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The refined crystal structure of the complex formed by bovine trypsin and *p*-guanidinobenzoate at 2.06 Å resolution

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The X-ray crystal structure of the complex of bovine β-trypsin with a synthetic inhibitor, 6-amidino-2-naphthyl-4-guanidinobenzoate (FUT), has been determined by the difference Fourier method at 2.06 Å resolution and refined to a crystallographic *R*-factor of 0.191 using the restrained least-squares method of Hendrickson and Konnert. The structure of trypsin is almost identical to previously published structures. A hydrolyzed fragment of the inhibitor, *p*-guanidinobenzoate, is covalently bound to the active site Ser^{195}-Oγ, forming an ester bond. The inhibitor fragment occupies the specificity pocket with a hydrogen-bonding pattern similar to that of benzamidine-inhibited trypsin.

Trypsin, bovine; Crystal structure; Inhibitor complex

1. INTRODUCTION

6-Amidino-2-naphthyl-4-guanidinobenzoate (FUT) is a potent trypsin inhibitor which has been used clinically to treat acute pancreatitis [1]. The guanidine group and the amidine group, located at opposite ends of the FUT molecule, might be responsible for the specific binding to trypsin. It has not been clear which of those two functional groups interacts with the specificity pocket of trypsin, with which the lysine or arginine side chain of a substrate has a specific interaction during the hydrolysis by trypsin. Furthermore, FUT has an ester bond which is slowly hydrolyzed by trypsin under physiological conditions (Noji, S., personal communication). In order to understand the inhibitory mechanism of FUT and the mechanism of its hydrolysis by trypsin, we have carried out a molecular dynamics study of the cmplex formed by trypsin and FUT [2]. At the same time, we have crystallized FUT-inhibited trypsin and performed an X-ray crystallographic analysis in order to obtain a detailed structure of the complex for correlation with the results of the molecular dynamics study. The X-ray crystallographic results are presented here.

2. MATERIALS AND METHODS

Bovine trypsin was purchased from Sigma (Type III). FUT was a gift from Eisai Pharmaceutical Company. Crystals of FUT-inhibited trypsin were grown by the vapor diffusion technique using the hanging-drop method. Conditions for the crystallization were similar to those used in the case of benzamidine-inhibited trypsin [3]. Droplets of mother liquor containing 2.6 mM trypsin, 40 mM FUT, 10 mM $CaCl_2$, and 0.6 M ammonium sulfate were slowly equilibrated at 4°C against 1.5 M ammonium sulfate solution. Long prismatic crystals up to 0.2 mm × 0.4 mm × 1.0 mm were obtained after one month.

X-ray diffraction experiments were done at the Photon Factory, National Laboratory of High Energy Physics (Tsukuba, Japan). The space group of the crystal was $P2_12_12_1$ with cell constants a = 54.96 Å, b = 58.50 Å and c = 67.35 Å, which are nearly identical to those of the orthorhombic crystal of benzamidine-inhibited trypsin. Intensity data were collected using a four-circle diffractometer with a wiggler beam monochromated to 1.1 Å wavelength. During the data collection, crystals were cooled to 5°C by blowing cool air over them. A total of 16 675 reflections within 10–2.06 Å resolution range were measured using three crystals. The intensity data of each crystal were corrected for absorption [4] and then all data were merged to yield 13 912 independent reflections. R_{merge}, defined as $\Sigma(I - \langle I \rangle)/\Sigma I$ summed over all measurements, was 0.041.

A first difference Fourier map was calculated between the observed structure factor amplitudes and the structure factor calculated from the trypsin moiety of benzamidine-inhibited trypsin [3]. All Fourier calculations were doing using the PROTEIN program package [5]. Within a specificity pocket, there was a positive difference density which was interpreted as being due to the *p*-guanidinobenzoate moiety of the inhibitor, based on its shape and size. At this stage, it was not clear from the density map whether the binding of the fragment was non-covalent or covalent. The crystallographic refinement was started from the benzamidine-inhibited trypsin structure omitting the bound inhibitor benzamidine. 12 936 reflection data from 6.0 Å to 2.06 Å resolution with intensity greater than 2σ were used for the calculation. The calculation was done with the restrained least-squares program by Hendrickson and Konnert [6]. After 7 cycles of refinement, the inhibitor fragment *p*-guanidinobenzoate was fitted to the electron density map to form two possible models, a covalent model and a non-covalent model. After several further cycles on those two models and examinations of electron density maps, the covalently bonded model was selected as preferable. This final *R*-factor was 19.1%, including 12 atoms of the inhibitor fragment, one calcium ion, and 77 water molecules.

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Abbreviations: FUT, 6-amidino-2-naphthyl 4-guanidinobenzoate

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3. RESULTS AND DISCUSSION

Overall folding of FUT-inhibited trypsin is almost identical to the previously published orthorhombic crystal structure of benzamidine-inhibited trypsin [3]. When the FUT-inhibited trypsin is superposed on the benzamidine-inhibited trypsin at the peptide backbone atoms (Cα, C, N, O), the root-mean-square differences between the two structures are 0.13 Å for backbone atoms and 0.23 Å for all atoms.

Fig.1 shows the superposed structures of the inhibitor and adjacent residues of the FUT-inhibited trypsin and benzamidine-inhibited trypsin. In the FUT-inhibited trypsin, the hydrolyzed fragment of FUT, namely *p*-guanidinobenzoate, binds to the specificity pocket of trypsin, which is supposed to interact with the lysine or arginine side chain of a peptide substrate. The inhibitor fragment is covalently attached to trypsin through an ester bond between inhibitor carboxylate and Ser^{195}-Oγ. The conformations in the region of the inhibitor are almost identical in the two structures except for somewhat larger movements of side chain atoms that are in close contact or are hydrogen-bonded to inhibitor atoms. Those movements are probably due to the larger size of *p*-guanidinobenzoate compared to benzamidine.

The hydrogen-bonding scheme around *p*-guanidinobenzoate is shown in fig.2 with corresponding interatomic distances. The guanidine group is hydrogen-bonded to Asp^{189}-Oδ1 and -Oδ2, Ser^{190}-Oγ, and a main chain carbonyl oxygen of Gly^{219}. A similar hydrogen-bonding scheme has been observed in benzamidine-inhibited trypsin, in which an amidine group participates in the hydrogen bonds instead of the guanidine group. But there is a difference in that the FUT-inhibited trypsin has bifurcated hydrogen bonds of Gly^{219} oxygen to two nitrogens of the guanidine group.

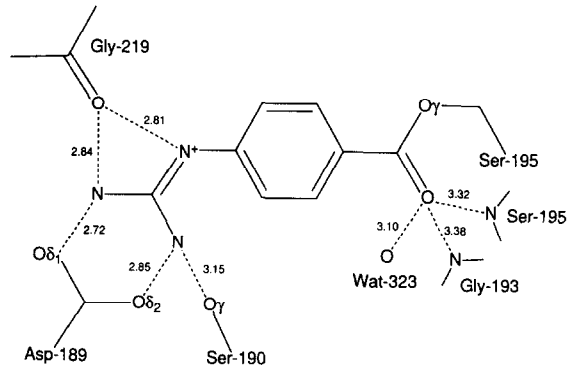

Fig.2. Hydrogen-bonding scheme and distances around the covalently bound inhibitor *p*-guanidinobenzoate in FUT-inhibited trypsin. Hydrogen bonds are shown as dashed lines with distances in Å.

In the commonly accepted hydrolysis mechanism of trypsin, main chain amide nitrogens of Gly^{193} and Ser^{195} form a so-called 'oxyanion hole' and stabilize negative charge on the substrate carbonyl oxygen during the nucleophilic attack on carbonyl carbon [7]. In FUT-inhibited trypsin, the distances from inhibitor carbonyl oxygen to Gly^{193} N and Ser^{195} N are 3.4 Å and 3.3 Å, respectively, which are rather longer than typical hydrogen bonds. As compared to the side chains of lysine and arginine which are specific residues of trypsin-hydrolyzable substrates, *p*-guanidinobenzoate is rigid and has little conformational freedom. Furthermore, *p*-guanidinobenzoate is 'anchored' to the specificity pocket on both sides with several strong hydrogen bonds and with one covalent bond to trypsin. The approach of carbonyl oxygen to the oxyanion hole seems to be hindered by those conformational and geometrical constraints.

A kinetic study shows that hydrolysis of FUT by trypsin proceeds in two steps (Noji,S., personal communication). The first step is quite rapid and produces

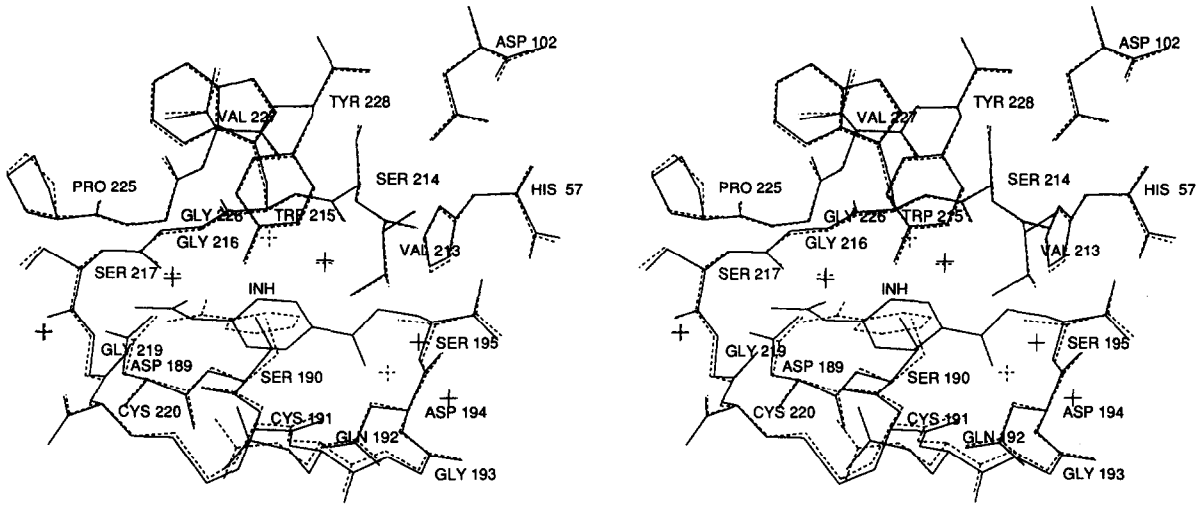

Fig.1. Stereo drawing of substrate binding site residues and bound inhibitors of FUT-inhibited trypsin (solid line) and benzamidine-inhibited trypsin (dashed line). Structures were superposed by a root-mean-square fit of trypsin backbone atoms. Small crosses are bound water molecules.

an acylated enzyme, which we have seen in this crystallographic study. The second step of the reaction, cleavage of the ester bond of the acylated enzyme, is very slow under physiological conditions. FUT may be regarded as a 'suicide inhibitor' to some extent. Our result suggests that the slow rate of deacylation of the FUT-inhibited enzyme may be due to the less favorable interaction between the carbonyl oxygen of the acyl group and the oxyanion hole of trypsin.

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