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Crystallization and preliminary X-ray crystallographic studies of Pz peptidase A from *Geobacillus collagenovorans* MO-1

Pz peptidase A is an intracellular M3 metallopeptidase found in the thermophile *Geobacillus collagenovorans* MO-1 that recognizes collagen-specific tripeptide units (Gly-Pro-Xaa). Pz peptidase A shares common reactions with mammalian thimet oligopeptidase (TOP) and neurolysin, but has extremely low primary sequence identity to these enzymes. In this work, Pz peptidase A was cocrystallized with a phosphine peptide inhibitor (PPI) that selectively inhibits TOP and neurolysin. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 56.38$, $b = 194.15$, $c = 59.93$ Å, $\beta = 106.22^\circ$. This is the first crystallographic study of an M3 family peptidase–PPI complex.

1. Introduction

We have studied the degradation of collagen by the thermophilic bacterium *Geobacillus collagenovorans* MO-1 (Okamoto *et al.*, 2001; Miyake *et al.*, 2005; Itoi *et al.*, 2006). The strain MO-1 produces three distinct enzymes related to collagen degradation. One is an extracellular and collagenolytic protease that can specifically bind to and digest collagen at multiple sites but not at the numerous repetitions of a collagen-specific tripeptide sequence (Gly-Pro-Xaa; Okamoto *et al.*, 2001). The others are two intracellular M3 metallopeptidases that recognize collagen-specific tripeptide units and are called Pz peptidases A and B after the synthetic substrate 4-phenylazobenzyl-oxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Pz-PLGPR), which contains the collagen-specific sequence (Miyake *et al.*, 2005). An enzyme called thimet oligopeptidase (TOP) or endopeptidase 24-15 (EC 3.4.24.15) that hydrolyzes synthetic oligopeptide substrates at the same sites as those of bacterial Pz peptidases is known to occur in mammalian cells (Tisliar, 1993; Barrett & Chen, 2004). Interestingly, the main role of TOP is not to metabolize collagens in mammalian cells, but is to extinguish the signalling action of short neuropeptides by hydrolyzing the oligopeptides. The molecular structure of TOP was recently revealed by X-ray crystallographic analysis at 2.0 Å resolution using the molecular-replacement method with the data of neurolysin (or endopeptidase 24-16), a highly homologous neuro-peptidase that shares more than 60% amino-acid sequence identity to TOP (Brown *et al.*, 2001; Ray *et al.*, 2004), as a model. TOP and neurolysin were both found to have a deep channel running through the entire molecule as an active site. However, TOP shares only 11% and 14% identity to Pz peptidases A and B, respectively; there is 22% identity between Pz peptidases A and B (Miyake *et al.*, 2005). Therefore, the active-site structures of the Pz peptidases presumably differ from the characteristic channels of TOP and neurolysin. The differences in structure and function between the two Pz peptidases, TOP and neurolysin are the focus of our interest at this time. As a first step toward understanding these differences, we found common phosphine inhibitors that showed potent inhibition with K_i values in the range 10–100 nM of Pz peptidases as well as TOP and neurolysin (Sugihara *et al.*, 2007). As a second step toward revealing the structures of Pz peptidases, Pz peptidase A was crystallized with one of the inhibitors and a preliminary X ray crystallographic analysis was performed in this study.

Table 1

X-ray diffraction data statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	FR-E
Wavelength (Å)	1.5418
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 56.38$, $b = 194.15$, $c = 59.93$, $\beta = 106.22$
Unique reflections	109618
Completeness (%)	96.2 (93.5)
Resolution range (Å)	19.97–1.80 (1.90–1.80)
R_{merge} (%)	6.2 (17.2)
Multiplicity	6.0 (5.9)
$I/\sigma(I)$	22.8 (7.0)

2. Materials and methods

2.1. Crystallization

Pz peptidase A was overexpressed in *Escherichia coli* BL21 (DE3) harbouring the plasmid pETA-1 at 310 K in LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin. Purification was carried out according to the method described by Sugihara *et al.* (2007). Purified Pz peptidase A was concentrated to 20 mg ml^{-1} in 50 mM Tris–HCl pH 7.5 prior to crystallization and was incubated with a phosphine peptide inhibitor [PPI; benzyloxycarbonyl-(L,D)-Phe-(PO₂CH₂)-(L,D)-Ala-Lys-Ser] at 500 μM for 24 h at 277 K to produce the complex. The solution containing the Pz peptidase A–PPI complex was subjected to crystallization trials. The initial crystallization screening was performed by the hanging-drop vapour-diffusion method at 293 K using Crystal Screens I and II (Hampton Research) by mixing 1 μl Pz peptidase A–PPI complex solution with 1 μl reservoir solution (Jancarik & Kim, 1991; Cudney *et al.*, 1994). Crystal Screen condition I-18 [20% (w/v) PEG 8000, 0.2 M magnesium acetate and 0.1 M sodium cacodylate pH 6.5] produced plate-shaped crystals in 3 d. After optimization of the crystallization conditions, the best crystals of the complex, with dimensions of about $1.20 \times 0.50 \times 0.10$ mm, were obtained from 12% (w/v) PEG 4000, 0.5 M magnesium acetate and 0.1 M Tris–HCl pH 7.0 within 5 d (Fig. 1).

2.2. Data collection and analysis

The crystal was transferred into a cryoprotectant consisting of 14% (w/v) PEG 4000, 0.5 M magnesium acetate, 0.1 M Tris–HCl pH 7.0 and 10% (v/v) 2-propanol. Several minutes later, it was scooped up in a cryoloop and frozen in liquid nitrogen. It was then mounted on a goniometer in a nitrogen stream at 93 K. X-ray diffraction was

Figure 1

A crystal of the recombinant Pz peptidase A–PPI complex obtained from 12% (w/v) PEG 4000, 0.5 M magnesium acetate and 0.1 M Tris–HCl pH 7.0. The crystal dimensions are about $1.20 \times 0.50 \times 0.10$ mm. The scale bar is 0.5 mm in length.

detected on an R-Axis VII imaging-plate system attached to a Rigaku Cu $K\alpha$ radiation rotating-anode generator (FR-E) with a crystal-to-detector distance of 120 mm. Data were collected to 1.8 Å resolution (0.5° frames) with an exposure time of 1 min. Data were indexed and integrated with *MOSFLM* (Leslie, 1992) and were scaled using *SCALA* from the *CCP4* program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The crystal of the Pz peptidase A–PPI complex belongs to the monoclinic space group $P2_1$, with unit-cell parameters $a = 56.38$, $b = 194.15$, $c = 59.93$ Å, $\beta = 106.22^\circ$. The crystal diffracted to 1.80 Å resolution (Fig. 2). Assuming the presence of two molecules in the asymmetric unit, the calculated Matthews coefficient (V_M) value was 2.37 Å³ Da^{−1} (Matthews, 1968). The solvent content of the crystal was calculated to be 48.1%. Data-collection statistics are given in Table 1.

Molecular-replacement calculations were performed on the Pz peptidase A–PPI complex using the program *MOLREP* (Vagin & Teplyakov, 2000). The putative oligoendopeptidase F from *G. stearothermophilus* was found to share 77% amino-acid sequence identity to Pz peptidase A and its structure (PDB code 2h1n) has recently been deposited. Employing the structure of *G. stearothermophilus* oligoendopeptidase F as a model, the data of the Pz peptidase A–PPI complex at 4.0 Å resolution were subjected to calculations of the rotation and translation functions for an initial search. The best solution was obtained with a correlation coefficient of 0.446 and an R factor of 0.485 (10–4.0 Å) from the translation-function calculation. The first electron-density map obtained by rigid-body refinement was insufficient to trace the loop regions of Pz peptidase A. However, repeated cycles of model fitting and refinement using the programs *REFMAC* (Murshudov *et al.*, 1997) and *Coot* (Emsley & Cowtan, 2004) allowed us to trace the entire Pz

Figure 2

X-ray diffraction pattern from a crystal of the Pz peptidase A–PPI complex. Diffraction spots are observed to a resolution of 1.8 Å.

peptidase A. Electron density corresponding to PPI was found to be located in the active site. Further refinements of the structural model are currently in progress, along with the crystallization of Pz peptidase B.

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