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Crystallization and preliminary X-ray diffraction analysis of a thermostable D-hydantoinase from the mesophilic *Bacillus* sp. AR9

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D-Hydantoinase catalyzes the conversion of DL-hydantoin derivatives to the corresponding optically pure *N*-carbamoyl amino acids, the first step in the industrial preparation of optically pure amino acids using biological catalysts. A thermostable D-hydantoinase from the mesophilic bacteria *Bacillus* sp. AR9 has been crystallized in three different crystal forms. The hexagonal faced crystals were the best looking, but did not diffract. One of the crystal forms is star-shaped and appeared to be twinned, but diffracted as a single crystal to a resolution of 2.3 Å. These crystals belong to space group $P6_4$ and have unit-cell parameters $a = b = 129.55$, $c = 102.86$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$.

1. Introduction

Synthesis of chiral compounds by organic synthetic methods always yields racemic mixtures. The use of enzymes to produce chiral molecules is increasing owing to biotechnological advances (Margolin, 1993). D-Hydantoinase (EC 3.5.2.2) is one such enzyme that is used in stereoselective hydrolysis of D- or L-hydantoin derivatives to optically pure *N*-carbamoyl amino acids. The product can be further hydrolyzed to optically pure amino acids enzymatically using carbamoylase (Olivieri *et al.*, 1981). Hydantoinases are useful in the cost-effective synthesis of natural and non-natural amino acids on an industrial scale.

All D-hydantoinases are reported to have broad substrate specificity and are able to cleave a number of natural substrates as well as many five-substituted hydantoin derivatives, including alkyl- or aryl-substituted hydantoins (Syldatk *et al.*, 1990). Biochemical studies and sequence analyses show that they belong to the aminohydrolases, which are functionally related to each other and are structurally related to urease (Kim & Kim, 1998; Holm & Sander, 1997). Very recently, D-hydantoinase structures from *Thermus* sp. (Abendroth, Niefind & Schomburg, 2002) and *Bacillus stearothermophilus* (Cheon *et al.*, 2002) have been reported. The structure of the L-hydantoinase from *Arthobactor aurescens* has also been reported (Abendroth, Niefind, May *et al.*, 2002). The molecular structure of a D-hydantoinase from a further *Bacillus* sp. will increase understanding of the function and mechanism of the enzyme and may allow it to be engineered in order to enhance its activity. Here, we report the preliminary X-ray analysis of a thermostable D-hydantoinase from the mesophilic *Bacillus* sp. AR9 (Sharma & Vohra, 1997).

2. Materials and methods

The D-hydantoinase (HYD) was purified from *Bacillus* sp. AR9, identified and characterized as described by Sharma & Vohra (1997). Briefly, the HYD gene was cloned by PCR amplification into the *Nhe*I and *Eco*RI sites of pET28a vector with a N-terminal histidine tag. The enzyme was expressed under the T7 promoter in *Escherichia coli* BL21(DE3) and induced with 1 m*M* IPTG. The expressed enzyme was purified on a Ni–NTA column. The protein was dialyzed against the crystallization buffer and concentrated. The protein concentration used for crystallization was 5 mg ml^{-1} in 30 m*M* Tris buffer pH 8.0 with 0.5 m*M* $MnCl_2$. Crystallization of HYD was set up at 300 K using the hanging-drop vapour-diffusion method with a sparse matrix of premixed solutions (Jancarik & Kim, 1991). Three different crystal morphological forms were observed using different crystallization conditions (Fig. 1). Star-shaped crystals (from 100 m*M* Tris buffer pH 8.5, 200 m*M* $MgCl_2$, 20% PEG 400) started appearing within 24 h and reached their final size in about a week (Fig. 1*a*). Rod-shaped crystals (from 100 m*M* imidazole pH 8.0, 200 m*M* CsCl, 20% PEG 3350; Fig. 1*c*) and hexagonal-faced crystals [from 100 m*M* sodium acetate buffer pH 4.1, 0.8 *M* $(NH_4)_2PO_4$; Fig. 1*b*] also appeared under different conditions after two and half months. Star-shaped crystals are of good quality and were above 0.5 mm in all dimensions (Fig. 1*a*). Although the hexagonal-faced crystals were about 0.6 mm in size, they did not show any diffraction. The rod-shaped crystals were very small (0.1 mm) and did not diffract.

Diffraction data from the star-shaped crystals (Fig. 1*a*) were initially collected on a MAR Research MAR300 image-plate detector to 2.8 Å at 300 K. The crystals appear to be

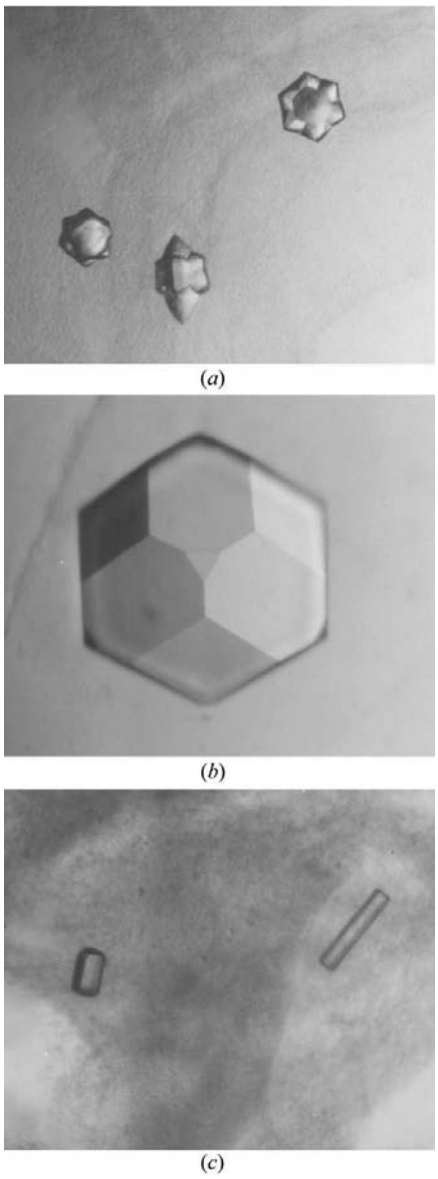

Figure 1
The three different crystal forms of D-hydantoinase (see text for crystallization conditions). (*a*) Star-shaped crystals, shown in two orientations. In one of the orientations the crystals look like bullets. (*b*) The hexagonal-faced crystals have multiple faces within one of the faces and are hexagonal, square or octagonal. (*c*) Small rod-shaped crystals, which are the smallest of the crystal forms obtained.

twinned. A grid search failed to produce crystals that were not star-shaped. However, the diffraction experiments proved that they were single crystals. The diffraction data were verified for merohedral twinning using *XDLDATAMAN* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) as well as using the twinning server of Yeates (1997). Both verification methods eliminated the possibility of merohedral twinning. Data to 2.0 Å were collected on an in-house MAR345 detector mounted on a Rigaku Ultrax 80s rotating-anode X-ray generator. The data were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997).

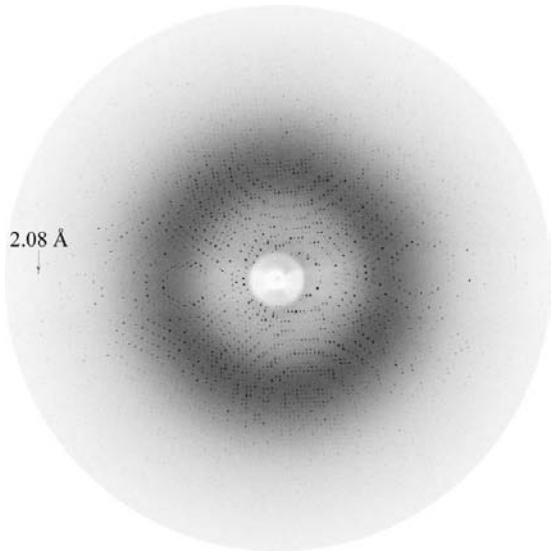

Figure 2
An X-ray diffraction pattern from a star-shaped D-hydantoinase crystal (oscillation range 1.0°). One of the maximum resolution spots is identified.

Table 1
Data-collection statistics for star-shaped HYD crystals.

Values in parentheses are for the data in the outer resolution shell.

Unit-cell parameters	
$a = b$ (Å)	129.55
c (Å)	102.86
$\alpha = \beta$ (°)	90
γ (°)	120
Resolution (Å)	49.0–2.30 (2.38–2.30)
Completeness (%)	98.4 (97.3)
No. observations [$I > 0\sigma(I)$]	136224
No. unique reflections [$I > 0\sigma(I)$]	42941
$\langle I\rangle/\langle\sigma(I)\rangle$	9.1 (2.5)
$R_{\rm merge}$†	0.12 (0.37)

3. Results and discussion

Although the hexagonal-faced crystals appeared to be of good size and quality for data collection, they did not diffract. The star-shaped crystals yielded 2.0 Å resolution data, but the statistics were only good to 2.3 Å (Fig. 2; Table 1). X-ray intensity data showed that the crystals were of hexagonal symmetry and that the space group was $P6_4$. The unit-cell parameters are $a = b = 129.55$, $c = 102.86$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Other parameters of the data-collection statistics are given in Table 1. Two molecules per asymmetric unit gave a Matthews coefficient of 2.46 Å^3 Da^{-1}, with a solvent content of 50% (Matthews, 1968).

HYD requires divalent cations such as Co, Mn, Mg and Zn ions for activity (Stallings *et al.*, 1985). Several attempts were made to prepare heavy-atom derivatives so that the structure could be solved by multiple isomorphous replacement (MIR). A mercury derivative was obtained by soaking in 5 m*M* $HgCl_2$. It was observed that HYD in the presence of mercuric compounds has zero relative activity to that of native HYD (Sharma, 1998). Therefore, it was thought that the Hg ions might be binding in the active site and acting as metal inhibitors. Attempts are under way to obtain other heavy-atom derivatives in order to solve the structure by the MIR method and by the molecular-replacement method using the published coordinates.

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