Crystallization and preliminary X-ray crystallographic studies of dipeptidyl aminopeptidase BII from *Pseudoxanthomonas mexicana* WO24

Yasumitsu Sakamoto,^a Yoshiyuki Suzuki,^b Ippei Iizuka,^a Chika Tateoka,^a Saori Roppongi,^a Hirofumi Okada,^b Takamasa Nonaka,^a Yasushi Morikawa,^b Kazuo T. Nakamura,^c Wataru Ogasawara^b and Nobutada Tanaka^{c*}

^aSchool of Pharmacy, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba, Iwate 028-3694, Japan, ^bDepartment of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan, and ^cSchool of Pharmacy, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Correspondence e-mail:
ntanaka@pharm.showa-u.ac.jp

Received 29 November 2013
Accepted 24 December 2013

Dipeptidyl aminopeptidase BII from *Pseudoxanthomonas mexicana* WO24 (DAP BII) is able to cleave a variety of dipeptides from the amino-terminus of substrate peptides. For crystallographic studies, DAP BII was overproduced in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. X-ray diffraction data to 2.3 Å resolution were collected using an orthorhombic crystal form belonging to space group $P2_12_12_1$, with unit-cell parameters $a = 76.55$, $b = 130.86$, $c = 170.87$ Å. Structural analysis by the multi-wavelength anomalous diffraction method is in progress.

1. Introduction

Dipeptidyl aminopeptidase (DAP or DPP; EC 3.4.14) catalyses the removal of dipeptides from the amino-terminus of peptides and proteins. In mammals, several enzymes with DPP activities have been purified from various tissues and they have been classified into four groups according to their selectivity for particular artificial substrates, pH optima and catalytic classification (Lee & Snyder, 1982). DPP I (cathepsin C) cleaves Gly-Arg-β-naphthylamide (Gly-Arg-βNA) and Gly-Phe-βNA at pH 6, DPP II cleaves Lys-Ala-βNA with a pH optimum of 5, DAP III degrades Arg-Arg-βNA with a pH optimum of 8–9 and DPP IV hydrolyses Gly-Pro-βNA at pH 8. DPP I is a cysteine protease, whereas DPPs II–IV are serine proteases. Among the DPPs, DPP IV has been most extensively studied because some DPP IV inhibitors have been accepted for the treatment of type 2 diabetes.

We have reported the identification, purification and characterization of DAP BI (bacterial dipeptidyl aminopeptidase; Ogasawara, Ochiai et al., 1996; Ogasawara et al., 1998), DAP BII (Ogasawara, Kobayashi et al., 1996), DAP BIII (Ogasawara, Kobayashi et al., 1996) and DAP IV (Ogasawara, Ogawa et al., 1996; Ogasawara et al., 2005) from *Pseudoxanthomonas mexicana* WO24, which is a Gram-negative aerobic bacterium isolated from the wastewater of a bean curd (tofu) factory. On the basis of the enzymological data that we obtained, we proposed that bacterial DAPs should be classified in a manner different from that of mammalian DPPs, except for DAP IV (Ogasawara, Kobayashi et al., 1996). We have also characterized a prolyl oligopeptidase (POP) from *P. mexicana* WO24 (unpublished data) and demonstrated that DAP BI, DAP BIII, DAP IV and POP belong to the POP family (Kanatani et al., 1991; Rawlings et al., 1991) and are classified into clan SC, family S9 in the MEROPS database (Rawlings et al., 2012). Recently, we have successfully determined the complete nucleotide sequence of DAP BII from *P. mexicana* WO24 (accession No. AB889525), and the predicted amino-acid sequence of DAP BII revealed that the enzyme is a 79 kDa (722 amino-acid) serine protease classified into clan PA, family S46, completely different from the POP family. The only two dipeptidylaminopeptidases, DPP7 (Banbula et al., 2001) and DPP11 (Ohara-Nemoto et al., 2011) from *Porphyromonas gingivalis*, were characterized in family S46.

To date, several crystal structures of family S9 enzymes have been reported, for example those of mammalian DPP IV (Engel et al., 2003; Hiramatsu et al., 2003; Rasmussen et al., 2003) and POP (Fülöp et al., 1998). However, a crystal structure analysis of a family S46 enzyme has not yet been reported. Thus, the three-dimensional structure of DAP BII is essential not only to understand the catalytic

mechanism of family S46 enzymes but also to clarify the structural origin of the exo-type peptidase activities of these enzymes. Here, we report the crystallization and preliminary X-ray crystallographic analysis of DAP BII from *P. mexicana* WO24.

2. Materials and methods

2.1. Overproduction and purification

Overproduction and purification of DAP BII were performed as described by Suzuki *et al.* (2014). Briefly, an *Escherichia coli* Rosetta 2(DE3)pLacI transformant harbouring the mature DAP BII sequence (Gly25–Lys722) with the signal peptide of DAP BIII from *P. mexicana* WO24 (Ogasawara *et al.*, 1998) inserted into pET-22b expression plasmid was used for the production of recombinant DAP BII. The mature DAP BII was composed of 698 amino acids with a theoretical molecular mass of 76 299.4 Da and an isoelectric point of 6.30. Cells were grown in 2×YT medium at 298 K to an OD₆₀₀ of 0.6. Overproduction of DAP BII was induced by 0.2 mM IPTG for 16 h at 298 K. After this period, the cells were harvested by centrifugation at 8000g. The cells were disrupted using BugBuster Protein Extraction Reagent (Novagen). The cell extract was obtained by centrifugation at 27 000g for 30 min.

DAP BII was precipitated by 70% saturated ammonium sulfate and the precipitate was then dissolved in 30% saturated ammonium sulfate solution in 50 mM Tris–HCl pH 9.0 (buffer A). The enzyme solution was filtrated with a 0.22 µm pore filter and applied onto a 20 ml HiPrep 16/10 Butyl column (GE Healthcare) equilibrated with 30% saturated ammonium sulfate in buffer A. The column was washed with two column volumes of wash buffer (30% saturated ammonium sulfate in buffer A). After washing, DAP BII was eluted with five column volumes of elution buffer (30–0% gradient of ammonium sulfate in buffer A). The fractions containing DAP BII were pooled and applied onto a 50 ml HiPrep 26/10 Desalting column equilibrated with 20 mM Tris–HCl pH 9.0 (buffer B). The DAP BII was then applied onto a 1 ml Mono Q 5/50 GL anion-exchange chromatography column equilibrated with buffer B. The column was washed with five column volumes of buffer B. After washing, DAP BII was eluted with 30 column volumes of elution buffer (0–0.2 M gradient of sodium chloride in buffer B). The fractions containing DAP BII were pooled, buffer-exchanged to 80 mM Tris–HCl pH 8.5 and concentrated to 10 mg ml^{−1} using a Vivaspin 20 concentrator

Figure 1
An orthorhombic crystal of DAP BII.

(GE Healthcare). The protein concentration was determined by the Bradford assay using bovine serum albumin as a standard (Bio-Rad).

2.2. Crystallization

Initial sparse-matrix crystal screening (Jancarik & Kim, 1991) was conducted using Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, PEG/Ion, PEG/Ion 2 and Index from Hampton Research, Wizard I, II and III, Ozma 1k, 4k, 8k and 10k, and Cryo I and II from Emerald BioSystems, and PACT and JCSG+ from Qiagen. Crystallization was carried out by the hanging-drop method, in which 1 µl protein solution was mixed with the same volume of reservoir solution and incubated at 293 K. The drops were suspended over 500 µl reservoir solution in 24-well plates.

2.3. X-ray data collection

Data collection was performed by the rotation method at 100 K using an ADSC Q315r CCD detector with synchrotron radiation ($\lambda = 1.000 \text{ \AA}$) on beamline 17A of the Photon Factory (PF), Tsukuba, Japan. The Laue group and unit-cell parameters were determined using the *HKL-2000* package (Otwinowski & Minor, 1997). Self-rotation and native Patterson functions were calculated using the programs *POLARRFN* and *FFT* from the *CCP4* suite (Winn *et al.*, 2011).

3. Results and discussion

3.1. Overproduction, purification and crystallization

DAP BII was successfully cloned, overproduced and purified to homogeneity. SDS–PAGE of the purified enzyme revealed a single 73 kDa protein band by Coomassie Brilliant Blue staining. The hydrolytic activities of recombinant DAP BII for synthetic substrates were $3.4 \pm 0.1 \text{ µmol min}^{-1} \text{ mg}^{-1}$ (U mg^{-1}) and $10 \pm 0.2 \text{ U mg}^{-1}$ for Gly-Phe-pNA and Ala-Ala-pNA, respectively, which were compar-

Figure 2
X-ray diffraction image from a DAP BII crystal. The circles indicate resolutions of 11.24, 5.67, 3.83, 2.93 and 2.40 Å.

able to those of native DAP BII (3.3 ± 0.1 and 9.5 ± 0.2 U mg⁻¹ for Gly-Phe-pNA and Ala-Ala-pNA, respectively). The recombinant enzyme was equally stable to the native enzyme at temperatures below 293 K for 30 min (Ogasawara *et al.*, 1998).

Initial crystal screening produced several microcrystals within one week. Thin plate-shaped crystals grew from condition No. 35 (20% PEG 8000, 0.05 M sodium acetate) of Ozma 8k (Emerald Bio-Systems) and condition Nos. 7 [20% (m/v) PEG 8000 in 0.1 M CHES pH 9.5], 25 [20% (m/v) PEG 8000 and 0.2 M sodium chloride in 0.1 M phosphate-citrate pH 4.2] and 87 [25% (m/v) PEG 3350 in 0.1 M trisodium acetate pH 4.5, 0.1 M bis-tris pH 5.5] of JCSG+ (Qiagen). Trials to improve the crystallization conditions were performed by varying the pH, buffer system, crystallizing agent concentration and protein concentration. To obtain crystals suitable for X-ray analysis, a droplet was prepared by mixing equal volumes (2 μ l + 2 μ l) of the protein solution (working solution) described above and reservoir solution [18% (m/v) PEG 8000, 20% (v/v) glycerol, 2 mM zinc chloride in 0.08 M CHES pH 9.5] and was suspended over 500 μ l reservoir solution in 24-well plates. Thin plate-shaped crystals with typical dimensions of approximately $1.0 \times 1.0 \times 0.01$ mm grew within one week (Fig. 1).

3.2. X-ray analyses

Since the crystallization conditions of DAP BII described above contained 20% (v/v) glycerol in the reservoir solution, X-ray data

Figure 3 Stereographic projection of the self-rotation function in spherical polar angles at $\kappa = 180^\circ$ for the DAP BII crystal. The self-rotation search was carried out with the program *POLARFN* from the *CCP4* suite (Winn *et al.*, 2011) using data in the 30.0–3.5 Å resolution shell and an integration radius of 25.0 Å. The three strongest peaks [relative height of 100; (ω, φ) = (0.0°, 0.0°), (90.0°, 0.0°) and (90.0°, 90.0°)] indicate the crystallographic twofold symmetry (origin peak). Contour lines are drawn at an increment of 10% of the origin peak, starting at 30% of the origin peak. The program produces a constant rotation angle κ for different axis directions defined by ω (the angle from the pole) and φ (the angle around the equator). The φ angles are marked on the circumference and the ω angles are defined as 0° or 180° at the centre and 90° around the edge. Noncrystallographic twofold rotation axes (57% height of the origin peak) are found at (ω, φ) = (30.4°, 0.0°), for example. Since the noncrystallographic symmetry (NCS) twofold axis is parallel to the *ac* plane and perpendicular to the *b* axis of the unit cell, a secondarily generated NCS twofold axis exists, for example (ω, φ) = (120.4°, 0.0°).

Table 1

Data-collection statistics for DAP BII.

Values in parentheses are for the outer shell.

Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å)	<i>a</i> = 76.55, <i>b</i> = 130.86, <i>c</i> = 170.87
No. of subunits in asymmetric unit	2 [1 dimer]
Solvent content (%)	56.1
X-ray source	Beamline 17A, PF
Detector	ADSC Q315r
Wavelength (Å)	1.000
Resolution range (Å)	50.0–2.30 (2.34–2.30)
No. of observed reflections	543533 (18285)
No. of unique reflections	76838 (3657)
Multiplicity	7.1 (5.0)
Mean <i>I</i> /σ(<i>I</i>)	50.3 (14.6)
<i>B</i> factor (Wilson plot) (Å ²)	26.8
<i>R</i> _{merge} † (%)	5.8 (16.9)
Completeness (%)	99.7 (96.0)

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

collection could be performed under cryogenic conditions without further addition of cryoprotectant. Crystals from the hanging drop were directly mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K just prior to data collection. The Laue group of the DAP BII crystals was found to be *mmm* and the unit-cell parameters were *a* = 76.55, *b* = 130.86, *c* = 170.87 Å. Only reflections with *h* = 2*n*, *k* = 2*n* and *l* = 2*n* were observed along the (*h*00), (0*k*0) and (00*l*) axes, respectively, indicating the orthorhombic space group *P*2₁2₁2₁. The current best diffraction data from a DAP BII crystal were collected to 2.3 Å resolution (Fig. 2). Data-collection statistics are summarized in Table 1. Assuming the presence of two or three subunits per asymmetric unit led to empirically acceptable *V_M* values of 2.80 or 1.87 Å³ Da⁻¹, corresponding to solvent contents of 56.1 or 34.2% (Matthews, 1968), respectively. Calculation of the self-rotation function (Fig. 3) showed a clear (57% height of the origin peak) noncrystallographic twofold axis, indicating that the number of molecules in the asymmetric unit of the DAP BII crystal would be an even number. This observation is consistent with the dimeric nature of DAP BII (Ogasawara *et al.*, 1998) and indicates that the number of molecules in the asymmetric unit of the DAP BII crystal must be two. A search for heavy-atom derivatives intended for phasing by multiple isomorphous replacement and preparation of selenomethionine-substituted DAP BII using LeMaster medium (Hendrickson *et al.*, 1990) and *E. coli* B834(DE3) cells for phasing by the Se-MAD/SAD method are under way.

We thank Drs Y. Yamada and N. Matsugaki of the Photon Factory for their help with data collection at the synchrotron facility. This work was supported in part by the Program for the Strategic Research Foundation at Private Universities (to NT and Y. Sakamoto) and a Grant-in-Aid for Scientific Research (C) (to Y. Sakamoto) from the MEXT of Japan.

References

- Banbula, A., Yen, J., Oleksy, A., Mak, P., Bugno, M., Travis, J. & Potempa, J. (2001). *J. Biol. Chem.* **276**, 6299–6305.
- Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H.-U. & Brandstetter, H. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 5063–5068.
- Fülpö, V., Böcskei, Z. & Polgár, L. (1998). *Cell*, **94**, 161–170.
- Hendrickson, W. A., Horton, J. R. & LeMaster, D. M. (1990). *EMBO J.* **9**, 1665–1672.
- Hiramatsu, H., Kyono, K., Higashiyama, Y., Fukushima, C., Shima, H., Sugiyama, S., Inaka, K., Yamamoto, A. & Shimizu, R. (2003). *Biochem. Biophys. Res. Commun.* **302**, 849–854.
- Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

- Kanatani, A., Masuda, T., Shimoda, T., Misoka, F., Lin, X. S., Yoshimoto, T. & Tsuru, D. (1991). *J. Biochem.* **110**, 315–320.
- Lee, C. M. & Snyder, S. H. (1982). *J. Biol. Chem.* **257**, 12043–12050.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Ogasawara, W., Kobayashi, G., Ishimaru, S., Okada, H. & Morikawa, Y. (1998). *Gene*, **206**, 229–236.
- Ogasawara, W., Kobayashi, G., Okada, H. & Morikawa, Y. (1996). *J. Bacteriol.* **178**, 6288–6295.
- Ogasawara, W., Ochiai, K., Ando, K., Yano, K., Yamasaki, M., Okada, H. & Morikawa, Y. (1996). *J. Bacteriol.* **178**, 1283–1288.
- Ogasawara, W., Ogawa, Y., Yano, K., Okada, H. & Morikawa, Y. (1996). *Biosci. Biotechnol. Biochem.* **60**, 2032–2037.
- Ogasawara, W., Tanaka, C., Suzuki, M., Kobayashi, G., Ogawa, Y., Okada, H. & Morikawa, Y. (2005). *Protein Expr. Purif.* **41**, 241–251.
- Ohara-Nemoto, Y., Shimoyama, Y., Kimura, S., Kon, A., Haraga, H., Ono, T. & Nemoto, T. K. (2011). *J. Biol. Chem.* **286**, 38115–38127.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Rasmussen, H. B., Branner, S., Wiberg, F. C. & Wagtmann, N. (2003). *Nature Struct. Biol.* **10**, 19–25.
- Rawlings, N. D., Barrett, A. J. & Bateman, A. (2012). *Nucleic Acids Res.* **40**, D343–D350.
- Rawlings, N. D., Polgar, L. & Barrett, A. J. (1991). *Biochem. J.* **283**, 647–648.
- Suzuki, Y. *et al.* (2014). Submitted.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.