Multiple Binding of Inhibitors in the Complex Formed by Bovine Trypsin and Fragments of a Synthetic Inhibitor, 4-[4-(*N*,*N*-Dimethylcarbamoylmethoxycarbonylmethyl)phenoxycarbonylphenyl]guanidinium Methanesulfonate (FOY-305)

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The crystal of bovine trypsin complexed with a potent inhibitor, 4-[4-(*N*,*N*-dimethylcarbamoylmethoxycarbonylmethyl)phenoxycarbonylphenyl]guanidinium methanesulfonate (FOY-305) in the novel orthorhombic from with a low molecular packing density was studied by the X-ray diffraction method. Using synchrotron radiation, the intensity data were collected to 1.8 Å resolution. The structure was solved by molecular replacement methods, and refined to an *R*-factor = 18.0% for 14364 reflections by the restrained least-squares method. The final difference Fourier maps revealed that hydrolyzed inhibitor fragments bind with the protein at multiple sites around the active center of trypsin. The structural feature in the crystalline state probably corresponds to a statistical average of several complexes which would be formed between the inhibitor and trypsin during the binding and releasing process in solution.

Keywords trypsin; inhibitor; X-ray; complex

4-[4-(*N*,*N*-Dimethylcarbamoylmethoxycarbonylmethyl)phenoxycarbonylphenyl]guanidinium methanesulfonate (FOY-305) is a potent inhibitor which is able to bind very tightly with trypsin.[1] The *p*-guanidinobenzoyloxy group is similar to a synthetic inhibitor, ethyl *p*-guanidinobenzoate, which is known to form an acyl–enzyme complex with trypsin.[2] The *p*-guanidinobenzoyloxy group interacts with the active center of trypsin, and it is supposed that these inhibitors form a similar acyl–enzyme complex. On the other hand, the characteristic inhibition mechanism of the present inhibitor was considered to depend on a very slow releasing process of the inhibitors from trypsin, as observed *in vitro*.[3] Thus, this inhibitor probably forms a Michaelis complex with trypsin in the process, although the structural aspect is not obvious. The present X-ray study was, therefore, undertaken in order to obtain direct evidence of the inhibitor binding to trypsin, and to reveal its binding features.

Bovine trypsin is known to crystallize in three distinct crystal forms: two orthorhombic forms with high and low molecular packing densities, and another trigonal form.[4] The high density form of a benzamidine complex[5] and an amidinophenylpyruvate complex[6] and the trigonal form of a synthesized thrombin inhibitor, MQPA, complex[7] have been studied so far. In the present study, a crystal of the low density form was used because the complex crystal can be more easily obtained by the soaking method than the other two crystal forms. However, since the complex in this form would be damaged within a short time, strong synchrotron radiation was used for the data collection in the X-ray diffraction experiment.

Experimental

Trypsin (Type I, powder from bovine pancreas) and benzamidine were purchased from Sigma Chem. Co., and 4-[4-(*N*,*N*-dimethylcarbamoylmethoxycarbonylmethyl)phenoxycarbonylphenyl]guanidinium methanesulfonate (FOY-305) was provided by the Ono Pharmaceutical Company. The purchased trypsin was purified using the method of Schroeder and Shaw[8] in order to exclude the cleavaged trypsins. To avoid the self-digestion of trypsin, a weak inhibitor (benzamidine hydrochloride, 10 mg/ml) and $CaCl_2$ 1 mg/ml were mixed into the trypsin solution. The crystals of the weak inhibitor complex were grown using the vapour diffusion technique. *ca.* 50 μl drops of a filtered 0.6 M ammonium sulfate solution at pH 8.0 were put in small plastic wells which were placed in a sealed plastic box with an external ammonium sulfate solution. The crystals were most effectively grown under the condition of 1.4 M external ammonium sulfate at pH 8.0, and adequate crystals (0.5 × 0.5 × 1.0 mm) were obtained after a few weeks. The obtained crystal was soaked in the 3.0 M ammonium sulfate at pH 8.0 in the presence of 5 mM FOY-305 at approximately 20 °C for a week.

The crystal has a space group $P2_12_12_1$, and cell dimensions, $a=63.50$, $b=69.05$, $c=63.71$ Å, $Z=4$, $V=279347.64$ Å^3, which are comparable with the $a=54.9$, $b=58.5$, $c=67.8$ Å, $V=217749.87$ Å^3 for the high density form (Stroud *et al.*[9]). The intensity data were collected with Weissenberg-camera[10] equipped for the synchrotron radiation beam from the BL6A2 station of the Storage Ring at the Photon Factory of the National Laboratory for High Energy Physics, Tsukuba, Japan; the beam current was 165—170 mA with a wave length of 1.004 Å at 8.0 °C. The crystal was rotated around the c-axis in an oscillation angle of 10.5° with a coupling constant of 3.0°/mm. Diffraction intensities within 1.8 Å resolution were recorded on Imaging Plates (IP)[11] mounted on a cassette with a 286.7 mm radius, with a total exposure time of 648 s (54 s × 12 IP). The spot densities on the IP were read with a Fuji BA100 system, and the data sets were processed using the WEIS program.[12] The number of reflections counted on each plate ranged from 4525 to 5827 (R_{sym} varied between 5.27 and 10.21%). The number of total observed reflections was 97940, and the number of independent reflections was 21009 ($R_{merge}=6.55\%$).

Molecular orientation was determind by the rotation function (PROTEIN program system[13]) using 1294 peaks within 5—20 Å shell in a calculated Patterson map based on the known bovine trypsin structure in the high density form[5] and an intramolecular vector set in a cubic cell with 70 × 80 × 70 Å^3 in an observed Patterson map synthesized with the intensity data of the 3—20 Å resolution. The position of the trypsin molecule in the unit cell was determined by application of the translation function program.[14] The obtained rotational and translational parameters were refined with the Fourier-transform fitting program TRAREF.[15] These rigid-body refinements gave an *R*-value of 30.5% for 2.5 Å resolution data. The refined rotation angles (151.16, 35.06, −122.18) in the definition by Huber,[16] and the translation vector (0.1091, 0.1312, 0.2474) are in good agreement with those previously determined by Matsushima & Bode (unpublished data) and Bartunik *et al.*[17] independently. The Hendrickson–Konnert restrained least-squares refinement program PROLSQ[18] was used throughout the refinement stage. During the course of refinement, the model was revised. The side chains of the amino acid residues were fitted into the $2F_O-F_C$ density map on a DAIKIN DS-300 interactive graphic computer.[19] A number of the isolated peaks were identified as water molecules when they had a well-defined electron density and were within an acceptable distance from at least one of the polar protein groups or from another water molecule. The improved structure (1677 atoms including 48 water molecules) was refined to the *R*-value = 18.0% for 14369 reflections in a range of 1.8—5.0 Å. The parameters used in the final stage of restrained refinement were 0.020 Å for bond lengths, 0.040 Å for bond angles, and 0.050 Å^3 for chiral volumes. The final route mean squares (r.m.s) deviations were correspondingly 0.010, 0.033 and 0.110 Å^3. The program was run on a FACOM VP-400E of the Kyoto University Data Processing Center. Altogether, more than 900 cycles were calculated, and

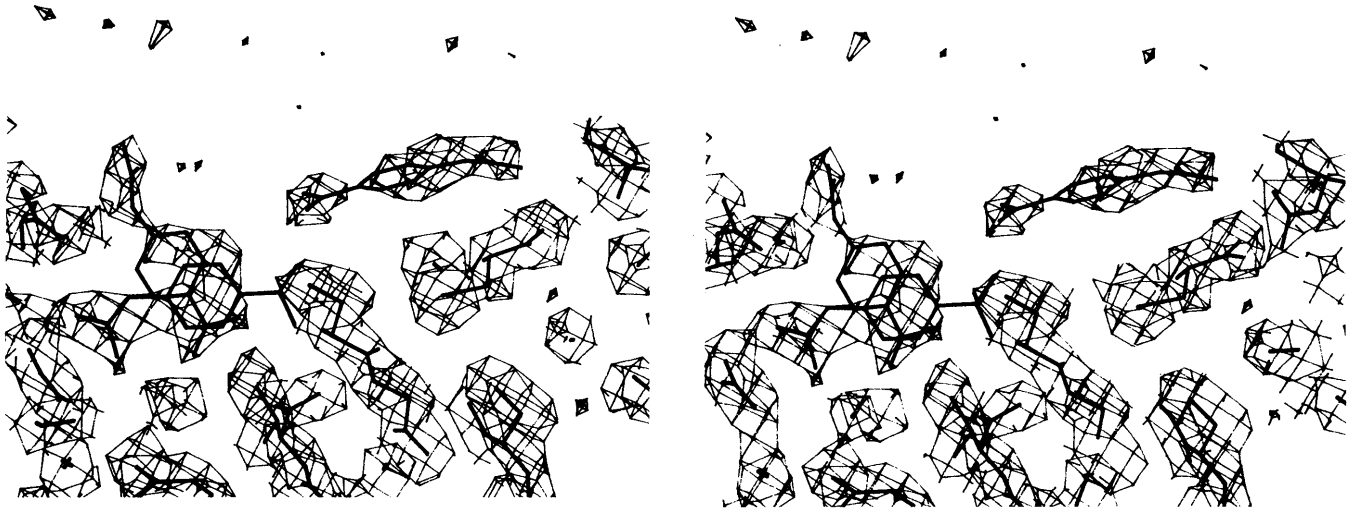

Fig. 1. Stereoscopic Drawing of the Final $2F_O-F_C$ Map around the Active Site of Trypsin with Hypothetical Inhibitor Fragment Models
The relative contour level is 0.85σ.

one cycle of least-squares refinement took about six seconds of central processing unit time.

Results and Discussion

The overall conformation of the trypsin molecule itself, determined here, is very similar to those of the other two crystal forms. When the Cα model was optimally superimposed on those of the native structures in the other orthorhombic form[5] and the trigonal form,[20] the equivalent atoms agreed with each other within the r.m.s deviation less than 0.33 Å. A cleft on the surface of the globular protein exists around the active center of Ser 195 Oγ. The $2F_O-F_C$ maps synthesized at the last stage showed comparatively high residual electron densities in the inside of the cleft. The residual electron densities had not been observed in the difference map synthesized by the use of data from the inhibitor-free trypsin crystal,[21] and the peaks could be properly interpreted by the inhibitor fragments which were assigned to the hydrolyzed portion of the inhibitor, *p*-guanidinobenzoate (Fig. 1). Although the model fitting had fairly large ambiguity, three fragment models could be fitted to the electron density peaks as shown schematically in Fig. 2. Two disordered fragments could be located inside the *P*1 pocket, and another fragment could locate out of the *P*1 pocket. Both aromatic rings of the fragments inside the *P*1 pocket had hydrophobic interactions with the nonpolar groups of the pocket wall, while the guanidyl group of one fragment had an electrostatically favorable interaction with the Asp 189 carboxyl group at the bottom of the pocket. Similar interactions of an inhibitor with the Asp 189 of trypsin have been observed in other complexes, *e.g.* the benzamidine complex[5] and amidinophenylpyruvate complex.[6] The electrostatic interaction is similar to that observed in the single crystal of the inhibitor itself, in which all protons of the guanidyl group participate in the hydrogen bonds formed with the surrounding polar groups.[22] The carboxylate groups of the inhibitor fragments inside the *P*1 pocket locate at the edge of the pocket, while the oxygen atom of one carboxylate group is close to the active center of Ser 195 Oγ. The third fragment of the inhibitor separates from the first and second disordered fragments. The fragment exists near His 57. The

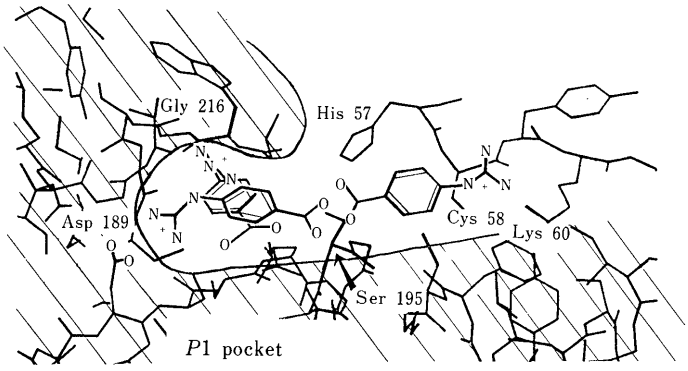

Fig. 2. Schematic Drawing of Three Inhibitor Fragment Models at the Active Site of Trypsin

carbonyl group is also close to the Ser 195 Oγ, but the guanidyl group has electrostatic interactions with Cys 58 and Lys 60 out of the *P*1 pocket. Such multiple binding of small synthetic inhibitors with a protein has been occasionally observed in a crystalline state. For instance, in the peptidyl inhibitor–chymotrypsin complex, two inhibitors bind to different sites of chymotrypsin.[23]

According to our recent ^{1}H-nuclear magnetic resonance (^{1}H-NMR) study of the mixture between the inhibitor and trypsin, it was clear that hydrolyzed fragments of the inhibitor bound with trypsin in the solution, and the signal change during incubation at 37 °C reflected a successive process of partial release of the *p*-guanidinobenzoyloxy fragment and their weak recombination with enzymes around the active site.[24] The NMR result suggests that trypsin has at least three binding modes for the inhibitor fragments, and that the inhibitor fragments would be trapped at the binding sites during the releasing process. Since the present complex studied by X-ray diffraction was obtained by soaking the crystal in an almost-saturated inhibitor solution, the inhibitors would be bound with the protein at every possible site in the soaking process. The structural feature in the crystalline state, therefore, probably corresponds to a statistical average of several complexes which would be formed between the inhibitor and trypsin. The location and orientation of each fragment observed in

the $2F_O - F_C$ map seems to reflect a trace of the fragments in the binding or releasing process, and the multi-binding structural aspect might be strongly related to the extreamly slow releasing process of this inhibitor. Consequently, the present X-ray study gave appropriate structural evidence for the unique interactions between the potent inhibitor (FOY-305) and trypsin.

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References

1) Y. Tamura, M. Hirado, K. Okamura, Y. Minato, and S. Fujii, *Biochim. Biophys. Acta*, **484**, 417 (1977).
2) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **242**, 5782 (1967).
3) N. Kayama, K. Sakaguchi, and S. Fujii, *Gendai-Iryou*, **12**, 1 (1989).
4) W. Bode and P. Schwager, *J. Mol. Biol.*, **98**, 683 (1975).
5) W. Bode and P. Schwager, *J. Mol. Biol.*, **98**, 693 (1975).
6) J. Walter and W. Bode, *Hoppe-Seyler's Z. Physiol. Chem.*, **364**, 949 (1983).
7) T. Matsuzaki, C. Sasaki, C. Okamura, and H. Umeyama, *J. Biochem.* (Tokyo), **105**, 949 (1989).
8) D. D. Schroeder and E. Shaw, *J. Biol. Chem.*, **243**, 2943 (1968).
9) R. M. Stround, L. Kay, and R. E. Dickerson, *J. Mol. Biol.*, **83**, 185 (1974).
10) N. Sakabe, *J. Appl. Cryst.*, **16**, 542 (1983).
11) J. Miyahara, K. Takahashi, Y. Amemiya, N. Kamiya, and Y. Satow, *Nucl. Instrum. Methods*, **A246**, 572 (1986).
12) T. Higashi, *J. Appl. Cryst.*, **22**, 9 (1989).
13) W. Steigemann, Ph. D. Thesis, Technische Universität München, 1974.
14) E. E. Lattmann and W. E. Love, *Acta Crystallogr., Sect. B*, **26**, 1854 (1974).
15) R. Huber and M. Schneider, *J. Appl. Cryst.*, **18**, 165 (1985).
16) R. Huber, *Acta Crystallogr., Sect. A*, **19**, 353 (1965).
17) H. D. Bartunik, L. J. Summers, and H. H. Bartsh, *J. Mol. Biol.*, **210**, 813 (1989).
18) W. A. Hendrickson and J. H. Konnert, "Computing in Crystallography," ed. by R. Diamond, S. Ramaseshan and K. Venkatesan, Indian Academy of Sciences, Bangalore., 1980, pp. 13.01—13.23.
19) The utility programs for molecular graphics were originally developed in the Kyoto Univ. Lab.
20) M. Marquart, J. Walter, J. Deisenhofer, W. Bode, and R. Huber, *Acta Crystallogr., Sect. B.*, **39**, 480 (1983).
21) O. Matsumoto, T. Higashi, T. Taga, M. Matsushima and K. Machida, unpublished data.
22) O. Matsumoto, T. Taga, and K. Machida, *Acta Crystallogr., Sect. C*, **45**, 913 (1989).
23) E. F. Meyer Jr., R. Radhakrishnan, G. M. Cole, and L. G. Presta, *J. Mol. Biol.*, **189**, 533 (1986).
24) O. Matsumoto, H. Masuda, T. Taga, Y. Kuroda, and K. Machida, *Chem. Pharm. Bull.*, **38**, 2015 (1990).