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The Effect of the Hydrogen Bond Network in the S_1-pocket on Catalytic Activity of Serine Protease, *Achromobacter* Protease I (API)

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Abstract Crystal structural analyses of the API-TLCK complex revealed that the ε-amino group (NZ) of the lysyl part of TLCK forms hydrogen bonds with OD1 of Asp^{225} which is a substrate specificity determinant of API, OG of Ser^{214}, O of Ser^{214}, OG1 of Thr^{189}, and O of Thr^{189}. The β-carboxyl oxygen of Asp^{225} forms hydrogen bonds with the NE1 of Trp^{182}. From these observations, it is thought that besides Asp^{225}, Thr^{189}, Ser^{214}, and Trp^{182} may also contribute to the steric specificity for lysine and high proteolytic activity of API. The side-chain hydroxyl groups of Thr^{189} and Ser^{214} were removed to elucidate the role of these hydrogen bonds in the S_1-pocket. The k_{cat}/K_m of T189V, S214A, and T189V·S214A were decreased to 1/4, 1/3, and 1/46, respectively, of the value for native API. The decreased activities were mainly due to the increase of K_m. The CD and fluoroscence spectra of the three mutants were similar to those of wild-type API. With regards to the kinetic parameters (K_i and k_2) of mutants for the reaction involving TLCK and DFP, k_2 decreased by increase of K_i only. These results suggest that the decreased catalytic activity of these mutants is caused by the partial loss of the hydrogen bond network in the S_1-pocket. On the other hand, the similarity of enzymatic properties between W182F and the native enzyme suggests that the hydrogen bond between OD2 of Asp^{225} and NE1 of Trp^{182} is not directly related to the reaction of Asp^{225} with the substrate.

Key words: API, API-TLCK complex, S_1-pocket, hydrogen bond, kinetic parameter

Achromobacter protease I (API, EC. 3.4.21.50), an extracellular protease of M_r 30,000, was isolated from the culture broth of *Achromobacter lyticus* M497-1. The protease specifically hydrolyzes lysyl bonds including the Lys-Pro bond [9]. In addition to its narrow substrate specificity for lysine, the protease has the following characteristic properties. (a) Its proteolytic activity is an order of magnitude higher than that of bovine trypsin; (b) it has a broad pH optimum, ranging from 8.5 to 10.7, for casein; and (c) it exhibits full activity in the presence of 5 M urea and 0.1% sodium dodecylsulfate. API reacts rapidly with diisopropyl fluorophosphate (DFP), while slowly with phenylmethanesulfonyl fluoride (PMSF). Both reactions take place stoichiometrically, resulting in complete loss of protease activity. The protease reacts very rapidly with N^{α}-tosyl-L-lysylchloromethylketone (TLCK) and does not react at all with its arginine counterpart. These results suggest that the protease is a serine protease specific for lysine.

To understand the structural basis of these functional characteristics, the primary structure of API was previously determined [9]. API consists of a single peptide chain of 268 amino acid residues with three disulfide bonds and is a member of the trypsin family. Furthermore, we previously identified three catalytic triad constituents and Asp^{225} essential for the function of this lysine-specific serine protease by means of site-directed mutagenesis [4]. The results suggest that the side chain of lysine in the bound substrate can fit into the pocket of API and interact with Asp^{225} by electrostatic force. The strict specificity of API for lysyl bonds is primarily based on the presence of Asp^{225}.

Recently, crystal structural analysis of the complex between API and N^{α}-tosyl-L-lysylchloromethylketone [1] has revealed that the carboxyl oxygen of Asp^{225} forms several hydrogen bonds with its surrounding amino acid residues and a water molecular (W420) in the S_1-pocket. The OD2 of Asp^{225} forms hydrogen bonds with OG1 of Thr^{189}, the OG of Ser^{214}, and the NE1 of Trp^{182}. W420 also forms hydrogen bonds with the OD1 of Asp^{225}, the CO of Thr^{189}, the CO of Ser^{214}, and the OG of Ser^{214}.

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When TLCK binds to API, W420 is replaced by the ε-amino group (NZ) of the lysyl part of TLCK (Fig. 1). From these observations, it is thought that, besides Asp^{225}, Thr^{189}, Ser^{214}, and Trp^{182} may also contribute to the steric specificity for lysine and the high proteolytic activity of API. In this paper, in order to elucidate whether the network of these hydrogen bonds in the S_1-pocket is one of lysine recognition operations in addition to electrostatic interaction, mutants eliminating the hydrogen bond(s) were successively constructed by site-directed mutagenesis and the importance of the hydrogen bonds for catalytic activity and substrate specificity in API were investigated.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized with an Applied Biosystems model 380B DNA synthesizer. The following materials were obtained from the indicated sources: all restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and the 7-deaza sequencing kit from Takara Shuzo Co. (Japan); *in vitro* mutagenesis system (version) and radio-labelled deoxyribonucleotides from Amersham Co.; Bacto-tryptone and Bacto-yeast extract from Sanraku Inc. (Japan); ovomucoid from hen egg-white from Seikagaku Co. (Japan); Boc-Val-Leu-Lys-MCA from Peptide Institute Co. (Japan); polyvinylidene difluoride membranes from Millipore Co.; SDS-PAGE molecular standards and goat antirabbit IgG horseradish peroxidase conjugate from Bio-Rad; Cosmosil-5Diol-120A column for HPLC gel filteration from Nakarai Tesque (Japan); reversed phase HPLC columns, μBondasphere 5 μC18 300 Å and 15 μ C4-300 Å from Waters; S-3-(trimethylamino) propylated lysozyme from Seikagaku Kogyo (Japan); lysyl endopeptidase from *Achromobacter lyticus* and Thermolysin, and diisopropyl fluorophosphate (DFP) from Wako Pure Chemical Industries (Japan); N^{α}-p-tosyl-L-lysinechloromethylketone (TLCK) from Sigma. All other reagents used were of the highest grade available.

Strains

The *E. coli* strain JM109 (*recA*1, *endA*1, *gyrA*96, *thi*△, *hsdR*17, *supE*44, *relA*1, (*lac-proAB*)/F'[*traD*36, *proAB*⁺, *lacI*^{q}, *lacZ*△M15]) was used for the preparation of plasmid and phage DNA. *E. coli* strains JA221 ([*hsdR, trpE*5, *leuB*6, *lacY, recA*1]/F'*lacI*^{q}, *lac*$^+$, *pro*$^+$) and TG1 (*supE, hsd*△5, *thi*△, [*lac-proAB*]/F'[*traD*36, *proAB*$^+$, *lavI*^{q}, *lacZ*△M15]) were used for mutagenesis of the API gene.

Construction of Mutants

DNA fragments encoding target amino acids were cut from the pSN8004 [4] expression vector with appropriate restriction enzymes and ligated into M13mp18. Then, the codons of the appropriate amino acids were mutated using a set of synthetic oligodeoxyribonucleotides. Synthetic oligodeoxyribonucleotides were used to generate the mutants of candidate residues in the S1-pocket. The mutated codons of the mutants are underlined.

T189V; 5'-ACCCGGCTCGACCACGCCGCC-3'
S214A; 5'-CCTGCAGCTGGCCGGGCCGCC-3'
W182F; 5'-CGAGGGCTGAAACTGCACGTT-3'

After undergoing mutation, the DNA fragment was cut from M3mp18 with the same restriction enzymes and religated into the expression vector pSN8004 [4].

Expression of API Gene

Expression of wild-type or mutated API genes was carried out with the trc promoter in the expression vector pKK233-2 [5]. The API gene was inserted downstream of the trc promoter. Transformed *E. coli* JA221 bearing the expression vector was incubated at 25°C overnight in L-broth. After incubation, the periplasmic proteins were extracted by the osmotic shock method [2].

Western Blot Analysis of Gene Products

SDS-PAGE and western blot analyses were performed by the method of Laemmli [3] and Towbin *et al.* [8], respectively. To inactivate API, formic acid (98~100%) was added to the periplasmic fraction to about 50% (v/v), allowed to stand for 5 min at ambient temperature, and then evaporated. The proteins were subjected to SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and exposed to anti-API rabbit sera.

Purification of Expressed API and its Mutants

Expressed wild-type or mutated API were purified by affinity chromatography on a hen's egg white ovomucoid. A periplasmic fraction prepared from culture media (1 liter) was passed through a DEAE-cellulose column (3.6×50 cm) equilibrated with 10 mM Tris·HCl (pH 9.0). After the pH of the unadsorbed fraction had been adjusted to 8.0 by adding glacial acetic acid, the fraction was applied to an ovomucoid-Sepharose 4B column (chicken ovomucoid content, 0.3 mmol/min, wet gel; 1.6×25 cm) equilibrated with 50 mM Tris·HCl (pH 8.0). After the column had been successively washed with 50 mM Tris·HCl (pH 8.0) and distilled water, the adsorbed wild-type or mutated API was eluted with 10 mM ammonium acetate (pH 3.0). The eluted solution was immediately neutralized by carefully adding diluted ammonium hydroxide since API is unstable below pH 4.0.

Amino Acid Composition and Sequence Analyses

Amino acid compositions were determined on a Hitachi L-8500S automatic amino acid analyzer. Proteins were hydrolyzed in twice-distilled 5.7 M HCl in evacuated

tubes at 110°C for 24 h. Protein sequence analysis was performed on a 477A Applied Biosystems Sequencer equipped with an on-line phenylthiohydantoin-derivative analyzer (120A). Phenylthiohydantoin-derivatives were eluted with a modified isocratic system.

Peptide Mapping

S-3-(trimethyl amino) propylated lysozyme (2.5 nm) was digested with wild-type or mutated API for 12 h at 37°C in 0.2 M Tris·HCl (pH 9.0; 0.1 ml) at a molar enzyme to substrate rato of 1:400. A portion of the digest (1.0 nmol) was chromatographed on a C18 μBondasphere column (3.9×450 mm) equilibrated with 0.1% trifluoroacetic acid using a 60 min linear gradient of 0~60% 2-propanol/acetonitrile (7:3, v/v) containing 0.08% trifluoroacetic acid at a flow rate of 0.8 ml/min.

Kinetic Parameters

Hydrolytic activity toward lysyl bonds was measured with Boc-Val-Leu-Lys-MCA as substrate. The tripeptide substrate was dissolved in *N,N*-dimethylformamide and diluted with 0.2 M Tris·HCl (pH 9.0) to a final *N,N*-dimethylformamide concentration of 1%. After incubating for 10 min at 37°C, a portion (2 ml) of the solution was transferred to a cuvette, and 50 μl of 2 nM enzyme solution was added. Increased fluoroscence from liberated 7-amino-4-methylcoumarin was measured at 440 nm upon excitation at 380 nm with a Hitachi fluoroscence spectrometer (F-4000, Japan). Substrate and enzyme concentrations were quantified by amino acid analysis of their hydrolysates. The kinetic constants K_m and k_{cat} were obtained from Lineweaver-Burk plots, and standard deviations for these values were less than 10%.

Circular Dichroism

Circular dichroism (CD) spectra were recorded from 260 to 210 nm on a JASCO model J-600s (Japan) spectropolarimeter. Protein samples were dissolved in 50 mM Tris·HCl (pH 8.0). The concentration of API and mutated APIs were 3.31 μM. All spectra were recorded at room temperature.

Spectrometry

All fluoroscence spectra were recorded on a Hitachi model F-400 fluoroscence spectrophotometer at 25°C. Protein samples (0.6 μM) in 50 mM Tris·HCl (pH 8.0) were excited at 278 nm and fluoroscence emission spectra were recorded from 300 to 500 nm. Fluoroscence intensity was expressed in relative values, which were obtained by taking the value for native API as 100.

Inhibition of the Enzymes with TLCK or DFP

A 200 μl sample of purified API or mutated API (0.2 μM) in 0.1 M Tris·HCl (pH 6.9), containing 0.015~0.236 μg DFP, was incubated at 25°C. Samples (20 μl) were taken after different incubation times and added to 2 ml 0.1 M Tris·HCl (pH 6.9) containing 10 μM Boc-Val-Leu-Lys-MCA as substrate. Hydrolytic activity was measured by the method described in Kinetic parameters. Inhibition constants were determined.

RESULTS AND DISCUSSION

Mutation at Thr^{189}, Ser^{214}, and Trp^{182}

Thr^{189} and Ser^{214} were substituted with Val and Ala, respectively, in order to elucidate the roles of Thr^{189} and Ser^{214} in catalytic activity. The hydrogen bond between the ε-amino group of substrate lysine and OG1 of Thr^{189} or OG of Ser^{214} should be eliminated by introduction of methyl groups or loss of the hydroxy group of Thr^{189} or Ser^{214} (Fig. 1). The mutated DNA segment was chemically synthesized and integrated into the API gene. When the mutated API genes coding for the proteins T189V, S214A, or T189V · S214A were each expressed in the *E. coli* strain JA221, T189V and S214A were each secreted into the periplasm in which a 30 kDa protein was clearly detected by SDS-PAGE and the western blot (Fig. 2). The lysylendopeptidase activity of these two proteins were approximately 1/10 that of the native enzyme, suggesting that hydrogen bonds may be important factors for the catalytic activity of native API. The T189V·S214A mutant

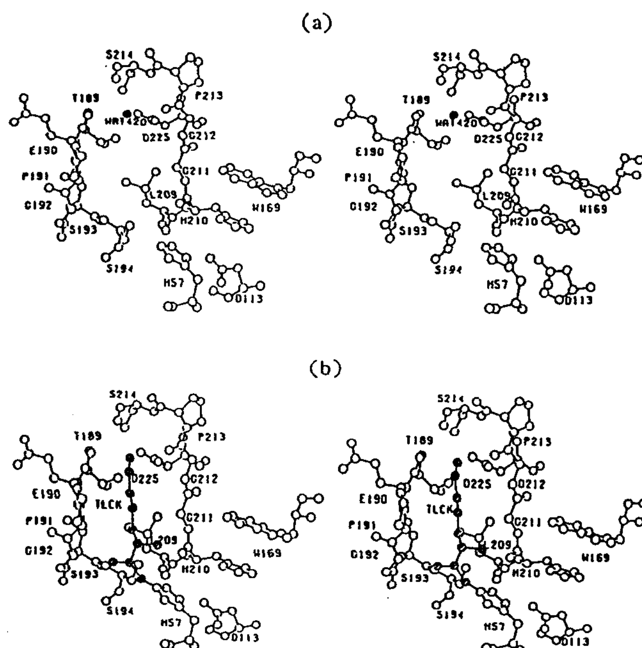

Fig. 1. Three-dimentional structure of active site of API (a) and API-TLCK (b).
Hatched circles indicate the atoms in the water molecule (WAT420) in (a) and the TLCK molecule with the tosyl group eliminated in (b).

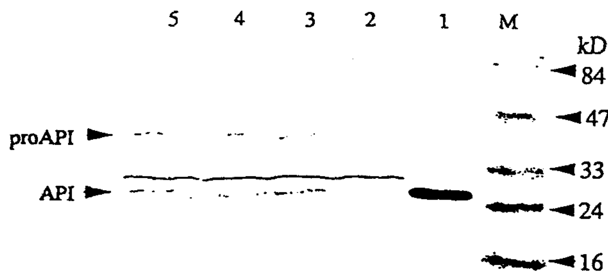

Fig. 2. Western blot analysis of periplasmic proteins from *E. coli* transformed by an expression vector bearing native or mutated API gene.

Lane 1, native enzyme; lane 2, without any API genes; lane 3, with the T189V gene; lane 4, with the S214A gene; lane 5, with the T189VS·214A; M, molecular weight markers.

sample showed faint bands at 45 kDa and 30 kDa by western blot (Fig. 2). This double mutant in the periplasm showed very low lysylendopeptidase activity (1/250 that of the native enzyme). However, the activity increased 2-fold after the sample was dialysed overnight at 4°C. Norioka *et al.* [4] showed that the 45-kDa protein is pro-API.

Trp[182] was substituted with phenylalanine in order to elucidate the role of the hydrogen bond between OD2 of Asp[225] and NE1 of Trp[182] in catalytic activity. The mutant W182F was secreted into the periplasm in which a 30 kDa was clearly detected by western blot (data not shown).

Characterization of Mutated APIs

All active mutants were purified to homogeneity by several types of chromatography (Figs. 3, 4) and analyzed.

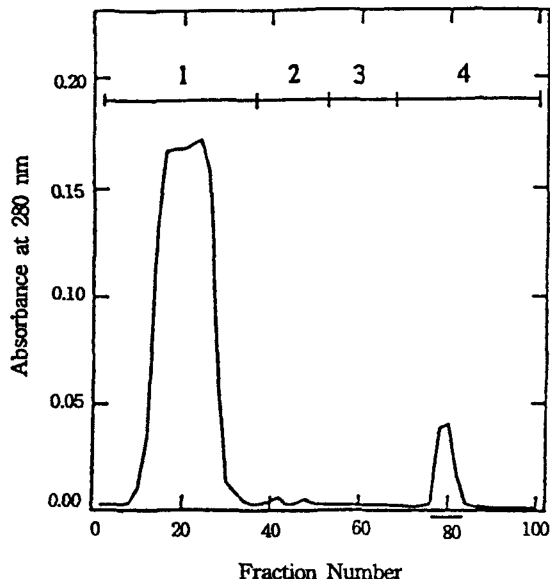

Fig. 3. Affinity chromatography of mutated API on ovomucoid-sepharose 4B column.

1, 0.05 M Tris-HCl (pH 8.0); 2, 0.05 M Tris-HCl (pH 8.0) containing 0.5 M NaCl; 3, distilled water; 4, 0.01 M ammonium acetate (pH 3.0); —, active fractions.

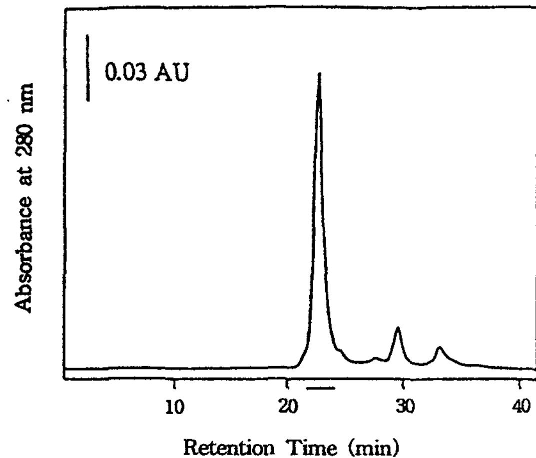

Fig. 4. Purification of mutated API by gel filteration HPLC.

The chromatography was performed on COSMOSIL column (7.5×600 nm) with 0.1 M ammonium acetate (pH 6.8) at a flow rate of 0.8 ml/min; —, active fractions.

The proteins were identified as purified enzymes by SDS-PAGE analysis (Fig. 5) and by examining their specific lysylendopeptidase activities (date not shown). In addition, the amino-terminal sequences of the proteins were the same as that of the native API (date not shown). The peptide maps of S-3-(trimethyl amino) propylated lysozyme obtained after digestion with the active mutants T189V, S214A, T189V·S214A, and W182F are presented in Fig. 6. The maps were identical to that of the wild-type protease, showing that lysine specificity was retained in all four samples. Conformations of mutated APIs were analyzed by circular dichroic and fluoroscence spectroscopy. The CD spectra of the three mutants were quite similar to that of wild-type API below 230 nm (Fig. 7). Possible conformational changes brought about by mutation were not demonstrated by fluoroscence spectra (Fig. 8). The fluoroscence emission maximum (λ_{max}) of each mutant was the same as that of

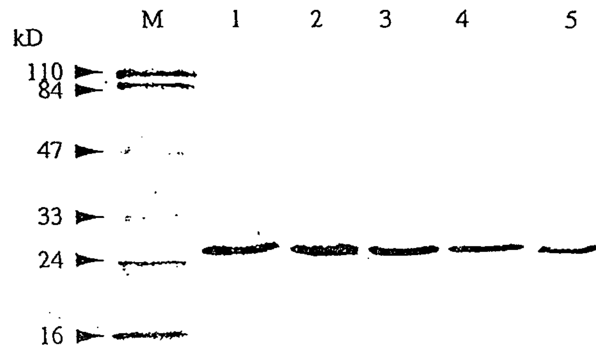

Fig. 5. SDS-polyacrylamide gel electrophoresis of five purified API mutants.

M, molecular weight markers; lane 1, native API; lane 2, wild-type API; lane 3, T189V; lane 4, S214A; lane 5, T189V·S214A; lane 5, W189F.

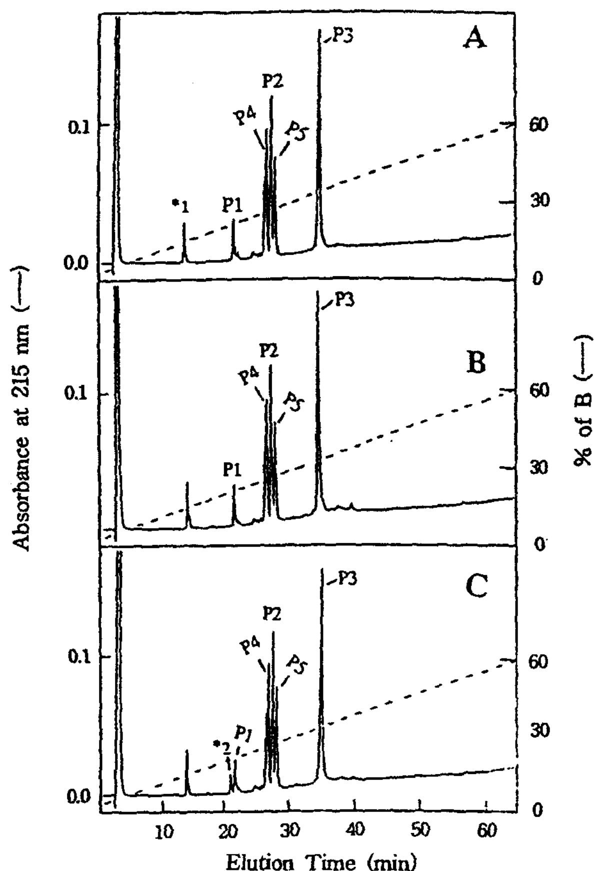

Fig. 6. Separation of peptides obtained by digestion of S-3-(trimethyl amino)propylated lysozyme with native and mutated APIs.

Added 2% formic acid, the digestion (1.3 nmol) were applied to an C18 Bondasphere column (3.9×150 mm) and eluted with a linear gradient of 0–60% 2-propanol/acetonitrile (7/3, v/v) in 0.1% trifluoroacetic acid for 1 h at a flow rate 1 ml/min. A, digestion with native API; B, with mutants (T189V, S214A, W182F); C, with T189V·S214A. P1, Val2-Lys13; P2, Arg14-Lys33; P3, Phe34-Lys96; P4, Ile98-Lys116; P5, Gly 117-Leu129; *1, no peptide peak; *2, Lys1-Lys13.

the wild-type and the relative fluoroscence intensity of the mutants was almost the same as that of the wild-type. These results indicate that the overall foldings of the three mutants are quite similar to that of the native enzyme.

Lysylendopeptidase Activity of Mutated APIs

The enzyme activities of all the mutants were examined using a synthetic substrate, Boc-Val-Leu-Lys-MCA. The k_{cat} values were not changed by the substitution of Thr^{189} with valine and Ser^{214} with alanine, but the K_m values increased by 4-, 3-, and 46-fold for T189V, S214A and T189V·S214A, respectively, (Table 1). The unchanged k_{cat} and changed K_m of mutants T189V and S214A indicate that the hydrogen bond between the protonated lysyl side chain and Thr^{189} and Ser^{214} exist in the S_1-pocket of API and that the hydrogen-bonding

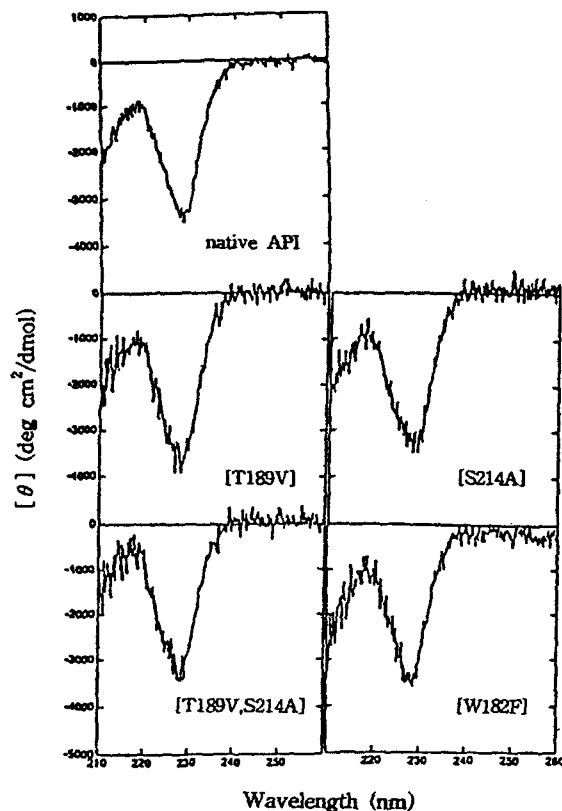

Fig. 7. CD spectra of wild-type and mutated APIs.

The spectra were recorded in 0.05 M Tris·HCl, pH 8.0, at room temperature.

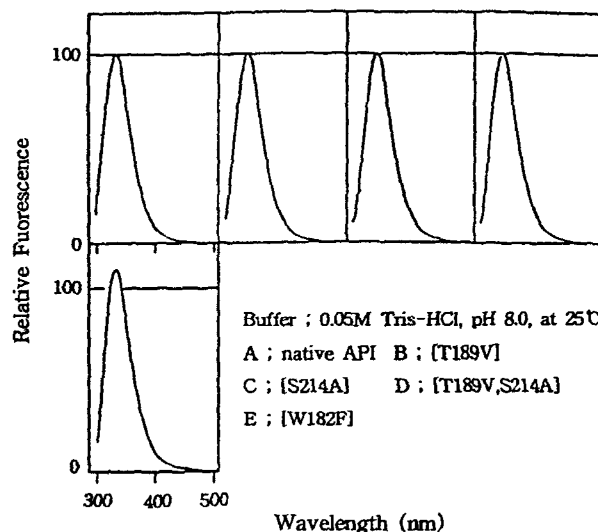

Fig. 8. Fluoroscence spectra of wild-type and mutated APIs.

The reaction mixture contained 0.12 μg enzyme and 10 μM of the substrate in a final volume of 2.022 ml.

networks affect substrate binding affinity. It has been reported that the mutant D225E has very low catalytic activity compared with that of native enzyme [2]. The significant decrease of activity in the D225E mutant in

Table 1. Kinetic constants of wild-type and mutated APIs for hydrolysis Boc-Val-Leu-Lys-MCA.

Enzymes	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot sec^{-1}$)	Main product
Wild-type	2.5	82	33.0	Mature
[T189V]	10.0	82	8.0	Proform and mature
[S214A]	7.0	82	12.0	Proform and mature
[T189V, S214A]	115.0	82	0.7	Proform and mature
[W182F]	2.7	72	27.0	Mature

Enzyme activity was assayed in 0.2 M Tris-HCl at pH 9.0 and 37°C.

spite of the essential negative charge in the S_1-pocket may be due to elimination of hydrogen bonds between the lysyl substrate and Thr^{189} and Ser^{214} in the S_1-pocket. However, the substitution of Trp^{182} with phenylalanine, which eliminated the hydrogen bond to the β-carboxyl group of Asp^{225}, had no effect on catalytic activity (Table 1). This result indicates that this hydrogen bond does not contribute to the accurate positioning of the side chain of Asp^{225}. In the case of trypsin, the negative charge of $Asp^{(189)}$ is thought to be precisely positioned in the S_1-pocket of the enzyme so that it can bind lysine or arginine substrate and that the distal ionic interaction between the P1 substrate side chain and $Asp^{(189)}$ contributes to precise positioning of the substrate scissile bond relative to the catalytic machinery [6].

Inhibition Experiment of Mutated APIs

In order to check whether the reduction of activity in the mutated APIs is due to comformational changes of the catalytic triad or substrate binding pocket, inhibition experiments of the three mutants by TLCK and DFP were performed (Table 2). The K_i of T189V, S214A and T189V·S214A for TLCK were about 7-, 8- and 5000-fold higher than that of wild-type API, respectively, but the k_2 of the mutants were not different from that of wild-type API. These results were consistent with those for catalytic activities of these mutants for Boc-val-Leu-Lys-MCA (Table 1). DFP also reacted with the mutants as fast as with wild-type API. These results indicate that the catalytic triads of these mutants are as functional as those of wild-type API.

The specificity of a given enzyme for a peptidyl chloromethyl ketone is described by the parameter k_2/K_i,

Table 2. The kinetic parameter of mutants for the reaction with TLCK and DFP.

Enzymes	TLCK			DFP
	K_i (μM)	k_2 ($10^{-3}sec^{-1}$)	k_2/K_i ($M^{-1}sec^{-1}$)	k_2 ($M^{-1}sec^{-1}$)
Wild-type	0.7	9.8	13200	0.37
[T189V]	4.8	9.3	1950	0.78
[S214A]	5.3	8.5	1610	0.83
[T189V, S214A]	3630	10.5	3	0.89

Enzyme activity was assayed in 0.2 M Tris-HCl at pH 6.9 and 37°C.

which represents the apparent second-order rate constant for the reaction of free enzyme and inactivator. K_i is probably a combination of rate constants representing inactivator binding and reversible formation constant for alkylation of His^{57}. The specificity of peptidyl chloromethyl ketone inactivation generally correlates with the specificity of substrate hydrolysis for a serine protease [7]. Since the k_2/K_i for TLCK inhibition of T189V, S214A, T189V·S214A, and wild-type API correlate with the respective k_{cat}/K_m for hydrolysis of Boc-Val-Leu-Lys-MCA, it demonstrates that hydroxyl groups of Thr^{189} and Ser^{214} form hydrogen bonds with the lysine side chain. Therefore, the existence of the hydrogen bonds was confirmed by the inhibition experiments. Table 2 also indicates that the N1 of His^{57} and the hydroxyl group of Ser^{194} essentially retain the same reaction activity in the T189V, S214A, and T189V·S214A as in native API. That is, the catalytic triads of mutated APIs fulfill the function of the native enzyme. It can be deduced that the substitutions primarily affect the substrate binding affinity in the S_1-pocket and perhaps do not affect the function of the catalytic triad. The results indicate that the lower catalytic activity of T189V, S214A, and T189V·S214A is not due to changes in tertiary structure binding ability of the mutants.

If this definition is correct, it is possible to speculate why K_m of T189V is larger than that of S214A. In S214A, only the hydroxyl group of Ser^{214} is eliminated but in T189V, the hydroxyl group of Thr^{189} is eliminated and the hydrophobicity of a part in the S_1-pocket is increased as well. We propose that a decrease of substrate affinity in API can be caused by an increase of hydrophobicity around Asp^{225}. The decrease in this binding ability is remarkable in the double mutant with two substituted residues. K_m of T189V·S214A was much greater than the combined K_ms of T189V and S214A (46-fold of wild-type API). It is therefore suggested that the substrate binding ability of API is affected by amino acid residues affecting a special quality of Asp^{225}.

In conclusion, the hydrogen bonds between lysyl substrates and Thr^{189} and Ser^{214} are important factors governing the expression of high lysine activity and mechanism of substrate specificity of API. Therefore, the existence of hydrogen bonds in the S_1-pocket demonstrates

that API binds more effectively to lysine and has more strict substrate specificity for lysyl substrates than does trypsin.

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