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Crystallization, preliminary X-ray analysis of a native and selenomethionine D-hydantoinase from *Thermus* sp.

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A D-hydantoinase from *Thermus* sp. was expressed in *Escherichia coli*, purified to homogeneity and crystallized both as native and Se-Met labelled protein. The crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 125.9, b = 215.8, c = 207.5 Å. A three-wavelength MAD data set was collected to 2.5 Å resolution and a native data set was collected to 1.7 Å resolution. Crystal packing and self-rotation calculations led to the assumption of six protomers per asymmetric unit, corresponding to a V_M value of 2.28 Å^3 Da^{-1} and a solvent content of 46%. As each protomer contains nine Se-Met residues, 54 selenium sites per asymmetric unit were present and could be unambigously located in the course of the MAD experiment. This selenium substructure is one of the largest selenium substructures that have been solved to date. The resulting phases obtained at a high-resolution limit of 3.0 Å could be extended to 1.7 Å and refined by application of density-modification techniques, especially non-crystallographic symmetry.

1. Introduction

Hydantoinases are cyclic amidases (E.C. 3.5.2; Webbs, 1992). They catalyze the reversible hydrolytic ring-opening of five- or six-membered cyclic diamides such as 5′-monosubstituted hydantoins and dihydropyrimidines to the corresponding carbamoyl amino-acids and 3-ureidoacids, respectively (Fig. 1). According to their stereoselectivity, hydantoinases have been classified as L- (Yamashiro *et al.*, 1988), non- or D-enantiomer selective (Möller *et al.*, 1988; Yokozeki & Kubota, 1987).

D-Hydantoinases are considered to be the bacterial counterpart of dihydropyrimidinases, which are directly involved in the catabolism of pyrimidines (Vogels & van der Drift, 1976). The natural functions of both L- and non-specific hydantoinases still remain unclear. Hydantoinases are used as biocatalysts for the stereospecific production of non-proteinogenic amino acids (Syldatk & Pietzsch, 1995).

The biochemical properties and substrate specificities of numerous hydantoinases are well characterized in the literature (reviewed by Syldatk *et al.*, 1999), but to date no structural information at the atomic resolution level has been published.

In this study, we used a thermostable D-hydantoinase from *Thermus* sp. (Elard *et al.*, 1987). As no directly homologous structure is known to date, Se-Met MAD phasing techniques were applied.

2. Materials and methods

2.1. Expression and purification

The D-hydantoinase from *Thermus* sp. (D-Hyd) was expressed in methionine auxotroph *E. coli* B834 cells (Novagene). The transcription of the gene for D-Hyd was under control of the IPTG-inducible T5 promoter and was performed by the endogenous *E. coli* RNA polymerase. Each medium contained 150 mg l^{-1} ampicillin and 0.1 m*M* $MnCl_2$. The cultivation of the native D-Hyd was performed in LB medium. For the production of Se-Met-D-Hyd, a 20 ml pre-culture was grown overnight in LB medium, centrifuged at 3000*g* for 5 min and resuspended in cold M9 minimal medium in three cycles. The washed cells were used to inoculate the main culture in 1.2 l M9 medium supplemented with 50 mg ml^{-1} selenomethionine and all other amino acids. The main cultures were grown at 310 K in three 3 l Erlenmeyer flasks to an OD_{600} of 0.8. Expression was then induced with 1 m*M* IPTG for 4 h. The cells were then cooled on ice, harvested by centrifugation for 20 min at 5900*g*, resuspended in 15 ml 20 m*M* Tris pH 8.0 and kept at 253 K for further use.

After addition of a protease-inhibitor cocktail, the thawed cells were lysed by passing them three times through an ice-cold French press. Cell debris was removed by centrifugation at 10 000*g* for 30 min.

All buffers for the purification were supplemented with 0.1 m*M* $MnCl_2$ and, for the Se-Met-D-Hyd, additionally with 20 m*M* DTT; all purification steps were performed at 277 K. Purification was performed in three steps following a modified version of a previously published protocol (Abendroth *et al.*, 2000). The purification steps consisted of fixed-bed Streamline anion-exchange, hydrophobic interaction and anion-exchange chromatography. On average, 15 mg of homogenous protein could be obtained from 1.2 l cultures. Homogeneity was tested with standard SDS–PAGE, native PAGE, isoelectric focusing, dynamic light scattering, MALDI and ESI mass spectrometry.

The purified enzyme was rebuffered in 20 m*M* Tris pH 8 (plus 20 m*M* DTT for Se-Met) and concentrated to 5.5 (native) or 9.5 mg ml^{-1} (Se-Met) by ultrafiltration.

2.2. Crystallization

All crystallization experiments were performed at 293 K using the sitting-drop variant of the vapour-diffusion method. Initial crystallization conditions were screened using the sparse-matrix approach (Jancarik & Kim, 1991).

Microseeding appeared to be crucial for obtaining good crystals. Initial crystals of native D-Hyd were crushed with a needle and diluted in different amounts of 1.6 *M* ammonium sulfate, 2% PEG 400, 100 m*M* HEPES/NaOH pH 7.5; these served as seeding stocks. 0.5 µl of the different seeding stocks were added to a drop which consisted of 3 µl protein solution and 4.5 µl reservoir (Se-Met-D-Hyd: 3 µl plus 3 µl) and which had been equilibrated overnight.

As in the case of Se-Met-D-Hyd, some protein precipitated during the first 12 h; the 24 drops were then collected and centrifuged at 10 000*g*. The clear supernatant was distributed again on clean micro-bridges and equilibrated for 1 h prior to seeding. The optimized reservoir consisted of 1.65 *M* ammonium sulfate, 5% PEG 400 and 100 m*M* HEPES–NaOH pH 7.5 (Se-Met-D-Hyd: 1.65 M ammonium sulfate, 5% PEG 400, 20 m*M* DTT and 100 m*M* HEPES–NaOH pH 7.5). As the quality and the size of the crystals depended on the quality of the seed crystals, several cycles of seeding were performed.

Crystals grew to dimensions of 0.5 mm in each direction (Se-Met-D-Hyd: 0.3 mm) within several days. For Se-Met-D-Hyd, only native crystals could be used as seeds. Whilst initial crystalization led to non-regular crystals, severals cycles of seeding led to good crystals of prismatic shape (Fig. 2).

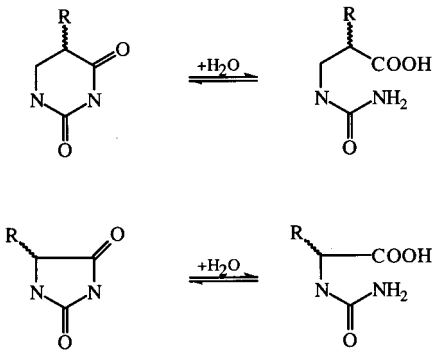

Figure 1
The reaction catalyzed by hydantoinases.

2.3. Diffraction experiments

For X-ray diffraction experiments at cryogenic temperatures, the crystals were transferred directly into a cryoprotectant buffer which consisted of 2 *M* Li_2SO_4, 5% PEG 400 and 100 m*M* HEPES/NaOH pH 7.5. The crystals were mounted in a nylon loop and flash-frozen in a nitrogen stream at 100 K (Oxford Cryosystems Cryostream). Both data sets were measured at the EMBL Outstation at DESY, Hamburg. The native data set was measured on beamline BW7B at 0.8439 Å using a MAR 345 detector. The diffraction limit was beyond 1.3 Å resolution; a complete data set was measured to 1.7 Å resolution. The MAD data set was measured on beamline X31 using a MAR 300 detector. The peak and inflection point were determined using EXAFS spectra. The native and the three MAD data sets were measured *en bloc* over approximately 95°.

All data were processed and scaled with *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997). The MAD data were not merged with *SCALEPACK* but with *SOLVE* (Terwilliger & Berendzen, 1999).

2.4. Solving the selenium substructure

The MAD data were merged with the local scaling routine of *SOLVE* (Terwilliger & Berendzen, 1999). The 54 Se-Met sites could be located using the automated protocol of *SOLVE* when the high-resolution limit was lowered to 3.0 Å. Initial phasing was also performed with *SOLVE*.

2.5. Subsequent calculations

Subsequent calculations were performed with programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994); for the calculation of the self-rotation function, the program *GLRF* (Tong & Rossman, 1990) was used.

Local symmetry operators were determined using the Se-Met positions with the *CCP*4 program *FINDNCS*. The initial phases from the MAD data set were combined with the structure-factor amplitudes of the native data set. Density modification was performed with the *CCP*4 program *DM*.

3. Results and discussion

Native and Se-Met-D-Hyd could be expressed in methionine auxotrophic *E. coli* cells. Because of the high expression level, these bacteria were also used for the native Cryostream enzyme. Both enzymes could be purified to homogeneity using a similar protocol. Approximately 15 mg of protein could be purified from a 1.2 l culture. ESI mass spectra of both native and Se-Met-D-Hyd were in accordance with the calculated molecular mass, indicating a complete substitution of Met by Se-Met. In both cases the N-terminal Met was post-translationally excized, which is common in *E. coli* when proline is the following residue (Hirel *et al.*, 1989).

The crystallization conditions were very similar for both variants as stated above. Several cycles of microseeding using seeds of native D-Hyd were crucial for the growth of well ordered and large crystals (Fig. 2). The crystals grew to their final size within a few days.

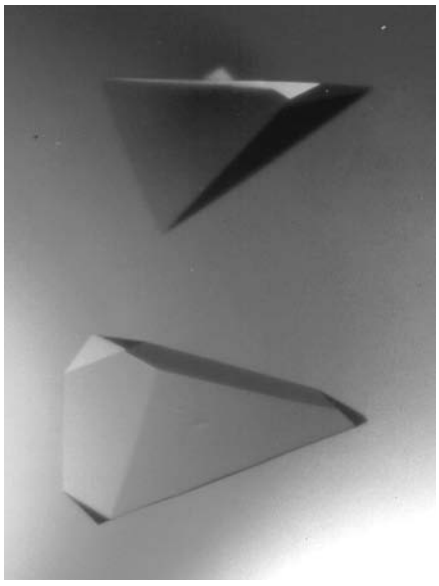

Figure 2
Orthorhombic crystals of native D-hydantoinase.

Table 1
Statistics of X-ray data sets of native and Se-Met-D-Hyd.

Values in parentheses refer to the highest resolution shell.

Data set	Wavelength λ (Å)	Resolution (Å)	Total No. of reflections	No. of unique reflections	$I/\sigma(I)$	Completeness (%)	Multiplicity (%)	R_{sym}†
Native	0.8439	20–1.7	1006898	293229	25.6 (9.5)	95.3 (89.9)	3.4	7.1 (10.2)
MAD remote	0.9184	25–2.5	350556	97226	16.4 (8.0)	99.8 (99.1)	3.6	5.9 (17.7)
MAD peak	0.9794	25–2.5	431701	97152	17.7 (9.2)	100.0 (99.9)	4.4	7.7 (16.9)
MAD inflection	0.9797	25–2.5	394476	96723	19.3 (9.0)	100.0 (99.8)	4.1	7.6 (14.8)

† $R_{sym} = \sum_{hkl} \sum_i I_i(hkl) - \langle I_i(hkl)\rangle / \sum_{hkl} \sum_i I_i(hkl)$.

Native crystals of D-Hyd diffracted to better than 1.3 Å resolution at beamline BW7B at DESY, Hamburg. Because of the size of the unit cell and the available beam time, the high-resolution limit was set to 1.7 Å in order to obtain a complete data set. A three-wavelength MAD data set was measured to 2.5 Å at beamline X31 at DESY, Hamburg.

X-ray intensity data showed orthorhombic Laue symmetry and systematic absences indicated the space group to be $C222_1$. The unit-cell parameters for the native enzyme are a = 125.9, b = 215.8, c = 207.5 Å and for Se-Met-D-Hyd, a = 125.1, b = 215.2, c = 207.6 Å. The data could be scaled to a symmetry R value of 7.1% for the native data set and 5.9–7.6% for the different MAD data sets. Statistics for all four data sets are summarized in Table 1.

Assuming six monomers in the asymmetric unit, the crystal packing parameter V_M (Matthews, 1968) is 2.28 Å^3 Da^{-1}, corresponding to a solvent content of 46%. During further analysis this value was corrected to 38%.

Most of the hydantoinases are described in the literature as forming tetramers and a few as forming dimers. Results of both gel-permeation chromatography and dynamic light scattering could be interpreted as either dimers, trimers or tetramers. The self-rotation function showed peaks at κ angles of 60, 120 and 180° (Fig. 3). Whereas the strongest peaks at $\kappa = 180°$ results from crystallographic symmetry, all others have non-cystallographic origin. Owing to the different possible quarternary structures of D-Hyd, the following interpretations were consistent with the self-rotation function: three dimers with their internal dyads lying in the *ab* plane, where the threefold and sixfold axes stem from mappings between these dimers in combination with the crystallographic symmetry; two trimers with the corresponding threefold axes and with twofold axes which map these trimers to each other; three tetramers with 222 symmetry on special positions.

One bottleneck in solving large structures of Se-Met-labelled proteins is the detection of the selenium sites. With the advent of new programs, a number of large selenium substructures have recently been solved (Deacon & Ealick, 1999). For solving this selenium substructure, the program *SOLVE* (Terwilliger & Berendzen) was used. *SOLVE* found all 54 sites without using information about local symmetry. For this purpose, the high-resolution limit had to be lowered from 2.5 to 3.0 Å where the data sets were complete, even if only data with $I/\sigma(I) \geq 3$ were considered. The substructure

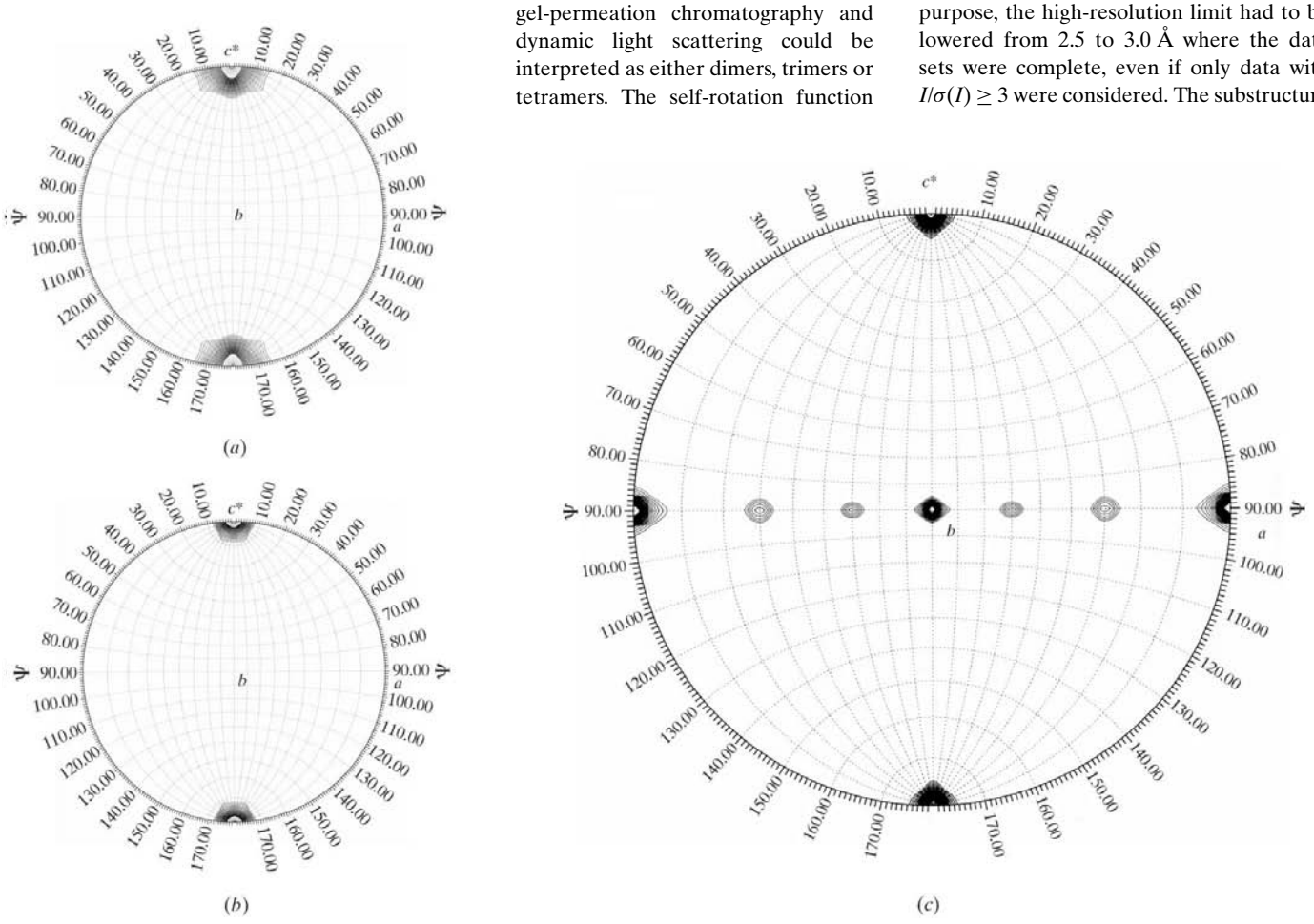

Figure 3
Self-rotation functions of D-Hyd with (*a*) κ = 60°, (*b*) κ = 120° and (*c*) κ = 180°.

solved here is one of the largest selenium substructures known in the literature to date (Deacon & Ealick, 1999). Initial maps after density modification seem to be traceable.

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