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

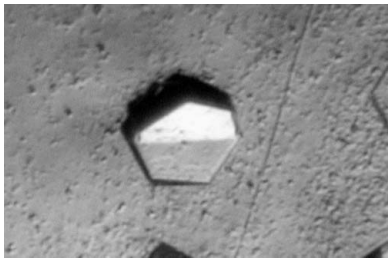
Crystallization and preliminary X-ray characterization of prolyl tripeptidyl aminopeptidase from *Porphyromonas gingivalis*

Yoshitaka Nakajima, Kiyoshi Ito,* Yue Xu, Nozomi Yamada, Yuko Onohara, Takashi Ito and Tadashi Yoshimoto

Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8521, Japan

Correspondence e-mail: k-ito@net.nagasaki-u.ac.jp

Received 20 September 2005
Accepted 1 December 2005
Online 5 November 2005



A recombinant form of prolyl tripeptidyl aminopeptidase from *Porphyromonas gingivalis* has been crystallized by the hanging-drop vapour-diffusion method using potassium sodium tartrate as a precipitating agent. The crystals belong to the hexagonal space group $P6_322$, with unit-cell parameters $a = b = 149.4$, $c = 159.7$ Å. The crystals are most likely to contain one subunit of a dimer in the asymmetric unit, with a V_M value of 3.14 Å^3 Da^{-1}. Diffraction data were collected to 2.1 Å resolution using synchrotron radiation at the BL5 station of the Photon Factory.

1. Introduction

Prolyl tripeptidyl aminopeptidase catalyzes the hydrolysis of peptide bonds when proline is located at the third position from an amino-terminus. This enzyme was isolated from *Porphyromonas gingivalis* (Cox & Eley, 1989; Banbula *et al.*, 1999), which is a major pathogen associated with periodontitis. This bacterium is asaccharolytic and needs to utilize external peptides as a carbon and energy source. It also produces collagenase (Grenier & McBride, 1987) and dipeptidyl aminopeptidase IV (Mayrand & Grenier, 1985). These peptidases are required in order to utilize the collagen, from which proline-rich peptides are produced as nutrients for the bacterium. It seems likely that these enzymes and prolyl tripeptidyl aminopeptidase play an important role in periodontitis. Prolyl tripeptidyl aminopeptidase and dipeptidyl aminopeptidase IV are homodimeric type II transmembrane proteins anchoring each N-terminal region and are both members of the prolyl oligopeptidase family. Several crystal structures of mammalian dipeptidyl aminopeptidase IV have been determined (Engel *et al.*, 2003; Hiramatsu *et al.*, 2003; Rasmussen *et al.*, 2003; Thoma *et al.*, 2003), but the structure of prolyl tripeptidyl aminopeptidase has not yet been solved. Dipeptidyl aminopeptidase IV is composed of an N-terminal β-propeller domain and a C-terminal catalytic domain with α/β-hydrolase topology and there is an active site on the domain boundary. In dipeptidyl aminopeptidase IV, it has been revealed that a substrate penultimate proline residue and an N-terminal amino group are respectively recognized by residues belonging to the catalytic domain and the β-propeller domain (Engel *et al.*, 2003; Rasmussen *et al.*, 2003; Thoma *et al.*, 2003). Prolyl tripeptidyl aminopeptidase was found to show 23.5, 16.2 and 14.8% sequence identity with *P. gingivalis*, human and porcine dipeptidyl aminopeptidase IV, respectively. The C-terminal catalytic domain of prolyl tripeptidyl aminopeptidase was then found to show 30.8, 25.2 and 26.3% sequence identity, respectively, with the equivalent domains of dipeptidyl aminopeptidase IV. Based on a comparison of the primary structure, the hydrophobic residues in the catalytic domain, which comprise a proline-recognized pocket, are well conserved between mammalian dipeptidyl aminopeptidase IV and prolyl tripeptidyl aminopeptidase. Since the catalytic domain of prolyl tripeptidyl aminopeptidase is considered to be similar to that of mammalian dipeptidyl aminopeptidase IV, it is expected that the distinct substrate specificities are achieved by the difference in β-propeller domain structure between the two enzymes.

In order to clarify the mechanisms of the highly specific recognition of the antepenultimate proline residue and tripeptidyl activity, we constructed a plasmid to overexpress the N-terminal truncated and soluble form of the enzyme and studied this enzyme by X-ray crys-

Table 1
Data-collection statistics.

Values in parentheses refer to the last resolution shell.

	Native	$(CH_3COO)_2Hg$
Data collection		
Space group	$P6_322$	$P6_322$
Unit-cell parameters		
a, b (Å)	149.4	150.0
c (Å)	159.7	160.9
Radiation source	Synchrotron radiation	Cu $K\alpha$
Wavelength	1.000	1.5418
Resolution range (Å)	50–2.1 (2.18–2.10)	40–3.1 (3.27–3.10)
No. of unique reflections	61466 (6027)	19892 (2838)
Completeness (%)	99.9 (100)	99.8 (100)
Redundancy	11.3 (10.8)	8.2 (8.3)
R_{merge}†	0.077 (0.271)	0.111 (0.302)
Mean $I/\sigma(I)$	85.0 (15.3)	18.7 (7.3)
Derivative data		
R_{diff}‡		0.166
No. of derivative sites		2
Figure of merit		0.207

† $R_{merge} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i I_{hkl,i}$, where I is the observed intensity and $\langle I\rangle$ is the average intensity for multiple measurements. ‡ $R_{diff} = \sum \big||F_{PH}| - |F_P|\big| / \sum |F_P|$, where $|F_{PH}|$ and $|F_P|$ are derivative and native structure-factor amplitudes, respectively.

tallography. This is the first report of the crystallization and the preliminary X-ray crystallographic analysis of prolyl tripeptidyl aminopeptidase.

2. Experimental

2.1. Expression and purification

As it has been hypothesized that the N-terminal hydrophobic region acts as an anchor to associate the enzyme with the membrane, an N-terminal truncated enzyme was expressed. To amplify the sequence lacking the region corresponding to residues 1–38, primer Ptp39Leu (5′-CGCGGATCC*CTGATGCCCGGAGGAAAAG*-3′), containing a *Bam*HI site, and primer PtpEndLeu (5′-GAAAA-AGCTT*CATAAGTGATC*-3′), containing a *Hin*dIII site, were synthesized. The sequences shown in italics correspond to Leu39–Glu45 and the Asp730-stop codon, respectively. PCR was performed using these primers and pGPTP as a template. The amplification condition consisted of an initial denaturation at 369 K for 5 min, 35 cycles of 367 K for 1 min, 333 K for 1 min and 345 K for 1 min and a final extension at 345 K for 7 min. The PCR product was digested with *Bam*HI and *Hin*dIII and was ligated into a similarly digested pQE30 (Qiagen) to produce pQE-ptp39.

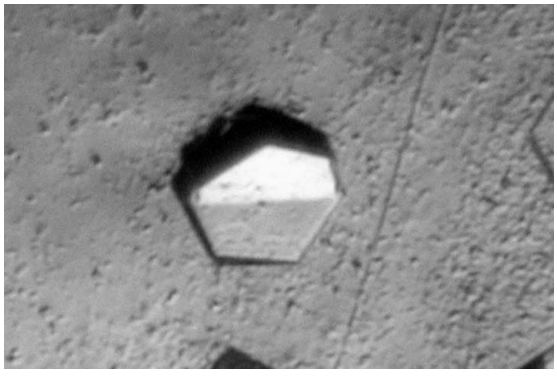


Figure 1
Crystals of prolyl tripeptidyl aminopeptidase as grown by the hanging-drop method. The average dimensions of these crystals were 0.3 × 0.2 × 0.2 mm.

Escherichia coli M15 transformed with pQE-ptp39 plasmid was aerobically cultured in 20 l N-broth containing 50 µg ml^{-1} ampicillin at 303 K for 18 h using a jar fermenter (MBS). The cells were suspended in 20 m*M* Tris–HCl buffer pH 7.5 and were disrupted by sonication in an ice bath using an Ultrasonic Disruptor (UD200, Tomy). The cell lysate was centrifuged at 12 000*g* for 20 min to remove cell debris. 2% protamine sulfate was added to the supernatant in a dropwise fashion, resulting in 16 mg per gram of wet cells, for the purpose of removing chromosomes and viscous materials. After centrifugation, the supernatant was fractionated with ammonium sulfate from 35 to 70% saturation. The precipitate was dissolved in 20 m*M* Tris–HCl buffer pH 7.0 containing 35% saturated ammonium sulfate and the enzyme solution was applied onto a Toyopearl HW65C column equilibrated with the same solution. Enzymes were eluted with a linear gradient of ammonium sulfate concentration from 35 to 0% saturation. The enzyme was then purified in a DEAE-Toyopearl column equilibrated with 20 m*M* Tris–HCl buffer pH 7.0 using a linear gradient from 0 to 1 *M* sodium chloride. The purified enzyme was homogeneous on SDS–PAGE. The purified enzyme was dialyzed against 200 m*M* potassium chloride with 20 m*M* Tris–HCl buffer pH 7.0, concentrated to 16.5 mg ml^{-1} using a Centriprep YM-30 (Millipore) and stored at 193 K.

2.2. Crystallization

Initial screening of the crystallization condition was performed by the microbatch method at 277 and 293 K using sparse-matrix screens (Jancarik & Kim, 1991) based on the commercially available Crystal Screens I and II (Hampton Research Inc.) and Wizard Screens I and II (Emerald Biosystems, Inc.). Several crystal forms were obtained and one of the promising crystallization conditions was optimized using the hanging-drop vapour-diffusion method at 293 K. A 2 µl droplet of 16.5 mg ml^{-1} protein solution mixed with the same amount of reservoir solution was equilibrated against 500 µl reservoir solution (1.1 *M* potassium sodium tartrate, 100 m*M* CHES buffer pH 9.0 and 200 m*M* lithium sulfate) to give crystals of prolyl tripeptidyl aminopeptidase.

2.3. Data collection

Data collection for the native crystal was performed at 100 K using a wavelength of 1.00 Å using the synchrotron-radiation source and an ADSC Quantum 315 CCD detector system at the Photon Factory BL5 station (Tsukuba, Japan). For data collection under cryogenic conditions, crystals were soaked for 1 min in a solution containing 30%(*v*/*v*) glycerol, 1.1 *M* potassium sodium tartrate, 100 m*M* CHES buffer pH 9.0 and 200 m*M* lithium sulfate. Crystals were mounted in a nylon loop and flash-cooled in a liquid-nitrogen stream at 100 K. The data for the crystals soaked in 1 m*M* mercury acetate for 12 h were collected to 3.1 Å resolution at 100 K on a Rigaku R-AXIS IV^{++} detector using Cu $K\alpha$ radiation generated by a Rigaku MicroMax007 rotating-anode X-ray generator with Osmic confocal focusing mirrors operated at 40 kV and 20 mA. The former data were processed and scaled using *HKL*2000 (Otwinowski & Minor, 1997) and the latter using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The open reading frame of pQE-ptp39 consists of 2079 bp coding for 693 amino-acid residues. The isoelectric value was calculated to be 6.17. The purified prolyl tripeptidyl aminopeptidase showed a single band of 83 kDa on SDS–PAGE, which is in good agreement with the

calculated value of 82 266 Da. From a gel-filtration experiment, it was suggested that the prolyl tripeptidyl aminopeptidase exists as a dimer, as does dipeptidyl aminopeptidase IV.

Hexagonal column-shaped crystals appeared within 3 d of incubation and grew to maximum dimensions of 0.3 × 0.3 × 0.2 mm (Fig. 1). From the data collection, the crystal was determined to belong to space group $P6_322$, with unit-cell parameters $a = b = 149.4$, $c = 159.7$ Å, $\gamma = 120^\circ$. Assuming the presence of one subunit of the dimer in an asymmetric unit, the Matthews coefficient V_M was calculated to be 3.14 Å^3 Da^{-1}, indicating a solvent content of approximately 61% in the unit cell (Matthews, 1968). These values are within the range typical for protein crystals (Kantardjieff & Rupp, 2003).

A native data set of 61 466 unique reflections was collected with high redundancy (11.3-fold), giving a data-set completeness of 99.9% in the resolution range 50.0–2.10 Å with an R_{merge} of 7.7% (Table 1). These data indicate that the crystals are of good quality for X-ray structural analysis. The crystals showed no significant decay upon exposure.

P. gingivalis prolyl tripeptidyl aminopeptidase shows sequence homology with human and porcine dipeptidyl aminopeptidase IV, the structures of which are known. We have tried to carry out molecular replacement with *AMoRe* (Navaza, 1994) using monomeric or partial models of mammalian dipeptidyl aminopeptidase IV as a search model. However, no solutions for either molecular orientation or molecular packing have been found.

Determination of two mercury sites, refinement of the heavy-atom parameters and calculations of the initial phases were performed with the program *SOLVE* (Terwilliger & Berendzen, 1999), which resulted in a mean figure of merit of 0.21 at 10–3.1 Å resolution. We tried to solve the phase problem by the single isomorphous replacement method, but the phasing power was not sufficient to determine the structure. We plan to solve the crystal structure using the multi-wavelength anomalous dispersion method using a synchrotron-radiation source or the multiple isomorphous replacement method resulting from a search for other heavy-atom derivatives.

References

Banbula, A., Mak, P., Bugno, M., Silberring, J., Dubin, A., Nelson, D., Travis, J. & Potempa, J. (1999). *J. Biol. Chem.* **274**, 9246–9252.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Cox, S. W. & Eley, B. M. (1989). *J. Periodontal Res.* **24**, 353–361.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

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.

Grenier, D. & McBride, B. C. (1987). *Infect. Immun.* **55**, 3131–3136.

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.

Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.

Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Mayrand, D. & Grenier, D. (1985). *Can. J. Microbiol.* **31**, 134–138.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

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.

Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.

Thoma, R., Loffler, B., Stihle, M., Huber, W., Ruf, A. & Hennig, M. (2003). *Structure*, **11**, 947–959.