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Crystallization and preliminary X-ray study of the deaminase AmnE from *Pseudomonas* sp. AP-3

The *amnE* gene from *Pseudomonas* sp. AP-3 has been verified as encoding a deaminase with 142 amino-acid residues. In order to change the substrate specificity *via* structure-based protein engineering, the *amnE* gene, after gene-code optimization, was chemically synthesized and cloned into the expression vector pET-28a. The protein was expressed in *Escherichia coli* BL21 (DE3) and purified by Ni²⁺-chelating affinity chromatography. Diffraction-quality crystals were obtained using the hanging-drop vapour-diffusion method and diffracted to a resolution of 2.09 Å. The crystals belonged to the orthorhombic space group *C*222₁, with unit-cell parameters *a* = 63.23, *b* = 88.93, *c* = 137.83 Å.

1. Introduction

Aniline compounds are major industrial raw materials that are widely used for the production of pesticides, dyes, plastics and other chemical products. The release of aniline compounds into the environment has enabled a number of microorganisms to mineralize these xenobiotic compounds (You & Bartha, 1982; Aoki *et al.*, 1983; Lyons *et al.*, 1984; Keskar *et al.*, 1990; Lechner & Straube, 1998). Many studies of the biodegradation of aniline compounds, such as aniline, chloroanilines, 3-aminophenol and 2-aminophenol, have been reported (You & Bartha, 1982; Aoki *et al.*, 1983; Lechner & Straube, 1998; Takenaka *et al.*, 1997, 1998, 2000).

2-Aminophenol, which is one of the intermediate metabolites in the biodegradation of nitrobenzene through the partial reductive pathway characterized by the release of nitrite, has been reported to be mineralized by *Pseudomonas* sp. AP-3 (Takenaka *et al.*, 1997, 1998, 2000). The metabolic pathway for 2-aminophenol in *Pseudomonas* sp. AP-3 is called the *meta*-cleavage pathway, in which 2-amino-2,4-pentadienoic acid is transformed into 2-oxopent-4-enoic acid by the deaminase AmnE. Compared with the metabolic pathway in *P. pseudoalcaligenes* JS45, the decarboxylation step occurs prior to the deamination reaction (He & Spain, 1999). In other bacterial metabolic pathways, it has been demonstrated that sequential enzyme-catalysed reactions can occur in either order (Hsu *et al.*, 1990). It is of interest to us to study the structure of deaminase AmnE in *Pseudomonas* sp. AP-3 to determine whether the sequence of deamination and decarboxylation is reversible. Based on the analysis of a three-dimensional structural model, the amino-acid residues in the catalytic domain of AmnE can be selected as mutation sites and can be replaced by residues that can reduce the substrate specificity. The redesigned AmnE protein is anticipated to be multi-specific and to interact with numerous partners, even 2-aminophenol. Here, we describe the cloning, expression, purification, crystallization and preliminary X-ray diffraction studies of the AmnE protein from *Pseudomonas* sp. AP-3.

2. Materials and methods

2.1. Cloning

The gene coding for the AmnE protein (GenBank accession No. BAB03536) was optimized and chemically synthesized for expression in *Escherichia coli* based on the released sequence in GenBank. The gene was then amplified by PCR using the forward primer 5'-GCTCCCATGGTTAGCAAAGCTGATA-3' and the reverse primer

5'-TTTGAGCTCGAGCGGCTTGTATG-3'. The *Nco*I and *Xho*I restriction sites are shown in bold. The PCR product was digested with *Xho*I and *Nco*I and subcloned into the pET-28a plasmid, which encodes a C-terminal His₆ tag. All plasmids were verified by sequencing (Genewiz, Beijing, People's Republic of China).

2.2. Expression and purification

The recombinant plasmid was transformed into *E. coli* BL21 (DE3) strain and grown in LB medium containing 50 µg ml⁻¹ kanamycin. Cells were then induced at an OD₆₀₀ of 0.6–0.8 by the addition of 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 16 h at 299 K. Cells harvested by centrifugation for 30 min at 8600g were resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 5% glycerol) and lysed using a high-pressure homogenizer (JNBio, People's Republic of China) on ice. The cell lysate was clarified by centrifugation for 30 min at 48 400g and 277 K; the supernatant was filtered and loaded onto an Ni²⁺-chelating affinity chromatography column (GE Healthcare, USA) equilibrated with lysis buffer and the unbound proteins were washed away with wash buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 5% glycerol, 50 mM imidazole). The tightly bound proteins, mainly comprising AmnE, were eluted with elution buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 5% glycerol, 100 mM imidazole). The AmnE protein was further purified by anion-exchange chromatography on a Resource Q column (GE Healthcare, USA). The protein was concentrated by ultrafiltration using a Millipore centrifugal ultrafiltration device (Amicon Ultra, 10 kDa cutoff) and analysed by SDS-PAGE. For crystallization purposes, the protein was concentrated to 10 mg ml⁻¹ in the buffer solution (20 mM Tris-HCl, 5% glycerol) by ultrafiltration without removal of the C-terminal His₆ tag.

2.3. Crystallization

Crystallization experiments were performed at 289 K using the hanging-drop vapour-diffusion method. Drops were prepared by mixing 1 µl purified protein solution and 1 µl reservoir solution on a cover slip and were equilibrated against 0.2 ml reservoir solution. Commercial crystallization kits (Crystal Screen, Crystal Screen 2, Index Screen and PEG/Ion Screen) from Hampton Research were

Figure 1
SDS-PAGE analysis of AmnE protein purified using a Resource Q column. Lane M, molecular-weight markers (labelled in kDa). Lane 1, purified AmnE protein.

Table 1

Diffraction data statistics.

Values in parentheses are for the outermost resolution shell.

Wavelength (Å)	1.5418
Space group	C222 ₁
Unit-cell parameters (Å)	<i>a</i> = 63.23, <i>b</i> = 88.93, <i>c</i> = 137.83
Resolution (Å)	50.00–2.09 (2.13–2.09)
<i>R</i> _{merge} † (%)	4.7 (19.6)
Average <i>I</i> /σ(<i>I</i>)	64.6 (6.9)
Completeness (%)	99.4 (95.4)
Resolution range (Å)	50.00–2.30
No. of observed reflections	22932 (752)
No. of unique reflections	14970 (708)
No. of molecules in the asymmetric unit	3
<i>V</i> _M (Å ³ Da ⁻¹)	3.15
Solvent content (%)	61.02

† $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the mean intensity of all symmetry-related reflections.

used for sparse-matrix screening of conditions and further rounds of optimization to produce diffracting crystals for data collection.

2.4. X-ray diffraction data collection and processing

The protein crystals were quick-soaked in reservoir solution containing 20%(v/v) glycerol as a cryoprotectant and were flash-cooled in liquid nitrogen. The preliminary X-ray diffraction analysis of crystals was performed using a Rigaku MicroMax-007 HF rotating-anode X-ray generator operated at 40 kV and 30 mA [Tianjin International Joint Academy of Biotechnology and Medicine (TJAB), People's Republic of China]. 720 frames were collected with 0.5° oscillation per image at 100 K. X-ray diffraction images were indexed, integrated and subsequently scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The data quality was assessed using *SFCHECK* (Vaguine *et al.*, 1999) and the solvent content was calculated using *MATTHEWS_COEF* from *CCP4* (Winn *et al.*, 2011).

3. Results

The *amnE* gene was cloned into the pET-28a vector and expressed in *E. coli* BL21 (DE3) cells with a yield of about 20 mg pure protein per litre of cell culture. After purification on a Resource Q column, the

Figure 2
Typical crystals of AmnE protein.

Figure 3
Typical X-ray diffraction pattern from an AmnE crystal.

AmnE protein was calculated to be >95% pure based on 12% SDS-PAGE analysis, showing a molecular mass of about 16 kDa, which is consistent with the calculated molecular mass of the AmnE protein with a C-terminal His₆ tag (Fig. 1).

Initial crystals of AmnE protein were obtained in PEG/Ion screen condition H1 after initial sparse-matrix screening, but failed to diffract. Following further optimization, rod-like crystals were obtained with mother liquor consisting of 0.2 M bis-tris pH 6.5, 0.2 M potassium chloride, 23%(w/v) polyethylene glycol 6000. The crystal dimensions were about 0.2 × 0.3 × 1.2 mm (Fig. 2).

The AmnE protein crystals diffracted to a resolution of 2.09 Å and belonged to space group *C*222₁, with unit-cell parameters *a* = 63.23, *b* = 88.93, *c* = 137.83 Å. Assuming the presence of three molecules per asymmetric unit, the *V_M* value is 3.1 Å³ Da⁻¹, corresponding to a solvent content of 61.02% (Matthews, 1968). A typical diffraction pattern for AmnE protein crystal is shown in Fig. 3 and data statistics are listed in Table 1.

AmnE protein shares 39% sequence identity with the putative translation initiation inhibitor Ph0854. Molecular replacement and structure refinement using Ph0854 (PDB entry 2dyj; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) and other related proteins as search models are in progress. The three-dimensional structures of the AmnE protein will provide insights into

the biochemical properties of this novel enzyme from *Pseudomonas* sp. AP-3.

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References

- Aoki, K., Shinke, R. & Nishira, H. (1983). *Agric. Biol. Chem.* **47**, 1611–1616.
- He, Z. & Spain, J. C. (1999). *Arch. Microbiol.* **171**, 309–316.
- Hsu, T., Lux, M. F. & Drake, H. L. (1990). *J. Bacteriol.* **172**, 5901–5907.
- Keskar, J. B., Charde, V. N., Parhad, N. M., Ingle, A. O. & Dagainawala, H. F. (1990). *Indian J. Microbiol.* **30**, 455–458.
- Lechner, U. & Straube, G. (1998). *J. Basic Microbiol.* **28**, 629–637.
- Lyons, C. D., Katz, S. & Bartha, R. (1984). *Appl. Environ. Microbiol.* **48**, 491–496.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Takenaka, S., Murakami, S., Kim, Y.-J. & Aoki, K. (2000). *Arch. Microbiol.* **174**, 265–272.
- Takenaka, S., Murakami, S., Shinke, R. & Aoki, K. (1998). *Arch. Microbiol.* **170**, 132–137.
- Takenaka, S., Murakami, S., Shinke, R., Hatakeyama, K., Yukawa, H. & Aoki, K. (1997). *J. Biol. Chem.* **272**, 14727–14732.
- Vaguine, A. A., Richelle, J. & Wodak, S. J. (1999). *Acta Cryst. D* **55**, 191–205.
- Winn, M. D. *et al.* (2011). *Acta Cryst. D* **67**, 235–242.
- You, I. S. & Bartha, R. (1982). *J. Agric. Food Chem.* **30**, 274–277.