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Crystallization and preliminary crystallographic studies of the recombinant L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43

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N-Carbamoyl-L-amino-acid amidohydrolases (L-*N*-carbamoylases; EC 3.5.1.87) hydrolyze the carbon–nitrogen bond of the ureido group in *N*-carbamoyl-L-α-amino acids. These enzymes are commonly used in the production of optically pure natural and non-natural L-amino acids using the 'hydantoinase process'. Recombinant L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 has been expressed, purified and crystallized by hanging-drop vapour diffusion. X-ray data were collected to a resolution of 2.75 Å. The crystals belonged to space group $P2_12_12$, with unit-cell parameters $a = 103.2$, $b = 211.7$, $c = 43.1$ Å and two subunits in the asymmetric unit.

1. Introduction

L-Amino acids are used as additives in animal and human foodstuffs, in the pharmaceutical and cosmetics industries and as chiral synthons in organic synthesis (Leuchtenberger *et al.*, 2005). Furthermore, non-natural L-amino acids are in increasing demand from the pharmaceutical industry as precursors of important biomedical drugs. By way of example, L-homophenylalanine is a constituent of several commercial antihypertensives (enalapril, lisinopril, quinapril, ramipril, trandolapril and benazepril, among others), L-DOPA has therapeutic uses to increase dopamine levels for the treatment of Parkinson's disease and L-*tert*-leucine appears in some HIV or hepatitis C protease inhibitors (atazanavir and boceprevir) and in matrix metalloproteinase inhibitors involved in connective tissue breakdown (Beckett *et al.*, 1999).

Biotechnological production of these compounds has been developed in recent decades (Leuchtenberger *et al.*, 2005). Among these processes, the 'hydantoinase process' is an inexpensive and environmentally friendly enzymatic method that is potentially applicable to the production of any optically pure natural or non-natural D- or L-amino acid from a wide spectrum of D,L-5-monosubstituted hydantoins as substrates (Altenbuchner *et al.*, 2001). The enantiomer obtained depends on the stereospecificity of the last enzyme in the reaction cascade (*N*-carbamoyl-D- or L-amino-acid amidohydrolases, also known as D- or L-*N*-carbamoylases). Microorganisms of the genera *Arthrobacter* (Wiese *et al.*, 2001), *Alcaligenes* (Ogawa *et al.*, 1995), *Bacillus* (Batisse *et al.*, 1997), *Blastobacter* (Yamanaka *et al.*, 1997), *Flavobacterium* (Yokozeki *et al.*, 1987), *Microbacterium* (Suzuki *et al.*, 2005), *Pseudomonas* (Ogawa & Shimizu, 1994) and *Sinorhizobium* (Martinez-Rodriguez *et al.*, 2005) have been reported to show L-*N*-carbamoylase activity.

Although their function is not completely clear, L-*N*-carbamoylases are thought to be detoxifying enzymes for the *N*-carbamoyl-L-α-amino acids formed between active carbamoyl groups, such as carbamoyl phosphate, and amino acids present in the cell (Gojkovic *et al.*, 2001). L-*N*-Carbamoylases from *Pseudomonas* sp. strains BS and ON-4a have also been described as being involved in the synthesis of L-cysteine in association with an L-2-amino-Δ^2-thiazolin-

4-carbonic acid hydrolase (Shiba *et al.*, 2002; Ohmachi *et al.*, 2002). Based on the comparison of their amino-acid sequence, L-*N*-carbamoylases belong to the peptidase family M20/M25/M40. They share a similar topology with allantoate amidohydrolase (PDB code 1z2l; Agarwal *et al.*, 2007), β-alanine synthase (PDB code 1r3n; Lundgren *et al.*, 2003), carboxypeptidase G2 (PDB code 1cg2; Rowsell *et al.*, 1997), peptidase T (PDB code 1fno; Hakansson & Miller, 2002) and peptidase V (PDB code 1lfw; Jozic *et al.*, 2002).

Despite the interest in this enzyme for its biotechnological applications, little attention has been paid to its functional mechanism or stability-related structural aspects. In previous work, we demonstrated the involvement of several conserved residues in all L-*N*-carbamoylases in the recognition and hydrolysis of their substrates, which led us to propose a hypothetical reaction mechanism based on the similarity to other enzymes of the peptidase family (Martinez-Rodriguez *et al.*, 2006). Here, we report the expression, purification, crystallization and preliminary crystallographic studies of recombinant L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar).

2. Materials and methods

2.1. Cloning, expression and purification

G. stearothermophilus CECT43 was used as the source of DNA encoding the L-*N*-carbamoylase. Two primers were designed based on the known sequence of L-*N*-carbamoylase from *G. stearothermophilus* NS1122A (GenBank accession No. S67784): forward primer 5′-AAACATATGATTCAAGGGGAACGTCTTTGG-3′ and reverse primer 5′-AAACTCGAGTTCCCCTTGGGCCAGTTGCCACAC-3′. An isolated bacterial colony was transferred from the LB plate to 50 µl MilliQ water using a sterile inoculating loop. Cells were lysed by boiling at 373 K for 10 min followed by immediate chilling on ice. After cooling, cell debris was removed by centrifugation. In order to amplify the gene encoding BsLcar by PCR, 5 µl of the supernatant containing genomic DNA was used. The amplified fragment was purified from agarose gel using QIAquick (Qiagen) and cleaved with *Nde*I and *Xho*I (Roche Diagnostic). The digested fragment was ligated into pET-22b(+) plasmid (Novagen) cleaved with the same enzymes to create plasmid pJAVI80. The sequence of the gene was determined using standard T7 promoter and terminator primers with the dye dideoxy nucleotide-sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems).

pJAVI80 was transformed into *Escherichia coli* BL21. A single colony was transferred into 10 ml LB medium supplemented with 100 µg ml^{-1} ampicillin and was incubated overnight at 310 K. 500 ml LB with the above-mentioned concentration of ampicillin was inoculated with 5 ml of the overnight culture and grown at 310 K to an OD_{600} of 0.4–0.6. For induction of expression of the L-*N*-carbamoylase gene, isopropyl-β-D-1-thiogalactopyranosidase was added to a final concentration of 0.2 m*M* and the culture was incubated at 310 K for a further 4 h. The cells were collected by centrifugation (Beckman JA2-21, 7000*g*, 277 K, 10 min), washed twice and resuspended in 50 ml wash buffer (300 m*M* NaCl, 0.02% NaN_3, 50 m*M* sodium phosphate pH 7.0). The cell walls were disrupted on ice by sonication using a UP 200 S Ultrasonic Processor (Dr Hielscher GmbH, Germany) for three periods of 30 s with pulse mode 0.5 and 60% sonic power. The pellet was removed by centrifugation (Beckman JA2-21, 10 000*g*, 277 K, 20 min) and discarded. The supernatant was applied onto a column with 2 ml of Talon metal-affinity resin (Clontech Laboratories), which was then washed four times with ten column volumes of wash buffer. After complete washing, BsLcar was eluted with 250 m*M* imidazole, 100 m*M* NaCl, 0.02% NaN_3 and 2 m*M* Tris pH 8.0. The purified enzyme was concentrated using an Amicon ultrafiltration system with Amicon YM-3 membranes and dialyzed against 20 m*M* Tris buffer pH 8.0. Recombinant His-tagged BsLcar was stored at 277 K for further crystallization experiments. The protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

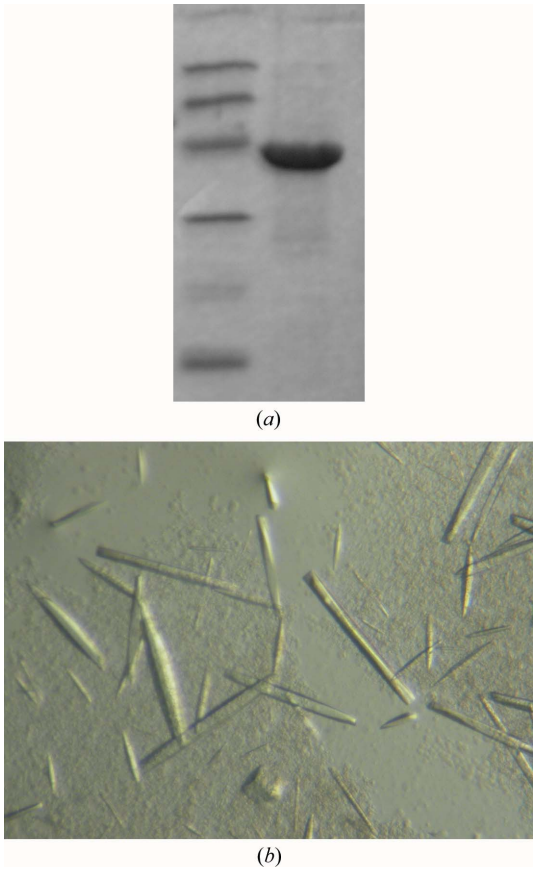

Figure 1
(*a*) SDS–PAGE analysis of purified BsLcar. Lane 1, standard molecular-weight markers: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa). Lane 2, purified L-*N*-carbamoylase (~8 µg). (*b*) Crystals of recombinant L-*N*-carbamoylase from *G. stearothermophilus* CECT43.

2.2. Molecular weight and subunit structure

The molecular weight of the monomeric form was estimated by SDS–PAGE using a low-molecular-weight marker kit (Amersham Biosciences, Barcelona). Size-exclusion chromatography–HPLC (SEC–HPLC) analysis was performed to estimate the molecular weight of the native enzyme using a gel-filtration standard (BioRad, Barcelona). The HPLC System (Breeze HPLC System, Waters, Barcelona) used a BioSEP-SEC-S 2000 column (Phenomenex, Madrid) and was equilibrated and eluted with 0.1 *M* potassium phosphate buffer pH 7.0 at a flow rate of 0.4 ml min^{-1}. 75 µl of a 1 mg ml^{-1} BsLcar solution was injected into the HPLC system and monitored at 280 nm.

2.3. Crystallization

Initial screening was carried out with the recombinant His-tagged BsLcar at a concentration of 15 mg ml^{-1} using a sparse-matrix method (Jancarik & Kim, 1991) with Hampton Research Crystal

Screens I and II at 277 and 293 K. The hanging-drop vapour-diffusion method was used, with drops made up by mixing equal volumes (2 µl) of enzyme solution and reservoir solution and suspended over a 1.0 ml reservoir.

2.4. Data collection

Crystals of L-*N*-carbamoylase were cryoprotected with 20%(*v*/*v*) glycerol in the crystallization solution. They were soaked for less than 60 s, after which they were placed in a cold nitrogen stream (Kryoflex) maintained at 110 K. X-ray diffraction data were recorded on a Bruker Smart6000 CCD detector with Kappa configuration (X8 Proteum) using Cu $K\alpha$ radiation from a Bruker Microstar microfocus (Montel Optics) rotating-anode generator operated at 45 kV and 60 mA. A total of 483 frames were collected with 120 s exposure per frame and a crystal-to-detector distance of 70 mm. Integrated intensity information was obtained for each reflection. This was then scaled with *SAINT* and corrected for absorption with *SADABS* from the *PROTEUM* software suite (Bruker AXS Inc.).

The allantoate amidohydrolase from *E. coli* K12 (Agarwal *et al.*, 2007; PDB code 1z2l; 33.1% identity with BsLcar) was used as the search model for molecular replacement using *MOLREP* (Vagin & Teplyakov, 1997). Initial refinement was carried out using simulated annealing with *phenix.refine* (Afonine *et al.*, 2005) from the *PHENIX* software suite (Adams *et al.*, 2002). Refinement is ongoing using *REFMAC*5 (Murshudov *et al.*, 1997) from the *CCP*4 software suite (Collaborative Computational Project, Number 4, 1994) and *Coot* (Emsley & Cowtan, 2004) for visualization and manual fitting.

3. Results

BsLcar was overexpressed and purified to over 95% purity in soluble form for crystallization (Fig. 1*a*). It showed an apparent molecular weight of 44 kDa, very similar to that deduced from its amino-acid sequence (~45 kDa). A protein sample of 15 mg ml^{-1} buffered in 20 m*M* Tris pH 8.0 was used to determine crystallization conditions with Hampton Research Crystal Screen I and II using the vapour-diffusion method. Crystals were obtained from drops made up of 2 µl protein solution and 2 µl 20% 2-propanol, 0.1 *M* HEPES pH 7.5, 0.2 *M* trisodium citrate at 293 K. Optimization resulted in well faceted crystals suitable for X-ray diffraction experiments, which were obtained using 15% 2-propanol, 0.1 *M* sodium cacodylate pH 6.5 and 0.6 *M* trisodium citrate. Maximum dimensions of 0.3 × 0.1 × 0.1 mm were attained in three weeks (Fig. 1*b*).

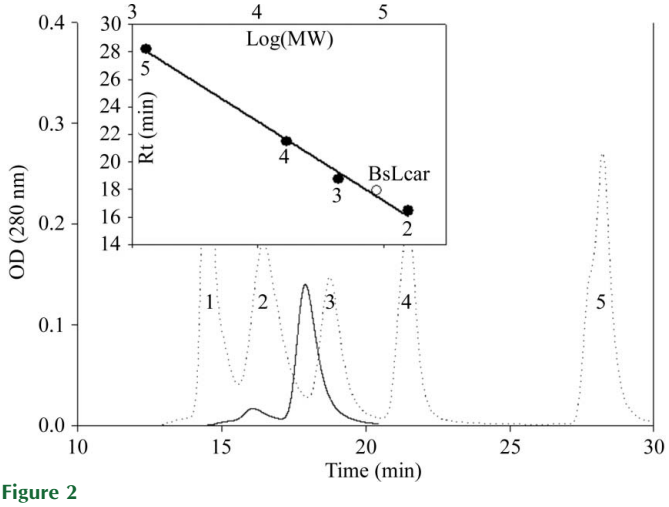

Figure 2
Size-exclusion chromatography of purified BsLcar (black line) and molecular-weight markers (dashed line). The molecular-weight markers consisted of (1) thyroglobulin (670 kDa), (2) bovine γ-globulin (158 kDa), (3) chicken ovalbumin (44 kDa), (4) equine myoglobin (17 kDa) and (5) vitamin B_{12} (1.3 kDa). The inset represents the fitting of log(MW) *versus* the retention time of the standards (black circles), together with the obtained data for BsLcar (white circle). The enzyme showed a homodimeric native structure.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.54
Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 103.2, b = 211.7, c = 43.1
Resolution range (Å)	73.9–2.75 (2.86–2.75)
Observed reflections	185586 (9098)
Independent reflections	25512 (2321)
Data completeness (%)	99.1 (91.5)
R_{merge}† (%)	12.4 (33.6)
Average $I/\sigma(I)$	12.2 (4.45)
Redundancy	3.4 (3.1)
Wilson B factor (Å^2)	19.9
Molecules per ASU	2
Matthews coefficient (Å^3 Da^{-1})	2.61
Solvent content (%)	52.9

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the weighted mean of all measurements.

BsLcar crystals belonged to the orthorhombic space group $P2_12_12$ and diffracted to 2.75 Å Bragg spacing, with an overall R_{merge} of 12.38% and of 33.6% for the 2.86–2.75 Å high-resolution shell (Table 1). The unit-cell parameters are a = 103.2, b = 211.7, c = 43.1 Å. The asymmetric unit contains two molecules, with a corresponding crystal volume per protein weight (V_M) of 2.61 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 52.9% (Westbrook, 1985). SEC–HPLC experiments conducted at pH 7.0 showed a molecular weight of approximately 90 kDa (Fig. 2) corresponding to a dimeric species.

Initial attempts using the complete dimeric structure of allantoate amidohydrolase from *E. coli* (Agarwal *et al.*, 2007) as a search model for molecular replacement were unsuccessful. The subunit of this enzyme is composed of two domains (known as the catalytic and dimerization domains for other members of the peptidase family; Martinez-Rodriguez *et al.*, 2006) linked by a hinge region. Therefore, search models were constructed from the two isolated domains. In this case, *MOLREP* was able to find a solution for both domain models. Inspection of the $|F_o - F_c|$ electron-density maps clearly shows two major peaks at the active site. As L-*N*-carbamoylases require divalent cations for enzymatic activity (Martinez-Rodriguez *et al.*, 2005), we suspect that these peaks correspond to the two metal ions known to bind in the active site; this hypothesis is also supported by the conservation of a metal-binding site in several amide-hydrolyzing enzymes (Martinez-Rodriguez *et al.*, 2006). One of the ions is located in the proximity of His79 and His189. The second ion is at binding distance from His380 and Glu125, while a fifth residue, Asp90, interacts with both metal ions, therefore acting as a bridging residue. After initial simulated annealing with *phenix.refine* (Afonine *et al.*, 2005), restrained positional refinement is being carried out with *REFMAC*5 (Murshudov *et al.*, 1997). After the first 20 cycles of positional refinement, the R and R_{free} values were 35.6% and 45.6%, respectively.

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