A preliminary neutron crystallography on the trypsin-bovine pancreatic trypsin inhibitor complex

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Abstract. Trypsin is one of serine proteases. BPTI (Bovine Pancreatic Trypsin Inhibitor) is a protein inhibitor, which binds trypsin tightly and inhibits cleavage of peptide bonds. X-ray structure determination of trypsin-BPTI complex could make clear the overview of the active site. However, information of hydrogen atoms related to catalytic mechanism has not been satisfied. In this study, the trypsin-BPTI complex structure has been determined by neutron diffraction data at 2.0 Å resolution. Deuterium atoms of catalytic triad, hydration structures in the binding pocket of trypsin and hydrogen bonds were observed. We would like to discuss details of hydrogen bonds in the interface between trypsin and BPTI and the adjacent water molecules including hydrogen atoms involved in the enzymatic reaction.

1. Introduction

Hydrogen atoms of dissociable groups and the hydride structures, deeply related to many chemical reactions in biological systems of several proteins, are directly observed by neutron diffraction experiments. In the active site, trypsin has three dissociable amino acids of His57, Asp102 and Ser195 (called catalytic triad), recognizes specifically the basic amino acid residues Lys or Arg and catalyzes the hydrolysis of peptide bond. The catalytic mechanism is explained by hydrogen bonds related to proton transfer among residues in the triad. The overview is as follows: Asp102 increases the basicity of the Nε2 of the imidazole of His57 by forming hydrogen bonding between the COO- of Asp102 and Nδ1H of the imidazole of His57. His57 then abstracts a proton from Ser195OH, thereby promoting its nucleophilic attack on the carbonyl carbon of the scissile peptide bond. The nucleophilic attack of Ser195Oγ results in formation of a tetrahedral intermediate of the peptide. In the tetrahedral...
intermediate, the carbonyl oxygen ion of the scissile peptide forms hydrogen bonds with the backbone NH groups of Gly193 and Ser195 [1]. To make clear the reason, it may be interesting to reveal these positions of hydrogen atoms in the active site. Also, to prevent cell destruction by autolysis of trypsin, pancreatic trypsin inhibitors invariably exists within organisms. Bovine pancreatic trypsin inhibitor (BPTI) has high inhibition effect for trypsin [2] [3]. The 58-residue BPTI has Lys15 (P1) whose side chain interacts electrostatically with Asp189 of specific binding site in trypsin. Several extensive studies of trypsin-BPTI complex have been performed at the atomic level [4]. To date, structures of trypsin-MIP (monoisopropyl ester phoshonic acid) complex [5][16] and BPTI alone [6] have been determined by neutron protein crystallography, but that of the trypsin-BPTI complex has not yet been determined. In this study, the trypsin-BPTI complex structure was determined by neutron diffraction data, and hydrogen atoms of the catalytic triad, hydration structures in the binding pocket of trypsin and hydrogen bonds were observed.

2. Materials and Methods

Recombinant bovine \( \beta \)-trypsin from corn and BPTI (trypsin inhibitor from bovine pancreas, type I-P) were purchased from Acris Antibody GmbH and Sigma-Aldrich, respectively. The BPTI was mixed with bovine \( \beta \)-trypsin in a 1.25:1 molar ratio [7] in a solution of 20 mM HEPES buffer (pH 7.5). The protein concentration was adjusted 55 mg ml\(^{-1}\). Crystallization was carried out by the sitting-drop method. The droplet was contained 1:1 mixture of protein solution and reservoir solution. The reservoir solution was 1.45 M ammonium sulfate, 200 mM HEPES buffer (pH 7.5), and 10 mM calcium chloride. The crystal grew to 2.2 mm\(^3\) (2.0 mm \( \times \) 1.1 mm \( \times \) 1.0 mm) for about 2 weeks. Thereafter, it was soaked in a D\(_2\)O solution at pH 7.9 for two weeks.

The neutron diffraction experiment was carried out at room temperature with the single-crystal diffractometer BIX-4, which is installed at the JRR-3 reactor of the JAEA [8]. A step scanning method with an interval of 0.3° was used to collect data and the exposure time was 4 hours per frame. The intensities of reflections were integrated and scaled by the program DENZO and SCALEPACK [9]. The statistics for the data reduction are summarized in Table 1. This neutron experiment was performed under the Common-Use Facility Program of JAEA (2008A-A06).

The neutron crystal structure refinement was carried out by a version of CNS [10] called nCNS modified for neutron refinement [14] and the graphics program was COOT [11]. To obtain an initial model for refinement, we solved the structure from X-Ray data. This data was collected from another crystal grown the same condition soaked in D\(_2\)O solution, refined by REFMAC of CCP4 packages [12] (initial model is PDB ID: 2PTC) [13]. In our neutron structure analysis, we included reflection data from 80 to 2.0 Å resolution in the neutron map calculation. The protonation or deprotonation states of dissociable groups were determined by difference Fourier maps for a model with omitted partial deuterium atoms.

3. Results and discussion

As the result of neutron structure refinement, the \( R \)\_crystal is 20.71%, and \( R \)\_free is 25.48% at resolution of 2.0 Å. The deuterium atom between His57N\( \varepsilon_2 \) and Ser195O\(^{\gamma} \) was observed as shown in Figure 1(a) and the initial stage of deprotonation from the serine was confirmed. The nuclear density map of the specific binding area of the BPTI is indicated in Figure 1(b). The refinement statistics for neutron structure of trypsin-BPTI complex are listed in Table 1.

3.1. Catalytic triad

A hydrogen atom binding His57N\(^{\delta_1} \) was exchanged for deuterium. The His57D\(^{\delta_1} \)- Asp102O\(^{\delta_1} \) and - Asp102O\(^{\delta_2} \) distances were 2.5 Å and 2.0 Å, respectively. A deuterium atom was identified between His57N\(^{\delta_2} \) and Ser195O\(^{\gamma} \). The distance between His57N\(^{\delta_2} \)-Ser195O\(^{\gamma} \) was 2.6 Å and the distances from the observed nuclear density map peak position of the deuterium atom to His57N\(^{\delta_2} \) and Ser195O\(^{\gamma} \) were
about 1.4 Å and 1.2 Å, respectively. These hydrogen bonds are short distance, which might facilitate the proton transfer during the catalytic mechanism. [15].

3.2. Specific binding site S1 Asp189 and P1 Lys15
In the X-ray result of trypsin-BPTI complex, two water molecules (oxygen atoms) have been assigned around ε-amine of Lys15, but the hydrogen atoms of the water molecules were identified. The hydrogen atoms of these water molecules were determined by neutron crystal structure analysis, and the exact hydrogen bonds scheme (donor and acceptor relations) were assigned clearly in this binding area as show in Figure 1(b). These hydrogen bond lengths indicated in Figure 1(b) were about 1.9 Å. The water molecules were locked tightly by the hydrogen bond.

Figure 1. Nuclear density map for (a) catalytic triad of trypsin and (b) specific binding site Asp189, Lys15 of inhibitor and two deuterium waters. Map colour of |Fo|-|Fc| map (3.0 σ) for model omitted deuterium atoms of catalytic triad is blue in Fig (a). 2|Fo|-|Fc| map (1.5 σ) for model included all hydrogen and deuterium atom is green colour in Fig (b). Hydrogen bonding is located with dot-line in this figure (a) and (b).

3.3. Waters are present in trypsin-BPTI binding area
Although the refinement is still underway, we could discuss the presence of water into the active site because the trypsin-BPTI complex crystal used in neutron diffraction experiment is soaked in D₂O solution. In general, it has been thought that water molecules cannot enter the active site of trypsin because the BPTI-binding loop is in close contact with the trypsin-binding area [1] [13]. In this study, hydrogen atoms in side chains of the catalytic triad were exchanged to deuterium atoms (occupancy of His57 D81 was 1.0) and also waters in specific binding area were exchanged to deuterium waters, which means that water molecules invaded in active site.
Table 1. Statistic data collection, reduction and refinement

| Data collection | Refinement |
|-----------------|------------|
| Space group     | I222       |
| Unit cell parameter (Å) | Resolution (Å) 80-2.0 |
| a               | 75.8       |
| b               | 85.6       |
| c               | 123.1      |
| Beam port/radiation | 1G, JRR-3 |
| Wavelength (Å)  | 2.6        |
| Diffractometer  | BIX-4      |
| Exposure time (h/fr) | 4 |
| Total frames    | 275        |
| Resolution (Å)  | 80-2.0     |
| Observed reflection | 48,815 |
| Unique reflection | 23,822    |
| $I/\sigma(I)$   | 4.8 (2.7)  |
| Completeness (%)| 86.7 (80.1)|

$R_{merge} = \sum |I-I>|/\sum I$, where I is the intensity of a reflection and $<I>$ is the average intensity.

$R_{free} = \sum |F_o|-|F_c|/\sum |F_o|$ calculated for the reflections of working and test sets, respectively. Values in parentheses are for the highest resolution shell.

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