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Crystallization and preliminary neutron diffraction studies of HIV-1 protease cocrystallized with inhibitor KNI-272

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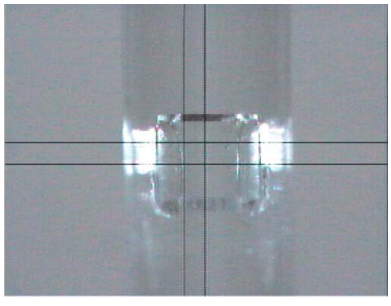

This paper reports the crystallization and preliminary neutron diffraction measurements of HIV-1 protease, a potential target for anti-HIV therapy, complexed with an inhibitor (KNI-272). The aim of this neutron diffraction study is to obtain structural information about the H atoms and to determine the protonation states of the residues within the active site. The crystal was grown to a size of 1.4 mm^3 by repeated macroseeding and a slow-cooling method using a two-liquid system. Neutron diffraction data were collected at room temperature using a BIX-4 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 $P2_12_12$, with unit-cell parameters a = 59.5, b = 87.4, c = 46.8 Å.

1. Introduction

Human immunodeficiency virus 1 (HIV-1), the cause of human acquired immunodeficiency syndrome, has been spreading among humans since the first half of the last century (Gao *et al.*, 1999; Hahn *et al.*, 2000; Korber *et al.*, 2000). HIV-1 protease (HIV-1 PR) is considered to be an effective target for anti-HIV therapy because it cleaves proteins that are essential for the proper assembly and maturation of infectious virions. HIV-1 PR is an aspartic protease and is a symmetric homodimer composed of two 99-residue polypeptides. Two catalytic aspartic acids (Asp25 and Asp125) are provided from each polypeptide and are positioned at the bottom of a deep cleft. Although the catalytic mechanism of HIV-1 PR has been extensively investigated by X-ray crystallography and other means (Brik & Wong, 2003), the proposed mechanism is not based on experimentally observed coordinates of H atoms.

To date, several inhibitors have been developed for HIV-1 proteases. Of these inhibitors, KNI-272, a transition-state mimetic tripeptide HIV-protease inhibitor, has previously been shown to have potent antiviral activity against HIV *in vitro* (Kageyama *et al.*, 1993) and *in vivo* (Kiso, 1996). A 2.0 Å resolution X-ray structure of the HIV-1 PR–inhibitor (KNI-272) complex has been reported (Baldwin *et al.*, 1995); however, no H-atom coordinates have been observed for the HIV-1 PR–inhibitor complex owing to the limited experimental resolution. It is thus intriguing to explore the location of the acidic proton of the HIV-1 PR–inhibitor complex, which would be helpful in designing highly potent and selective inhibitors through structure-based drug design. Neutron protein crystallography provides a powerful complement to X-ray analysis by enabling the visualization of H atoms. A major hurdle to neutron protein crystallography is that unusually large crystals (>0.5 mm^3) are required in order to compensate for the weak flux of the available neutron beam.

Macroseeding is frequently used to enlarge protein crystals. However, it is a difficult procedure because it requires multiple washes in a slightly dissolving solution (Stura, 1999). Extreme care must be taken

when handling the crystals in order to avoid the generation of microcrystals. If the crystals adhere tightly to the growth vessel, it becomes increasingly difficult to handle them without causing mechanical damage.

We have previously developed a two-liquid system for growing high-quality protein crystals (Adachi *et al.*, 2002, 2003, 2004). In this system, a protein/precipitant solution floats on a very dense insoluble liquid. Since crystals grow at the interface of the two liquids under optimized growth conditions by batch and slow-cooling methods, the system enables handling of the crystals without contacting the vessel. The crystals are easily removed without causing mechanical damage to them, in the same manner as in containerless techniques (Chayen, 1996; Lorber & Giegé, 1996). In addition, a seed crystal can be easily picked up and inserted onto the interface of the two liquids in the new vessel. However, the two-liquid system is not common in the field of protein-crystal growth, probably because vapour diffusion is the most commonly used crystallization method for proteins (McPherson, 1982, 1999).

In this study, we tested the use of the two-liquid system in crystallizing the HIV-1 PR–inhibitor (KNI-272) complex. By combining the two-liquid system and conventional methods such as a macroseeding procedure and a slow-cooling method, we were able to grow large high-quality crystals of the HIV-1 PR–inhibitor complex. Here, we report the successful collection of neutron diffraction data to 2.3 Å resolution.

2. Materials and methods

2.1. Preparation of HIV-1 protease

The DNA of HIV-1 protease (HIV-1 PR) was chemically synthesized (Takara) and the sequence was optimized for expression in *Escherichia coli* (Takara). The expression vector for HIV-1 PR was constructed by inserting the synthesized gene into the pET43.1a vector (Novagen) using the *Nde*I–*Eco*RI restriction sites. The resulting plasmid encodes a gene for an initial methionine and the 99 amino acids of HIV-1 PR (MPQITLWKRPLVTIKIGGQLKEALLDTGADDTVIEEMSLPGRWKPKMIGGIGGFIKVRQYDQIIIEIAGHKAIGTVLVGPTPVNIIGRNLLTQIGATLNF), including five mutations, Q7K, L33I, L63I, C67A and C95A, to prevent autoproteolysis and cysteine thiol oxidation. The ∼10 kDa protein was propagated in *E. coli* BL21 (DE3) and was expressed as inclusion bodies. Cells were induced in the mid-log phase by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 m*M*. After 3 h, the cells were collected by centrifugation and lysed by sonication in 20 ml lysis buffer (20 m*M* Tris–HCl pH 8.0). The inclusion bodies were washed with lysis buffer, pelleted by centrifugation at 13 000*g* for 20 min and solubilized in 50%(*v*/*v*) acetic acid. After centrifugation to remove denatured material, the supernatant was diluted with 0.1 *M* sodium acetate buffer pH 5.5 containing 5% ethylene glycol and 10% glycerol (Hui *et al.*, 1993). After the insoluble materials had been removed by centrifugation at 5000*g* for 20 min, the clear supernatant was applied onto a column (2 × 15 cm) of SP-Sepharose FF (GE Healthcare) pre-equilibrated with 50 m*M* sodium acetate buffer pH 5.0. The protein was eluted with a linear gradient of 0–1.5 *M* NaCl in 50 m*M* sodium acetate buffer pH 5.0. The eluted fraction containing HIV-1 PR was pooled and applied onto a prepacked column (0.64 × 10 cm) of Resource RPC (GE Healthcare) pre-equilibrated with 0.02% TFA. The protein was eluted with a linear gradient from 1% to 80% acetonitrile/2-propanol [70:30(*v*:*v*)] at a flow rate of 2 ml min^{-1}. The eluted protease was dialyzed against 0.1 *M* glycine buffer pH 2.8 and subsequently against 50 m*M* sodium acetate buffer pH 5.0 at 277 K. The protein solution was concentrated to 5 mg ml^{-1} with a Millipore Ultrafree filter (3 kDa cutoff). HIV-1 PR in 50 m*M* sodium acetate buffer pH 5.0 was mixed with inhibitor KNI-272 dissolved in dimethyl sulfoxide at a 1:5 molar ratio.

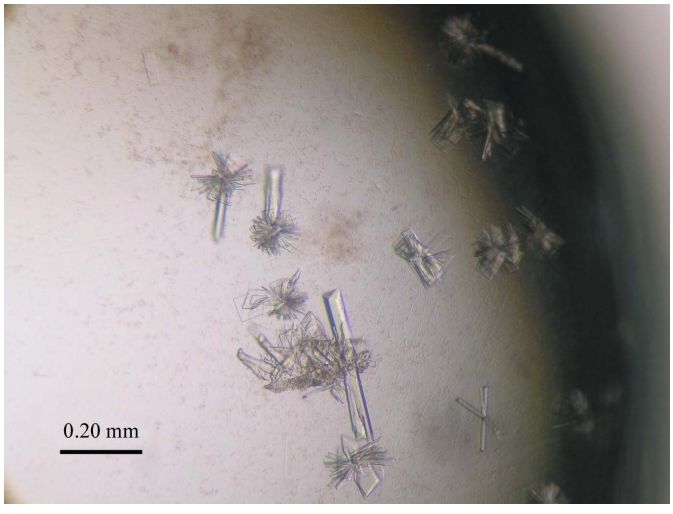

Figure 1
HIV-1 PR crystals obtained in an initial attempt.

2.2. Crystallization

At the beginning of our studies, crystallization of the HIV-1 PR–inhibitor (KNI-272) complex was attempted under conditions similar to those previously reported (Baldwin *et al.*, 1995). The initial condition, using the batch method, contained drops consisting of 2 µl 1.4 *M* ammonium sulfate, 65 m*M* citrate, 130 m*M* sodium phosphate pH 6.2 and an equal volume of the protein–inhibitor mixture. In this initial attempt to crystallize the HIV-1 PR–inhibitor complex the protein precipitated heavily in the drops, but microcrystals were observed in the precipitate (Fig. 1). Since this condition did not seem to be favourable for growing large high-quality crystals, the crystallization condition was refined. A search for a suitable precipitant solution for crystallization of the HIV-1 PR–inhibitor complex was performed in the range 0.05–1.0 *M* ammonium sulfate with pH values ranging from 4.0 to 8.0 at 293 K. The batch optimization experiments led to the following conditions: drops containing 5 µl protein at 2.5 mg ml^{-1} and 0.25 m*M* inhibitor in 50 m*M* sodium citrate–phosphate pH 5.5 were mixed with 5 µl precipitating buffer containing 0.061 *M* ammonium sulfate. The crystals typically grew to maximum dimensions of 0.3 × 0.3 × 0.1 mm over one week.

Further refinement of the crystallization conditions did not produce larger crystals; therefore, a macroseeding procedure was repeatedly performed using the crystals that were obtained by the optimized batch method described above as seeds. For the macroseeding procedure, a two-liquid batch method (Adachi *et al.*, 2004) was used as the crystallization method. This method is basically similar to the traditional batch method, except for the use of a very dense insoluble liquid (Fluorinert FC-70; 3M, USA). In this system, crystals grow at the interface of the protein/precipitant solution and the high-density liquid, which enables the convenient removal of large crystals without causing mechanical damage to them. In our experiments, 10 µl Fluorinert FC-70 and a 10 µl protein/precipitant droplet were used. Three rounds of macroseeding with the two-liquid

Table 1
Neutron diffraction data statistics.

Values in parentheses are for the outer shell. The values in the right-hand column are for data that were processed without any rejections.

Space group	$P2_12_12$	
Neutron source	JRR3 (BIX-4)	
Wavelength (Å)	2.6	
Resolution (Å)	2.3 (2.38–2.30)	
Observed reflections	15433	16285
Unique reflections	9473	9736
$\langle I/\sigma(I)\rangle$	7.7 (2.5)	7.9 (2.6)
Completeness (%)	83.4 (80.3)	85.7 (81.5)
$R_{\rm merge}$†	0.081 (0.226)	0.094 (0.286)

† $\sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of a reflection and $\langle I(hkl)\rangle$ is the average intensity.

system further increased the size of the seed crystals from 0.3 × 0.3 × 0.1 to 0.5 × 0.5 × 0.15 mm.

The procedure above was certainly effective, but it was too time-consuming to grow crystals of suitable size; for example, crystals of 0.3 × 0.3 × 0.1 mm grew to 0.5 × 0.5 × 0.15 mm over two months. In order to improve the crystal-growth rate, we applied a slow-cooling method (Adachi *et al.*, 2002) after three rounds of macroseeding with the two-liquid system. The temperature was manually decreased by 0.5 K from 293 to 289 K every 5 d to a final temperature of 287 K. The slow-cooling method accelerated the growth rate from 0.2 × 0.2 × 0.1 mm in seven weeks to 0.5 × 0.5 × 0.15 mm in just two weeks. Thus, the largest crystals grew to maximum dimensions of 1.9 × 1.8 × 0.4 mm (1.4 mm^3; Fig. 2).

2.3. Data collection and processing

To reduce the background from neutron dispersion, the H_2O in the crystal was exchanged to D_2O by vapour diffusion for one month and the crystals were subsequently soaked for 2 d in crystallization solution consisting of D_2O and 0.061 *M* ammonium sulfate. A 1.4 mm^3 D_2O-soaked crystal was mounted at 293 K in a quartz capillary tube and sealed with wax for data collection. Neutron diffraction data were collected at room temperature using a monochromatic neutron beam (λ = 2.6 Å) and recorded on a neutron imaging plate using a BIX-4 single-crystal diffractometer at JRR-3, JAEA (Kurihara *et al.*, 2004). Data were collected using the step-scanning method with an interval angle of 0.3° and 360 min exposure per frame. All diffraction data from a single crystal were autoindexed, integrated and corrected for Lorentz and polarization effects with the program *DENZO* (Otwinowski & Minor, 1997). Data were scaled and merged with the program *SCALEPACK* (Otwinowski & Minor, 1997).

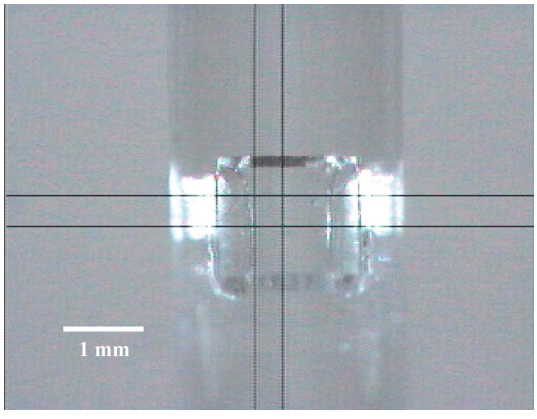

Figure 2
An HIV-1 PR crystal of dimensions 1.9 × 1.8 × 0.4 mm (1.4 mm^3) loaded into an X-ray quartz capillary 2.5 mm in diameter.

3. Results and discussion

A large high-quality HIV-1 PR crystal with dimensions of 1.9 × 1.8 × 0.4 mm (1.4 mm^3) was successfully obtained when macroseeding was employed in the two-liquid system and combined with the slow-cooling method. To our knowledge, this is the first experiment in which the two-liquid system has been applied to grow a large protein crystal, apart from the crystallization of hen egg-white lysozyme (HEWL; Adachi *et al.*, 2002, 2004). The system provides a new strategy for the convenient manipulation of a large protein crystal. This study has also demonstrated that the slow-cooling procedure effectively accelerates the growth rate of HIV-1 PR crystals, as previously described for HEWL crystals (Adachi *et al.*, 2002, 2004). Thus, the use of the two-liquid system combined with the slow-cooling procedure seems to be suitable for growing crystals of other proteins.

The crystal obtained was mounted in a quartz capillary tube and subjected to neutron diffraction using a BIX-4 single-crystal diffractometer (Kurihara *et al.*, 2004). The crystals diffracted to 2.3 Å resolution at room temperature. Diffraction data composed of 169 still images covering 50.7° were integrated and scaled to 2.3 Å resolution in space group $P2_12_12$, with unit-cell parameters a = 59.5, b = 87.4, c = 46.8 Å. The observed reflections were merged and scaled into 9473 unique reflections with an $R_{\rm merge}$ of 0.081 (9736 unique reflections; $R_{\rm merge}$ was 0.094 without rejections), as indicated by the data-reduction statistics for the neutron data set given in Table 1. The results of the preliminary neutron diffraction experiment suggest that the two-liquid system combined with the slow-cooling method does not cause any harmful side effects. This data set is currently being used for the refinement of the HIV-1 PR–inhibitor complex.

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