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Amphitrite ornata dehaloperoxidase: enhanced activity for the catalytically active globin using MCPBA

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Abstract

Dehaloperoxidase (DHP) from *Amphitrite ornata* is the only heme-containing, hydrogen peroxide-dependent globin capable of oxidatively dehalogenating halophenols to yield the corresponding quinones. To ascertain that this enzymatic activity is intrinsic to DHP, we have cloned and expressed the enzyme in *Escherichia coli*. We also find that an alternate oxygen atom donor, *meta*-chloroperbenzoic acid, gives appreciably higher activity than hydrogen peroxide. Under optimal turnover conditions (large peroxide/peracid excess), after an initial burst of activity, DHP appears to become trapped in a non-catalytic state (possibly Compound II) and is unable to fully convert all halophenol to product. However, full substrate conversion can be achieved under more physiological conditions involving a much smaller excess of oxygen atom donor. Parallel studies have been carried out using horseradish peroxidase and myoglobin to calibrate the activity of DHP versus typical peroxidase and globin proteins, respectively.
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Amphitrite ornata is a terebellid polychaete that generates no detectable volatile haloaromatic metabolites but often inhabits coastal sediments contaminated with such halometabolites [1–9]. Haloaromatic sediment contamination gives a competitive advantage to those invertebrate species that are capable of metabolizing noxious haloaromatics. In 1996, Chen et al. [10] reported that this organism contained a dehalogenating peroxidase, dehaloperoxidase (DHP). DHP was shown to contain two identical subunits with a total molecular weight of ∼31 kDa and to function as a peroxidase converting halogenated phenols to quinones in the presence of hydrogen peroxide Eq. (1). Spectroscopic studies revealed that DHP is a His-ligated heme-containing enzyme that is isolated in the oxyferrous state [11].

$$X\text{-phenol} + H_2O_2 \xrightarrow{\text{DHP}} \text{quinone} + H_2O + H^+X^- \quad (X = I, Br, Cl, F)$$

(1)
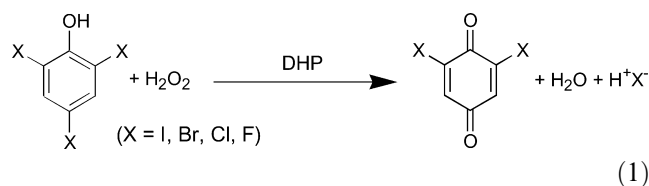
The crystal structure of native DHP has revealed that it has the same protein fold as myoglobin [12]. Halophenol substrates such as 4-iodophenol were shown to bind to DHP in the distal pocket rather than at the heme edge as in horseradish peroxidase (HRP), the prototypical peroxidase [13]. The HRP active site is considered to be too narrow for organic substrates to enter and come into close contact with the heme iron center. DHP is the first known peroxidase with a globin fold and the first identified enzymatically active globin [14].

Lignin peroxidase (LiP) [15] and HRP [16] also dehalogenate halophenols. Ferrari et al. [16] have

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proposed that the mechanism of dehalogenation of 2,4,6-trichlorophenol (TCP) by HRP is similar to that catalyzed by LiP and involves two one-electron steps via the two classical ferryl [oxo-iron(IV)] peroxidase heme intermediates, Compounds I and II, with the former being required to initiate the reaction by formation of a phenoxy radical. Such radicals are known intermediates in peroxidase-catalyzed phenol oxidation, and nucleophilic attack by water on a TCP radical cation is likely. However, the exact mechanism of oxidative dehalogenation by DHP has not been determined and may differ from that of the HRP and LiP systems [15,16], given the ability of DHP to bind substrate in close proximity to the heme iron. The oxidative dehalogenation of TCP could take place via a peroxygenase mechanism as a result of direct oxygen atom transfer from the oxygen donor co-substrate. *Caldariomyces fumago* chloroperoxidase and the cytochrome P450 class of heme enzymes catalyze peroxygenase reactions; however, DHP lacks structural homology to either of these enzymes [17,18].

The enzymatic assays that established the activity of DHP were carried out with native *A. ornata* DHP [10] and did not address the possibility of a contaminant peroxidase. This concern is even more serious in light of the substantially higher dehaloperoxidase activity seen with HRP (vide infra). Thus, expression of catalytically active recombinant DHP, reported herein from an *Escherichia coli* cell line, firmly establishes the protein as the active enzyme during catalysis and also lays the foundation for future mechanistic studies.

We also describe specific kinetic assay conditions with hydrogen peroxide and with an alternate oxygen atom donor co-substrate, *meta*-chloroperbenzoic acid (MCPBA), for either optimal turnover or product yield. The conditions of large oxygen atom donor excess that produce optimal initial turnover also lead to the formation of a non-catalytic state (possibly Compound II). However, maximal product yield can be achieved under more physiological conditions.

Materials and methods

Materials and instrumentation. Reagent grade chemicals (Aldrich, ACROS, or Fisher) were used without further purification except for potassium ferricyanide (recrystallized from water). Hydrogen peroxide and *meta*-chloroperbenzoic acid were taken from 30% and 10 mM stock solutions, respectively. HRP (type VI, Rz 2.7, Aldrich) was used without further purification. Myoglobin (horse heart type III, Sigma) was treated with potassium ferricyanide followed by desalting with a P-6 DG (Bio-Rad) column. UV–visible absorption spectra were measured using a Cary 400 spectrophotometer interfaced to an IBM PC.

Ligation of primers for the ribosome binding site sequence. In previous work [1], we cloned the DHP gene in a pBluescript vector. This construct was digested with *Pst*I and *Nco*I to release a 120 bp fragment upstream of the start codon and the remaining linear fragment was

purified. The primers used were the same as the ribosome binding site (RBS) sequence used for the synthetic myoglobin gene [19], except that they contained *Pst*I and *Nco*I sticky ends. For ligation of the RBS sequence to the DHP gene, the annealed primer (RBS), ligase buffer, T4 DNA ligase, and the purified linear plasmid DNA containing the DHP gene were mixed, allowed to ligate at 14 °C overnight, and then transformed into competent *E. coli* TB-1 cells.

Isolation of the DHP gene with the attached ribosome binding site. The plasmid DNA was digested using the restriction enzymes, *Pst*I and *Ssp*I, and then the DNA was isolated, and purified using standard methods. The resulting DNA was 580 bp long and contained the DHP gene with the new RBS. This DNA piece was then ligated to the DNA isolated from the myoglobin plasmid pUC19 digestion with *Pst*I and *Eco*RI. This final ligation yielded the pUC19 plasmid containing the DHP gene with a RBS sequence attached. This plasmid was then stored and transformed into competent *E. coli* TB-1 cells for growth and protein expression of the DHP enzyme. The resulting plasmid was also sequenced to confirm the correct base pair sequence. The plasmids were stored as glycerol stocks in −70 °C freezer.

Expression of the DHP enzyme from the pUC19 plasmid source. The plasmid carrying the DHP sequence was grown using 2XYT-ampicillin (amp) growth media for plates and liquid broth. The cells were streaked onto 2XYT agar plates and allowed to grow overnight at 37 °C, and a single colony was streaked and repeated twice to ensure purification of the cell colonies. Five hundred microliter aliquots of the resulting cell/broth mixture were used to inoculate six 1 L flasks of 2XYT-amp broth. The growth media and inoculate were allowed to grow at 37 °C overnight. The cells were collected by centrifugation for 45 min at 5000 rpm. These steps were repeated until an adequate amount of cell pellet ($\sim$20 g) was obtained for purification. The cell pellet was weighed and stored at −20 °C.

Purification of recombinant DHP. After sonication of the cell pellet, the supernatant was applied to an anion exchange column (DE52, Whatman, 300 mL) equilibrated at pH 5.0 in 50 mM sodium phosphate buffer. The column was washed and eluted with equilibration buffer. The absorbance was monitored at 416 nm (Soret peak of the oxyferrous state). Fractions with the best 416/280 nm absorbance ratio were combined, concentrated, and loaded onto a size exclusion column (P-100, Bio-Rad) equilibrated at pH 5.0 in 50 mM sodium phosphate buffer. The best fractions were again collected based on the 416/280 nm absorbance ratio. Samples with a ratio greater than 2.0 were found to be electrophoretically homogeneous.

Preparation of samples. Purified DHP was oxidized to the ferric state by treatment with potassium ferricyanide, followed by desalting with a P-6 DG (Bio-Rad) column. DHP, HRP, and myoglobin concentrations were determined using published molar absorptivities [11,20,21].

Peroxidase activity assay. UV absorption spectroscopy was used to assay for dehaloperoxidase activity, and hydrogen peroxide, or *meta*-chloroperbenzoic acid (400 μM final concentration) was used to initiate the reactions. Turnover measurements (enzyme conc. = 0.1 μM) were carried out directly in a cuvette by monitoring the change in absorbance at 272 nm ($\varepsilon = 14\ \mathrm{mM^{-1}\,cm^{-1}}$) for appearance of products. A 1000-fold excess of 2,4,6-trichlorophenol was used to maintain psuedo-first order conditions. For each set of reaction conditions, between 8 and 12 individual reactions were performed and then averaged to determine the turnover numbers, which were calculated from the initial linear portion of the trace at 272 nm.

Results and discussion

The cloning and expression of DHP from *A. ornata* enables us to demonstrate that the previously observed activity [10] is inherent to DHP and not the result of a
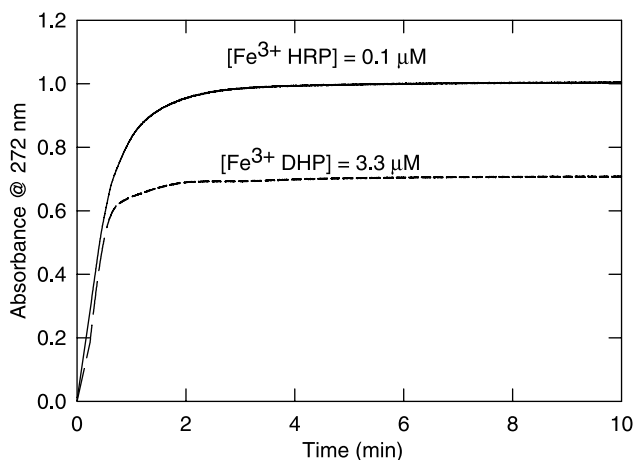
Fig. 1. UV absorption data monitored at 272 nm in 100 mM sodium acetate buffer (pH 5.4) for the oxidative dechlorination of 2,4,6-trichlorophenol (100 μM) catalyzed by horseradish peroxidase (solid line) (0.1 μM) or recombinant dehaloperoxidase (dashed line) (3.3 μM) initiated by hydrogen peroxide (400 μM).

small, highly active, peroxidase contaminant. The development of a recombinant form of DHP will make it possible to prepare mutant forms of the enzyme for future mechanistic investigations.

Fig. 1 shows the UV absorption spectral assays for the oxidative dechlorination of 2,4,6-trichlorophenol (TCP) at pH 5.4 by recombinant DHP compared to that catalyzed by horseradish peroxidase (HRP). The co-substrate for initiating these reactions is hydrogen peroxide Eq. (1). Using data such as those in Fig. 1, optimum turnover numbers for both enzymes for formation of the product, 2,6-dichloro-1,4-benzoquinone ($\lambda_{max} = 272$ nm), have been calculated from the initial linear trace as a result of the absorption spectral change at 272 nm (Table 1). In Fig. 1, the concentration of DHP is 33 times that of HRP because the turnover for reactions initiated by DHP is consistently ~33 times less than that by HRP. The turnover number for HRP is essentially identical to that previously reported by Ferrari et al. [16] and is approximately 33-fold higher than that for DHP. As has been previously reported [10,16],

the identity of the quinone product generated by HRP and DHP has been confirmed by mass spectrometry. The turnover numbers calculated for recombinant DHP are very similar to those calculated for native DHP (Table 1). No spectral change at 272 nm is seen in the absence of DHP or HRP.

With myoglobin, a very slow increase in absorbance at 272 nm is observed under similar conditions (data not shown). However, all attempts to directly confirm formation of the expected quinone product by extraction and mass spectral analysis have been unsuccessful. We conclude that myoglobin likely catalyzes the dehalogenation of halophenols under the proper conditions but not at appreciable rates.

Fig. 2 shows spectral assays for the oxidative dechlorination of TCP by recombinant DHP compared to that catalyzed by HRP using MCPBA as co-substrate. The respective turnover numbers for the two enzymes with this co-substrate are again listed in Table 1. The turnover number for the MCPBA-dependent oxidative dechlorination of TCP by recombinant DHP is over 3-
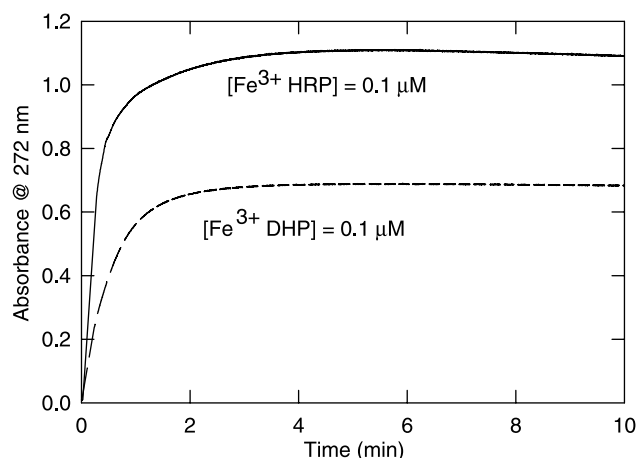

Fig. 2. UV absorption data monitored at 272 nm in 100 mM sodium acetate buffer (pH 5.4) for the oxidative dechlorination of 2,4,6-trichlorophenol (100 μM) catalyzed by horseradish peroxidase (solid line) (0.1 μM) or recombinant dehaloperoxidase (dashed line) (0.1 μM) initiated by *meta*-chloroperbenzoic acid (400 μM).

Table 1
Turnover numbers for the oxidative dechlorination of 2,4,6-trichlorophenol by recombinant dehaloperoxidase and horseradish peroxidase initiated by hydrogen peroxide and *meta*-chloroperbenzoic acid[a]

Enzyme	Turnover number (mol product) (mol enzyme)$^{-1}$ s^{-1} initiated by H_2O_2	Turnover number (mol product) (mol enzyme)$^{-1}$ s^{-1} initiated by MCPBA
Recombinant dehaloperoxidase	3.3 ± 0.8[b]	10.8 ± 0.7
Horseradish peroxidase	102 ± 5.9	48.5 ± 2.3

[a] The reaction was initiated by addition of H_2O_2 or *meta*-chloroperbenzoic acid (400 μM final concentration) and was carried out at pH 5.4 in 100 mM sodium acetate at 4 °C. Product formation was monitored at 272 nm. Enzyme concentration was 0.1 μM. 2,4,6-Trichlorophenol concentration was 100 μM.

[b] Numerous turnover studies carried out with electrophoretically pure native DHP consistently and repeatedly yielded turnover numbers that are essentially identical to those reported here for recombinant DHP (R.L. Osborne, Y.P. Chen, J.H. Dawson, unpublished results). The turnover numbers previously reported for native DHP [10] appear to be incorrect.

fold higher than the value obtained when the reaction is initiated by hydrogen peroxide. Because the turnover number for the MCPBA-dependent reaction with HRP is actually 2-fold lower than when initiated by hydrogen peroxide, the oxidative dechlorination activity of DHP, expressed as a percentage of the HRP value, is 7-fold higher when MCPBA is the co-substrate. With myoglobin, little evidence of MCPBA-dependent oxidative dechlorination of TCP has been observed. No spectral change at 272 nm is seen in the absence of DHP or HRP. The enhanced activity with MCPBA as a co-substrate suggests that the natural co-substrate for the DHP-catalyzed reaction may not be hydrogen peroxide and might instead be an alternative oxygen atom donor such as an alkyl hydroperoxide.

The kinetic assays shown in Figs. 1 and 2 have been designed to achieve a maximum turnover number for the reaction with each enzyme and co-substrate. One puzzling feature of the data in both figures is the observation that while the reactions with HRP go all the way to completion (full conversion of TCP to quinone product leads to an absorbance of ~1.0), the DHP-catalyzed reactions do not. Instead, the enzyme seems to become trapped in a non-catalytic state and the reaction only goes approximately 60% of the way to completion. Reasoning that this non-catalytic state might be the result of hydrogen peroxide-dependent formation of DHP Compound II, assay conditions involving a much smaller excess of hydrogen peroxide have been examined. As illustrated in Fig. 3, decreasing the ratio of hydrogen peroxide to enzyme permits the reaction to go to completion (albeit with a much lower turnover number). Similar results are observed with myoglobin (data not shown), confirming that it does catalyze the oxidative

dechlorination of TCP, although again at a very minimal rate. Optimum product yield for DHP is achieved when using a 20-fold excess of hydrogen peroxide, conditions that are in fact much more physiological than the huge excess of oxidant needed to achieve optimal turnover. Preliminary studies at higher DHP concentration under the conditions that lead to generation of the non-catalytic state indicate that the unknown species spectroscopically resembles myoglobin Compound II. In addition, the spectrum does not change in the presence of carbon monoxide, ruling out formation of a stable oxyferrous state (data not shown). The nature of this species will be the focus of future studies.

DHP catalyzes the oxidative dehalogenation of a variety of halophenols [10]. This activity is achieved, despite significant differences in the proximal His environment [12,22] relative to typical peroxidases. The distal pocket of DHP is almost as hydrophobic as seen in myoglobin, and DHP has a His residue in the distal pocket positioned more like the distal His of peroxidases [12], although it lacks the adjacent Arg that is proposed to play a role in the formation of HRP Compound I [17]. The availability of cloned DHP will make it possible to prepare mutants of the enzyme to better understand how the above-mentioned structural features promote peroxidase activity in a globin.

Summary

The activity studies of recombinant DHP reported herein clearly establish that it is an enzymatically active globin capable of rapidly catalyzing dehalogenation reactions. Perhaps not surprising, given that it is a globin, DHP is quite a bit less active than HRP at catalyzing the hydrogen peroxide-dependent oxidative dechlorination of TCP. Switching to MCPBA as co-substrate, however, results in a 7-fold increase in the rate of this reaction for DHP relative to HRP. This suggests that the natural co-substrate for DHP may not be hydrogen peroxide and could instead be an alternative oxidant such as an alkyl hydroperoxide. Finally, we have observed that under optimal turnover conditions, DHP becomes trapped in a non-catalytic state (possibly DHP Compound II). Full product conversion is achieved under more physiological conditions involving a much lower excess of oxidant co-substrate. Future experiments will address many of the remaining mechanistic ambiguities.
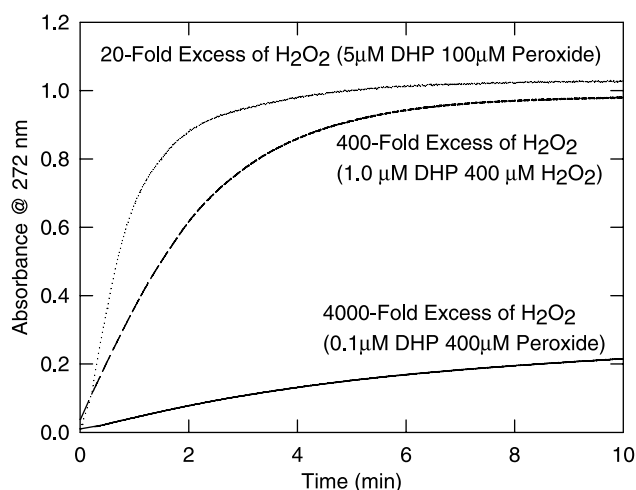

Fig. 3. UV absorption data monitored at 272 nm in 100 mM sodium acetate buffer (pH 5.4) for the oxidative dechlorination of 2,4,6-trichlorophenol (100 μM) catalyzed by recombinant dehaloperoxidase initiated by hydrogen peroxide. Solid line: [DHP], 0.1 μM; [H_2O_2], 400 μM; dashed line: [DHP], 1.0 μM; [H_2O_2], 400 μM; and dotted line: [DHP], 5.0 μM; [H_2O_2], 100 μM.

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