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Crystallization and X-ray diffraction analysis of nylon hydrolase (NylC) from *Arthrobacter* sp. KI72

Nylon hydrolase (NylC) encoded by *Arthrobacter* plasmid pOAD2 (NylC_{p2}) was expressed in *Escherichia coli* JM109 and purified by ammonium sulfate fractionation, anion-exchange column chromatography and gel-filtration chromatography. NylC_{p2} was crystallized by the sitting-drop vapour-diffusion method with ammonium sulfate as a precipitant in 0.1 M HEPES buffer pH 7.5 containing 0.2 M NaCl and 25% glycerol. Diffraction data were collected from the native crystal to a resolution of 1.60 Å. The obtained crystal was spindle shaped and belonged to the C-centred orthorhombic space group C222₁, with unit-cell parameters $a = 70.84$, $b = 144.90$, $c = 129.05$ Å. A rotation and translation search gave one clear solution containing two molecules per asymmetric unit.

1. Introduction

The biodegradation of unnatural synthetic compounds provides a suitable system for studying how microorganisms evolve the enzymes that are essential for the degradation of such xenobiotic compounds. We have studied the degradation of polymeric nylon-6 and the by-products of the manufacture of nylon-6, namely 6-aminohexanoate oligomers (nylon oligomers), as a model system (Okada *et al.*, 1983; Negoro, 2000). We found that three enzymes, 6-aminohexanoate-cyclic dimer hydrolase (NylA; Yasuhira *et al.*, 2010), 6-aminohexanoate-dimer hydrolase (NylB; Negoro *et al.*, 2005; Ohki *et al.*, 2006, 2009; Kawashima *et al.*, 2009) and nylon hydrolase (NylC), are responsible for the degradation of nylon-6 and its related compounds.

NylC is a member of the N-terminal nucleophile hydrolase superfamily and degrades cyclic and linear oligomers of 6-aminohexanoate with a degree of polymerization greater than three by an endo-type mode (Negoro *et al.*, 1992, 2012; Kakudo *et al.*, 1993, 1995; Yasuhira, Uedo *et al.*, 2007). NylC has been found in *Arthrobacter* sp. KI72 (pOAD2 plasmid-encoded NylC; NylC_{p2}), *Agromyces* sp. KY5R (NylC_A) and *Kocuria* sp. KY2 (NylC_K; Yasuhira, Uedo *et al.*, 2007). NylC_A and NylC_K have five and 15 amino-acid substitutions, respectively, relative to the NylC_{p2} sequence, and both have 10–20 K higher thermostability than NylC_{p2}. Interestingly, replacement of the NylC_{p2} sequence with the NylC_A/NylC_K sequences drastically alters the thermostability of the enzyme. In particular, two mutations (D122G/H130Y) from NylC_A and two further mutations (D36A/E263Q) from NylC_K in NylC_{p2} (heat denaturation temperature $T_m = 325$ K) enhanced the protein thermostability by 36 K ($T_m = 361$ K; Negoro *et al.*, 2012).

Knowledge of the crystal structure of NylC will allow us to study the molecular basis of the thermostability and catalytic mechanism of this enzyme. Recently, we reported the crystallization conditions and the structural analysis of NylC_A at 2.0 Å resolution (Yasuhira *et al.*, 2011; Negoro *et al.*, 2012). However, the obtained crystal contained 15 monomer molecules in a single asymmetric unit of space group I222, with unit-cell parameters $a = 155.86$, $b = 214.45$, $c = 478.80$ Å. Based on the structural analyses, the 15 monomer molecules constitute three tetramers, one dimer forming a tetramer that is related by a crystallographic twofold axis and one monomer forming a tetramer that is related by crystallographic 222 symmetry in a single asymmetric unit of NylC_A (Negoro *et al.*, 2012). In addition, most mutations which significantly affect protein stability among the NylC_{p2}, NylC_A and

NylC_K proteins were found to be located at the subunit interfaces generating the dimeric or tetrameric structures (Negoro *et al.*, 2012). Since crystals containing such a high number of molecules with heterogeneous oligomeric states in the asymmetric unit are inappropriate for further crystallographic analysis of the mutants, we newly screened the crystallization conditions of the NylC_{p2} protein to obtain a crystal with fewer molecules in an asymmetric unit.

2. Materials and methods

2.1. Expression and enzyme purification

The pBluescript II SK(+) expression vector (Stratagene, La Jolla, California, USA) was used to express the *nylC_{p2}* gene, which encodes a polypeptide of 355 amino acids, in *Escherichia coli*. Plasmid pSKFC4, in which a 1.1 kb *Bam*HI–*Pst*I fragment containing *nylC_{p2}* was inserted downstream of the *lacP* region in the vector (*Bam*HI/*Pst*I sites), was constructed (Fig. 1; Yasuhira, Tanaka *et al.*, 2007) identically to *nylC_A* (Yasuhira *et al.*, 2011). Competent cells of *E. coli* JM109 were prepared by the CaCl₂ method (Sambrook & Russell, 2001) and were stocked at 193 K before use. For purification of NylC_{p2}, the competent cells (100 µl) were newly transformed with pSKFC4 (10 µg). The cells were incubated at 273 K for 30 min, followed by heat-shock treatment at 315 K for 45 s (Sambrook & Russell, 2001). The cell suspension was transferred to 900 µl SOC medium (Sambrook & Russell, 2001) and incubated at 310 K for 1 h. Since prolonged cultivation at 303 K or higher temperatures resulted in insoluble NylC_{p2} in the cells, *E. coli* cells harbouring the hybrid plasmid were grown in 400 ml TB medium (Sambrook & Russell, 2001) containing 100 mg l⁻¹ ampicillin and 1 mM isopropyl β-D-1-thiogalactopyranoside at 298 K for 60 h. The cells were harvested by centrifugation at 10 000g for 10 min, washed with buffer A (20 mM phosphate buffer pH 7.3 containing 10% glycerol) and suspended in

20 ml buffer A. After disrupting the cells by ultrasonication (20 kHz, 5 min, four times), the cell lysate obtained by centrifugation at 30 000g for 30 min was used as the crude enzyme solution. To precipitate the NylC_{p2} fraction, ammonium sulfate (40% saturation) was added to the crude enzyme solution (22 mg ml⁻¹, 20 ml) and kept for 30 min on ice. The NylC_{p2} precipitate was obtained by centrifugation at 30 000g for 10 min and dissolved in 20 ml buffer A. To decrease the ammonium sulfate concentration in the sample, the enzyme solution was diluted to 140 ml with buffer A and the NylC_{p2} was purified by HiTrap Q Sepharose HP (two sequentially joined 5 ml columns; GE Healthcare) column chromatography with a 0.16–0.4 M NaCl linear gradient in a total volume of 160 ml. NylC_{p2} fractions were concentrated by centrifugation at 4000g for 40 min using an Amicon Ultra microconcentrator (membrane cutoff size 10 kDa; Millipore) and purified using Sephacryl S-200 HR column (16 × 650 mm; GE Healthcare) chromatography. All purification procedures were carried out at 277 K and 25 mg purified NylC_{p2} was obtained.

2.2. Crystallization

Crystallization tests were performed by the sitting-drop vapour-diffusion method in 96-well plates. Drops were prepared by mixing 2 µl purified NylC_{p2} solution (10 mg ml⁻¹ protein in buffer A) and 2 µl reservoir solution and were equilibrated against 100 µl reservoir solution at 283 K. To obtain crystals suitable for X-ray diffraction, a survey for optimum conditions was conducted by varying the concentration of ammonium sulfate (1.2–2.2 M in 0.2 M intervals) in the presence of buffers of varying acidity (0.1 M HEPES buffer pH 7.5–8.5 in 0.5 pH-unit intervals), salt solutions (0.2 M NaCl) and/or 25% glycerol.

2.3. Data collection and crystallographic analysis

Cryocooling was performed for data collection by placing the crystal in a cold nitrogen stream at 100 K. Diffraction data sets were collected on beamline BL41XU at SPring-8 (Hyogo, Japan) equipped with an ADSC Q315 CCD detector system. The following parameters were chosen for data collection: wavelength, 1.0000 Å; crystal-to-detector distance, 200 mm; oscillation range per image, 1.0°. Indexing,

Figure 1

Structure of the pSKFC4 plasmid. The pSKFC4 plasmid is a hybrid plasmid composed of a 1.1 kb *Bam*HI–*Pst*I fragment containing the *nylC_{p2}* gene and a 2.9 kb *Bam*HI–*Pst*I fragment of the pBluescript II SK(+) expression vector (Yasuhira, Tanaka *et al.*, 2007). The vector plasmid contains the ampicillin-resistance (*ampR*) gene as the selection marker for transformation of *E. coli*. The *nylC_{p2}* gene is expressed under the control of the *lacP* promoter of the vector region. The DNA region for the *Bam*HI site, ribosome-binding (Shine–Dalgarno; SD) sequence and initiation codon (ATG) of the *nylC_{p2}* gene are shown in the nucleotide sequence.

Figure 2

Native PAGE (a) and SDS-PAGE (b) of purified NylC. Lane M, molecular-size marker; lane 1, NylC_A; lane 2, NylC_{p2}. Molecular masses are labelled in kDa.

Table 1Data-collection statistics for NylC_{p2}.

Values in parentheses are for the outer resolution shell.

Space group	C222 ₁
Unit-cell parameters (Å)	$a = 70.84$, $b = 144.90$, $c = 129.05$
Wavelength (Å)	1.0000
Resolution (Å)	50–1.60 (1.64–1.60)
Total reflections	598801
Unique reflections	86374 (5510)
Completeness (%)	99.5 (96.3)
$R_{\text{merge}}^{\dagger}$ (%)	5.5 (45.1)
$\langle I/\sigma(I) \rangle$	30.4 (3.45)
Multiplicity	6.9 (5.9)

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

integration and scaling of reflections were performed using the *HKL-2000* program package (Otwinowski & Minor, 1997). Diffraction data were collected from the NylC_{p2} crystal to a resolution of 1.60 Å (Table 1). A rotation and translation search for molecular replacement was performed by *Phaser* (McCoy *et al.*, 2007) within the *CCP4* program suite (Winn *et al.*, 2011), using one protomer (chain A) of the NylC_A structure (PDB entry 3axg; Negoro *et al.*, 2012) as a template.

3. Results

The *nylC_{p2}* gene was cloned and expressed in *E. coli* followed by purification of the NylC_{p2} protein. The NylC_{p2} sample obtained at the final step of the purification procedure (the eluate from Sephacryl S-200 HR column chromatography) gave a single band on native PAGE. The mobility was identical to the mobility of purified NylC_A analysed as a control experiment (Fig. 2*a*). In contrast, SDS–PAGE of NylC_{p2} gave two bands [α subunit (27 kDa) and β subunit (9 kDa)], which were common to the corresponding bands derived from NylC_A (Fig. 2*b*). The auto-cleavage generating the α - and β -subunits from the inactive precursor (35 kDa) is a specific feature of the N-terminal nucleophile hydrolase family. From these analyses, we judged that the NylC_{p2} sample was purified to homogeneity and we used this sample for crystallization experiments.

The initial grid screening gave microcrystals after 1 week under the condition 1.4 M ammonium sulfate, 0.2 M sodium chloride, 0.1 M HEPES buffer pH 7.5. After optimization of the crystallization conditions, crystals with the highest quality were obtained using 1.6 M ammonium sulfate as a precipitant in 0.1 M HEPES buffer pH 7.5,

Figure 3

Crystals of NylC_{p2}. The crystal (0.2 × 0.1 × 0.1 mm) of NylC_{p2} was obtained using a reservoir solution consisting of 1.6 M ammonium sulfate, 0.1 M HEPES buffer pH 7.5, 0.2 M NaCl, 25% glycerol.

0.2 M NaCl and 25% glycerol. Spindle-like crystals grew to typical dimensions of 0.2 × 0.1 × 0.1 mm (Fig. 3).

Diffraction data for the NylC_{p2} crystal were collected to 1.60 Å resolution, higher than the previously reported NylC_A structure determined at 2.0 Å resolution (Yasuhira *et al.*, 2011; Negoro *et al.*, 2012). Neither a dose-dependent increase in R_{merge} nor a decrease in the signal-to-noise ratio [*i.e.* $\langle I/\sigma(I) \rangle$] per image were detected, indicating that there was no significant radiation damage during data collection. The symmetry of diffraction intensities and systematic absences along the 00*l* axis indicated that the crystal belonged to the C-centred orthorhombic space group C222₁. The unit-cell parameters of the crystal were $a = 70.84$, $b = 144.90$, $c = 129.05$ Å. A diffraction image is shown in Fig. 4 and diffraction data statistics are summarized in Table 1. There are likely to be two molecules in the asymmetric unit, as estimated from the Matthews coefficient (V_M) of 2.24 Å³ Da^{−1} (Matthews, 1968), corresponding to a solvent content of 45%. Indeed, the rotation search in *Phaser* gave two clear unique peaks with Z-scores of 5.08 and 4.87 above the default cutoff, and the subsequent translation search yielded a clear solution for two molecules. The crystal packing analysis revealed that each of the two NylC_{p2} monomers seems to extensively interact with the adjacent monomer related by the crystallographic twofold axis.

The oligomeric structure of NylC_{p2} in aqueous solution has been suggested previously based on estimation of the molecular weight of native NylC_{p2} by gel-filtration chromatography as 93 000 Da, while that of the polypeptide as deduced from the nucleotide sequence was calculated as 36 902 Da (Kakudo *et al.*, 1995). These biochemical and crystallographic analyses indicate that the enzyme is likely to exist as a dimer in solution. In the case of the NylC_A crystal, the protein molecules existed mainly as a tetramer (Negoro *et al.*, 2012). Each monomer of NylC_A contacts at two interfaces with the two adjacent subunits, with the extent of one contact (1451 Å³) being less than half that of the other (3121 Å³). The extent of contact seems to be related to the ability of oligomer formation and we therefore predict that the more extensive contact area contributes to dimer formation of NylC_{p2}. In the case of NylC_{p2} there were only two molecules in the asymmetric unit, compared with NylC_A which had 15 molecules (Yasuhira *et al.*, 2011). The higher resolution and the far lower

Figure 4

X-ray diffraction image of the native NylC_{p2} crystal. The detector edge corresponds to 1.60 Å resolution.

number of molecules per asymmetric unit of the NylC_{p2} crystal compared with the NylC_A crystal will benefit structural analysis of NylC_{p2} and its mutants.

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References

- Kakudo, S., Negoro, S., Urabe, I. & Okada, H. (1993). *Appl. Environ. Microbiol.* **59**, 3978–3980.
- Kakudo, S., Negoro, S., Urabe, I. & Okada, H. (1995). *J. Ferment. Bioeng.* **80**, 12–17.
- Kawashima, Y., Ohki, T., Shibata, N., Higuchi, Y., Wakitani, Y., Matsuura, Y., Nakata, Y., Takeo, M., Kato, D. & Negoro, S. (2009). *FEBS J.* **276**, 2547–2556.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Negoro, S. (2000). *Appl. Microbiol. Biotechnol.* **54**, 461–466.
- Negoro, S., Kakudo, S., Urabe, I. & Okada, H. (1992). *J. Bacteriol.* **174**, 7948–7953.
- Negoro, S., Ohki, T., Shibata, N., Mizuno, N., Wakitani, Y., Tsurukame, J., Matsumoto, K., Kawamoto, I., Takeo, M. & Higuchi, Y. (2005). *J. Biol. Chem.* **280**, 39644–39652.
- Negoro, S. *et al.* (2012). *J. Biol. Chem.* **287**, 5079–5090.
- Ohki, T., Shibata, N., Higuchi, Y., Kawashima, Y., Takeo, M., Kato, D. & Negoro, S. (2009). *Protein Sci.* **18**, 1662–1673.
- Ohki, T., Wakitani, Y., Takeo, M., Yasuhira, K., Shibata, N., Higuchi, Y. & Negoro, S. (2006). *FEBS Lett.* **580**, 5054–5058.
- Okada, H., Negoro, S., Kimura, H. & Nakamura, S. (1983). *Nature (London)*, **306**, 203–206.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd ed. New York: Cold Spring Harbor Laboratory Press.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Yasuhira, K., Shibata, N., Mongami, G., Uedo, Y., Atsumi, Y., Kawashima, Y., Hibino, A., Tanaka, Y., Lee, Y.-H., Kato, D., Takeo, M., Higuchi, Y. & Negoro, S. (2010). *J. Biol. Chem.* **285**, 1239–1248.
- Yasuhira, K., Shibata, N., Tanaka, Y., Kumagai, N., Tanaka, Y., Nagai, K., Kato, D., Takeo, M., Negoro, S. & Higuchi, Y. (2011). *Acta Cryst.* **F67**, 892–895.
- Yasuhira, K., Tanaka, Y., Shibata, H., Kawashima, Y., Ohara, A., Kato, D., Takeo, M. & Negoro, S. (2007). *Appl. Environ. Microbiol.* **73**, 7099–7102.
- Yasuhira, K., Uedo, Y., Takeo, M., Kato, D. & Negoro, S. (2007). *J. Biosci. Bioeng.* **104**, 521–524.