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Preliminary X-ray analysis of a new crystal form of the vanadium-dependent bromoperoxidase from *Corallina officinalis*

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Abstract

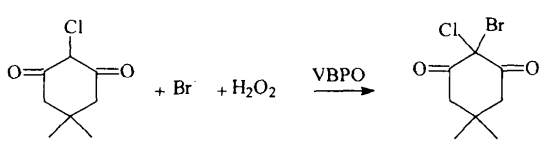
A new crystal form of the vanadium-dependent bromoperoxidase from *Corallina officinalis* has been obtained. The crystals exhibit a 'teardrop' morphology and are grown from 2 *M* ammonium dihydrogen phosphate pH 5 and diffract to beyond 1.7 Å resolution. They are in tetragonal space group $P4_222$ with unit-cell dimensions of $a = b = 201.9$, $c = 178.19$ Å, $\alpha = \beta = \gamma = 90°$. A 2.3 Å resolution native data set has been collected at the Hamburg Synchrotron. A mercury derivative data set has also been collected, and the heavy-atom positions have been determined. The self-rotation function and the positions of the heavy atoms are consistent with the molecule being a dodecamer with local 23 symmetry.

1. Introduction

Halometabolites, principally containing chlorine and bromine, occur extensively throughout nature and are also important synthetic organic tools (Gribble, 1992). The haloperoxidase enzymes are also widely distributed in nature having so far been isolated from fungi (Hollenberg & Hager, 1978), macroalgae (Hewson & Hager, 1980), bacteria (Van Peé & Lingens, 1985) and mammals (Agner, 1941). Haloperoxidase enzymes utilize hydrogen peroxide to catalyze the oxidation of halides and the halogenation of organic substrates, [1]. A typical reaction catalyzed by haloperoxidase enzymes is the bromination of monochlorodimedone to give dihalodimedone, [3]. If there is no available organic halogen acceptor then hypohalous acid is produced, [2].

$$RH + H_2O_2 + HX \rightarrow RX + 2H_2O \quad [1]$$

$$H_2O_2 + X^- + H^+ \rightarrow HOX + H_2O \quad [2]$$



Monochlorodimedone Dihalodimedone [3]

Haloperoxidase enzymes have been classified into three groups. Those which contain a heme group, those which contain a metal ion and those which contain no metals at all. Those enzymes which contain a metal other than as part of a heme unit usually contain vanadium as vanadate (Van Peé, 1990). The first vanadium-dependent haloperoxidase to be identified was isolated from the brown macroalga *Ascophylum nodosum* (Wever *et al.* 1985). Subsequently vanadium dependent haloperoxidases were identified in a number of different types of algae (Vollenbroek *et al.*, 1995) and also fungi (Van Schijndel *et al.*, 1993) and lichen (Plat *et al.*, 1987). The vanadium-dependent bromoperoxidase from *C. officinalis* has been well characterized (Sheffield *et al.*, 1993). It is thermostable retaining activity at 363 K and is stable in organic solvents such as ethanol, propanol and acetone (Rush *et al.*, 1995). As a result there is a great interest in the application of the enzyme in biotransformation reactions (De Boer *et al.*, 1987; Itoh *et al.*, 1988; Coughlin *et al.*, 1993). Halogenated nucleic acids have been synthesized using this enzyme, which are of use as anti-cancer and anti-viral agents.

The X-ray structure for the chloroperoxidase from the fungus *Curvularia inaequalis* has been reported by Messerschmidt & Wever (1996). This is a monomeric vanadium-dependent enzyme and may well differ in structure from the macroalgal bromoperoxidases. To date this is the only structure known for a vanadium-dependent haloperoxidase from any organism, although preliminary X-ray data exists for the monomeric enzyme from the phaeophyte *Ascophyllum nodosum* (Muller-Fahrnow *et al.*, 1988). Electron microscopy has been used to obtain structural information on the haloperoxidase from *Corallina pilulifera* (Itoh *et al.*, 1986). This has suggested that the *C. pilulifera* enzyme is a dodecamer of two superimposed hexagonal rings. The enzyme from *C. officinalis* is also believed to be a dodecamer of total molecular weight 740 kDa and subunit mass 64 kDa.

Cubic crystals have previously been grown from the *C. officinalis* enzyme using PEG 6000 and 0.4 *M* KCl in 50 m*M* Tris–H_2SO_4, pH 6.8 (Fig. 1*a*) (Rush *et al.*, 1995). These crystals were stable and diffracted beyond 2 Å resolution at a Synchrotron source. A native data set was collected to 2.8 Å resolution. The unit-cell dimensions were $a = b = c = 309.36$ Å and the space group was either $I23$ or $I2_13$. This crystal form was poorly reproducible.

Here we report a new crystal form of the enzyme which diffracts to high resolution. Phases have been determined from a mercury derivative of this tetragonal crystal form. These phases have subsequently been improved using sixfold averaging.

2. Methods and materials

The haloperoxidase enzyme was extracted from 700 g of the macroalgae *C. officinalis* collected from Ladrum Bay, Devon, UK, in mid-winter when enzyme activity is maximal. The fresh washed fronds were macerated using a mortar and pestle in buffer *A* (50 m*M* Tris–H_2SO_4, pH 8.0). Cell debris was removed by centrifugation at 4 000 rev min^{-1}, for 20 min. The resulting crude protein extract was made

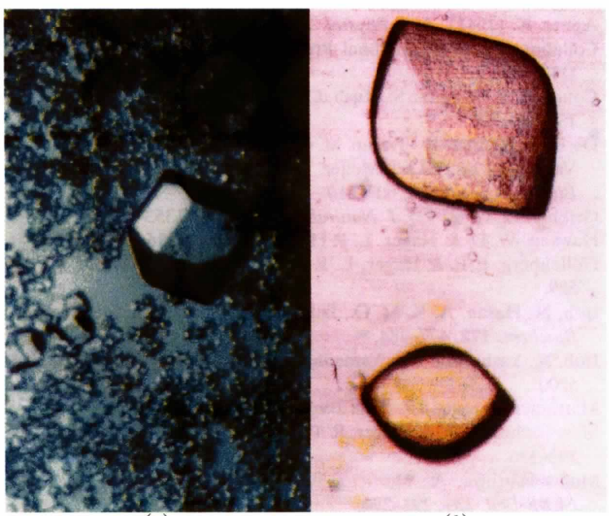
Table 1. *Data collection and processing statistics*

	Native	Mercury acetate derivative
Wavelength (Å)	0.93	0.99
Crystal-to-detector distance (mm)	200	200
Resolution (Å)	20–2.3	20–2.5
No. of measured reflections	622828	791552
No. of unique reflections	156862	126445
Completeness (all data) (%)	96.8	99.7
Completeness (highest resolution shell) (%)	95.2	99.7
$\langle I/\sigma(I)\rangle$ (all data)	6.62	9.29
$\langle I/\sigma(I)\rangle$ (highest resolution shell)	2.28	2.79
$I > 2\sigma(I)$ (%)	66.7	72.1
R_{sym} (all data)† (%)	15.7	16.4
R_{sym} (highest resolution shell)† (%)	51.3	53.8
R_{iso} (20–3 Å) (%)		16.8

† $R_{sym} = \sum(\sum_i |I_i - \langle I\rangle|)/\sum\langle I\rangle$ where $\langle I\rangle$ is the mean of the intensity measurements, I_i, and the summation extends over all reflections.

60% saturated with respect to enzyme-grade ammonium sulfate. After stirring for 1 h the extract was centrifuged at 9 600 rev min^{-1}, for 45 min. The pellet was resuspended in a minimum of buffer and dialysed, overnight, against buffer *A* containing 1 m*M* sodium vanadate. A FFQ™ Sepharose column (2.6 × 15 cm), equilibrated in buffer, was loaded with the dialysed sample. Protein was eluted with a linear gradient of 0–1 *M* KBr in buffer *A* over ten bed volumes, with the VBPO activity eluting at 0.3 *M* KBr. Active fractions were pooled and concentrated using 80% ammonium sulfate. This was then loaded onto a Phenyl Sepharose CL-4B column (2.6 × 15 cm) which had been equilibrated with 10% ammonium sulfate in buffer *A*. Protein was eluted with a negative linear gradient from 10 to 0% ammonium sulfate, with the VBPO activity eluting between 4 and 0% ammonium sulfate. Active fractions were again pooled and concentrated using 80% ammonium sulfate. The pellet was resuspended in a minimum of buffer *A* and applied to a Sepharose 6B gel-filtration column (1.5 × 30 cm). Fractions with activity were collected and applied to a Sephacryl S-1000HR gel-filtration column (2.6 × 25 cm). Active fractions from this final step were pooled and concentrated by centrifugation using Amicon centricons (10 kDa cut-off). The purified protein sample ran as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with a molecular weight of 64 kDa.

For the purposes of crystallization the protein was adjusted to 7 mg ml^{-1} in buffer *A*. Crystallization trials were initially conducted using the Hampton Crystal Screen I, Magic 50 conditions. The crystals used for data collection were grown by hanging-drop vapour-phase diffusion at 291 K from 2 *M* ammonium dihydrogen phosphate in 0.1 *M* Tris–HCl final pH 5. Crystals were harvested into a mother liquor containing 2.1 *M* ammonium dihydrogen phosphate, 0.1 *M* Tris–HCl pH 5. The mercury derivative was prepared by soaking a crystal harvested in mother liquor containing 10 m*M* mercury acetate for 1 h before data collection.



(*a*) (*b*)

Fig. 1. Two distinct crystal forms of vanadium dependent haloperoxidase from *C. officinalis*. (*a*) The previously reported cubic crystals, (*b*) the new 'teardrop' morphology crystals.

3. Results and discussion

Large crystals (0.6 × 0.6 × 0.4 mm) grew in three weeks from 2 *M* ammonium dihydrogen phosphate in 0.1 *M* Tris–HCl and continued to grow for a further eight months. Crystals had a 'teardrop' morphology, as shown in Fig. 1(*b*).

Data were collected in house on a Siemens HiStar Area Detector using Cu $K\alpha$ radiation from a rotating-anode generator. A complete data set was collected from a single large crystal to 3.7 Å resolution. Data were processed using the *SAINT* program (*SAINT*, 1993) and resulted in an R_{merge} of 12.1%. The unit-cell dimensions were determined to be $a = b = 201.9$, $c = 178.19$ Å, $\alpha = \beta = \gamma = 90°$, in either tetragonal space group $P4_222$ or $P4_22_12$.

The native Patterson contained a peak at position $\frac{1}{2}$, $\frac{1}{2}$, 0 with a peak height a third of the origin peak. This indicates that this crystal form has a pure translational non-crystallographic symmetry. This pseudo-*C*-centering results in alternate weak and strong reflections in the data.

The self-rotation function calculated using *POLARRFN* (Collaborative Computational Project, Number 4, 1994) at κ = 120° (Fig. 2) showed four very strong peaks at almost the same height as the crystallographic peaks for κ = 90° indicating the presence of four threefold non-crystallographic symmetry axes. This is not consistent with the dodecameric structure being composed of two stacked hexagonal rings as had originally been presumed. A model in which the molecules are arranged with local 23 symmetry is consistent with the self-rotation functions.

Calculations of the solvent content (Matthews, 1968) showed that there could be between three and eight molecules in the asymmetric unit. The presence of the non-crystallographic threefold axes and the pseudo-symmetry suggests that there are six molecules in the asymmetric unit with a solvent content of 48%.

Crystals diffracted to beyond 1.7 Å on the Wiggler beamline BW7B at Hamburg. The crystals were unstable to intense radiation and so only a few degrees of data could be collected from each crystal on the Wiggler beamline. Attempts to freeze the crystals for data collection resulted in a large increase in mosaicity of the crystals and poor diffraction. A complete native data set to 2.3 Å resolution was collected from a single crystal at room temperature on the less intense X-31 beamline at Hamburg. Screening for heavy-atom derivatives 'in house' showed that 10 m*M* mercury acetate was an isomorphous derivative. A complete data set for this derivative was also collected to 2.5 Å resolution. Data were collected at 0.93 Å for the native and at 0.99 Å for the mercury derivative in order to optimize the anomalous scattering. Data were processed and reduced using the *DENZO* program, (Otwinowski & Minor, 1997; Collaborative Computational Project, Number 4, 1994) the resulting statistics for the native and derivative data sets are given in Table 1. The R_{sym} values for both data sets are higher than usual for a data set collected from a synchrotron source. This is possibly a result of the pseudo-centering. Examination of the systematic absences showed that the space group was $P4_222$.

It is possible to use a reduced cell for the data where only the strong reflections are used. The reduced cell is tetragonal with dimensions $a = b = 143.8$, $c = 178.19$ Å. Heavy-atom positions were first determined in the reduced cell and then in the full cell using *SHELXS* (Sheldrick, 1990). These positions confirm the 23 local symmetry and that the molecule is a dodecamer. The phasing power for this derivative is 1.14 between 10 and 3.5 Å. Inspection of the electron density after sixfold averaging has revealed some secondary-structure elements. Model building is currently in progress. In addition a search for suitable freezing conditions for the crystals is being carried out in order to prolong their lifetime during data collection on high-intensity beamlines.

There are two mechanisms reported for the haloperoxidase enzymes, one involving an enzyme-bound intermediate and the other the production of hypohalous acid. Unravelling the potentially complex structure of the oligomeric bromoperoxidases will aid in the determination of the enzyme mechanism and establish the function of the vanadium within the enzyme. It will also aid in directing the use of this enzyme in biotransformation reactions.

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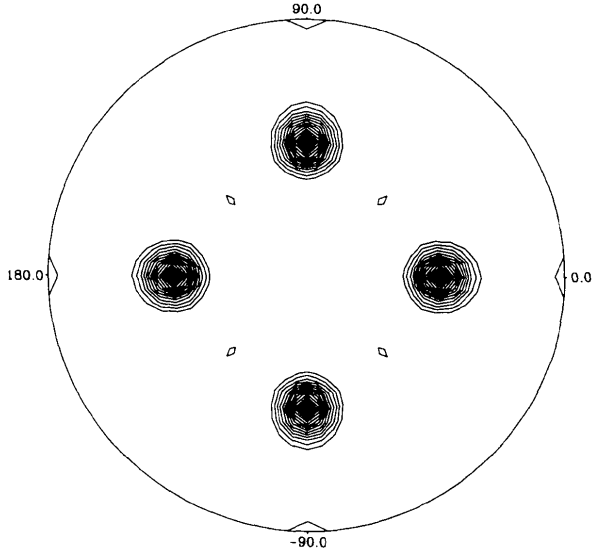

Fig. 2. A stereographic plot of the κ = 120° section of the self-rotation function for the vanadium-dependent bromoperoxidase native data (40–4 Å). The positions of the four non-crystallographic threefold axes can be clearly seen. Plot contoured at 5σ levels above 10σ.

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