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Crystallization of porcine pancreatic elastase and a preliminary neutron diffraction experiment

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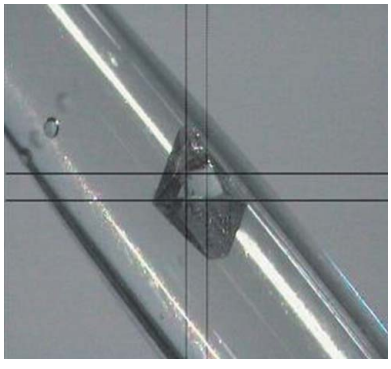

Porcine pancreatic elastase (PPE) resembles the attractive drug target leukocyte elastase, which has been implicated in a number of inflammatory disorders. In order to investigate the structural characteristics of a covalent inhibitor bound to PPE, including H atoms and the hydration by water, a single crystal of PPE for neutron diffraction study was grown in D_2O containing 0.2 *M* sodium sulfate (pD 5.0) using the sitting-drop vapour-diffusion method. The crystal was grown to a size of 1.6 mm^3 by repeated macroseeding. Neutron diffraction data were collected at room temperature using a BIX-3 diffractometer at the JRR-3 research reactor of the Japan Atomic Energy Agency (JAEA). The data set was integrated and scaled to 2.3 Å resolution in space group $P2_12_12_1$, with unit-cell parameters a = 51.2, b = 57.8, c = 75.6 Å.

1. Introduction

PPE is a serine protease that is classified into the chymotrypsin family and is one of the representative enzymes for which the catalytic mechanism has been thoroughly investigated by structural methods (Shotton & Watson, 1970; Sawyer *et al.*, 1978; Clore *et al.*, 1986). Recent interest in the serine proteases has focused on the low-barrier hydrogen-bonding interaction for acceleration of catalytic reaction (Cleland *et al.*, 1998) and on the substrate–inhibitor recognition for structure-based drug design (SBDD; Steinmetzer & Sturzebecher, 2004; Liu *et al.*, 2006). It is known that uncontrolled proteolytic degradation by pancreatic elastase (EC 3.4.21.36) causes the fatal disease pancreatitis. Inhibitors of PPE are promising candidates for SBDD, as elastases have active sites with similar structures. We have previously investigated the PPE–inhibitor interaction using X-ray crystallography (Nakanishi *et al.*, 2000; Kinoshita *et al.*, 2003, 2005, 2006). To date, most inhibitors designed by SBDD have used structures obtained by X-ray crystallography. Since *de novo* design using tertiary structure scaffolds of ligands or substrate-binding sites in drug-target proteins is still difficult, the crystal structures are mainly used to improve existing drug candidates through characterization of the interactions between the protein and drug candidates. A more detailed analysis of the interactions between the protein and the drug candidate, including hydrogen positions and hydration, will be likely to assist in the development of highly potent and selective inhibitors through SBDD. Tertiary structure analysis using neutron diffraction is a powerful method to determine the location of H atoms and the hydration in protein structures owing to the strong interaction of neutrons with H atoms (Myles, 2006). Thus, a crystal of PPE plus inhibitor large enough for neutron diffraction study was grown and we report the successful collection of neutron diffraction data to 2.3 Å resolution.

2. Materials and methods

2.1. Materials

Commercially available PPE was purchased from Worthington Biochemicals (Lakewood NJ, USA) and used without further purification. The peptidic inhibitor 4-({(*S*)-1-[((*S*)-2-{[(*RS*)-3,3,3-trifluoro-

Table 1
Neutron diffraction data statistics.

Data in the last column were processed without any rejections.

Space group	$P2_12_12_1$	
Neutron source	JRR3 (BIX3)	
Wavelength (Å)	2.9	
Resolution (Å)	2.3 (2.38–2.30)	
Observed reflections	36557	44479
Unique reflections	8638	8884
$I/\sigma(I)$	8.1 (4.4)	7.6 (4.2)
Completeness (%)	82.8 (71.1)	85.1 (74.2)
$R_{\rm merge}$†	0.141 (0.233)	0.219 (0.389)

† $R_{\rm merge} = \sum |I - \langle I\rangle| / \sum I$, where I is the intensity of a reflection and $\langle I\rangle$ is the average intensity.

1-isopropyl-2-oxopropyl]aminocarbonyl}pyrrolidin-1-yl-)carbonyl]-2-methylpropyl}aminocarbonyl)benzoic acid (FR130180), which is competitive at neutral pH and is covalently bound at pH 5.0, was supplied by Astellas Pharma Inc. (Tokyo, Japan). D_2O was purchased from Cambridge Isotope Laboratories Inc. (Andover MA, USA). Other chemicals were from Wako Pure Chemical Industries Ltd (Osaka, Japan).

2.2. Crystallization

Initial crystals of the PPE–FR130180 complex were obtained using a procedure similar to that reported previously (Shotton & Watson, 1970; Sawyer *et al.*, 1978; Kinoshita *et al.*, 2003). A search for an initial precipitant solution (pD 5.0) for crystallization of the PPE–FR130180 complex was performed in the range 0.2–0.3 *M* sodium sulfate at 293 K using the sitting-drop vapour-diffusion method. Briefly, 5 µl protein–inhibitor complex solution containing 20 mg ml^{-1} PPE and 1 m*M* inhibitor was mixed with 5 µl precipitant solution containing 50 m*M* D-substituted sodium acetate and 0.2–0.3 *M* sodium sulfate (pD 5.0). All solutions were prepared using D_2O in order to reduce the background noise from neutron scattering. The initial single crystal had dimensions of 0.1 × 0.06 × 0.06 mm and was obtained in 2 d.

Neither refinement of the crystallization conditions nor scale-up experiments produced larger crystals; therefore, a macroseeding procedure was repeatedly performed using the initial crystal as a seed in order to obtain larger crystals. The seed crystal was washed twice with precipitant solution and added to 100 µl precipitant solution with 10 µl PPE–inhibitor complex solution. Since larger seeds tend not to produce any small crystals at higher concentrations of precipitant solution, the concentration of sodium sulfate in the precipitant solution was increased by 0.1 *M* every 3 d until the concentration reached 0.7 *M* (total 15 d). Small crystals that often appeared around the seed crystal during crystal growth were removed using a sharp needle (Hampton Research) before the next seeding. After reaching 0.7 *M* sodium sulfate, seeding using the mixture of 100 µl precipitant solution containing 50 m*M* D-substituted sodium acetate and 0.7 *M* sodium sulfate with 10 µl complex solution containing 20 mg ml^{-1} PPE–inhibitor was repeated 30 times over three months.

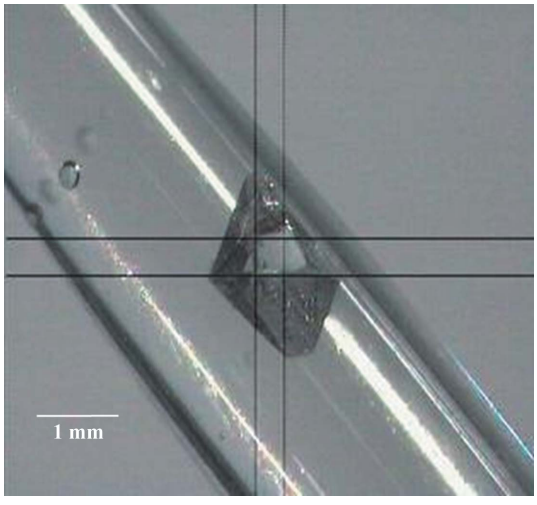

Figure 1
The PPE crystal, approximate dimensions 1.5 × 1.3 × 0.9 mm (∼1.6 mm^3), loaded into a quartz capillary.

2.3. Data collection and processing

The crystal thus obtained was sealed in a quartz capillary (Fig. 1) and used in the neutron diffraction study. Diffraction data were collected at room temperature using a monochromatic neutron beam (λ = 2.9 Å) and were recorded on a neutron imaging plate at a BIX-3 single-crystal diffractometer at JRR-3, JAEA (Tanaka *et al.*, 2002). Data collection was carried out by the step-scanning method with an interval angle of 0.3° and 50 min exposure per frame. Neutron diffraction data from the PPE–inhibitor complex were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

A crystal with dimensions of 1.5 × 1.3 × 0.9 mm (∼1.6 mm^3) was successfully obtained by repeated macroseeding. The crystal was sealed in a quartz capillary and subjected to neutron diffraction using a BIX-3 single-crystal diffractometer (Tanaka *et al.*, 2002). Diffraction data comprising 595 still images covering 178.5° were integrated and scaled to 2.3 Å resolution in space group $P2_12_12_1$, with unit-cell parameters a = 51.2, b = 57.8, c = 75.6 Å. The observed reflections were merged and scaled into 8638 unique reflections with an $R_{\rm merge}$ of 0.141 (8884 unique reflections with an $R_{\rm merge}$ of 0.219 without rejection). Data-collection and processing statistics are shown in Table 1. Although the data collection covers 180°, many reflections were rejected during data processing because of the weak diffraction signal under the limited exposure. The relatively low completeness of this neutron data may also be a consequence of the cylindrical shape of the detector.

The resulting 2.3 Å neutron data set has sufficient quality to determine the location of hydrogen and deuterium owing to their scattering lengths (Myles, 2006) as observed in previous reports (Blakeley *et al.*, 2004, 2006; Chatake *et al.*, 2005; Arai *et al.*, 2005). This neutron diffraction data should help in the elucidation of the strong inhibition mechanism of FR130180, which will be important information for redesigning the inhibitor.

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