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**The Crystal Structure‡ and Amino Acid Sequence of Dehaloperoxidase from *Amphitrite ornata* Indicate Common Ancestry with Globins*.**

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‡The atomic coordinates of the native protein and its complex with 4-iodophenol have been deposited to the Research Collaboratory for Structural Bioinformatics Protein Databank = PDB # XXXX and PDB # XXXX respectively.

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SUMMARY

The full length, protein coding sequence for dehaloperoxidase was obtained using a reverse genetic approach and a cDNA library from marine worm *Amphitrite ornata*. The crystal structure of the dehaloperoxidase was determined by the multiple isomorphous replacement method and refined at 1.8 Å resolution. The enzyme fold is that of the globin family and together with the amino acid sequence information indicates that the enzyme evolved from an ancient oxygen carrier. The peroxidase activity of DHP arose mainly through changes in the positions of the proximal and distal histidines relative to those seen in globins. The structure of a complex of DHP with 4-iodophenol is also reported and it shows that in contrast to larger heme peroxidases DHP binds organic substrates in the distal cavity. The binding is facilitated by the histidine swinging in and out of the cavity. The modeled position of the oxygen atom bound to the heme suggests that the enzymatic reaction proceeds via direct attack of the oxygen atom on the carbon atom bound to the halogen atom.
Polychlorinated phenols and other polychlorinated aromatics of anthropogenic origin have been widely dispersed and constitute significant environmental problems. It is less known that bromoaromatics of biotic origin are also widespread and secreted as chemical warfare by a number of marine organisms. Dehalogenating enzymes are used as the first line of defense against these toxicants by organisms that live in such contaminated environments (1). We have recently discovered and characterized by a number of techniques (2-4) an enzyme with a novel function: dehaloperoxidase (DHP). DHP is isolated from *A. ornata*, a terebellid polychaete. This species does not produce halogenated compounds itself but usually co-habits estuarine mud flats with other polychaete worms, such as *Notomastus lobatus*, and hemichordata such as *Saccoglossus kowalewskyi*, which secrete large quantities of brominated aromatics and other halometabolites as repellents (5,6). The levels of DHP are very high as it represents approximately 3% of the soluble protein in crude extracts of *A. ornata*. The enzyme catalyses the oxidative dehalogenation of polyhalogenated phenols in the presence of hydrogen peroxide at a rate at least ten times faster than all known halohydrolases of bacterial origin, according to the equation:

\[
\begin{align*}
\text{Br}_3\text{OHBr}_{\text{Br}} + \text{H}_2\text{O}_2 & \rightarrow \text{Br}_{\text{Br}}\text{O}_{\text{Br}} + \text{H}^+ + \text{Br}^- + \text{H}_2\text{O} \\
\end{align*}
\]

The oxidative potential of hydrogen peroxide likely allows for the unusually high rate of this reaction as well as for the unique ability of DHP to dehalogenate fluorophenols. The enzyme has activity towards substrates with different number and positions of halogen substituents (2).
The binding of oxygen and peroxide ligands and their activation is due to the presence of heme in a variety of oxygen carriers and enzymes. This is also true for DHP, which contains one heme per subunit (3) and a histidine as the proximal Fe ligand (4). The propensity of peroxidases (and oxygenases, which tend to have a cysteinate proximal ligand) to cleave the oxygen-oxygen bond and form a high-valent iron-oxo intermediate, as opposed to globins, has been explained by the “push-pull” theory (7,8), in which crucial roles have been assigned to the proximal histidine and polar residues in the distal pocket. Peroxidases form a strong hydrogen bond between the N$_\delta$$_1$ atom of the proximal histidine and a nearby glutamate conveying partial histidinate character to the proximal histidine and making it a better electron donor (providing a better electron “push”). Conversely, globins lack a glutamate in the proximal pocket and the N$_\delta$$_1$ atom of the axial histidine only forms a weak hydrogen bond (7,8). However, more recent studies of cytochrome c peroxidase mutants indicate that the electronic “push” plays only a minor role in its catalytic activity (9). On the other hand the “pull” effect survives well a scrutiny of site directed mutagenesis (10). Here, we report structural analyses of DHP and their implications for evolutionary relationship between DHP and oxygen carriers. We also discuss how well the “push-pull” theory explains the catalytic properties of DHP. Preliminary data have been briefly communicated previously (11).

**MATERIALS AND METHODS**

*N-terminal amino acid sequencing.* Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were electroblotted onto polyvinylidene difluoride (PVDF) membrane. Edman Degradation of the PVDF blots was performed using an
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Applied Biosystems 492 Procise™ Protein Sequencing System and 610A Data Analysis software to determine NH$_2$-terminal protein sequence. The sequence of first 64 amino acids was established.

**Cloning and cDNA sequence.** A cDNA library was constructed from *A. ornata* collected at North Inlet, Georgetown, SC, USA in the Uni-ZAP XR vector (Stratagene, La Jolla, CA). A 125 bp fragment of the DHP gene was amplified from the library by PCR using primers designed from the N-terminal amino acid sequence. The fragment was labeled with fluorescein-11-dUTP using random priming (Amersham Life Science, England) and used to screen the cDNA library. Positive plaques were purified through three rounds of repeated screening. Plasmid DNA was excised using ExAssist helper phage with the SOLR strain (Stratagene, La Jolla, CA) and the sequence determined with an automated DNA sequencer (Li-COR, Lincoln, NB). The nucleotide sequence was translated into an amino acid sequence using the GCG software (12).

**DHP structure determination.** Protein was purified and assayed for activity using the same procedures as published before. Crystals with two subunits in an asymmetric part of the unit cell were obtained as reported previously (3). Briefly, an unbuffered solution containing 30% PEG8000 and 200 mM ammonium sulfate was used as the precipitant in the hanging-drop vapor diffusion method. Crystals belong to space group $P2_12_12_1$ with unit cell dimensions $a=68.5$, $b=68.4$, $c=61.1$ Å.

Even in the absence of sequence information, we suspected a structural relation to the globin family based on the protein size and the presence of the heme. However, the molecular replacement method with a myoglobin model did not provide a solution. The DHP structure was determined by MIRAS using a mercury acetate derivative (0.1 mM, 1 hour soak), a 4-iodophenol derivative (saturated, overnight soak), and anomalous scattering from heme iron atoms. Two
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mercury sites were located in a difference Patterson map and used to phase difference-Fourier and anomalous-Fourier maps in which the positions of I and Fe atoms were located. Structure-factor phases were calculated with MLPHARE (13,14) yielding a figure of merit of 0.58 and improved with solvent flattening and histogram matching in DM (14,15). An electron density map calculated at 3.0 Å resolution revealed the orientation of heme groups and location of several helices. At this stage, the similarity of DHP to the globin structure became apparent. A model of myoglobin was used to construct the molecular envelope and electron density maps were optimized using non-crystallographic symmetry averaging, solvent flattening, and phase extension to the maximum resolution of the native data set, 1.8 Å, with DM. The resulting electron density was readily interpretable. The initial model was built with only partial sequence information. The identification of residues in the stretch where the sequence was not known, based on the shape of side chains electron density and hydrogen bonding pattern turned out to be successful for ca 60% of the residues. The final model included all amino acids (137 per subunit), hemes, two sulfate ions, and 107 water molecules and was optimized using simulated annealing, positional, and B-refinements of the CNS (16) software without non-crystallographic symmetry restraints. The structure of iodophenol complex was obtained by rebuilding the native structure and refinement with the CNS. The crystallographic results are summarized in Table 1.

Insert table 1.

Illustrations. Figure 1 was prepared with the GCG software (12) and edited to include the structural information. Figures 2, 3, and 4 were prepared with the CHAIN program(17).

RESULTS AND DISCUSSION
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Amino acid sequence. Oxygen carriers in *A. ornata* have not been characterized in detail. We have isolated a protein with N-terminal sequence, DCNALDRIKVLDQQI, which is 53% identical to earth worm giant hemoglobin; others have reported a tetrameric hemoglobin with four different N-terminal sequences(18), one of which agrees with our giant hemoglobin data. These sequences, 3-4 amino acids, were too short to show any relation to DHP. *A. ornata* also has a monomeric, coelomic hemoglobin (19); the sequence of which has not yet been determined. However, the N-terminal 28 amino acid sequence of the coelomic hemoglobin from another polychaete worm, *Enoplobranchus sanguineus* (19), is 32% identical to DHP indicating that DHP evolved from an oxygen carrier. DHP has retained its ability to bind oxygen and when isolated it is in the oxy-ferrous state (4), but, in contrast to the coelomic hemoglobin, is dimeric (2). The complete sequence closest to DHP found in the SwissProt database is that of *Aplysia limacina* (sea hare) myoglobin (MBA), with 20.6% identity in a 126 amino acid overlap. Interestingly, MBA is a globin that has had its distal histidine replaced by valine (20). The alignment of the amino acid sequence of DHP with those of MBA and sperm-whale myoglobin (MYO) as well as information on 3D-structure superpositions, is shown in Fig. 1.

Insert figure 1.

Polychaete worms are well represented by the Middle Cambrian (21) about 530 million years ago, so the dehalogenase activity is probably younger than that. On the other hand the oxygen carrier function of globins is considerably older and universal (22), so it is highly likely that the DHP activity arose by a globin gene duplication and divergence.

Structure. The overall fold of DHP closely resembles that of globins despite the relatively low sequence identity. The structural differences are on the same order of magnitude as the differences between distantly related globins (Fig. 1). A least-squares superposition of the
structures of DHP and MYO, shown in Fig. 2, yielded an rms. distance between the positions of C<sub>α</sub> of 1.8 Å.

Insert figure 2.

In solution DHP is dimeric (2) and crystallizes with the dimer in an asymmetric part of the unit cell. The two subunits of DHP are very similar; their least-squares superposition shows that they are related by a two-fold non-crystallographic symmetry axis with an rms. distance between C<sub>α</sub>s of 0.25 Å and the largest differences, ca 0.85 Å, at residues 39-41, which form lattice contacts. Intersubunit contacts are relatively limited; the dimer interface involves hydrophobic interactions of the side chains of Val74 from both subunits and hydrogen bonding between the side chain of Asp72 from one subunit and the side chains of Arg112 and Asn 126 from the other. The formation of the dimer reduces the surface area by 464 Å<sup>2</sup>. The residues at the interface do not correspond to those in tetrameric and dimeric hemoglobins and the spatial arrangement of dimers is completely different.

Mechanism. The catalytic mechanism of DHP is probably similar to that to that employed by other peroxidases at least as far as the key role of a high-valent iron-oxo intermediate formed upon addition of hydrogen peroxide to the enzyme and subsequent heterolytic cleavage of the O-O bond (8). Since the reaction does not take place at the heme edge but in the distal pocket and the enzyme is isolated in the ferrous state, it may be speculated that the intermediate does not carry the oxidative equivalent in the form of porphyrin radical (Compound I) as found in peroxidases. If so, both oxidative equivalencies of peroxide would be contained on the heme iron, which cycles between II and IV oxidation states, and thus the intermediate would be Compound II. Such reactivity has previously been observed in model systems (23).
The “pull” component in peroxidases is created by the distal histidine functioning as an acid/base in proton transfer to the leaving water molecule with the guanidinium moiety of an arginine stabilizing the developing negative charge (7,8). This arrangement is functional because peroxidases do not utilize the distal cavity as an organic substrate binding-pocket but rather the reaction takes place at the heme edge (24). Cytochromes P-450, which bind organic substrates next to heme, appear to depend largely on the “push” created by the proximal cysteinate ligand (8,25). It is likely that the DHP’s utilization of the distal cavity is dynamic, and combines elements of mechanisms of both cytochrome P-450 and peroxidases. In short, DHP binds peroxide and uses the distal histidine as the “pull” to accomplish the heterolytic cleavage of the oxygen-oxygen bond. When the high-valent iron-oxo intermediate is ready, the distal histidine swings out of the cavity enabling the substrate to enter the distal pocket and undergo oxidation. This hypothesis finds strong support in the observation that the distal histidine, His 55, in the native DHP is disordered between two positions: one outside the distal pocket, the other inside, Fig. 3b. The distances between the N$_{ε2}$ atom of the distal histidine and the heme-Fe in globins are 4.1-4.6 Å while in peroxidases are larger, 5.5-6.0 Å (10). In DHP, this distance is 5.4 Å for the position inside the distal cavity. It appears that the ability of His 55 to function in the in/out mode arose as a result of a difference in the position of its C$_{α}$ when compared to MYO. This difference is ca 1 Å when the superposition that minimizes distances between equivalent C$_{α}$s of DHP and MYO is used, or 2.4 Å when the hemes are superimposed. The shift appears to be generated by the replacement of Gly that follows the distal His in MYO with Thr in DHP. This residue is located on a sharp turn of the main chain and the larger side chain of threonine pushes the main chain towards the entrance of the distal cavity creating the shift, as shown in Fig. 3b. This residue, Gly 65 in MYO, is almost completely conserved in globins that utilize distal His.
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The out of pocket location of distal histidine is not unique to DHP. It was observed in low pH (4.0) structure of myoglobin (27) in which the distal histidine was likely to be protonated.

Insert figure 3.

The content of the distal pocket in the native DHP is partially disordered and probably correlated with the disorder of the distal histidine. Nevertheless, it is apparent that the heme iron is pentacoordinated. In the structure of the complex with 4-iodophenol, Fig. 4, the positions of I-atom (11σ in the difference Fourier map) and O-atom are well established, however, the orientation of the plane of the phenyl ring is based on steric considerations. It appears to form an angle of about 45° with the plane of the heme. The distal histidine is in the position outside the distal cavity.

Insert figure 4.

On the proximal side of the heme there are significant differences between DHP and both globins and peroxidases. In DHP, the proximal histidine does not form a strong hydrogen bond to a nearby carboxylate as is in peroxidases. Instead, the imidazole ring is rotated by a ca 60° with respect to the position in globins and its H-atom points directly into the oxygen atom of the Leu 83 peptide carbonyl. The resulting H-bond is shorter, and likely stronger, than in myoglobin where this H-bond is bifurcated, Fig. 3a. The reorientation of the imidazole ring is facilitated by the replacement of the last turn of the F-helix of myoglobin with a very short 3₁₀ helix and, remarkably, a shift of the proximal histidine in the sequence by two residues, Fig. 1. It may also be suspected that the lone electron pairs of S₅ of Met 86, one of which is in contact with His 89, contribute to the “push” through charge transfer. Studies using vibrational spectroscopy (27) have shown that the Fe-N bond, the strength of which reflects the electron “push”, is indeed
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stronger in DHP than in globins, although not as strong as in peroxidases where the proximal histidine has a partial histidinate character.

DHP offers a valuable perspective, as a chimera of sort, on one of the most thoroughly studied structure-function relationship, that of the protoporphyrin IX properties in globins versus peroxidases. The distal pocket is almost as hydrophobic as in globins but the distal histidine is positioned more like in peroxidases. It appears that the lack of an auxiliary, polarizing arginine has to be compensated on the proximal side. Indeed, there are large differences there between classical globins and DHP. They suggest, however, that this is not so much electronic “push” but rather the different coupling of protein molecular dynamics to the heme through repositioning of the proximal histidine. Such concept is in agreement with the studies of the variants of peroxidases with altered proximal environment of the heme and supports the proposition that the mechanical coupling of the heme iron plays an important role (9). Thus perhaps it is time to replace the “push and pull” theory in peroxidases with a “pull and pull” approach.

It is likely that the DHP function arose to dehalogenate biogenic bromophenols, but the enzyme also can dehalogenate trichlorophenols and a number of other anthropogenic pollutants. The enzyme efficiency does not change significantly with the position and number of halogen atoms at the phenol ring (2). It appears that the enzyme simply binds the organic substrate close to a very active oxygen atom and works without a system of complicated hydrogen bonds and other interactions that are characteristic of enzymes with more sophisticated mechanisms. This also offers hope that appropriate modifications of the residues lining the distal cavity can broaden or modify its specificity. Efforts in other laboratories to enhance the peroxidase activity of MYO have yielded only limited success (10). The structure of DHP offers excellent clues how to proceed towards this goal.
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Footnotes

1The abbreviations used are: DHP, dehaloperoxidase from *Amphitrite ornate*; MBA, myoglobin from *Aplysia limacina* (sea hare); MYO, myoglobin from sperm whale; PVDF polyvinylidene difluoride.
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Figure captions

Fig. 1. Alignment of the amino acid sequences of DHP, MYO, and MBA. Underlined are conserved structural elements, defined as those for which least-squares superpositions gave a distance between the corresponding Cα-atoms less than 2 Å. Bold letters indicate distal cavity residues, bold letters in italics the proximal histidine (89) and its neighbors (84, 86, 88, and 97).

Fig. 2. Stereo view of the least-squares superposition of Cα-plots of DHP (in bold) and MYO.

Fig. 3. Comparison of the heme environment in DHP and globins. Stereo views shows the least-squares superposition of the heme of DHP (in bold) and MYO (Protein Data Bank entry code 1mbo). a) The distal environment of the heme. Electron density is from a map calculated with F0 – Fc coefficients with the imidazole ring of His 55 omitted from the model. It is contoured at 2.3 σ level and shows the two alternative positions of the distal His 55. b) The proximal environment of the heme. In DHP the distance between Nδ1 of His 89 and O of Leu 89 is 2.7 Å and the hydrogen atom (not shown) points into the acceptor. In MYO the corresponding hydrogen bond is bifurcated with distances from Nδ1 to O of Leu 89 and Oγ of Ser 92 both equal 2.9 Å and the hydrogen atom pointing in between the receptors. Large differences in the conformation of the main chain are apparent. c) Electron density map calculated with 2F0 – Fc coefficients and contoured at 0.9 σ level for the part of the model shown in b.
Fig. 4. Stereo view of 4-iodophenol bound in the distal pocket. The density, contoured at 2.0 \( \sigma \) level, is from an annealed omit map calculated with \( F_o-F_c \) coefficients. The distal histidine, His55, is out of the pocket.
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Table 1. Crystallographic data collection, phasing, and refinement statistics.

|                              | DHP    | DHP Hg\(^{2+}\) | DHP 4-iodophenol |
|------------------------------|--------|-----------------|------------------|
| Resolution of data (Å)       | 1.78   | 2.1             | 2.5              |
| R\(_{\text{merge}}\) (%)     | 5.1    | 6.2             | 6.8              |
| Reflections total/unique     | 66,563/24,637 | 42,634/17,139 | 20,811/8,709    |
| R\(_{\text{iso}}\)           |        | 16.4            | 18.6             |
| Resolution for MIR/AS (Å)    | none/10-4.5 | 10-3/10-4.5     | 10-3/10-4.5      |
| R\(_{\text{Cullis}}\) (acen)/R\(_{\text{Cullis}}\) (cen)/R\(_{\text{Cullis}}\) (ano) (%) | 0/0/85 | 62/55/80        | 91/72/90        |
| Number of sites iso/ano      | 0/2    | 2/4             | 2/4              |
| R\(_{\text{cryst}}\)/R\(_{\text{free}}\) | 0.197/0.225 | 0.201/0.219    |                  |
| Rms. deviation bond lengths/angles | 0.005/1.0 |                  | 0.011/1.9       |
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1 51
dhp GF KQDIATIRGD LRTYAQDFIFL AFLNKYPDER RYFKNYVG.K SDQELKSMAK
mba SLSAAEADLA GKSAPVFAN KNANGLDFLV ALFKEFPDSA NEFADPKG.K SVADIKASP
myo VLEGQWGLVL VHVWAKVEAD VAGHQDILIL RLFKSHPETL EKFDRFKLK TEAEMKASED

1 60
52 108
dhp FGDHTKEVFN LMMEVADRAT DCVPLASDAN TLVQMKQH...SSLTTGNFE KLFWALKVEYM
mba LRDVSSRIFT RLENFVNNAA NAGKMSAMLS QFAKE...HV GFGVQSAQFE NVRSMFGFV
myo LKKHGVVTLL ALGAILKKK...GHHEAELK PLAQSHAT KHKIPIKYLE PISEAIIHV

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