Acta Cryst. (1996). D**52**, 1191–1193

Crystallization and initial spectroscopic characterization of the heme-containing dehaloperoxidase from the marine polychaete *Amphitrite ornata*

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(*Received 14 February 1996; accepted 14 June 1996*)

Abstract

The heme-containing dehaloperoxidase from *Amphitrite ornata* was crystallized from an unbuffered solution containing 30% PEG 8000 and 200 m*M* ammonium sulfate by the hanging-drop vapor-diffusion method. Dark-red bipyramidal crystals are orthorhombic in space group $P2_12_12_1$ with unit-cell dimensions $a = 68.5$, $b = 68.4$ and $c = 61.1$ Å. The asymmetric unit contains two subunits related by a non-crystallographic twofold axis. The crystals scatter beyond 2 Å resolution. The native data have been collected and one single-site mercury derivative has been found. SIRAS phasing was used to determine the positions of the heme Fe atoms and structure determination is in progress. A preliminary spectroscopic investigation indicates that the heme is protoporphyrin IX and its coordination sphere resembles that of a typical heme peroxidase, *i.e.* histidine ligated. Detailed spectroscopic and electrochemical studies are now under way.

1. Abbreviations

DHP, the dehaloperoxygenase from *A. ornata*; MIR, multiple isomorphous replacement; SIR, single isomorphous replacement; SIRAS, single isomorphous replacement supplemented with anomalous scattering; SAS, single-wavelength anomalous scattering; PEG, polyethylene glycol; MCD, magnetic circular dichroism.

2. Introduction

Many species of marine worms occupying coastal sediments produce and secrete a variety of haloaromatic secondary metabolites. These compounds include bromophenols, bromopyrroles, bromoindoles, bromohydroquinones, and bromobenzylalcohols (Gribble, 1994; King, 1986; Woodin, 1991; Woodin, Marinelli & Lincoln, 1993), all of which have characteristic unpleasant odors and are presumably toxic to many other organisms. Sediments contaminated with these bromoaromatic toxins are repellent to juveniles of non-bromometabolite-producing invertebrate species (Woodin *et al.*, 1993). As a consequence, areas inhabited by bromometabolite-producing worm species and contaminated with these noxious compounds may select for organisms tolerant of these toxins.

Amphitrite ornata is a terebellid polychaete which produces no detectable volatile halometabolites (Lincoln, Fielman, Marinelli & Woodim, unpublished data) but often inhabits sediments contaminated with bromometabolites from other species. Recently, Chen, Woodin, Lincoln & Lovell (1996) have isolated from this organism a protein with novel enzymatic activity: dehaloperoxidase (DHP). They proposed that this activity is responsible for the tolerance of *A. ornata* to haloaromatics. DHP catalyzes a H_2O_2-dependent oxidative dehalogenation of a variety of halophenols, including mono-, di- and tri-substituted halophenols with iodine, bromine, chlorine and fluorine substituents.

DHP is a homodimer of molecular weight 31 kDa and subunit $M_r = 15\,529$ Da as determined by electrospray mass spectroscopy. A search of the Swiss-Prot database using the sequence of first 41 amino acids has not shown any proteins with significant sequence similarity. Although there are several heme proteins of similar size with known tertiary structures, none of them is an enzyme. Interestingly, the chloroperoxidase from *Notomastus lobatus* has a heme-containing subunit of similar size (Chen, Lincoln, Woodin & Lovell, 1991). Other heme peroxidases with structure determined by X-ray crystallography all have much larger molecular weights and it is unlikely that they are homologous to the *A. ornata* DHP. It is thus of interest to determine whether the enzymatic activity of DHP arose from a molecular scaffolding similar to that of the cytochromes or globins, or if it involves a new heme-binding fold. We initiated the crystallographic determination of the DHP structure in order to study the mechanism of catalysis and to elucidate its evolutionary relationship to other heme proteins. Magnetic circular dichroism spectroscopy has an electronic fingerprinting ability much more powerful than electronic absorption spectroscopy (Sono & Dawson, 1987; Dawson & Dooley, 1989) and is used here as a probe of the axial ligation of the heme in DHP.

3. Methods

DHP was purified from extracts of *A. ornata* as previously described (Chen *et al.*, 1996). The vapor-diffusion method in the hanging-drop setup (McPherson, 1990) was used to scan through a variety of crystallization conditions. Typically, a 5 µl drop of DHP solution containing 5–10 mg ml^{-1} protein was mixed with the same volume of precipitant solution and equilibrated in Linbro plates against 0.6 ml of precipitant solution at 277 K. Various buffers, pH values and precipitants were tested and the best results were obtained using an unbuffered solution of 200 m*M* ammonium sulfate and 30% PEG 8000. This solution (Crystal Screen No. 31 from Hampton Research, Jancarik & Kim, 1991) has a pH of 6.5.

Preliminary X-ray diffraction experiments were carried out using a Huber oscillation-precession camera and graphite-monochromated Cu $K\alpha$ radiation generated by a Rigaku RU200 rotation-anode source. A data set of the native enzyme was collected with a R-AXIS II area detector positioned at $\theta = 0°$ and a crystal-to-detector distance of 90 mm.

Spectroscopic samples were handled at 277 K at concentrations of approximately 45 µ*M* in 100 m*M* potassium phosphate

buffer pH 5.0. Heme identification (as iron protoporphyrin IX) and concentration determinations were accomplished by the pyridine hemochromogen method (Antonini & Brunori, 1971). Electronic absorption spectra were obtained with a Cary 210 spectrophotometer interfaced to an IBM PC. MCD spectra were recorded at a magnetic field strength of 1.41 T with a JASCO J500A spectropolarimeter equipped with a JASCO MCD 1B electromagnet and interfaced to a Gateway 2000 4DX2–66V PC through a JASCO IF-500–2 interface unit. All spectroscopic data handling was accomplished using custom data manipulation software and has been described elsewhere (Huff, Chang, Cooper, Smith & Dawson, 1993).

4. Results and discussion

The crystals appeared within a few days, typically after one week, and grew for a period of about three weeks. They were red to black in color depending on their thickness, with bipyramidal, pseudo-octahedral morphology, reaching up to 0.4 mm. The crystals were obtained from several protein preparations under the same crystallization conditions. Still photographs recorded with a crystal-to-film distance of 100 mm showed diffraction to a resolution better than 2.8 Å. The initial precession photographs taken at 6 Å resolution were interpreted as a tetragonal lattice with $a = b = 68$ and $c = 61$ Å. A total of 48 629 reflections with $F^2/\sigma > 1.0$ were measured to 2.0 Å resolution. An attempt to average reflections in the tetragonal system using either $4/mmm$ or $4/m$ Laue symmetry yielded $R_{merge} = 0.3$. However, when the symmetry was lowered to orthorhombic, only 206 reflections were rejected, yielding $R_{merge} = 0.055$ and 17 759 independent observations in an averaging procedure. The data set is 90% complete to 2.0 Å; in the last shell, 2.25–2.01 Å, completeness is 85% with an average $F^2/\sigma = 5$. The crystals belong to the space group $P2_12_12_1$ with $a = 68.5$, $b = 68.4$ and $c = 61.1$ Å. Assuming two subunits per asymmetric part of the unit cell and using $M_r = 16$ kDa per subunit gave 45% of solvent in the crystals (Matthews, 1968). The self-rotation function, calculated with 10–4 Å resolution data, showed a peak of a height 0.56 of the crystallographic twofold peak. This indicated the presence of a non-crystallographic twofold axis parallel to [110] direction.

Since the size of the molecule, the presence of the heme, and the MCD data suggested some similarity to myoglobin, we attempted to solve the structure using the molecular replacement method with a model of myoglobin. There were no significant peaks in the rotation function so we proceeded to solve the phase problem with the MIR method. The crystals were soaked in a number of heavy-atom reagents to obtain suitable derivatives. Mercury acetate yielded a useful derivative when a relatively short soaking time, 1 h, was used. The Patterson map was interpreted with two sites per asymmetric unit. Anomalous difference Fourier maps, calculated at 3.5 Å resolution, phased with SIRAS of the mercury derivative clearly showed the positions of the heme Fe atoms in both native and derivative data. The Fe peaks were the two strongest peaks and were 1.6 and 1.3 times the highest noise in the native data and 1.6 and 1.4 in the Hg derivative data. The phasing was improved by the incorporation of Fe atoms into the phasing model and including the anomalous signal for both data sets. The absolute configuration was established using the method described by Blundell & Johnson (1976). A difference anomalous Fourier map for the derivative was recalculated and a comparison of the heights of the Fe and Hg peaks was used to estimate the occupancies for the Hg atoms at about 0.6 and 0.3. A native Fourier map calculated with the SIRAS/SAS phases showed disk-shaped electon density for hemes and fragments in which the peptide has clearly α-helical conforma-

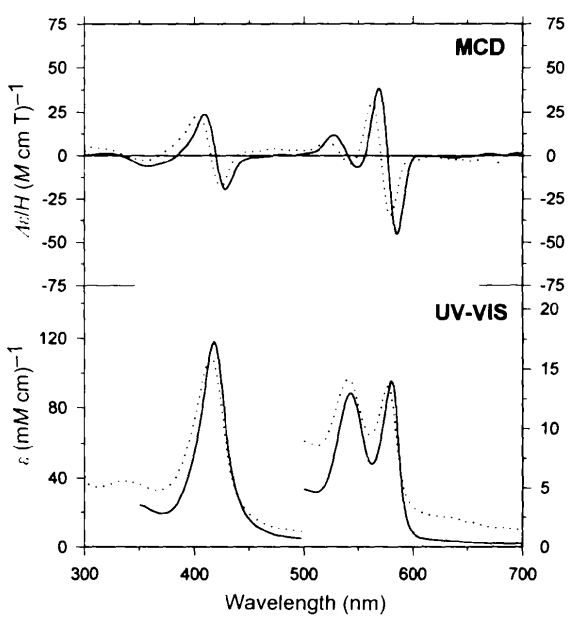

Fig. 1. MCD and electronic absorption spectra of native (as isolated) DHP (45 μ*M*) (. . .) in 100 m*M* potassium phosphate pH 5.0 and of oxyferrous horseradish peroxidase (55.9 μ*M*) (—) in 100 m*M* potassium phosphate pH 7.0 (as previously reported by Dawson, Kadkhodayan, Zhuang & Sono, 1992).

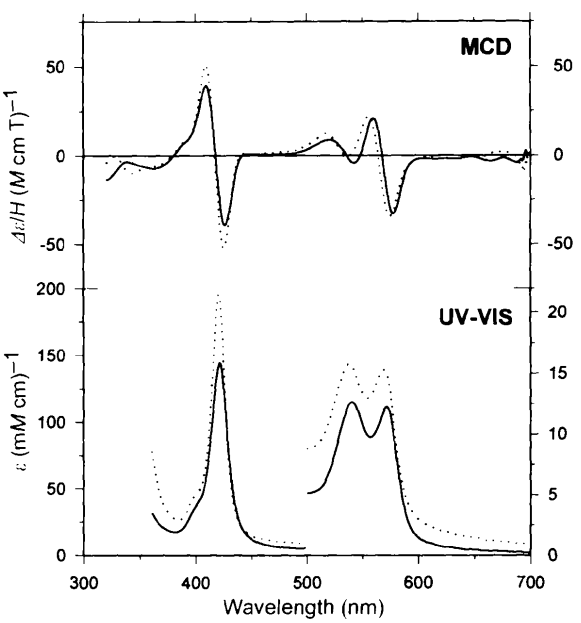

Fig. 2. MCD and electronic absorption spectra of carbonmonoxyferrous DHP (45 μ*M*) (. . .) in 100 m*M* potassium phosphate pH 5.0 and of carbonmonoxyferrous horseradish peroxidase (55.9 μ*M*) (– –) in 100 m*M* potassium phosphate pH 7.0 (as previously reported by Dawson *et al.*, 1992)

tion. The program system *PHASES* (Furey, 1994) was used for these calculations.

Initial tests suggested that the protein also has chloroperoxidase activity. During the heavy-atom search we therefore soaked crystals in 100 m*M* sodium iodide. No binding was detected. Crystals were also soaked in saturated solution of 4-iodophenol, which is only poorly soluble in the artificial mother liquor, 35% PEG 8000. A difference Fourier map and also an anomalous difference Fourier map indicated binding at two sites that was estimated at 0.3 and 0.15 occupancy. The peaks were close to the positions of the Fe atoms. While the incorporation of this information into phasing did not improve the quality of the native Fourier map significantly, it clearly confirmed the binding of a possible substrate.

The positions of heavy atoms were used to determine the the location of the non-crystallographic twofold axis. The transformation is **R**(–0.0733, 0.9973, –0.0097, 0.9973, 0.0733, –0.0104, –0.0097, –0.0104, –0.0097, –0.0104, –0.9999), **T**(–15.96, 14.99, 16.85). The transformation places α-helices modelled in one subunit into suitable electron density of the other. Efforts to determine the molecular envelope and use non-crystallographic symmetry averaging to improve electon-density maps are under way.

The heme in DHP was identified through the pyridine hemochromogen method as protoporphyrin IX (data not shown). Typical heme-containing peroxidases such as horseradish peroxidase employ histidine as the fifth ligand to the heme iron (protoporphyrin IX) and are isolated in the ferric state (Theorell, 1947). Electronic absorption and MCD spectra of native DHP (as isolated) instead resemble those of the oxyferrous adduct of horseradish peroxidase (Fig. 1). Confirmation that native DHP exists in the oxyferrous state was obtained by the immediate formation of a carbonmonoxyferrous adduct (as judged by its spectroscopic similarity to carbonmonoxyferrous horseradish peroxidase, Fig. 2) upon bubbling of a solution of the native enzyme with carbon monoxide. Carbon monoxide only binds to ferrous heme (Antonini & Brunori, 1971). The spectroscopic similarities between the parallel derivatives of ferrous horseradish peroxidase and DHP (Figs. 1 and 2) provide evidence for proximal histidine ligation in the latter. The fact that the enzyme is isolated in the oxyferrous state suggests that the heme iron may have an unusually high reduction potential since no exogenous reductants are present during purification of the enzyme.

Detailed spectroscopic and electrochemical studies of DHP's heme prosthetic group are now underway to complement the ongoing crystallographic study in efforts to understand the enzymatic activity and evolutionary origins of this unusual enzyme.

We thank Dr Hengmin Ke and Dr Alexander Tulinsky for help with the data collections carried out in their laboratories as well as Eric D. Coulter and Dr Masanori Sono for helpful discussions. This work was supported in part by the National Science Foundation (grants DMB-9018114 and OCE-9201857), National Institutes of Health (GM26730) and Environmental Protection Agency (grant R82–4776–010).

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