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Crystallization and preliminary X-ray diffraction analysis of L-aminoacylase from the hyperthermophilic archaeon *Thermococcus litoralis*

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The enzyme L-aminoacylase catalyses the hydrolysis of *N*-acyl-L-amino acids from peptides or proteins. The recombinant enzyme from the hyperthermophilic archaeon *Thermococcus litoralis* has been purified to homogeneity. This zinc-containing enzyme has been crystallized from ammonium sulfate using the sitting-drop vapour-diffusion method. The crystals diffract to 2.8 Å resolution and belong to the rhombohedral space group *R*32, with unit-cell parameters $a = b = 102.4$, $c = 178.5$ Å, $\gamma = 120^\circ$ in a hexagonal lattice setting. The asymmetric unit contains one enzyme monomer, containing a single zinc ion. Two synchrotron data sets have been collected at a remote wavelength and at the maximum f' wavelength for zinc. This has allowed the position of the metal to be identified in anomalous Patterson maps.

1. Introduction

Aminoacylase I (*N*-acylamino acid amidohydrolase; EC 3.5.1.14) catalyses the hydrolysis of *N*-acyl-L-amino acids from peptides or proteins. Aminoacylase I has been identified in eukaryotic species as well as eubacteria and archaea. Although these enzymes have been known for many years, their physiological role is still not fully understood. It has been proposed that they are involved in the catabolism of terminal *N*-acyl peptides or in the recycling of *N*-acylated amino acids (Gade & Brown, 1981). Aminoacylases are known to play a role in xenobiotic detoxication and bioactivation and are important in the inter-organ processing of xenobiotic derived amino-acid conjugates. Mercapturates (S-substituted *N*-acetyl-L-cysteine conjugates), which are formed by the acetylation of L-cysteine-S-conjugates obtained from the hydrolysis of glutathione-S-conjugates, are cleaved by aminoacylases (Anders & Dekant, 1994). Human aminoacylase I has been assigned to distal chromosome 3p21.1. This region is deleted in several neoplasms, including small-cell lung cancer (Scaloni *et al.*, 1992) and renal cell carcinoma. It has been suggested that the acylase I and small-cell cancer tumour suppressor genes are closely linked (Miller *et al.*, 1989) and that the processing of *N*-acylated peptides may influence growth regulation (Tsunasawa, 1992).

The mammalian aminoacylases are the most extensively studied, with the enzymes from human (Cook *et al.*, 1993) and pig (Birnbaum *et al.*, 1952) being characterized. The major interest in the bacterial/archaeal enzymes is their importance industrially, as they can be used for resolution of racemic *N*-acylated amino acids (Chenault *et al.*, 1989). Amino-acid sequence identity between the members of the aminoacylase I family is low; mammalian enzymes show only 12% identity to eubacterial and archaeal aminoacylases. However, the amino-acid residues proposed to be involved in metal binding are conserved throughout the family. All of the aminoacylases characterized to date are homodimers with a subunit molecular weight between 42 and 45 kDa, containing one zinc ion per subunit. It was generally thought that this zinc was involved directly in enzymatic catalysis as a Lewis acid; however, there is a growing consensus that it plays only a structural role (Heese *et al.*, 1990). Paramagnetic and kinetic studies on the porcine kidney aminoacylase suggest that the metal centre is too far from the ligand-binding site to be involved directly in substrate binding or catalysis (Heese *et al.*, 1990). It has been proposed that the presence of the metal ion helps to maintain the conformation of the active site in a strained state – the entactic state required for catalysis (Weiss *et al.*, 1995). Studies involving circular dichroism and FT-Raman spectroscopy (Tang *et al.*, 1995) have shown that there is a difference between the holoenzyme and the apoenzyme amino-acylase structures, since removal of the zinc results in a decrease in the extent of ordered structure.

The hyperthermophilic archaeal *T. litoralis* L-aminoacylase has been cloned and over-expressed in *Escherichia coli* (Toogood *et al.*, 2002). The molecular mass of the enzyme was calculated to be 43 814 Da according to its sequence. The *T. litoralis* L-aminoacylase is

thermostable, with a half-life of 25 h at 343 K. This makes it suitable for industrial biotransformation reactions at elevated temperatures.

The *T. litoralis* L-aminoacylase has 82% sequence identity to a bifunctional carboxypeptidase/aminoacylase from *Pyrococcus horikoshii* OT3 (Ishikawa *et al.*, 2001) and 45% sequence identity to a carboxypeptidase from *Sulfolobus solfataricus* (Colombo *et al.*, 1995). However, the *T. litoralis* enzyme does not exhibit detectable carboxypeptidase activity (Toogood *et al.*, 2002).

A zinc carboxypeptidase G2 from *Pseudomonas* sp. strain RS-16 for which the three-dimensional structure is available (Rowsell *et al.*, 1997) has a distant sequence similarity (17% amino-acid identity) to the *T. litoralis* L-aminoacylase. The overall sequence identity between these two enzymes is low; however, reiterative BLAST search (Altschul *et al.*, 1997) analysis suggests they are both members of the same protein family, peptidase M20. Carboxypeptidase has two zinc ions bound in this region, whereas the aminoacylase has only one.

There is no three-dimensional information available for any aminoacylase enzyme to date and therefore the elucidation of the X-ray structure of the *T. litoralis* enzyme will further our understanding of the catalytic mechanism and evolution of this group of metalloproteins.

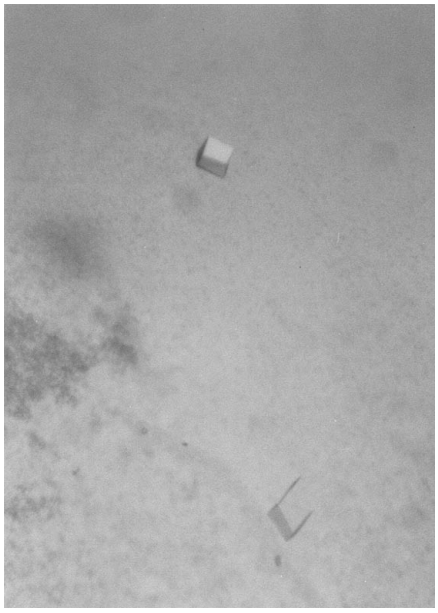

Figure 1
Rhombohedral crystals of recombinant *T. litoralis* L-aminoacylase. Typical dimensions are approximately 0.1 × 0.1 × 0.2 mm.

2. Materials and methods

2.1. Protein purification

The aminoacylase protein was purified from *E. coli* harbouring the gene for the enzyme in the overexpression vector pUCcer11 (Toogood *et al.*, 2002). *E. coli* cells containing the expressed protein were resuspended in 40 ml 100 m*M* Tris–HCl pH 8.0 and sonicated for 2 min on ice. The insoluble cell debris was removed by centrifugation at 30 000*g* and the supernatant was decanted into a sterile universal tube and heated to 343 K in a water bath for 20 min. The precipitated protein was removed by centrifugation at 30 000*g* for 30 min at 277 K.

Ammonium sulfate (enzyme grade) was added slowly to the supernatant to 60%(*w*/*v*) saturation with continuous stirring. The precipitated protein was removed by centrifugation (10 000*g*, 20 min) and redissolved in 10 ml 100 m*M* Tris–HCl pH 8.0, 0.4 *M* ammonium sulfate (buffer *A*) before application to a hydrophobic interaction chromatography column (phenyl Sepharose CL 4B, Pharmacia) pre-equilibrated with buffer *A*. An isocratic gradient was carried out over five column volumes leading to 100% buffer *B* (30% 2-propanol, 10 m*M* Tris–HCl pH 8.0) at a flow rate of 2 ml min^{-1} and was collected in 10 ml fractions. Active fractions were pooled and dialysed against 5 l of 10 m*M* Tris–HCl pH 8.0 (buffer *C*) overnight. The protein was precipitated with 80% ammonium sulfate, centrifuged at 10 000*g* for 20 min and redissolved in 2 ml of buffer *C* and applied to a gel-filtration column (Superdex 200 Hiload 16/60, Pharmacia) pre-equilibrated with buffer *C*. Protein was eluted with buffer *C* at 0.7 ml min^{-1} and collected in 5 ml fractions. All purification steps were carried out at 277 K unless otherwise stated. All buffers contained 1×10^{-5} *M* phenylmethylsulfonyl fluoride and 2×10^{-5} *M* benzamidine as protease inhibitors. The purity of the protein was analysed by SDS–PAGE and the concentration of the protein was determined by the Bradford assay (Bradford, 1976). Bovine serum albumin (Sigma–Aldrich Co.) was used to generate a standard curve.

2.2. Enzyme activity

L-Aminoacylase activity was determined by an HPLC method. The substrate *N*-Ac-DL-tryptophan was used in the aminoacylase activity because of its ease of detection. The reaction mixture contained 0.89 ml 0.1 *M* Tris–HCl pH 8.0, 100 µl 100 m*M* *N*-Ac-DL-Trp and 10 µl of enzyme combined in a microcentrifuge tube which was incubated at the required temperature for 1 h. The reaction was stopped by addition of 1 µl 200 m*M* EDTA. A 100 µl aliquot of the stopped reaction mixture was added to 900 µl of HPLC mobile phase (45% methanol, 1 ml l^{-1} H_3PO_4) and mixed. An aliquot of 1 µl was injected into a Hypersil C18 BDS column using a Beckman System Gold HPLC with a flow rate of 2.0 ml min^{-1} using a run time of 4 min and a detection wavelength of 225 nm. The substrate retention time was 3.02 min and the product 1.98 min.

2.3. Sedimentation equilibrium analysis

Sedimentation equilibrium analysis of the purified L-aminoacylase enzyme was carried out by the BBSRC National Centre for Macromolecular Hydrodynamics, University of Nottingham, UK. The L-aminoacylase sample was monitored by sedimentation equilibrium at concentrations of 0.5 and 1 mg ml^{-1} in the buffer 10 m*M* Tris–HCl pH 7.0.

2.4. Crystallization

Crystallization was carried out by the method of sitting-drop vapour diffusion. Prior to crystallization, the protein was concentrated using 30 kDa molecular-weight cutoff Microsep microconcentrators (Filtron Technology Corp., Northborough, MA, USA) to 7.5 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0. 2 µl aliquots of protein solution were mixed with 2 µl of the reservoir solution. The reservoir contained 47% (saturation) ammonium sulfate, 100 m*M* MES pH 6.0, 1% β-octyl glucoside.

2.5. Crystallographic data collection

Data were collected at the DESY Synchrotron, Hamburg, EMBL station BW7A using a single cryocooled crystal at 100 K and a MAR Research CCD detector. The cryogenic liquor consisted of 57% (saturation) ammonium sulfate, 30%(*v*/*v*) glycerol, 100 m*M* MES pH 6.0 and 1% β-octyl glucoside. The data were collected at a remote wavelength of 0.9793 Å and at a maximal f' wavelength for zinc of 1.2824 Å.

3. Results and discussion

The purified recombinant L-aminoacylase migrated on SDS–PAGE as a single band of 43 kDa. However, gel-filtration analysis carried out in 10 m*M* Tris–HCl pH 8.0 estimated the size of the enzyme to be 170 kDa,

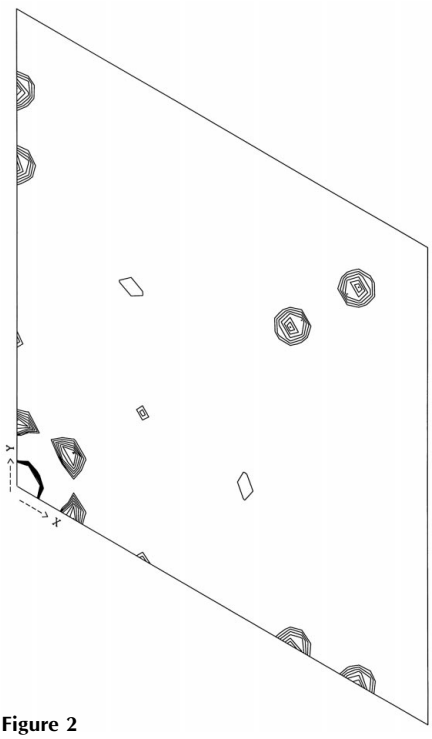

Figure 2
$w = 0$ section of the anomalous difference Patterson synthesis calculated with Fourier coefficients $(F_r - kF_1)^2$ at 10–5 Å resolution, where F_r is the structure factor at a remote wavelength, F_1 is the structure factor at a maximal f' wavelength for zinc of 1.2824 Å and k is the scale coefficient.

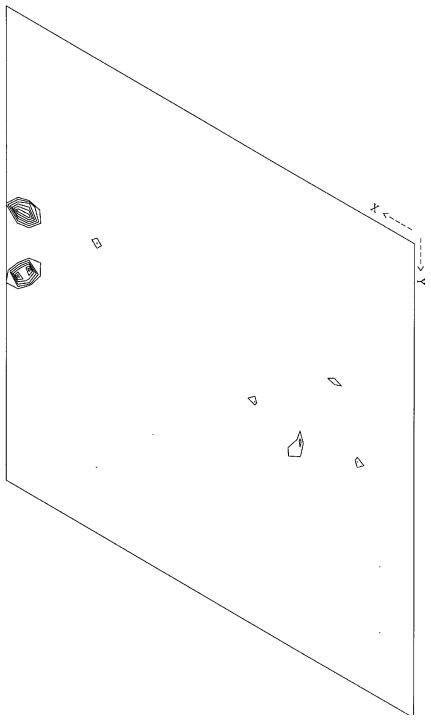

Figure 3
$w = 0.066$ section of the anomalous difference Patterson synthesis calculated at 10–5 Å resolution with Fourier coefficients $(F_{1+} - F_{1-})^2$, where F_1 is the structure factor at a maximal f' wavelength for zinc of 1.2824 Å.

suggesting that the enzyme exists as a homotetramer. The sedimentation studies carried out at 0.5 and 1 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.0 also showed that the enzyme exists predominantly as a tetramer. The specific activity using the substrate *N*-Ac-DL-Trp was found to be 0.2 µmol min^{-1} per milligram of protein.

A shower of crystals was formed when crystallization was carried out in the absence of β-octyl glucoside. Addition of the detergent decreased the amount of nucleation, subsequently increasing the size of the crystals. Crystals grew in 1–2 d and reached dimensions of 0.1 × 0.1 × 0.2 mm (Fig. 1). The crystals diffracted to 2.8 Å using synchrotron radiation and belonged to the rhombohedral space group *R*32, with unit-cell parameters $a = b = 102.4$, $c = 178.5$ Å, $\gamma = 120^\circ$ in the hexagonal lattice setting, $a = b = c = 83.9$ Å, $\alpha = \beta = \gamma = 75.3^\circ$ in the rhombohedral lattice setting.

Data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). A summary of the X-ray data statistics is shown in Table 1. A twinning test using the program *SFCHECK* (Vaguine *et al.*, 1999) gave average values of $\langle I^2\rangle/\langle I\rangle^2 = 1.97$, suggesting the crystal was not twinned, a possibility in the *R*32 space group. The solvent content of the crystals that contain a monomer in the asymmetric unit has been estimated at 41%, with $V_M = 2.06$ Å^3 Da^{-1} (Matthews, 1968). It is possible, owing to the high salt content of the crystallization solution, that intersubunit ionic bonds could have been disrupted, resulting in a monomer or a dimer in the crystal rather than the tetramer observed in solution studies. A tetramer would be inconsistent with the observed crystallographic symmetry.

The position of the zinc in the asymmetric unit has been found in the anomalous difference Patterson map using data collected at a wavelength of 1.2824 Å in the resolution range 15–5 Å using the program *RSPS* (Collaborative Computational Project, Number 4, 1994). The resulting zinc ion fractional coordinates are $(x, y, z) = (0.512, 0.483, 0.144)$, which agree with the anomalous difference Patterson maps calculated with Fourier coefficients $(F_r - kF_1)^2$ (Fig. 2) and $(F_{1+} - F_{1-})^2$ (Fig. 3), where F_r is the structure factor at a remote wavelength, F_1 is the structure factor at the maximal f' wavelength for zinc of 1.2824 Å and k is the scale coefficient. Heavy-atom refinement and phasing was performed in parallel by *MLPHARE* (Collaborative Computational Project, Number 4, 1994), *SHARP* (de La Fortelle & Bricogne, 1997) and by the fast maximum-likelihood heavy-atom refinement algorithm (A. Lebedev, unpublished data). The phases from *SHARP* and the fast maximum-likelihood heavy-atom refinement algorithm had a difference of 60°. The phases obtained from *MLPHARE* were not consistent with any other phases. Density modification of the phases from the fast maximum-likelihood heavy-atom refinement algorithm was performed using the program *DM* (Cowtan, 1994). Some short α-helices and a β-sheet could be observed in the resulting synthesis, which allowed the determination of the hand of the map. Phases from *SHARP* were modified using the program *SOLOMON* (Abrahams & Leslie, 1996). Several secondary-structure elements could be also observed in the resulting synthesis. However, the quality of neither of the two maps was good enough to trace the aminoacylase main chain.

The molecular-replacement studies were carried out with the programs *MOLREP* (Vagin & Teplyakov, 1997) and *AMoRe* (Navaza, 1994) using the whole structure or the two separate domains of carboxypeptidase G2 (Rowsell *et al.*, 1997). The molecular-replacement approach has so far failed to find a solution that could be refined. Phased molecular replacement is currently being attempted using the anomalous phases and the carboxypeptidase G2 structure with the program *MOLREP* (Vagin & Teplyakov, 1997). In addition, heavy-atom derivatives are also being employed in an effort to solve the structure of this aminoacylase enzyme.

Table 1
Processing statistics for native and anomalous data sets.

Values in parentheses are for the highest resolution shell.

	Data set	
Parameter	Native	Anomalous
Wavelength (Å)	0.9793	1.2824
Oscillation range (°)	0.5	0.5
Resolution range (Å)	15–2.9 (2.95–2.9)	15–3.0 (3.04–3.0)
No. of measured reflections	26083	30612
No. of unique reflections	7218	7354
Completeness (%)	88.7 (93.0)	99.7 (100.0)
$I > 2\sigma(I)$ (%)	72.7	85
$\langle I\rangle/\sigma\langle I\rangle$	17.8	15.8
R_{sym}† (%)	6.1 (49.7)	7.7 (53.5)

† $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where I is the intensity of reflection.

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