Unique Binding of a Novel Synthetic Inhibitor, *N*-[3-[4-[4-(Amidinophenoxy)carbonyl]phenyl]-2-methyl-2-propenoyl]-*N*-allylglycine Methanesulfonate, to Bovine Trypsin, Revealed by the Crystal Structure of the Complex†

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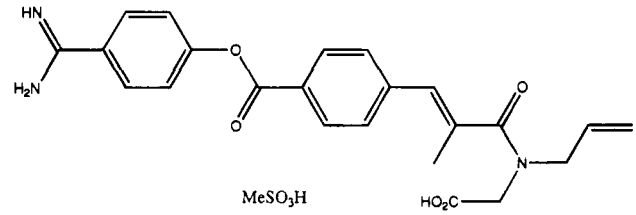
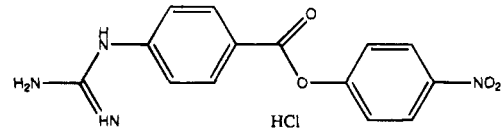
ABSTRACT: Trypsin and *N*-[3-[4-[4-(amidinophenoxy)carbonyl]phenyl]-2-methyl-2-propenoyl]-*N*-allylglycine methanesulfonate (**1**), a newly designed and orally active synthetic trypsin inhibitor, were cocrystallized. The space group of the crystal is $P2_12_12_1$ with cell constants $a = 63.74$ Å, $b = 63.08$ Å, and $c = 69.38$ Å, which is nearly identical to that of the orthorhombic crystal of guanidinobenzoyltrypsin. The structure was refined to a crystallographic residual $R = 0.176$. The refined model of the **1**−trypsin complex provides the structural basis for the reaction mechanism of **1**. On the basis of the present X-ray results, it is proposed that the potent inhibitory activity of **1** is mainly due to the formation of an acylated trypsin through an "inverse substrate mechanism" and its low rate of deacylation.

The molecular mechanism of enzyme inhibition is a subject of fundamental interest to researchers in a variety of fields, and many three-dimensional structures of trypsin−inhibitor complexes have been determined by X-ray crystallographic methods.

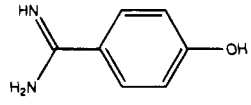
Although a number of benzamidine (Markwardt et al., 1972), arginine (Matsuzaki et al., 1988), and guanidinobenzoate (Mares-Guia & Shaw, 1967) derivatives have been reported to be potent trypsin inhibitors, the number of orally active anti-trypsin drugs is very small. Recently, a potent trypsin inhibitor, *N*-[3-[4-[4-(amidinophenoxy)carbonyl]-phenyl]-2-methyl-2-propenoyl]-*N*-allylglycine methanesulfonate (**1**) was designed and then synthesized. It is very different from other trypsin inhibitors in both its structure and its biological properties. It consists of *p*-amidinophenol and a uniquely oriented acyl moiety and exhibits much stronger inhibitory activity ($IC_{50} = 4.13$ nM against trypsin compared with that of synthetic substrate), together with an unusually long-lasting activity ($t_{1/2} > 60$ min for plasma, liver, and small intestine homogenates of rat and dog), than NPGB[1] (**2**) (an orally active and substrate-type inhibitor) (Senokuchi et al., 1995). These novel characteristics of **1** prompted us to investigate its mechanism of action at a molecular level. In this paper, we report results of X-ray crystal analysis of a trypsin complex crystallized from solution after reaction of trypsin with **1**. A marked difference between the trypsin-binding modes of **1** and **2** is delineated in this paper. On the basis of the present results, moreover, a possible inhibitory mechanism of **1** is discussed. The chemical structures of **1** and **2** are shown in Figure 1.



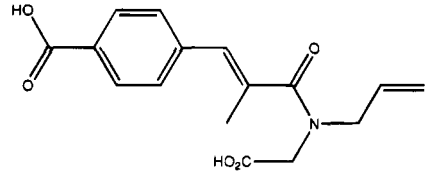
N-[3-[4-[4-(amidinophenoxy)-carbonyl]phenyl]-2-methyl-2-propenoyl]-*N*-allylglycine methanesulfonate **1**



NPGB: *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride **2**



p-amidinophenol **3**



N-[3-(4-carboxyphenyl)-2-methyl-2-propenyl]-*N*-allylglycine **4**

FIGURE 1: Chemical structures of **1** and **2**, together with those of the products of hydrolysis of **1**, *i.e.*, **3** and **4**.

EXPERIMENTAL PROCEDURES

Preparation of the 1−Trypsin Complex Crystal. Bovine trypsin (Type III) was purchased from Sigma Co. (U.S.A.).

† The atomic coordinates of the complex structure have been deposited in the Brookhaven Protein Data Bank under file name 1BTP.

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[1] Abbreviations: NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; GB-trypsin, guanidinobenzoyltrypsin; Boc, *t*-butoxycarbonyl; AMC, 7-amino-4-methylcoumarin; Asp, aspartic acid; Ser, serine; His, histidine; Tyr, tyrosine; Lys, lysine; Phe, phenylalanine; Arg, arginine.

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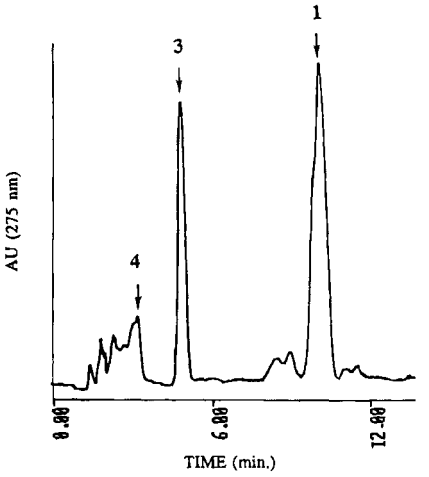

FIGURE 2: Chromatogram of the mother liquor used for crystallization of the trypsin−**1** complex.

The method of Senokuchi et al. (1995) was used to synthesize **1**. Crystals of the trypsin−**1** complex were grown by the vapor diffusion technique using the hanging-drop method; droplets of mother liquor containing 50 mM acetic acid buffer (pH 5.5), 60 mg/mL trypsin, 5 mg/mL **1**, 2 mg/mL $CaCl_2$, and 0.8 M ammonium sulfate were slowly equilibrated at 4 °C against 1.7 M ammonium sulfate solution. Long prismatic crystals up to 0.3 mm × 0.4 mm × 0.8 mm in size were obtained after 1 week.

HPLC Analysis of Crystallization Solution. After the complex crystal was obtained, ion-paired HPLC analysis of the mother liquor was carried out under the following conditions. A Hitachi HPLC system equipped with a YMC pack ODS AM-312 instrument was used and set at an absorbance wavelength of 275 nm. A solution consisting of methanol:distilled water:acetic acid:5% sodium *n*-dodecyl sulfate in H_2O−MeOH (1:1):5% sodium *n*-heptyl sulfate in H_2O−MeOH (1:1) = 330:210:1:3:8 was used as a mobile phase at a flow rate of 1.2 mL/min. Three major and several minor peaks were observed (Figure 2). These major peaks were assigned to the products of hydrolysis of **1** (*i.e.*, **3** and **4**, Figure 1) and intact **1** and were identified by comparison of their retention times with those of the synthetic authentic compounds (Senokuchi et al., 1995).

Diffraction Data Collection and Refinement. X-ray diffraction data were collected on an RAXIS IIc (Rigaku) diffractometer. The space group of the crystal was $P2_12_12_1$ with cell constants a = 63.74 Å, b = 63.08 Å, and c = 69.38 Å. A total of 12 752 reflection data up to 2.2 Å resolution with intensity greater than $2\sigma(F_o)$ were used for the structure determination and refinement. Since the crystal data were nearly identical to those of the orthorhombic crystal of guanidinobenzoyl GB-trypsin (Mangel et al., 1990), which was prepared from the reaction of trypsin with **2**, we calculated the first difference Fourier map using the atomic coordinates of the trypsin moiety of GB-trypsin; the atomic coordinates (PDB1TLD.ENT) were obtained from the Brookhaven Protein Data Bank (Bartunik et al., 1989). All Fourier calculations were carried out using the X-PLOR (Brunger, 1992) program package. In the vicinity of the active site of trypsin, there was a positive electron density which could be interpreted as a part of **1**, as judged from its shape and size. At this stage, it was not clear whether the binding of the fragment was noncovalent or covalent. In order to clarify the electron density of **1**, restrained refinement calculations were performed repeatedly with X-PLOR. After several cycles of refinements, it became clear that the inhibitor fragment was covalently attached to the trypsin *via* an ester bond between the carbonyl carbon of **1** and Oγ of Ser195 (Figure 3). The final R factor was 17.6% for 12 593 reflections up to 2.2 Å resolution, including the 20 atoms of the **1** fragment, 1 calcium ion, and 50 water molecules. The root mean square deviation from the ideal value was 0.017 Å for bond lengths and 3.2° for bond angles. The bond length between the Ser195 Oγ and the carbonyl carbon of **1** was 1.45 Å.

RESULTS

A stereoscopic view of the overall interface between the inhibitor and trypsin is shown in Figure 4.

S1 and S1′ Sites. In a number of peptidic inhibitor−trypsin complexes, the basic moiety of the inhibitor has been considerd to be inserted into a narrow cleft at the S1 site consisting of the 189−192 and 214−217 residues of trypsin to form an ion pair with Asp189 at the bottom of the S1 site. The synthetic substrate-type inhibitor **2** is not an exception (Otsuki et al., 1989). The guanidinobenzoyl moiety linked to the Ser195 Oγ atom is tightly anchored to Asp189 to form an ion pair with two hydrogen bonds, as is illustrated in Figure 5a, and the corresponding side chain is oriented forward of the S1 site and shows strong van der Waals interaction with the substrate (Mangel et al., 1990). This is in contrast with the case of the present trypsin−**1** complex. Since **1**, which has a charged amidinium function in its leaving group, has been regarded as an "inverse substrate-type" inhibitor, its inhibition mechanism is con-

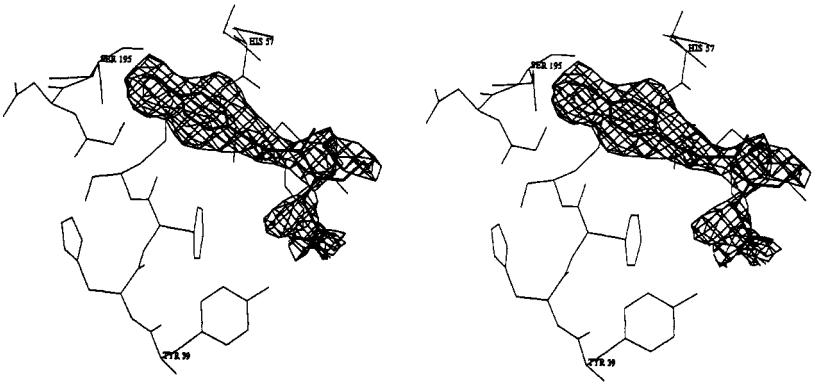

FIGURE 3: Electron density map of **1** binding to the trypsin binding pocket at the final stage of refinement. The position of **1** on the map is shown with thick lines. The thin lines represent the amino acid residues of trypsin.

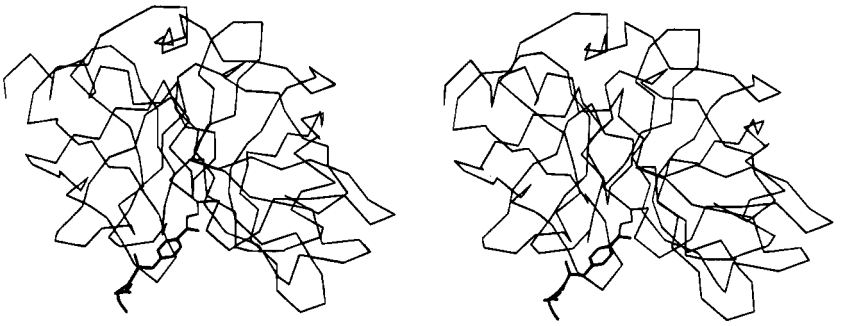

FIGURE 4: Stereoscopic view of the overall structure of the trypsin−**1** complex. The trypsin Cα atoms and the acylated **1** molecule (thick lines) are shown. The covalent bond between Ser195 and the acylated part of **1** is also shown.

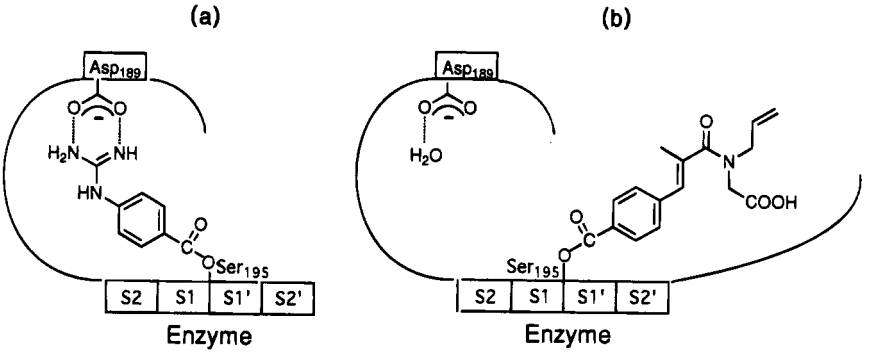

FIGURE 5: Schematic representation of the interaction modes with the trypsin active site of (a) the guanidinobenzoyl moiety in the GB-trypsin complex and (b) the [(*p*-carbonylphenyl)-2-methyl-2-propenoyl]-*N*-allylglycine in the **1**−trypsin complex.

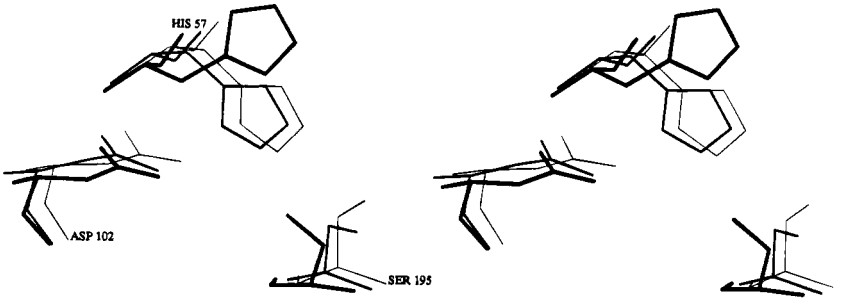

FIGURE 6: Stereoscopic superposition of the spatial orientation diagrams of Ser195, His57, and Asp102 residues constituting the catalytic sites in native trypsin (thin lines), GB-trypsin (intermediate lines), and **1**−trypsin (thick lines).

sidered to be unusual (Tanizawa et al., 1977). The present X-ray results (Figure 3) clearly showed the formation of an acyl-enzyme intermediate *via* the ester bond formation between the carbonyl carbon of **1** and Oγ of Ser195 without involvement of the *p*-amidinophenol fragment after the hydrolysis of the internal ester bond in **1**. Unexpectedly, a water molecule existed at the S1 site instead of the amidinophenol moiety of **1**, and the remaining acyl part of **1** was oriented in the same direction as the P1′ side chain (Figure 5b).

Because of the different binding modes between the acyl moieties of **1** and **2**, a marked difference was observed at both the Ser195 and His57 side chains (Figure 6). The dihedral angles N1−Cα−Cβ−Cγ and Cα−Cβ−Cγ−Nδ1 of His57 and N1−Cα−Cβ−Oγ of Ser195 in the **1**−trypsin complex are −176, −153, and 101°, respectively, while those in the GB-trypsin complex are 85, −94, and −77°, respectively.

Catalytic Triad. The catalytic triad, which is formed among His57, Asp102, and Ser195 in the present trypsin−**1** complex, is different from that in the GB-trypsin complex or intact trypsin (Marquart et al., 1983) (Figure 7). While a hydrogen bond exists directly between the Asp102 carboxyl oxygen forms a hydrogen bond with the imidazole nitrogen of His57 *via* a water molecule in the **1**−trypsin complex. It is noteworthy that the acyl moiety of **1** is stabilized by His57, because the distance between the His57 imidazole ring and the **1** acyl moiety is within the range in which van der Waals interaction is expected to occur; the distance between the nearest atoms of the **1** phenyl ring and the His57 imidazole ring is 4.1 Å, and the dihedral angle between these planes is about 40°.

Possible hydrogen bonds of **1** in the trypsin binding pocket are schematically shown in Figure 8. The carbonyl oxygen atom of the acylated **1** is located in the oxyanion hole (Robertus et al., 1972) and forms hydrogen bonds with NH groups of Gly193 and Ser195. The acyl moiety of **1** is in van der Waals contact with the sulfur atom of the S−S bond formed between Cys42 and Cys58, as well as with the imidazole ring of His57. Two carboxylic oxygen atoms of the *N*-allylglycine of **1** form hydrogen bonds with the hydroxyl group of Tyr39 and Nε amino group of Lys60.

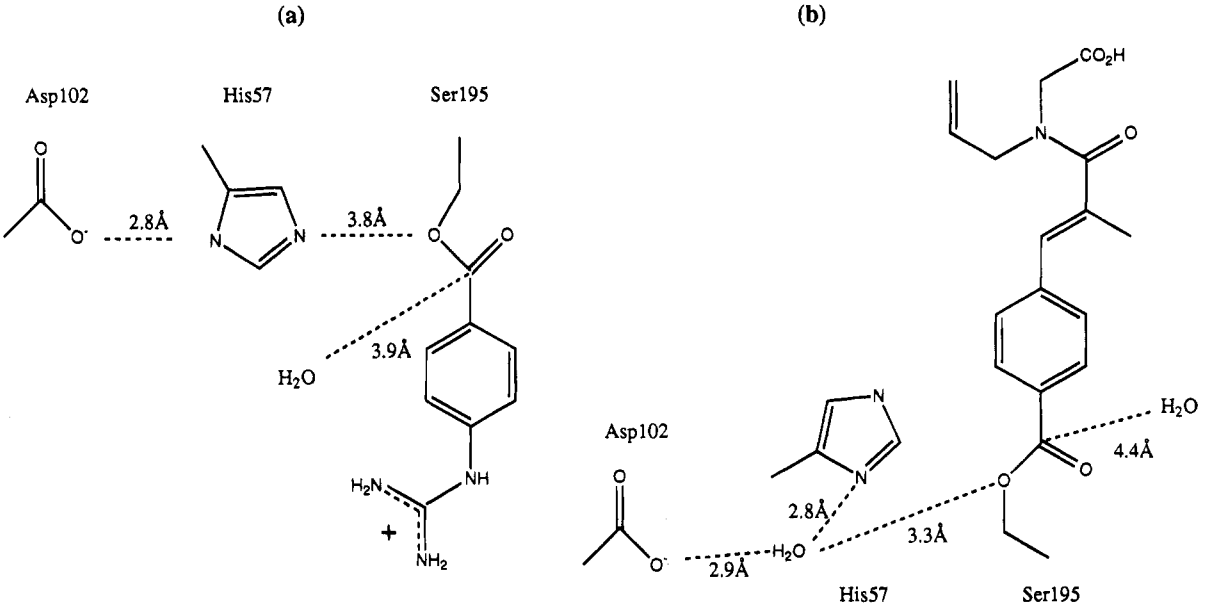

FIGURE 7: Schematic comparison of the catalytic triad moieties in trypsin acylated with **2** (a) and **1** (b).

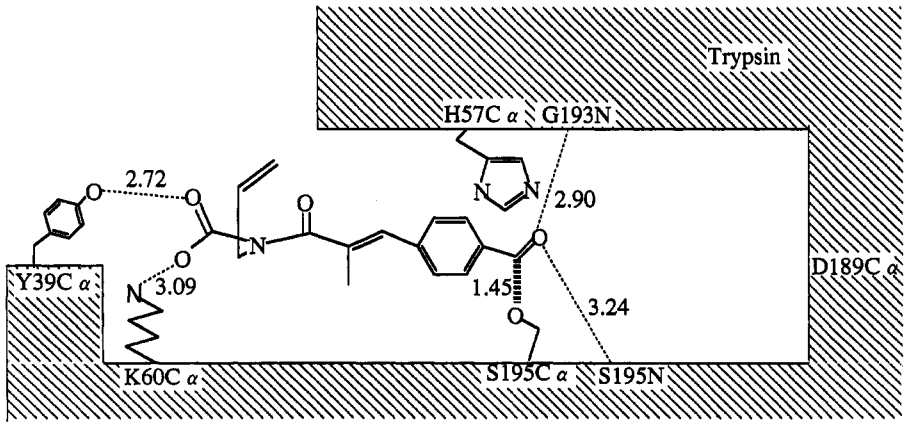

FIGURE 8: Schematic hydrogen bond network formed in the trypsin−**1** complex.

DISCUSSION

Trypsin cleaves specifically the peptide bond where a basic residue such as arginine is present on the acyl side of the substrate; the amide bond of Arg-X (X, any peptide) is cleaved by trypsin, while that of X-Arg is not in general. On the other hand, Tanizawa et al. (1977) reported several acyl derivatives of *p*-hydroxybenzamidine among which the inverse substrate-type ester inhibitors have an amidine group in the leaving group, and these are characterized by rates of deacylation that are lower than those of the normal substrate-type inhibitors such as *p*-nitrophenyl guanidinobenzoate **2**. Similarly, **1** is an inverse substrate-type inhibitor characterized by a low rate of deacylation. The processes of hydrolysis of the inverse and normal substrate-type inhibitors by trypsin are compared in Figure 9a,b), respectively. Judging from the present X-ray analysis results, **1** and trypsin might form a Michaelis complex (**Ia**) and then an acylated trypsin (**IIa**). This is supported by the presence of *p*-amidinophenol (**3**) in the mother liquor, which was detected by HPLC analysis. Trypsin would then be reactivated by the deacylation of the acylated complex, where the deacylation is considered to proceed through a tetrahedral intermediate formed by the addition of a water molecule to the ester carbonyl carbon of the acylated Ser195.

The behavior of the hydrolytic water molecule during the deacylation of the GB-trypsin complex was shown by time-resolved Laue crystallography (Singer et al., 1992, 1993; Perona et al., 1993). The hydrolytic water molecule was located above the ester carboxylate plane suitable for the formation of a tetrahedral intermediate and was stabilized by hydrogen bonding and/or electrostatic interactions with the Ser195 carbonyl oxygen, His57 imidazole nitrogen, and/or neighboring water molecule oxygen atoms (Figure 7a); the formation of the catalytic triad is thought to facilitate the proton trnasfer between His57 and the water molecule in the deacylation process.

In contrast with the case of the GB-trypsin complex, the acylation of the **1**−trypsin complex, which occurs even though the ester carbonyl oxygen atom in the complex, as well as in the GB-trypsin complex, is located in the oxyanion hole (Robertus et al., 1972), caused a marked conformational change of His57 and Ser195 in the catalytic triad (Figure 6) and the participation of one water molecule in the hydrogen bond network of the catalytic triad (Figure 7b), where the

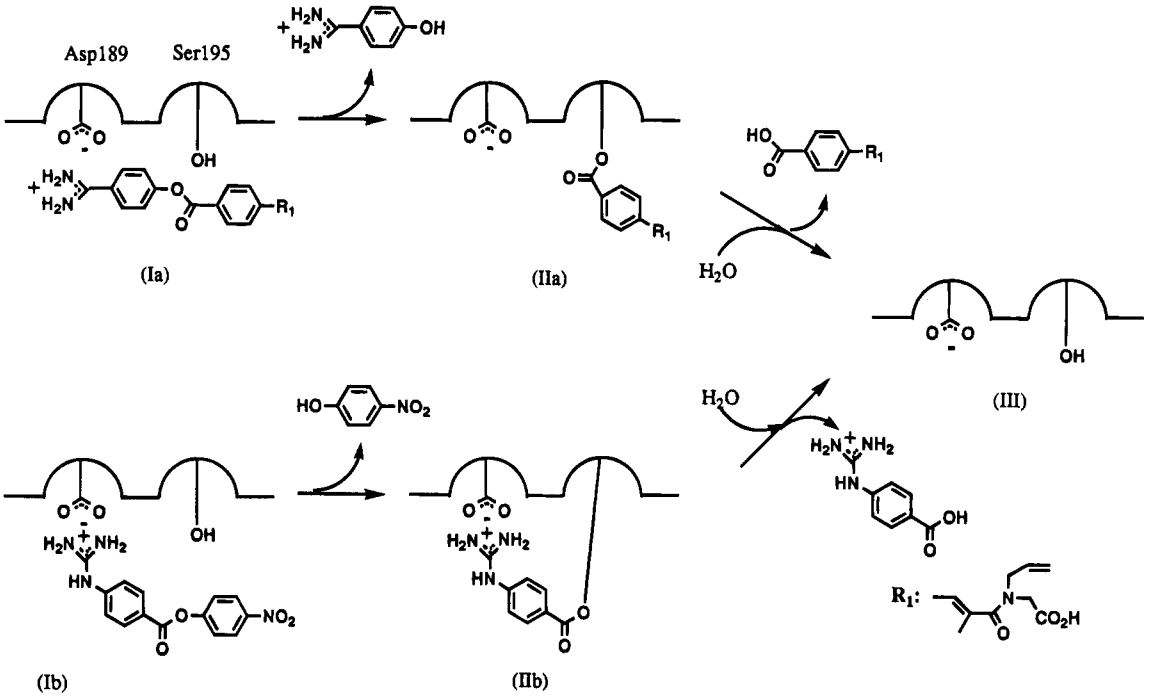

FIGURE 9: Proposed processes of hydrolysis of the inverse substrate-type inhibitor **1** (a) and the normal substrate-type inhibitor **2** (b) by trypsin.

distance between the other water molecule and the ester carbonyl carbon increases to 4.4 Å. The formation of this unique hydrogen bond network is due to the inverse substrate-type function of **1**. The orientation of the covalently bound **1** is different from that of the usual inhibitor; namely, **1** is not held in the canonical conformation formed by having a positively charged residue at the S1 site (Figures 5 and 7). Consequently, His57 and Asp102 are forced to adopt conformations which prevent them from facilitating the hydrolysis of the covalently bound intermediate (Figure 6). The terminal *N*-allylglycine moiety of **1** is located at the mouth of the cleft of the trypsin binding site and is bordered by solvent molecules (Figure 4); this is one reason why the electron density corresponding to this terminal moiety (Figure 3) was insufficiently clear to allow unequivocal determination of its position. However, the best fit to the electron density obtained by crystallographic refinement showed that two hydrogen bonds were present between the terminal carboxylic oxygen atoms of **1** and the hydroxyl group of Tyr39 and Nϵ amino group of Lys60 (Figure 8), thus contributing to the stabilization of the terminal moiety of the acylated **1**.

The comparison of the crystal structure of the **1**−trypsin complex with that of the GB-trypsin complex indicates that the hydrogen bond network at the catalytic triad which is usually observed in the substrate−trypsin complex is not in the case of the complex of trypsin with the inverse substrate-type inhibitor. Instead, the three residues of His57, Asp102, and Ser195 are linked to one another by hydrogen bonds *via* one water molecule. This leads us to speculate that the rate of hydrolysis of the **1**−trypsin complex is lower than that of the GB-trypsin complex.

In conclusion, the reaction mechanism of the inverse substrate-type inhibitor **1**, which exhibits potent and long-lasting activity, might involve the following four sequential steps: (1) recognition of the basic group by trypsin, (2) formation of a Michaelis complex, (3) formation of an acylated trypsin, and (4) slow deacylation, accompanied by formation of a tetrahedral intermediate by the addition of a water molecule to the ester carbonyl carbon atom of the **1** fragment linked to Ser195.

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