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Expression, crystallization and preliminary X-ray diffraction studies of *N*-carbamyl-D-amino-acid amidohydrolase from *Agrobacterium radiobacter*

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The *Agrobacterium radiobacter* CCRC 14924 *N*-carbamyl-D-amino-acid amidohydrolase, the enzyme used for production of D-amino acids, was overexpressed in *Escherichia coli* JM109. The expressed protein was crystallized by vapour diffusion using lithium sulfate as precipitant. It crystallizes in space group $P2_1$ with unit-cell parameters a = 69.8, b = 67.9 and c = 137.8 Å and β = 96.4°. There are four molecules per asymmetric unit. Crystals diffract to 2.8 Å resolution using a rotating-anode source at cryogenic (113 K) temperatures.

1. Introduction

N-Carbamyl D-amino-acid amidohydrolase (carbamylase) catalyzes the hydrolysis of *N*-carbamyl D-amino acids to optically active D-amino acids, which are valuable intermediates in the production of pharmaceutical chemicals including β-lactam antibiotics, small peptide hormones and pesticides (Syldatk *et al.*, 1990). In cooperation with D-specific hydantoinase, which catalyzes the cleavage of the specific hydantoin into a D-carbamyl derivative, the hydantoinase–carbamylase reaction process (Takahashi *et al.*, 1979; Olivieri *et al.*, 1981) is now a primary method for industrial production of D-amino acids owing to its lower reaction temperature, higher yield, rapidity and much lower amount of waste.

Carbamylases are found in microorganisms including *Agrobacterium* (Olivieri *et al.*, 1981; Runser *et al.*, 1990), *Arthobacter* (Möller *et al.*, 1988) and *Comamonas* (Ogawa *et al.*, 1993). In addition, several thermotolerant microorganisms have recently been isolated and found to possess thermostable carbamylase activity (Ogawa *et al.*, 1994; Nanba *et al.*, 1998), which might be useful for more efficient production of D-amino acids. Biochemical characterization of different carbamylases has been studied in some detail. It appears that carbamylase forms a dimer (Grifantini *et al.*, 1996) or a trimer (Ogawa *et al.*, 1994) of identical subunits of ~34 kDa. The D-enantiomer of the *N*-carbamyl amino acid is required for carbamylase activity (Ogawa *et al.*, 1993). Moreover, activity and stability of carbamylase is negatively affected by oxidizing conditions (Grifantini *et al.*, 1996), by thiol reagents, Cu^{2+}, Hg^{2+}, Ag^{+} and ammonia ions (Nanba *et al.*, 1998).

Despite tremendous efforts towards the isolation and biochemical characterization of different carbamylases, there is essentially no structural information available for carbamylase. In this paper, we describe the expression, crystallization and preliminary X-ray diffraction of carbamylase from *Agrobacterium radiobacter* CCRC 14924 as the first step towards an understanding of the substrate

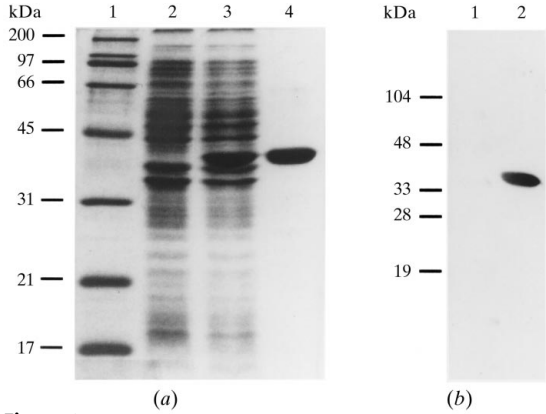

Figure 1
SDS–PAGE (*a*) and Western blot (*b*) analysis of *Agrobacterium radiobacter* carbamylase expressed in *E. coli* JM109. (*a*) Lane 1, protein markers; lane 2 total proteins prepared from *E. coli* JM109 (pQE30); lane 3, total proteins prepared from *E. coli* JM109 (pQE30-NCA); lane 4, purified carbamylase. (*b*) Lane 1, total proteins prepared from *E. coli* JM109 (pQE30); lane 2, purified carbamylase.

Table 1
Data-collection statistics of native carbamylase crystal.

Resolution limits (Å)	I/σ	R_{sym}†	Completeness (%)
40.00–6.03	25.0	0.041	94.9
6.03–4.79	19.2	0.059	96.5
4.79–4.18	19.5	0.065	96.3
4.18–3.80	14.8	0.085	94.9
3.80–3.53	10.4	0.119	94.4
3.53–3.32	7.5	0.157	93.5
3.32–3.15	5.3	0.206	93.0
3.15–3.02	3.5	0.297	91.1
3.02–2.90	2.6	0.387	90.1
2.90–2.80	2.2	0.470	87.1

† $R_{sym} = \sum_{hkl} |I_i - I_{avg}| / \sum_{hkl} \sum |I_i|$.

specificity, catalytic mechanism and thermostable properties of this enzyme.

2. Methods

2.1. Expression and purification

Carbamylase was overexpressed using *Escherichia coli* JM109 (pQE30-NCA) containing the full-length DNA clone of the enzyme. The expressed protein with a six-histidine-residue tag at the amino-terminal end was then purified by immobilized nickel-ion chromatography. Fractions containing the enzyme were collected and the sample was concentrated. Protein purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Enzyme activity was determined by high-performance liquid chromatography using *N*-carbamyl-D-*p*-hydroxyphenylglycine as substrate (Louwrier & Knowles, 1996).

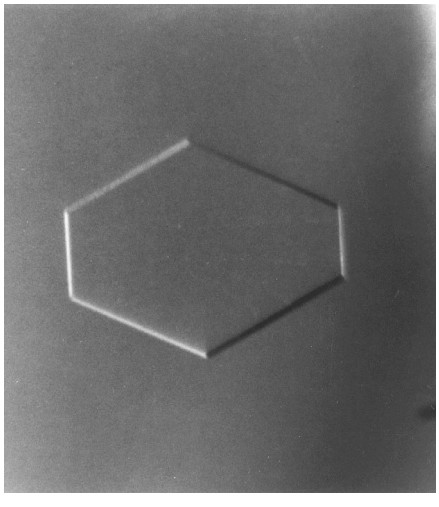

Figure 2
Crystal of carbamylase.

2.2. Crystallization

Crystallization was conducted by the hanging-drop vapour-diffusion method (Wlodawer & Hodgson, 1975) using 24-well tissue-culture plates. A 2 µl droplet containing protein solution (15 mg ml^{-1}) in 10 m*M* HEPES (pH 7.5), 1 m*M* EDTA and 0.02% sodium azide was mixed with an equal volume of the well precipitant. Initial crystallization was attempted using a screen described by Jancarik & Kim (1991) at a constant temperature of 296 K.

2.3. X-ray crystallography

Native data were collected on a R-AXIS IV imaging-plate system using double-mirror-focused Cu $K\alpha$ X-ray radiation generated from a Rigaku RU-300 rotating-anode generator. Oscillation images with a 2.5° oscillation range were processed with *DENZO* and *SCALEPACK* (Otwinowski, 1993). Data collection at room temperature indicated a rapid decay of the crystals upon exposure to X-rays. Cryo-cooling conditions have therefore been developed as follows: the crystals were soaked in synthetic mother liquor containing 15% PEG 400 and 5% glycerol for a couple of minutes and the data were collected at 113 K using an MSC X-Stream cryo-system.

3. Results and discussion

Approximately 10 mg of pure soluble carbamylase per litre of culture broth were obtained. Purified enzyme migrates with an apparent molecular mass of ~36 kDa and is >95% pure as judged by a Coomassie-stained SDS–PAGE gel (Fig. 1). Gel-filtration experiments carried out on a Superose 12 column (Pharmacia) showed that the expressed protein exists as a dimer in solution, consistent with the observation by Grifantini *et al.* (1996). The K_m and V_{max} values for the expressed carbamylase were 19 m*M* and 12 units mg^{-1}, respectively.

Crystals of carbamylase were grown within two weeks using lithium sulfate as a precipitant. The optimal precipitating condition consists of 1.15 *M* lithium sulfate and 100 m*M* HEPES buffer pH 7.0. The crystals reach a maximum size of about 0.5 × 0.4 × 0.1 mm in two months at 296 K (Fig. 2). At room temperature the crystals diffract to 2.3 Å using a rotating-anode generator. However, only a partial data set (~50%) could be collected using a single crystal, and a clear decay of the diffraction intensities was observed. By use of the cryo method, the quality of the data was greatly improved and the resolution remained the same.

The autoindexing procedure of *DENZO* indicated that the crystals belong to the monoclinic space group with unit-cell parameters a = 69.8, b = 67.9, c = 137.8 Å and β = 96.4°. The systematic absences indicated the space group to be $P2_1$. A 2.8 Å native data set has been collected. Table 1 summarizes the data-collection statistics. Based on average volume-to-mass ratios (Matthews, 1968), the asymmetric unit of the crystal is estimated to contain between three and five molecules. Since the protein acts as a dimer in solution, it is expected that there are four molecules per asymmetric unit and the crystal volume per protein mass (V_m) is 2.4 Å^3 Da^{-1}. Heavy-metal soaks are in progress in order to solve the structure by multiple isomorphous replacement.

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