Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and X-ray diffraction analysis of 6-aminohexanoate-dimer hydrolase from *Arthrobacter* sp. KI72

Taku Ohki,[a] Nobuhiro Mizuno,[b] Naoki Shibata,[c,d] Masahiro Takeo,[a] Seiji Negoro[a] and Yoshiki Higuchi[c,d]*

[a]Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2201, Japan, [b]Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan, [c]Department of Life Science, Graduate School of Science, University of Hyogo, 3-2-1 Koto, Kamigori-cho, Ako-gun, Hyogo 678-1297, Japan, and [d]RIKEN Harima Institute/SPring-8, 1-1-1 Koto, Mikazuki-cho, Sayo-gun, Hyogo 679-5248, Japan

Correspondence e-mail: hig@sci.u-hyogo.ac.jp

Received 8 August 2005
Accepted 12 September 2005
Online 30 September 2005

To investigate the structure–function relationship between 6-aminohexanoate-dimer hydrolase (EII) from *Arthrobacter* sp. and a cryptic protein (EII′) which shows 88% sequence identity to EII, a hybrid protein (named Hyb-24) of EII and EII′ was overexpressed, purified and crystallized using the sitting-drop vapour-diffusion method with ammonium sulfate as a precipitant in MES buffer pH 6.5. The crystal belongs to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 96.37$, $c = 113.09$ Å. Diffraction data were collected from native and methylmercuric chloride derivative crystals to resolutions of 1.75 and 1.80 Å, respectively.

1. Introduction

Microbial degradation of manmade synthetic compounds provides examples for investigating how microorganisms have evolved enzymes responsible for the degradation of these specific compounds. The bacterium *Arthrobacter* sp. KI72 (formerly *Flavobacterium* sp.) has been utilized as a model system to investigate the degradation of byproducts of nylon, including various oligomers of 6-amino-hexanoate (nylon oligomer; Negoro, 2000). Previous biochemical studies have revealed that the existence of three enzymes encoded on a plasmid pOAD2 is responsible for the degradation of nylon oligomers. These enzymes are 6-aminohexanoate-cyclic dimer hydrolase (EI; Kinoshita *et al.*, 1977), 6-aminohexanoate-dimer hydrolase (EII; Kinoshita *et al.*, 1981) and *endo*-type 6-amino-hexanoate-oligomer hydrolase (EIII; Kakudo *et al.*, 1993). A protein homologous to EII also exists (EII′); it is located on a different region of pOAD2 from the other three enzymes and shows 88% sequence identity to EII, but has only 0.5% of the activity of the EII enzyme (Negoro *et al.*, 1983; Kato *et al.*, 1995; Fig. 1*a*). Amino-acid substitutions at just two positions in the EII′ protein, G181D (Gly to Asp at position 181) and H266N (His to Asn at position 266), are sufficient to restore the enzyme activity to that of the parental EII enzyme (Kato *et al.*, 1991). Based on these results, it is believed that the EII gene has evolved by gene duplication followed by nucleotide-base substitution from an ancestral gene (Okada *et al.*, 1983).

Knowledge of the three-dimensional structures of the EII and EII′ enzymes will enhance our understanding of the catalytic mechanism and the evolution of nylon oligomer-degrading enzymes. A comparison of the structures of EII and EII′ with those of related proteins with similar structures is likely to aid efforts to improve enzyme function through protein engineering. Here, we report on the crystallization and preliminary crystallographic analysis of an EII-EII′ hybrid protein (Hyb-24). The amino-acid sequence of the first 24 N-terminal amino acids in Hyb-24 is identical to the N-terminal sequence of EII and the remainder of the Hyb-24 sequence is identical to the corresponding sequence of EII′ (Fig. 1). The results reported here will be used as a basis for the determination of the crystal structure of 6-aminohexanoate-dimer hydrolase.

2. Materials and methods

2.1. Expression and purification

To construct plasmid pHY3, which expresses high levels of the EII′-type protein (Hyb-24), pKT1, a hybrid plasmid composed of

pKP1500 and the 1421 bp *Eco*RI–*Hin*dIII fragment containing the *nylB* gene, was digested with *Pvu*II and *Hin*dIII and the 2778 bp *Pvu*II–*Hin*dIII fragment in the plasmid pKT1 was combined with the 1232 bp *Pvu*II–*Hin*dIII fragment containing the *nylB′* gene (Fig. 1*b*). As a result, the gene product (Hyb-24) includes five-amino-acid replacements, *i.e.* T3A [from Thr (EII′) to Ala (EII) at position 3], P4R, T5S, S8Q and D15G in the EII′ sequence, but shows the same activity level as EII′. pHY3 was transformed into *Escherichia coli* KP3998 and the transformed bacteria were grown in a 500 ml culture of Terrific broth medium (12 g Bacto tryptone, 24 g Bacto yeast extract, 4.0 g glycerol, 2.3 g KH_2PO_4, 12.5 g K_2HPO_4, 1000 ml distilled water, pH 7.0) with ampicillin (100 µg ml^{-1}) at 310 K until the cell density (OD_{600}) reached 0.6. Expression of the Hyb-24 protein was induced by addition of isopropyl-1-thio-β-galactoside to a final concentration of 1 m*M*. After 24 h of cultivation, cells were harvested by centrifugation at 3980*g* for 10 min at 277 K, washed with buffer *A* (20 m*M* phosphate buffer containing 10% glycerol pH 7.3) and suspended in 60 ml buffer *A*. The cell suspension was lysed by sonication and the lysate was centrifuged at 32 000*g* for 30 min at 277 K. The supernatant was loaded onto a Hi-Trap Q-Sepharose (Amersham Biosciences) column (5 ml) equilibrated with buffer *A*. After washing with buffer *A*, the enzyme was eluted with a total of 300 ml of the same buffer containing a linear NaCl gradient from 0 to 0.5 *M* at a flow rate of 3 ml min^{-1}. Fractions, each containing a volume of 6 ml, were collected. The fractions containing the enzyme were pooled and concentrated to a volume of 1 ml using a Centriprep YM-10 (Millipore Inc.). The concentrated enzyme was applied onto a Sephacryl S-200 High-Resolution gel-filtration (Amersham Biosciences) column (1.6 × 40 cm) equilibrated with buffer *A* and eluted with the same buffer at a flow rate of 0.5 ml min^{-1}. 1 ml fractions were collected. The fractions containing Hyb-24 were pooled and the enzyme solution was again applied onto a Hi-Trap Q-Sepharose column. Elution was performed as described above.

The purity was assessed by Coomassie-stained SDS–PAGE (12.5%) and native PAGE. The purified protein solution was concentrated using a Centriprep YM-10 and a Centricon YM-3 microconcentrator (Millipore Inc.) for the crystallization trials. All purification and concentration steps were carried out at 277 K. A typical yield of 10 mg of Hyb-24 per 500 ml of culture was achieved.

2.2. Crystallization

Initial crystallization trials were performed using Wizard I and Wizard II (Emerald Biostructures) screening kits and by implementing the sitting-drop vapour-diffusion technique in 96-well plates. Protein droplets prepared by mixing 1 µl protein solution (17 mg ml^{-1} protein in buffer *A*) and 1 µl reservoir solution were equilibrated against 100 µl reservoir solution at 283 K. Crystals were obtained from both Wizard I (condition Nos. 20 and 27) and Wizard II (condition Nos. 4, 7, 18, 24, 28 and 35) solutions within 48 h.

To obtain crystals suitable for X-ray diffraction, the protein concentration, the precipitant, salt, glycerol concentration and the

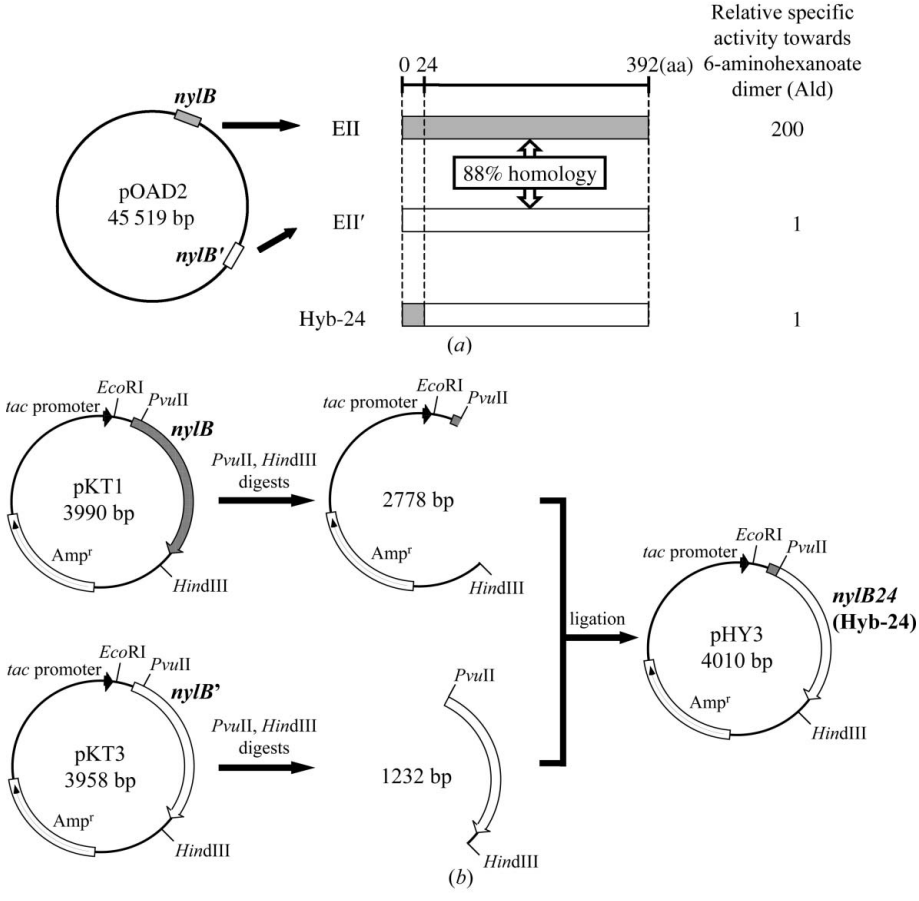

Figure 1
Structure of nylon-oligomer-degrading plasmid pOAD2 and construction of a hybrid plasmid. (*a*) The functional enzyme (EII; *nylB* gene product) and its analogous protein (EII′; *nylB′* gene product) are encoded on plasmid pOAD2 of *Arthrobacter* sp. KI72. Shaded boxes and open boxes represent the regions of EII and EII′, respectively. (*b*) Scheme for constructing a hybrid plasmid pHY3 encoding the *nylB24* gene.

Figure 2
Crystals of Hyb-24 obtained using a reservoir solution consisting of 2.0 *M* ammonium sulfate, 0.2 *M* lithium sulfate, 0.1 *M* MES buffer pH 6.5 and 15% glycerol.

pH of the buffer solutions were further optimized. Protein droplets were then prepared by mixing 2 µl protein (34 mg ml^{-1}) and 2 µl reservoir solution and were equilibrated against 800 µl reservoir solution at 283 K.

2.3. Data collection and crystallographic analysis

For data collection, native crystals were soaked in a cryoprotectant solution [2.4 *M* ammonium sulfate, 30%(*v*/*v*) glycerol and 0.1 *M* MES buffer pH 6.5] for 24 h prior to freezing in a nitrogen cold stream. Methylmercuric chloride (CH_3HgCl) derivative crystals were prepared by soaking crystals for 24 h in cryoprotectant solution containing 0.1 m*M* methylmercuric chloride. X-ray diffraction data were collected from native and heavy-atom derivative crystals at 100 K using synchrotron radiation with an ADSC detector on the BL44B2 beamline at SPring-8 (Hyogo, Japan). The wavelength employed for these diffraction experiments was 1.0000 Å and the crystal-to-detector distance was maintained at 150 mm with an oscillation range per image of 1°, covering a total oscillation range of 180°. Determination of the unit-cell parameters and integration of reflections were performed using the *HKL*2000 program package (Otwinowski & Minor, 1997).

3. Results

The *nylB24* gene encoding a polypeptide of 392 amino acids was cloned and expressed in *E. coli* and the Hyb-24 protein was purified to homogeneity. The purity of the protein samples used for crystallization was confirmed by SDS–PAGE and native PAGE. The highest quality crystals were obtained using solutions consisting of 2.0–2.2 *M* ammonium sulfate, 0.1–0.2 *M* lithium sulfate and 15–25% glycerol in 0.1 *M* MES buffer pH 6.5. Hexagonal-shaped crystals grew to typical dimensions of 0.3 × 0.3 × 0.3 mm (Fig. 2). Diffraction data were collected from native and methylmercuric chloride derivative crystals to 1.75 and 1.80 Å, respectively. Radiation damage was not observed during data collection. The crystals belong to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 96.37$, $c = 113.09$ Å. Crystal parameters and diffraction data statistics are summarized in Table 1.

Table 1
Data-collection statistics.

Values in parentheses are for the outer resolution shell.

Data collection	Native	CH_3HgCl derivative
Space group	$P3_121$ or $P3_221$	$P3_121$ or $P3_221$
Unit-cell parameters		
$a = b$ (Å)	96.370	96.318
c (Å)	113.090	113.045
Wavelength (Å)	1.0000	1.0000
Resolution (Å)	30–1.75 (1.81–1.75)	30–1.80 (1.86–1.80)
Total reflections	663588	617198
Unique reflections	61867 (6013)	56718 (5587)
Completeness (%)	99.8 (98.4)	100.0 (100.0)
$R_{\rm merge}$ (%)	5.2 (40.8)	6.4 (36.4)
$\langle I/\sigma(I)\rangle$	54.6 (5.9)	47.1 (6.4)

We would like to express our sincere thanks to Dr Y. Shiro of RIKEN Harima Institute/SPring-8 and Dr H. Axelrod of the Joint Center for Structural Genomics at the Stanford Synchrotron Radiation Laboratory for their helpful discussions. This work was supported in part by grants from the 21st Century COE Program, the National Project on Protein Structural and Functional Analyses and the JAXA project.

References

Kakudo, S., Negoro, S., Urabe, I. & Okada, H. (1993). *Appl. Environ. Microbiol.* **59**, 3978–3980.
Kato, K., Fujiyama, K., Hatanaka, H. S., Prijambada, I. D., Negoro, S., Urabe, I. & Okada, H. (1991). *Eur. J. Biochem.* **200**, 165–169.
Kato, K., Ohtsuki, K., Koda, Y., Maekawa, T., Yomo, T., Negoro, S. & Urabe, I. (1995). *Microbiology*, **141**, 2585–2590.
Kinoshita, S., Negoro, S., Muramatsu, M., Bisaria, V. S., Sawada, S. & Okada, H. (1977). *Eur. J. Biochem.* **80**, 489–495.
Kinoshita, S., Terada, T., Taniguchi, T., Takene, Y., Masuda, S., Matsunaga, N. & Okada, H. (1981). *Eur. J. Biochem.* **116**, 547–551.
Negoro, S. (2000). *Appl. Microbiol. Biotechnol.* **54**, 461–466.
Negoro, S., Taniguchi, T., Kanaoka, M., Kimura, H. & Okada, H. (1983). *J. Bacteriol.* **155**, 22–31.
Okada, H., Negoro, S., Kimura, H. & Nakamura, S. (1983). *Nature (London)*, **306**, 203–206.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.