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Crystallization and preliminary X-ray diffraction analysis of two lysinal derivatives of *Achromobacter* protease I

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Abstract

Two crystal forms of lysinal derivatives of *Achromobacter* protease I have been obtained. The first, modified by benzyloxycarbonyl-Val-lysinal crystallizes in the monoclinic space group $P2_1$ with unit-cell dimensions of $a = 39.6$, $b = 71.2$, $c = 45.6$ Å and $\beta = 98.4°$. The second, modified by benzyloxycarbonyl-Leu-Leu-lysinal crystallizes in the orthorhombic space group $I222$ (or $I2_12_12_1$) with unit-cell dimensions of $a = 98.7$, $b = 102.2$ and $c = 55.8$ Å. The space groups and the unit-cell dimensions of the present two lysinal derivatives are different to those of the protease and TLCK-modified one. The space group of the protease is $P1$ with cell dimensions $a = 39.53$, $b = 40.34$, $c = 43.92$ Å, $\alpha = 114.81$, $\beta = 113.75$ and $\gamma = 74.00°$ and that of the TLCK-modified one is also $P1$ with cell dimensions of $a = 37.30$, $b = 42.74$, $c = 48.02$ Å, $\alpha = 120.10$, $\beta = 112.81$ and $\gamma = 68.54°$. Diffraction to 1.9 Å resolution for the Val-lysinal modified crystal and to 2.2 Å resolution for the Leu-Leu-lysinal modified crystal has been observed using a rotating-anode X-ray generator. Full structure determinations of these lysinal-modified protease crystals may lead to an understanding of the molecular basis of enzyme–substrate interactions in the catalytic process of this protease.

1. Introduction

Achromobacter protease I (API, E.C. 3.4.21.50) is one of the serine proteases produced by *Achromobacter lyticus* M497-1 (Masaki, Nakamura, Isono & Soejima, 1978; Masaki, Fujihashi, Nakamura & Soejima, 1981). The protease specifically hydrolyzes the peptide bonds at the carboxyl side of lysine residues. In addition to the strict substrate specificity for the lysyl bond, API has several unique properties such as a relatively higher protease activity, a wide pH optimum ranging from 8.5 to 10.7 and structural stability against denaturation with 0.1% sodium dodecyl sulfate and 4 *M* urea (Masaki, Tanabe, Nakamura & Soejima, 1981). Because of these special properties, API has been an effective tool for the peptide fragmentation in protein sequence analysis.

API consists of a single chain of 268 amino-acid residues with three disulfide bonds (Tsunasawa, Masaki, Hirose, Soejima & Sakiyama, 1989) and shows low sequence homology (approximately 10%) with other serine proteases. The catalytic triad of serine proteases has been assigned to Ser194, His57 and Asp113 based on a comparison of primary structures. Furthermore, subsite-mapping experiments using synthetic peptide substrates revealed the presence of three subsites, S1, S2 and S3 at the N-terminal side of the scissile peptide bond. These residues have been tentatively assigned to His210, Gly211 and Gly212, respectively (Sakiyama, *et al.*, 1990).

We have determined the crystal structure of API and its derivative alkylated by *N*α-tosyl-lysine chloromethyl ketone (Kitagawa *et al.*, 1996) and their coordinates have been deposited in Protein Data Bank (Bernstein *et al.*, 1977).‡ These structural studies of API and its derivatives have provided the first view of the active site and the basic proteolytic mechanism of this protease.

Several lysinal derivatives with the C-terminal aldehyde group were synthesized and their inhibition against API was tested (Masaki, Tanaka, Tsunasawa, Sakiyama & Soejima, 1992). Since lysinal derivatives selectively bind to the hydroxyl group of Ser194 in the active site (Ser195 for chymotrypsin numbering), the lysinal-modified API may be a practical model for enzyme/substrate interaction in the enzymatic reaction. Among the lysinal derivatives, benzyloxycarbonyl(Z)-Val-lysinal and Z-Leu-Leu-lysinal are relatively more potent inhibitor against API (Masaki *et al.*, 1992). In this paper, we describe the crystallization and preliminary X-ray diffraction analysis of API modified by two lysinal derivatives, Z-Val-lysinal and Z-Leu-Leu-lysinal, which crystal forms are different from the ones we have already obtained in the native API and the TLCK-modified one.

2. Materials and methods

API was purified as described previously (Masaki *et al.*, 1981). Purified API was stored at 277 K and concentrated to approximately 20 mg ml^{-1} using Centricon 10 tubes (Amicon) before crystallization.

Z-Val-lysinal and Z-Leu-Leu-lysinal were synthesized as described previously (Masaki *et al.*, 1992).

3. Crystallization and preliminary X-ray characterization of API modified by Z-Val-lysinal

Z-Val-lysinal was dissolved in a small amount of 100 m*M* Tris–HCl pH 8.0 buffer solution. API modified by Z-Val-lysinal was prepared by mixing the Z-Val-lysinal solution into

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‡Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1ARB, 1ARC, R1ARBSF, R1ARCSF). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: GR0617).

the protein solution previously prepared in 100 m*M* Tris–HCl pH 8.0. The protein concentration in the mixture was 20 mg ml^{-1} (0.7 m*M*) and the Z-Val-lysinal concentration was 7.7 m*M* which was 11 times molar excess to the protein content in the solution. The mixture solution was kept overnight at 277 K and was used for the crystallization experiment.

Crystallization was performed using the standard hanging-drop vapor-diffusion technique. The reservoir solution was 15–20%(*w*/*v*) polyethylene glycol 6000 in 100 m*M* Tris–HCl buffer pH 8.0. The equi-volume mixture of protein solution and reservoir solution was equilibrated at room temperature over 1 ml of reservoir solution in a tissue-culture plate. Plate-like crystals appeared in a few days, reaching their maximum size of 0.4 × 0.4 × 0.1 mm over a further week (Fig. 1). A crystal was mounted and sealed in a glass capillary with a small amount of mother liquor. The precession photographs were then taken using Ni-filtered Cu $K\alpha$ radiation from a rotating-anode X-ray generator operated at 40 kV 100 mA (Fig. 2). The precession photographs of *hk*0 and *h*0*l* zones showed that the crystal apparently belongs to space group $P2_1$, with unit-cell dimensions of $a = 39.6$, $b = 71.2$, $c = 45.6$ Å and $\beta = 98.4°$.

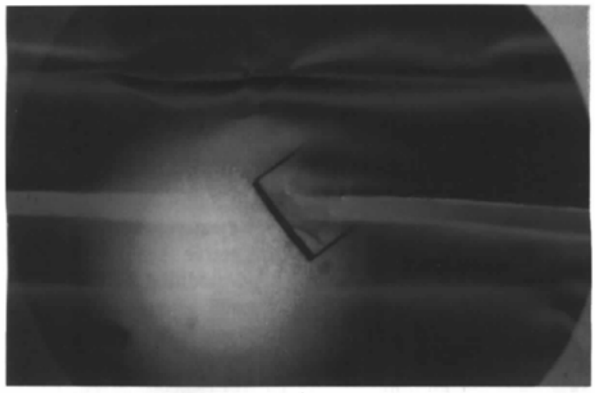

Fig. 1. A crystal of API modified by Z-Val-lysinal. The *c** axis is approximately perpendicular to the square plane of the crystal.

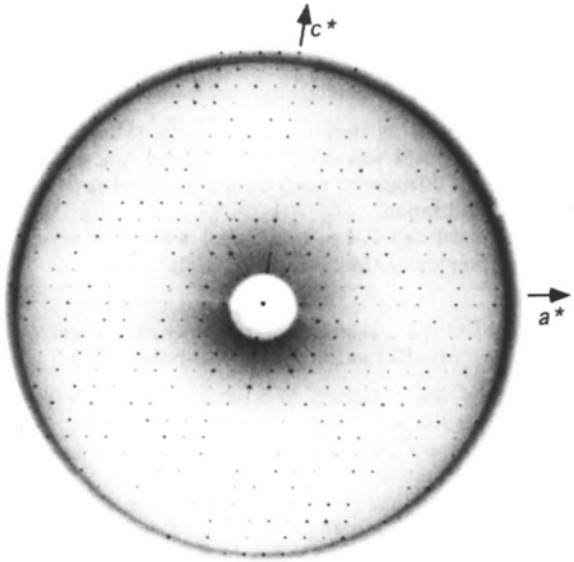

Fig. 2. A precession photograph of *h*0*l* zone of Z-Val-lysinal crystal. The horizontal direction is *a** and the vertical roughly *c**. The edge of the photograph is 3 Å resolution.

Three-dimensional intensity data were collected using R-AXIS IIc (Rigaku) imaging-plate system on a rotating-anode X-ray generator operated at 40 kV 100 mA. The crystal diffracted up to 1.9 Å resolution. We collected and processed the intensity data to 1.9 Å resolution. These data gave an R_{merge} of 9.6% for intensities and the completeness of 89%.

4. Crystallization and preliminary X-ray characterization of API modified by Z-Leu-Leu-lysinal

Z-Leu-Leu-lysinal is sparingly soluble in water and so a small amount of *N*,*N*-dimethylformamide (DMF) was used as a solvent. The dissolved Z-Leu-Leu-lysinal in DMF was then mixed in Tris–HCl buffer pH 8.0 with the final buffer concentration of 100 m*M*. API modified by Z-Leu-Leu-lysinal was prepared by mixing the Z-Leu-Leu-lysinal solution in the protein solution previously prepared in 100 m*M* Tris–HCl pH 8.0 with a concentration of 20 mg ml^{-1}. The Z-Leu-Leu-lysinal concentration in the mixture was 5.5 m*M* which was eight times molar excess to the protein content in the solution. The DMF concentration was finally 4.7%(*w*/*v*) in the solution. The mixture was kept overnight at 277 K and subsequently used for crystallization.

Crystallization was performed by both hanging- and sitting-drop vapor-diffusion methods. The best crystal was obtained with the reservoir solution, 18–20%(*w*/*v*) polyethylene glycol 6000 in 25 m*M* Tris–HCl buffer pH 8.0, and was equilibrated at room temperature. Thin plate-like crystals appeared within a week and the size of the biggest one was 0.8 × 0.2 × 0.05 mm. A crystal was mounted and sealed in a glass capillary with a small amount of mother liquor. The precession photographs were then taken using Ni-filtered Cu $K\alpha$ radiation from a rotating-anode X-ray generator operated at 40 kV 100 mA. The precession photographs showed that the crystal apparently belongs to the orthorhombic space group with unit-cell dimensions of $a = 98.7$, $b = 102.2$, $c = 55.8$ Å. The reflections of $h + k + l =$ odd were not recorded on the films, so the crystal was body centered. The space group of this crystal was determined to be $I222$ (or $I2_12_12_1$).

Three-dimensional intensity data were collected using an R-AXIS IIc (Rigaku) imaging-plate system on a rotating-anode X-ray generator operated at 40 kV and 100 mA. The crystal diffracted up to 2.2 Å resolution. We collected and processed the intensity data to 2.2 Å resolution. These data gave an R_{merge} of 11% for intensities and the completeness of 84%.

5. Concluding remarks

Assuming one molecule per asymmetric unit, the V_m values are 2.3 Å^3 Da^{-1} for Z-Val-lysinal modified API and 2.6 Å^3 Da^{-1} for Z-Leu-Leu-lysinal modified API, which lie within the range (1.68–3.53 Å^3 Da^{-1}) observed for globular proteins (Matthews, 1968). Both lysinal derivative crystals are suitable for structural studies and preliminary efforts are being directed towards the solution of the structure using molecular-

replacement techniques. The X-ray studies on lysinal modified API crystals will play a vital role in developing a full understanding of the enzyme/substrates structures especially of the transition tetrahedral intermediate and will enable comparison to be made with the serine protease super family. Z-Leu-Leu-lysinal is one amino acid longer than Z-Val-lysinal, so the correlation between the subsite interaction and the inhibition ability may be also clarified after the full structure determination of these lysinal modified API's.

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