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Crystallization and preliminary X-ray data of the recombinant peptide amidase from *Stenotrophomonas maltophilia*

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The peptide amidase from *Stenotrophomonas maltophilia* selectively hydrolyses the C-terminal amide bond in peptide amides. Crystals have been obtained by sitting-drop vapour diffusion from solution containing polyethylene glycol (PEG) 6000, HEPES pH 7.5, glycerine and sodium azide (NaN_3). The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 74.18, b = 62.60, c = 101.91 Å, $\beta = 90^\circ$. X-ray data from these crystals diffracted at the European Synchrotron Radiation Facility (ESRF, France) ID14-1 beamline to 1.4 Å.

1. Introduction

The peptide amidase from the Gram-negative bacterium *S. maltophilia* has been purified and characterized by Stelkes-Ritter *et al.* (1995). Isolation of the gene as well as expression and characterization of the recombinant peptide amidase has been carried out by Neumann & Kula (2002). The peptide amidase has a molecular mass of 53.5 kDa. An N-terminal signal peptide has been found in the gene (identified by the SignalP algorithm; Nielsen *et al.*, 1997), which is cleaved off in the processed protein, as shown by N-terminal protein-sequence analysis. The enzyme selectively hydrolyses the C-terminal amide bond in peptide amides, *i.e.* it is a hydrolase that acts on carbon–nitrogen bonds other than peptide bonds in linear amides. The biological function of this enzyme is still unknown as the biological substrate is unidentified. The EC number of the peptide amidase is EC 3.5.1.-.

L-Alanyl-L-phenylalaninamide is hydrolysed with v_{max} = 194 U mg^{-1} and K_M < 0.5 m*M*. Peptide bonds or amides in the side chain of glutamine or asparagine are not attacked. The temperature optimum lies between 319 and 327 K; the pH optimum lies between 7.0 and 8.2. Since *S. maltophilia* yields only 4 U per litre of culture volume, an efficient expression system in *Escherichia coli* was established. The natural function of the peptide amidase is not known. Sequence analysis showed that the peptide amidase belongs to the amidase signature family first described by Mayaux *et al.* (1991). This family is defined in the Pfam database (at The Wellcome Trust Sanger Institute, Cambridge, England) by a conserved amino-acid sequence, the amidase signature sequence (G-[GA]-S-[GS]-[GS]-G-*x*-[GSA]-[GSAVY]-*x*-[LIVM]-[GSA]-*x*(6)-[GSAT]-*x*-[GA]-*x*-[DE]-*x*-[GA]-*x*-S-[LIVM]-R-*x*-P-[GSAC]). This enzyme family comprises at least 200 proteins from over 90 organisms (BLAST search, NCBI, Maryland, USA), which are spread over all three domains of living organisms: bacteria, archaea and eukarya. Until now, no structure of any protein from this family has been published, although a successful crystallization experiment for an archaeal amidase signature enzyme has been reported recently (Nastopoulos *et al.*, 2001).

2. Experimental procedures and results

2.1. Expression and purification

The peptide amidase has been expressed under the control of a T7 promotor in *E. coli* Origami (DE3) (Novagen) from a construct without its N-terminal signal peptide but containing a C-terminal His_6 tag. The gene product has been purified by affinity chromatography using a Ni–NTA superflow column (Qiagen) and subsequent gel-filtration chromatography (G-25, Pharmacia) to remove imidazole, which was used as eluent in the preceding purification step. At this point the peptide amidase was >95% pure as determined by SDS–PAGE (silver stained). A detailed description of cloning, expression, purification and characterization of the recombinant enzyme will be published elsewhere (Neumann & Kula, 2002). The recombinant amidase consists of 520 amino acids and its molecular weight is 55.5 kDa. The protein solution was concentrated by ultrafiltration with a 30 kDa cutoff membrane (ultrafiltration cell from Amicon). For crystallization experiments, the concentration of the peptide amidase was adjusted to 9–24 mg ml^{-1} in 10 m*M* potassium phosphate buffer pH 7.5.

2.2. Crystallization

Initial crystallization experiments were performed with 98 different buffers from Crystal Screen kits 1 and 2 from Hampton Research (Jancarik *et al.*, 1991; Cudney *et al.*, 1994). The sitting-drop vapour-diffusion method was applied using Cryschem Plates with sealing tape and crystallization plates with microbridges sealed with cover slips (Hampton Research). Each drop was prepared by mixing 5 µl of protein solution with an equal volume of reservoir solution; the reservoir volume was 1 ml. After 48 h, crystals appeared under the following conditions: (*a*) 10% PEG (polyethylene glycol) 8000, 0.1 *M* HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) pH 7.5, 8% ethylene glycol, 277 K; (*b*) 20% PEG 10 000, 0.1 *M* HEPES pH 7.5, 277 K; (*c*) 12% PEG 20 000, 0.1 *M* MES [2-(*N*-morpholino)ethanesulfonic acid] pH 6.5, room temperature; (*d*) 20% PEG 10 000, 0.1 *M* HEPES pH 7.5, room temperature.

Crystals grown under the initial crystallization conditions were not stable in cryoprotectants. An extensive optimization screening was performed based on the initial conditions. Different parameters were varied: molecular weight of PEG (200–20 000 Da), concentration of PEG [8–20%(*w*/*v*)], concentration of protein (9–24 mg ml^{-1}), pH value (6.5–8.0), incubation temperature (277, 289 K and room temperature) and glycerine concentration (0–20%).

Crystals displaying the desired properties were obtained under the following conditions: 13% PEG 6000, 0.1 *M* HEPES pH 7.5, 20% glycerine, 0.02% sodium azide (NaN_3), 289 K. The drop volume was increased to 20 µl; the protein concentration was 24 mg ml^{-1}. Fig. 1 shows a crystal obtained under the optimized conditions. These crystals are also stable in solution containing 1 m*M* $HgCl_2$ (24 h soaking time). The pseudo-orthorhombic crystal shape is pinacoid. Under crossed polarizers in the microscope, one of the extinctions is tilted in the *ac* plane perpendicular to the needle axis, which indicates a monoclinic point group.

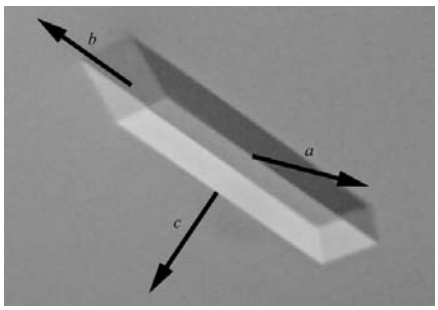

Figure 1
Crystal of the peptide amidase. The crystal size corresponds to approximate 0.36 × 0.07 × 0.05 mm.

3. X-ray data collection and analysis

Measurements were made at 100 K. The mother liquor was suitable for cryocooling. Native and initial derivative data sets were collected using a synchrotron radiation source at the European Synchrotron Radiation Facility (ESRF, France, beamline ID14-1) and a rotating-anode X-ray generator (Enraf–Nonius FR571, 40 kV and 50 mA) on a MAR CCD detector and a MAR image-plate area detector, respectively. The data evaluation was performed with the programs *MOSFLM* (Leslie, 1992) and *SCALA*/*TRUNCATE* from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994). The native crystals diffract to 1.4 Å resolution with a mosaicity of 0.45°. The relevant native data statistics are summarized in Table 1.

Amidase crystallizes in the monoclinic space group $P2_1$, but the diffraction patterns are close to the orthorhombic Laue group *mmm* (the overall R_{sym} value for an orthorhombic space group is 10.5%). The unit-cell parameters are a = 74.18, b = 62.60, c = 101.91 Å, β = 90°. The self-rotation functions (Tong & Rossmann, 1990) show local symmetry axis along the cell edges (Table 1; Fig. 2). The difference in orientation for the local rotation axis compared with the orthorhombic case appears to be negligible. Therefore, it must be concluded that the lowering of the symmetry from orthorhombic to monoclinic is a consequence of the translational component of the local symmetry. This conclusion had been verified by a difference Patterson synthesis of a $HgCl_2$ derivative. Using the program *FINDNCS* (Collaborative Computational Project, Number 4, 1994), the non-crystallographic translation was calculated from six Hg sites to be t_1 = 0.500, t_2 = 0.596, t_3 = 0.501 (fractional units) (Table 1). A V_M value of 2.2 Å^3 Da^{-1} is consistent with the presence of two molecules in the asymmetric unit. The solvent content is estimated to be 41.9%.

Table 1
Diffraction data statistics for amidase crystallized in space group $P2_1$.

Values given in parentheses refer to reflections in the outer resolution shell.

Compound	Native	Native + $HgCl_2$
Space group	$P2_1$	$P2_1$
Unit-cell parameters		
a (Å)	74.18	73.96
b (Å)	62.60	62.50
c (Å)	101.91	101.70
β (°)	90.0	90.0
Resolution (Å)	1.4	2.3
Observed reflections	1739422	284030
Unique reflections	183606	40849
Completeness (%)	99.3 (95.0)	98.2 (96.8)
R_{sym}† (%)	9.4 (14.0)	5.0 (11.1)
$\langle I/\sigma(I)\rangle$ last shell	5.1	8.9
Average B factor‡ (Å^2)	13.5	23.8
NCS operator§	r_{11} = 1.0, r_{12} = 0.0, r_{13} = 0.0 r_{21} = 0.0, r_{22} = −0.99, r_{23} = 0.0 r_{31} = 0.0, r_{32} = 0.0, r_{33} = −1.0 t_1 = 0.500, t_2 = 0.596, t_3 = 0.501	
R_{iso}¶	—	17.3

† Defined as $R_{sym} = 100\% \times |I_{i,h} - \langle I\rangle_h|/\sum\langle I\rangle_h$. $I_{i,h}$ is the intensity of the ith observation of reflection h, $\langle I\rangle_h$ is the mean intensity value of the reflection and the summation is over all reflections. ‡ Determined from a Wilson plot. § Determined by a self-rotation function and *FINDNCS* (t_1, t_2 and t_3 are fractional units). ¶ $R_{iso} = \sum|F_P - F_{PH}|/\sum F_P$, where F_P and F_{PH} are the structure-factor amplitudes of the native and derivative data, respectively.

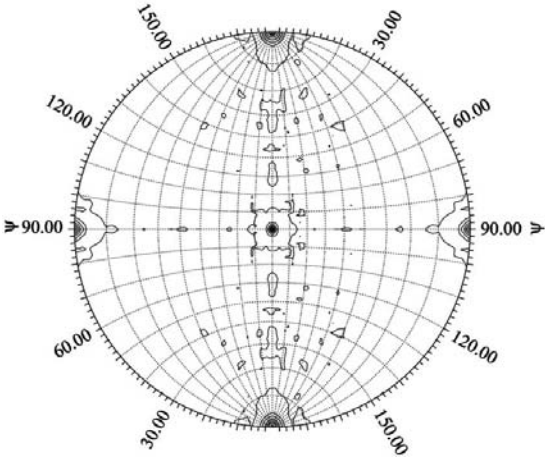

Figure 2
Origin-removed self-rotation function of amidase; 30–1.4 Å; number of reflections, 71 911; radius, 30 Å; contour, 20% to maximum in 10% intervals.

A further search for suitable heavy-atom derivatives to solve the structure by multiple isomorphous replacement methods is currently in progress.

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