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Binding Diversity of a Noncovalent-Type Low-Molecular-Weight Serine Protease Inhibitor and Function of a Catalytic Water Molecule: X-Ray Crystal Structure of PKSI-527–Inhibited Trypsin

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PKSI-527 is a noncovalent-type low-molecular-weight inhibitor. The X-ray crystal structure of the trypsin-PKSI-527 complex revealed a binding mode (Form II) different from the previously reported one (Form I) [Nakamura, M. *et al.* (1995) *Biochem. Biophys. Res. Commun.* 213, 583–587]. In contrast to the previous case, the electron density of the inhibitor revealed the whole structure clearly. Each structural part of the inhibitor in Forms I and II was differently located at the active site, although the modes of binding of the terminal amino group to the Asp189 carboxyl group were similar. This binding diversity, which is a characteristic of the noncovalent-type low-molecular-weight inhibitor, provides a suitable example for estimating the possible mechanism toward the enzymatic inhibition, together with the structural basis necessary for a specific inhibitor. The mode of binding in Form II reflects the inhibitor-specific situation and is in contrast with the substrate-mimetic binding mode for Form I. Based on the generally accepted catalytic mechanism for serine protease, we propose that a water molecule located at the catalytic site plays an important role in blocking the catalytic function of the reactive Ser193 OH group.

Key words: binding diversity, crystal structure, noncovalent inhibitor, trypsin.

Trypsin is a prominent member of the serine protease family, and forms the functional principle of some large and highly specific proteases, involved in precesses ranging from digestion to key regulatory mechanisms such as coagulation and hormone release, that serve to maintain a favorable extracellular environment (*1–3*). Since it is generally accepted that an imbalance of their enzymatic activities causes serious diseases, inhibitors that can selectively control their proteolytic activities are very useful for the prevention of such diseases (*4–6*). In particular, the development of noncovalent-type low-molecular-weight inhibitors is of considerable importance in view of their potential therapeutic value as drugs.

PKSI-527 (*trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanyl-4-aminophenyl acetic acid; Fig. 1) is now used as a representative noncovalent-type and low-molecular-weight inhibitor for the serine protease family (*7*). Since elucidation of the inhibitory mechanism of PKSI-527 at the atomic level could provide important information for further development of a potent inhibitor specific for each protease, we previously analyzed the X-ray crystal structure of the bovine pancreas trypsin–PKSI-527 complex (*8*). Although the results confirmed the main function (P1–S1 interaction) of PKSI-527 as an inhibitor, the structural roles of the P1′ and P2′ sites remained unclear because of a lack of data regarding their electron densities. This could be mainly due to their insufficient and/or flexible docking, and appears to be a typical feature of a noncovalent-type inhibitor exhibiting modest activity.

On the other hand, the binding diversity at the catalytic pocket could often be observed for the low-molecular-weight noncovalent inhibitor. A study on this binding diversity appears to be important, because this information is absolutely necessary for designing a clinically usable drug. Thus, various attempts to obtain complex crystals in which PKSI-527 was fully inserted into trypsin have been performed. In this paper, we report the mode of binding of PKSI-527 located at the catalytic pocket in trypsin, which is considerably different from the previous binding mode. Based on the results of comparison of these binding modes, we also discuss the time-resolved catalytic/inhibitory step of trypsin and the importance of a catalytic water molecule.

MATERIALS AND METHODS

Materials and Inhibitory Assay—Bovine pancreas trypsin was purchased from Sigma (Type III). The synthesis of PKSI-527 and assaying of its inhibitory activity toward trypsin were performed according to the literature (*7*).

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Abbreviations: PKSI-527, *trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanyl-4-aminophenyl acetic acid; rmsd, root mean square deviation.

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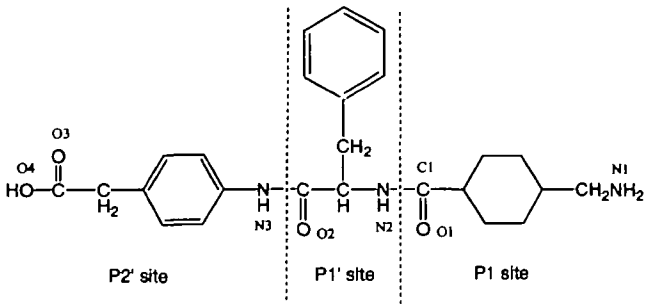
Crystallization and X-Ray Data Collection—A 20 mg/ml trypsin solution was prepared with 0.1 M sodium acetate buffer (pH 5.5) containing 6 mM $CaCl_2$. A 3-fold molar excess of PKSI-527, which was solubilized in a small amount of dimethylsulphoxide, was included in the solution; this molar ratio was shown to be sufficient for complete inhibition of the enzyme by the inhibitory assay.



PKSI-527

Fig. 1. **Chemical structure of PKSI-527, together with the numbering for the C1, N, and O atoms.** The site number (P1, P1', and P2' sites) of each residue is labeled, based on the binding mode elucidated from the present crystal structure.

Crystals of the complex were obtained within a week using the vapor diffusion hanging drop method at room temperature (14°C), where drops of 5.5 μl of the complex solution were equilibrated against the same amount of the reservoir solution of 0.1 M Tris-HCl buffer (pH 8.0) containing 2.2–2.6 M $(NH_4)_2SO_4$. Since these crystals exhibited about 50% of the enzymatic activity of trypsin, as estimated by assay measurement, they were subjected to soaking for 3 days at 14°C in a 2.5 M $(NH_4)_2SO_4$ solution, buffered with 0.1 M sodium acetate (pH 5.5), containing 5 mM $CaCl_2$ and 1 mg/ml of PKSI-527 which was solubilized in a small amount of dimethylsulphoxide. The thus prepared crystals exhibited complete inhibition.

The crystal belongs to orthorhombic space group $P2_12_12_1$. The X-ray diffraction intensities at 293 K were collected with a Rigaku RAXIS-IIc diffractometer on a rotating anode generator (Cu Kα radiation). The X-ray crystallographic data are as follows: a = 55.05 Å, b = 58.57 Å, and c = 67.55 Å, total number of reflections = 17318, resolution up to 1.85 Å, completeness = 89.8%, and R_{merge} = 4.33%.

Structure Determination and Refinement—The crystal structure of the complex was solved by the molecular replacement method using the atomic coordinates of the previously analyzed trypsin (*8*) as a starting model. The model was oriented, translated and refined with program CNS (*9*) using a combination of simultaneous annealing and con-

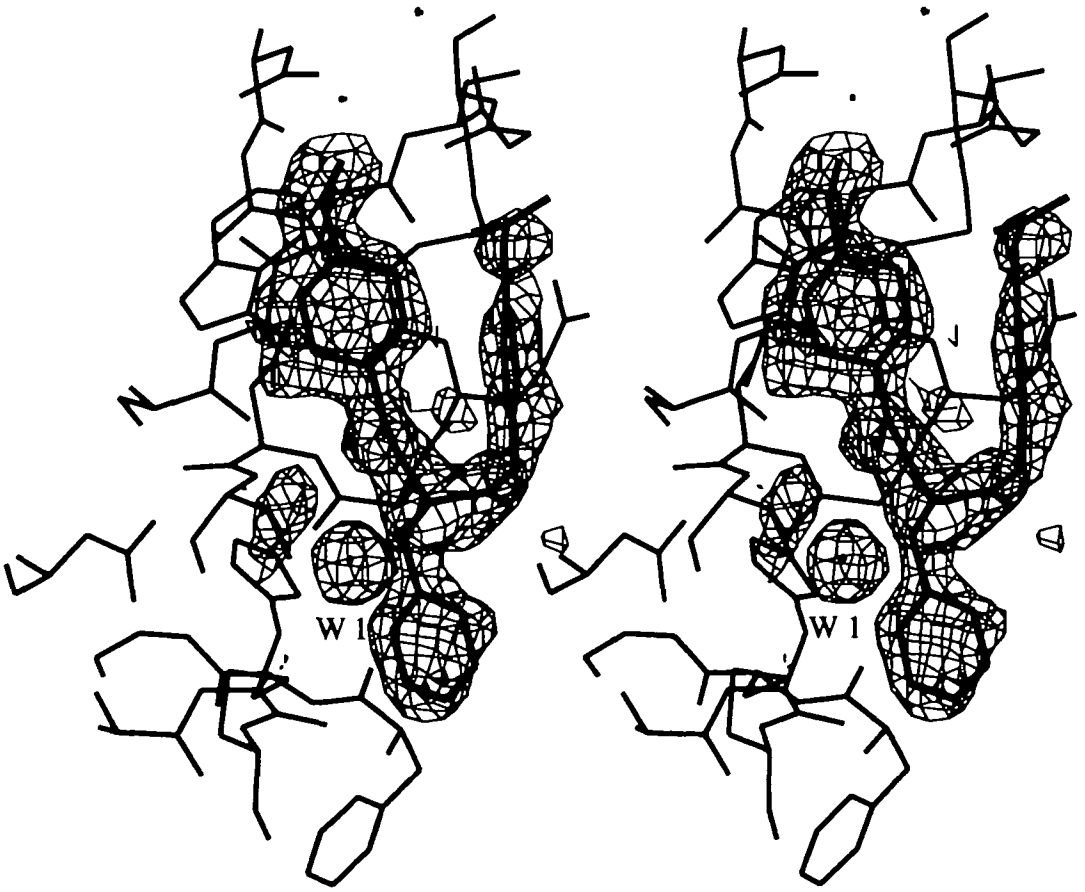


Fig. 2. **A F_o–F_c electron density map (in stereo) of PKSI-527 bound to the trypsin active site.** The electron density of W1 (water molecule) is also shown. The molecular position of the inhibitor is shown by thick lines on the map. Thin lines represent the partial amino acid residues of trypsin.

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ventional restrained refinement technique. Successive difference Fourier syntheses and consequent model fittings were carried out using the TURBO-FRODO program (*10*). The present state for the structural refinement is as follows: R = 18.4% at 30–1.85 Å for 17,279 independent reflections, R_{free} = 21.1% for 10% of total data with the same resolution range, number of atoms = 1625 (including one Ca^{2+} ion), number of solvents = 63, and temperature factors of inhibitor = 18.7–29.7 Å^2. The rmsds from the ideal bond distances and angles were 0.005 Å and 1.318°, respectively.

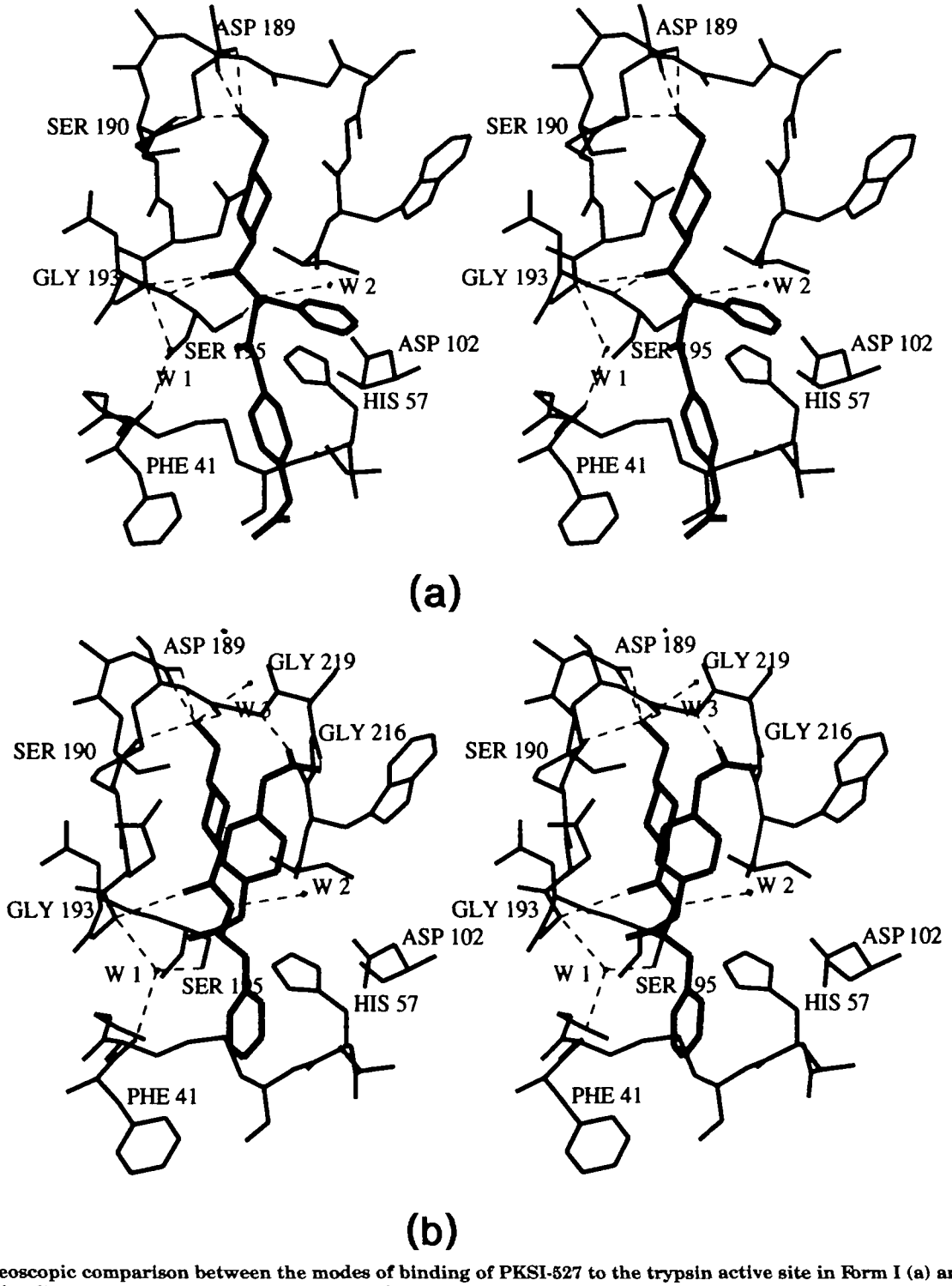

Fig. 3. **Stereoscopic comparison between the modes of binding of PKSI-527 to the trypsin active site in Form I (a) and Form II (b).** The broken lines represent short contacts between PKSI-527 and trypsin or water molecules. Hydrogen bonds between W1 with trypsin are also shown.

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RESULTS AND DISCUSSION

An electron density map of PKSI-527, which was noncovalently bound to the trypsin active site, is shown in Fig. 2, where the chemical structure of inhibitor is depicted by thick lines and W1 represents water solvent. In contrast to the previously analyzed complex structure (*8*) (named Form I), the whole structure of the inhibitor was clearly observed in this structure (named Form II). Furthermore, a remarkable difference was observed for the overall folding of PKSI-527 and its binding mode at the active site (Fig. 3). This diversity obviously results from the different conditions used for preparing the trypsin-PKSI527 complex crystal. As was stated in the experimental section, the crystals of Form II were prepared by further soaking of Form I crystals in the crystallization solution, in which a high concentration of inhibitor was dissolved to obtain complete inhibition.

The modes of binding of PKSI-527 in Forms I and II are shown in Fig. 3, a and b, respectively. Short interatomic distances in both forms are summarized in Table I. It is characteristic that the terminal amino group is similarly located at the so-called "specificity pocket" of the Asp189 carboxyl group through hydrogen bonds, suggesting an important structural requisite for the inhibitor. In contrast, the remaining parts of PKSI-527 are differently located. The most notable difference is the disposition of Phe and 4-carboxymethyanilide moieties with respect to the aminomethylcyclohexane ring at the binding pocket. The different ϕ and ψ torsion angles of Phe (119.9° and 158.2° for Form I, and –87.1° and –51.8° for Form II, respectively) lead to such a reverse disposition. This means that the spaces of the S1′ and S2′ subsites are large enough to accommodate the interchange of P1′ and P2′ side groups. In other words, the pocket of the trypsin S1′ subsite would not be so rigidly constructed as to impose any substrate-specificity and allows the location of the inhibitor P1′ group at the S2′ subsite. On the whole, the P1′ and P2′ sites of PKSI-527 are located at the entry of the cleft of trypsin and are bordered by solvent molecules. Therefore, the binding pattern at these sites appears not to be uniform, but to be dependent on the conditions for the complex formation. On the other hand, a remarkable difference was also observed concerning the electron density of the inhibitor in the two forms. The electron densities corresponding to Phe and carboxymethyanilide moieties were not so clear as their unequivocal positioning in Form I was possible. This is in contrast with the rich density in Form II. Although clear reasons for the poor electron densities in Form I are not known at present, the insufficient complex formation due to the incomplete inhibition could be at least responsible for Form I. The hydrogen bonds of (i) O1 to NHs of Gly193 and Ser195 ("oxyanion hole"), and (ii) N2H to Ser195 O^{γ} (active residue) formed in Form I were not observed in Form II. Instead, hydrogen bonds of (i) O1 to Gly193 NH, (ii) O3 to Gly219 NH, and (iii) O4H to Gly216 O are formed in Form II. It could be said that the hydrogen bonds of N1H···O^{δ}(Asp189), O3···NH (Gly219), and O4H···O (Gly216) importantly contribute to the fixation of the whole structure of the inhibitor at the active site because these coupled hydrogen bonds at both terminal groups are lacking in Form I.

It appears interesting to consider the biological meaning of the different binding modes observed for Forms I and II. According to the catalytic mechanism generally accepted for the serine protease (*11*), the catalytic reaction is initiated by the nucleophilic attack by the Ser195 O^{γ} atom of the carbonyl carbon of a basic amino acid residue such as Lys or Arg in the substrate peptide. In the case of PKSI-527 (Fig. 1), the C1 atom corresponds to this carbonyl carbon. It is remarkable that the C1–O^{γ} and O1–O^{γ} (Ser195) (2.79 and 2.95 Å) distances in Form I are considerably shorter than those in Form II (4.21 and 4.05 Å). On the other hand, the importance of a water molecule in the catalytic reaction has been proposed (*12*, *13*). W1 in Forms I and II corresponds to this water, and is visualized at an equivalent position, in available crystal structures of trypsin–substrate analog/inhibitor complexes. The W1 molecule is sta-

TABLE I. **Interatomic short contacts (Å) of PKSI-527 with trypsin observed in Forms I and II.**

PKSI-527	Trypsin	Distance
Form I		
N1	Asp189 $O^{\delta1}$	2.88
N1	Asp189 $O^{\delta2}$	3.12
N1	Ser190 O	2.70
O1	Gly193 NH	2.97
O1	Ser195NH	3.03
N2	Ser195 O^{γ}	3.05
N2	W2[a]	3.26
Form II		
N1	Gly219 O	3.12
N1	Asp189 $O^{\delta1}$	3.22
N1	Asp189 $O^{\delta2}$	2.37
N1	Ser190 O	2.54
N1	W3	2.96
O1	Gly193 NH	3.12
N2	W2	3.31
O3	Gly219 NH	2.61
O4	Gly216 O	2.58

[a]W represents water solvent. The atomic number of the inhibitor is shown in Fig. 1.

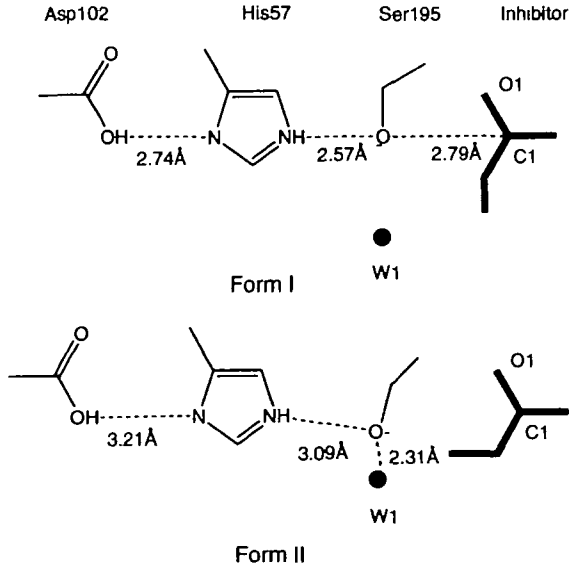

Fig. 4. **Schematic comparison of the interaction among the catalytic triad residues (Ser195, His57, and Asp102), W1, and the inhibitor in Forms I and II.** The dotted lines show possible hydrogen bonds. The carbonyl moiety of the inhibitor is shown by thick lines.

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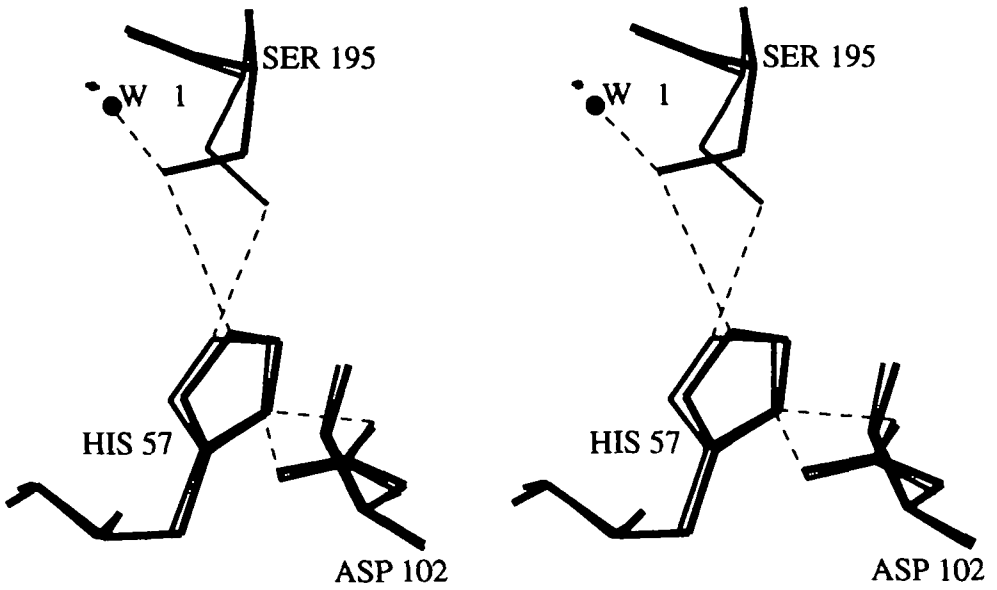

Fig. 5. **Stereoscopic superposition of spatial orientation diagrams of Ser195, His57, and Asp102 constituting the catalytic sites in Form I (thin line) and II (thick line).** W1 molecules in Forms I and II are also shown as small and large filled circles, respectively.

bilized by the two hydrogen bonds of (Gly193) NH and (Phe41) O (3.27 and 2.63 Å for Form I, and 3.29 and 3.00 Å for Form II, respectively) The mode of interaction of the W1 molecule in the catalytic site is shown in Fig. 4. Interestingly, the W1 water molecule fixes the Ser195 O^{γ} atom through a strong OH···O hydrogen bond (2.31 Å) in Form II, and consequently prevents the nucleophilic attack by the anionic O^{γ} atom of the carbonyl C1 carbon. Since such a situation does not occur in Form I (OH···O = 3.88 Å), it may be said that W1 plays a role in controlling the catalytic function of Ser195. In fact, the spatial orientation of the Ser195 side chain was significantly different between Forms I and II, as compared with those of the remaining residues (Fig. 5). The observation of the (W1) OH···O^{γ} (Ser195) hydrogen bond appears to be the first example to our knowledge. As judged from the above-mentioned mutual disposition among the catalytic triads (Asp102, His57, and Ser195), the W1 molecule, and the inhibitor, it would be reasonable to consider that the binding mode in Form I is close to the substrate-mimetic intermediate state, whereas that in Form II reflects the inhibitor-intrinsic state at the catalytic pocket. It is obvious from the complete lack of enzymatic activity that the binding mode in Form II completely blocks the approach of substrate to the active site.

The crystal structures of many trypsin-inhibitor complexes have been reported so far. As for the crystal structures of trypsin inhibited by low-molecular-weight inhibitors, a number of derivatives such as benzamidine (*14*), *p*-guanidinobenzoate (*15*, *16*), N^{α}-(2-naphthylsulphonyl-glycyl)-DL-*p*-amidinophenylalanylpiperidine (*17*), *N*-[3-[4-[4-(amidinophenoxy) carbonyl]phenyl-2-methyl-2-propenoyl]-*N*-allylglycine (*18*), and leupeptin (*19*) have been reported. However, all of them were covalently bonded to the O^{γ} atom of the Ser side chain located at the active center. Thus, the crystal structures of the trypsin-PKSI527 complex appear to be the first example of the noncovalent binding of a low-molecular-weight inhibitor to trypsin to our knowledge. Generally, in the case of a low-molecular-weight inhibitor, noncovalent binding to the active center appears to be rarely achieved, because of a lack of any specific driving force which is sufficient to lead to direct binding to the active pocket (*20*). Also, the present complex is a rather special case, and the 'specificity pocket' consisting of Asp189 and Gly219 could be the main reason for this. Thus the present results would be useful for effectively designing a noncovalent-type low-molecular-weight trypsin-specific inhibitor.

REFERENCES

1. Horn, H. and Heidland, A. (eds.) (1982) Proteases: *Potential Role in Health and Disease*, Plenum Press, New York and London
2. Cunningham, D.D. and Long, G.L. (eds.) (1987) *Proteinases in Biologic Control and Biotechnology*, Alan R. Liss, New York
3. Patthy, L. (1990) Evolutionary assembly of blood coagulation proteins. *Semin. Thromb. Hemostasis* **16**, 245–259
4. Laskowski, M., Jr. and Kato, I. (1980) Protein inhibitors of proteinases. *Annu. Rev. Biochem.* **49**, 593–626
5. Katunuma, N. (1990) New biological functions of intracellular proteases and their endogenous inhibitors as bioreactants. *Adv. Enzyme Regul.* **30**, 377–392
6. Stenman, U.H., Koivunen, E., and Itkonen, O. (1991) Biology and function of tumor-associated trypsin inhibitor, TATI. *Scand. J. Clin. Lab. Invest. Suppl.* **207**, 5–9
7. Wanaka, K., Okada, Y., Tsuda, Y., Okamoto, U., Hijikata-Okunomiya, A., and Okamoto, S. (1992) Synthesis of *trans*-4-aminomethylcyclohexanecarbonyl-L- and -D-phenylalanyl-4-carboxymethyl-anilide. *Chem. Pharm. Bull.* **40**, 1814–1817
8. Nakamura, M., Tomoo, K., Ishida, T., Taguchi, H., Tsuda, Y., Okada, Y., Okunomiya, A., Wanaka, K., and Okamoto, S. (1995) Design of noncovalent trypsin inhibitor based on the X-ray crystal structure of the complex. *Biochem. Biophys. Res. Commun.* **213**, 583–587
9. Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.-S., Kuszewski, J., Niges, M., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T., and Warren, G.L. (1998) Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D* **54**, 905–921
10. Jones, T.A. (1978) A graphics model building and refinement system for macromolecules. *J. Appl. Cryst.* **15**, 23–31
11. Blow, D.M., Birktoft, J.J., and Hartley, B.S. (1969) Role of a bur-

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ied acid group in the mechanism of action of chymotrypsin. *Nature* **221**, 337–340
12. Singer, P.T., Smalas, A., Carty, R.P., Mangel, W.F., and Sweet, R.M. (1993) The hydrolytic water molecule in trypsin, revealed by time-resolved Laue crystallography. *Science* **259**, 669–673
13. Perona, J.J., Craik, C.S., and Fletterick, R.J. (1993) Locating the catalytic water molecule in serine proteases. *Science* **261**, 620
14. Fehlhammer, H. and Bode, W. (1975) The refined crystal structure of bovine beta-trypsin at 1.8 Å resolution. I. Crystallization, data collection and application of patterson search technique. *J. Mol. Biol.* **98**, 683–692
15. Tomioka, N., Satow, Y., and Itai, A. (1989) The refined crystal structure of the complex formed by bovine trypsin and p-guanidinobenzoate at 2.06 Å resolution. *FEBS Lett.* **258**, 153–155
16. Mangel, W.F., Singer, P.T., Cyr, D.M., Umland, T.C., Toledo, D.L., Stroud, R.M., Pflugrath, J.W., and Sweet, R.M. (1990) Structure of an acyl-enzyme intermediate during catalysis: (guanidinobenzoyl)trypsin. *Biochemistry* **29**, 8351–8357
17. Bode, W., Turk, D., and Sturzebecher, J. (1990) Geometry of binding of the benzamidine- and arginine-based inhibitors N^{α}-(2-naphthylsulphonyl-glycyl)-DL-*p*-amidinophenylalanyl-piperidine (NAPAP) and (2*R*,4*R*)-4-methyl1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulphonyl)-L-arginyl]-2-piperidine carboxylic acid (MQPA) to human α-thrombin: X-ray crystallographic determination of the NAPAP-trypsin complex and modeling of NAPAP-thrombin and MQPA-thrombin. *Eur. J. Biochem.* **193**, 175–182
18. Odagaki, Y., Nakai, H., Senokuchi, K., Kawamura, M., Hamanaka, N., Nakamura, M., Tomoo, K., and Ishida, T. (1995) Unique binding of a novel synthetic inhibitor, *N*-[3-[4-[4-(amidinophenoxy)carbonyl]phenyl-2-methyl-2-propenoyl]-*N*-allylglycine methansulfonate, to bovine trypsin, revealed by the crystal structure of the complex. *Biochemistry* **34**, 12849–12853
19. Kurinov, I.V. and Harrison, R.W. (1996) Two crystal structures of the leupeptin-trypsin complex. *Protein Sci.*, **5**, 752–758
20. Yamamoto, A., Tomoo, K., Doi, M., Ohishi, H., Inoue, M., Ishida, T., Yamamoto, D., Tsuboi, S., Okamoto, H., and Okada, Y. (1992) Crystal structure of papain-succinyl-Gln-Val-Val-Ala-Ala-*p*-nitroanilide complex at 1.7 Å resolution: noncovalent binding mode of a common sequence of endogenous thiol protease inhibitors. *Biochemistry* **31**, 11305–11309

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