrations: 1 - 1 M acetate; 2 - 0.6 M; 3 - 0.4 M.

2.2. Electrophoresis

Denaturing polyacrylamide gel electrophoresis in the presence of SDS was performed by the Laemmli method [10].

2.3. Enzyme assay and protein measurement

Brominating activity was measured spectrophotometrically at 290 nm with monochlorodimedon (48 μM, $\varepsilon = 19000\ M^{-1} \times cm^{-1}$), hydrogen peroxide (8.8 mM), sodium bromide (100 mM) and azide (10 mM) in a medium of 1 M sodium acetate (pH 5.5) [11].

Phosphatase activity was registered after the incubation (37°C) of CPO (50 μl) with *p*-nitrophenylphosphate (*p*NPP; 6 mM, 50 μl). The reaction was stopped by adding 1 ml of 0.1 M NaOH and the amount of *p*-nitrophenol was measured spectrophotometrically at 410 nm ($\varepsilon = 18300\ M^{-1} \times cm^{-1}$) [12].

Protein concentrations were determined spectrophotometrically at 280 and 260 nm using the following equation: protein (mg ml^{-1}) = $(1.55\ A_{280} - 0.76\ A_{260})$ [13].

3. Results and discussion

The first attempts at discovering phosphatase activity of a representative of bacterial non-heme haloperoxidases were unsuccessful [14]. Acetate was originally used for the isolation and purification of bacterial non-heme haloperoxidases [8]. To find phosphatase activity, acetate has been substituted by phosphate because of appreciable inhibition of the phosphoester hydrolysis in acetate medium. The addition of 1 M

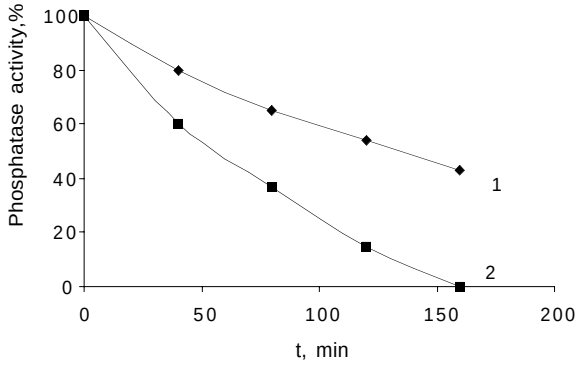


Fig. 4. Inhibition of phosphatase activity of CPO by PMSF. 1: In the medium containing phosphate anions (0.1 M); 2: in non-phosphate medium. PMSF was added to CPO for a final concentration of 2 mM and the sample was incubated for 3 h. Aliquots from the sample were taken at 40 min intervals to determine phosphatase activity.

Table 1
Purification of chloroperoxidase from *S. marcescens* in potassium phosphate

Purification steps	Total protein, mg	Total activity, U	Specific activity, U mg^{-1}	Recovery, %	Purification (*n*-fold)
Crude extract	372.00 ± 20.00	53.5 ± 1.4	0.14 ± 0.02	100.0	1
Sulfate ammonium fractionation	63.70 ± 0.90	41.4 ± 1.0	0.65 ± 0.05	78.0	5
DEAE–Sephadex	3.08 ± 0.05	210.0 ± 1.1	68.20 ± 0.60	394.0	477
Sephadex G-200	1.36 ± 0.04	213.4 ± 0.9	157.00 ± 12.00	400.0	1095
Blue Sepharose	0.50 ± 0.03	108.6 ± 0.7	217.1 ± 20.00	203.7	1518

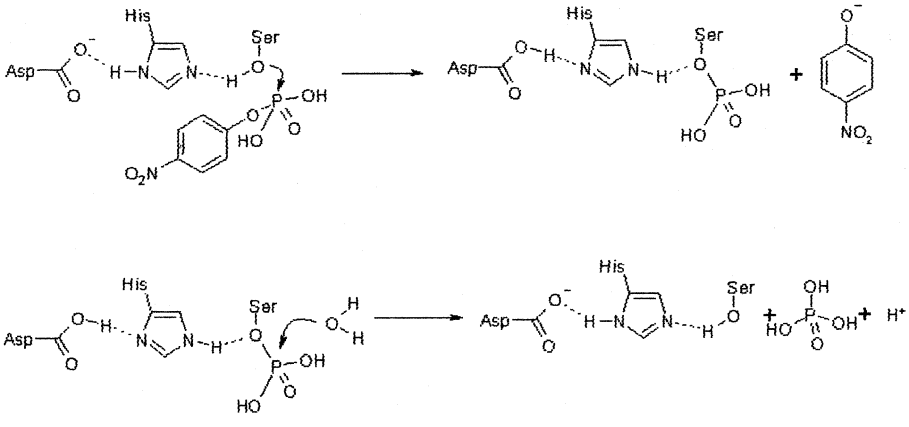
acetate causes a decrease of 60% in CPO phosphatase activity. It should be noted that acetate ions take part in the halogenation reaction and the most acceptable concentration of them is rather high – not less than 1 M [15]. As a result, the phosphatase activity of CPO isolated and purified without acetate is twice as high (0.06 U mg^{-1}) as that purified in the presence of acetate (about 0.03 U mg^{-1}). The fractions containing haloperoxidase activity after the last step of purification – affinity chromatography – exhibit phosphatase activity as well (Fig. 1). The electrophoresis in PAGE with SDS for the enzyme obtained after precipitation, anion-exchange, gel-filtration and affinity chromatography showed a single protein band corresponding to a molecular mass of 29 kDa (Fig. 2), so phosphatase activity belongs to the CPO named.

Phosphatase activity of CPO being determined by the method described above can be measured only after anion-exchange chromatography due to the presence of pigment prodigiosin in crude extract. Prodigiosin gives an additional peak of absorption at 410 nm and pH > 10, so the results of purification presented are shown for haloperoxidase activity (Table 1). As well as for the other bacterial non-heme haloperoxidases [16], a spasmodic increase of activity in some intermediate steps of purification is observed. This can be explained by the removal of an unknown inhibitor. The method of purification developed allows one to obtain about 0.5 mg of protein from 12 g of cells (wet weight). The enzyme purified has both a high halogenating and a rather high phosphatase activity that depends on the presence of acetate ions. Now the existence of cloned and sequenced appropriate genes does show non-heme bacterial haloperoxidases to be quite available [17–19].

The *p*NPP hydrolysis by CPO takes place in acid media (optimum activity is between 5.2 and 6.1; not shown). It can proceed at comparatively high temperatures for enzyme reaction: till 65°C the decrease in *p*NPPase activity for CPO is not observed what is very important for preparative purposes (not shown). Analogous curves were obtained for halogenating activity of this enzyme [15].

The presence of such ions as Ni^{2+}, Zn^{2+}, Cu^{2+} in the assay mixture influences essentially the phosphatase activity of CPO. At a concentration of 0.01 M they decrease activity to 10% from initial figures. Ba^{2+} (0.01 M) and $[Fe(CN)_6]^{4-}$ (0.01 M) have a slight effect – 90% of the activity remains. The influence of phosphatases' inhibitors was as follows: WO_4^{2-} (1 mM), 23% of residual activity, tartrate (20 mM), 90% of residual activity. The degree of activation by alkaline phosphatase activator Mg^{2+} depends on the concentration: 5 mM Mg^{2+} – 132% in comparison with initial 100%, 30 mM – 200%. However, it cannot be said that phosphatase activity is completely dependent on the presence of Mg^{2+} as is reported by some authors [20]. Mg^{2+} is not necessary for the exhibition of phosphatase activity by CPO. An inhibitor of alkaline phosphatase F^- in the concentration of 0.3 M has an activating effect on the *p*NPPase activity of CPO investigated – the *p*NPPase activity increases by 32%. Interestingly, halogenating activity of CPO is inhibited by F^-; however, the inhibition is not competitive [15].

The K_m value for *p*NPP determined from the Lineweaver–Burk plot is 1.8 ± 0.1 mM at pH 5.7, with the V_{max} value accounted for – 0.085 μmol/min per mg protein (not shown). The K_m value obtained seems rather high for acid phosphatases (usually 0.1–0.2 mM) [9], which allows the proposition that the CPO investigated exhibits phosphatase activity as supplementary. In addition to the inhibitory effect of acetate ions on the *p*NPPase activity of CPO, inhibition of the mixed type of haloperoxidase activity of CPO by phosphate ions



Scheme 2.

(K_i 75 mM at pH 6) was also found. These data together with the absence of inhibition of haloperoxidase activity by *p*NPP at a saturating concentration of acetate ions (1 M) (Fig. 3) give basis to the supposition that phosphoester hydrolysis proceeds in the same active site that is responsible for the exhibition of haloperoxidase activity. The above presented data are confirmed by an irreversible inhibition of *p*NPPase activity by the modificator of Ser residue phenylmethanesulfonyl fluoride (PMSF) (Fig. 4). It depends on time and proceeds much faster in the medium containing no phosphate ions. This can be easily explained by the competition between phosphate and PMSF to bind them with the serine residue in the active site.

The phosphate anion that split off seems likely to attach covalently to the hydroxyl group of serine in the active site of CPO according to Scheme 2.

Thus, our investigations have shown that not only carboxyl esters but also phosphoesters of aromatic derivatives are subject to the nucleophilic attack of serine residue. The data obtained from the exhibition of phosphatase activity of non-heme CPO from *Serratia marcescens* are in accordance with the scheme of halogenation mechanism postulated for bacterial heme- and metal-independent haloperoxidases.

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