

Elucidation of the cause for reduced activity of abnormal human plasmin containing an Ala⁵⁵-Thr mutation: importance of highly conserved Ala⁵⁵ in serine proteases

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Received 23 February 1998

Abstract In serine proteases, Ala⁵⁵ is highly conserved and located just behind the catalytic triad. That the activity of human plasmin is reduced by the A55T substitution indicates the importance of Ala⁵⁵ in catalysis. In the present study, the 3-D model of A55T human plasmin shows that an unusual hydrogen bond between Thr⁵⁵ Oγ1 and His⁵⁷ Nε2 alters His⁵⁷ into an inactive conformation in which His⁵⁷ cannot accept a proton from Ser¹⁹⁵ as a catalytic base. Our results demonstrate that Ala⁵⁵ contributes heavily to the active conformation of His⁵⁷ and ensures the proton transfer from Ser¹⁹⁵ to His⁵⁷.

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Key words: Serine protease; Plasmin; Homology modeling; Molecular dynamics simulation

1. Introduction

Throughout the large family of trypsin-like serine proteases, the catalytic triad of His⁵⁷, Asp¹⁰² and Ser¹⁹⁵ (Fig. 1; chymotrypsinogen numbering is used in the present paper) is completely conserved. The catalytic roles of these residues have been well established by experimental [1–4] and theoretical [5,6] evidence. Serine proteases catalyze the hydrolysis of amides and esters by nucleophilic attacks on the carbonyl carbon of the scissile bond by the hydroxyl group of Ser¹⁹⁵. His⁵⁷ acts as a catalytic base which enhances the nucleophilicity of Ser¹⁹⁵ and assists proton transfer from the serine hydroxyl to the substrate leaving group. Asp¹⁰² Oδ atoms (Oδ1 and Oδ2) stabilize the side-chain conformation of His⁵⁷ that is required for catalysis by accepting hydrogen bonds from His⁵⁷ Nδ1.

In addition to the catalytic triad, neighboring residues, such as Cys⁴², Gly⁴³, Ala⁵⁵, Cys⁵⁸, Tyr/Phe/Trp⁹⁴, Gly¹⁹⁶, Gly¹⁹⁷ and Ser²¹⁴, are almost completely conserved in both the primary and tertiary structures [7,8]. These residues together with the catalytic triad stabilize one another by intramolecular interactions conserved in serine proteases, which maintain the active conformation of the catalytic triad (Fig. 1). Among the conserved neighboring residues, Ala⁵⁵ seems to be very important for catalysis, because X-ray structures show that Ala⁵⁵ Cβ is located just behind the catalytic His⁵⁷ and Asp¹⁰². Interestingly, there are many reports that reduced

activity of human plasmin (HUPL) in patients with venous thrombosis and with retinohoroidal vascular disorders results from replacing Ala⁵⁵ with Thr in the serine protease domain [9–14]. Previous experimental studies of A55T HUPL demonstrated that the patients possess normal levels of plasminogen antigen, and that its Michaelis constant is very similar to normal ones. In spite of these normal properties, A55T HUPL has low activity on the synthetic substrates [15,16]. These experimental results indicate that the cause of the reduced activity of A55T HUPL is localized in the catalytic site, whereas the overall structure of A55T HUPL is normal. Therefore, we investigated the structural features of the catalytic sites of normal and A55T HUPLs to reveal the role of the residue at position 55.

In the present paper, since the X-ray structures of normal and A55T HUPLs have yet to be solved, we constructed 3-D structures by homology modeling method. The catalytic sites of the models were evaluated using molecular dynamics (MD) simulations. Our results indicate that there are clear structural differences between the catalytic sites of normal and A55T HUPLs. The reduced activity of A55T HUPL probably results from the inability of His⁵⁷ to act as the catalytic base.

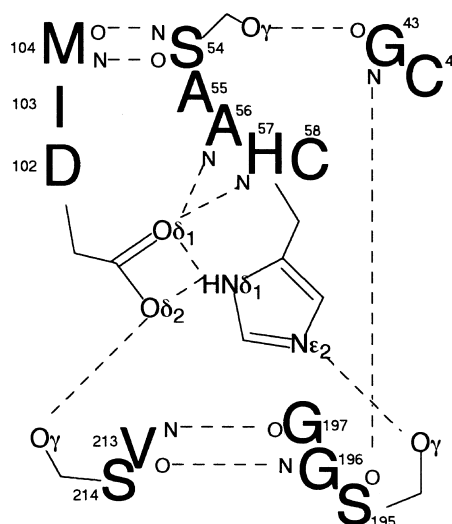


Fig. 1. Schematic representation of the hydrogen bonding network in the catalytic site of BOTR. In order to act as the catalytic base, His⁵⁷ must conform to the following conditions: (i) monoprotonated His⁵⁷ at optimum pH must be protonated at Nδ1 not Nε2 and (ii) His⁵⁷ must be stabilized in the conformation in which Nε2 can accept a proton from Ser¹⁹⁵ Oγ.

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Abbreviations: BOTR, bovine trypsin; HUPL, serine protease domain of human plasmin; MD simulation, molecular dynamics simulation; loop 5–6, loop between 5 and 6 β-strands in the N-terminal domain of serine protease

	16	26	36	46	56
HUPL	VVG	GCVA	HPHS	WPVQ	VS
BOTR	IVG	GYTC	GANT	VPYQ	VS
					*
		66	76	86	
HUPL	HCLE	KSPR	SSYK	VILGA	HQ
BOTR	HCY---	KS--	GIQV	RLGED	NIN
		*			
	96	106	116	126	
HUPL	-----	RK	DIAL	LKLS	SPAV
BOTR	YNS	NTLN	NDIM	LKLS	ASAS
		*			
	136	146	156	166	
HUPL	ECF	ITGW	GETQ	---TF	GAGL
BOTR	QCL	ISGW	GNTK	SSGTS	YPDV
					*
	176	186	196	206	
HUPL	GRV	QSTEL	CAGH	LAGG	TDS
BOTR	GQIT	SNMFC	AGYLE	GGK	DSC
					*
	216	226	236		
HUPL	TSW	GLGC	ARP	NKPG	VYVR
BOTR	VSW	GGSC	AKN	KPGV	YTKV

Fig. 2. Sequence alignment of HUPL and BOTR. Chymotrypsin numbering is indicated above the sequences. Residues marked with asterisks are catalytic triad and Ala⁵⁵. Loop 5–6 is indicated by bold letters.

2. Materials and methods

2.1. Construction of the models

The 3-D structure of HUPL was constructed with the CHIMERA modeling system [17] based on the sequence of the serine protease domain of HUPL (residues 562–791, plasminogen numbering) [18]. The main chain was constructed by connecting the fragments of reference X-ray structures obtained from the Brookhaven Protein Data Bank (PDB) [19]: residues 16–18 (bovine trypsin; PDB code 4PTP), 19–41 (bovine chymotrypsin; 5CHA), 42–54 (4PTP), 55–64 (human u-PA; 1LMW), 65–67 (4PTP), 68–80 (human neutrophil elastase; 1HNE), 81–118 (4PTP), 119–138 (5CHA), 139–156 (4PTP), 157–180 (1LMW), 181–198 (4PTP), 199–210 (5CHA) and 211–245 (4PTP). Two loop searches in the PDB were performed at residues 92–101 and 145–150. The final structure derived from CHIMERA was refined

by energy minimization with an AMBER united-atom force field [20] using the program APRICOT [21]. The program PROCHECK [22] was used to evaluate the stereochemical quality of the model. The A55T HUPL model was constructed based on the HUPL model by replacing Ala⁵⁵ with Thr, and was energy minimized using APRICOT.

2.2. Molecular dynamics simulation in the catalytic region

A cap of TIP3P water molecules [23] was placed 22 Å from His⁵⁷ Ce1 of the models. First the water molecules were energy minimized and then the whole system was energy minimized. All residues which lie 15 Å or more from His⁵⁷ Ce1 were fixed, while the residues within 15 Å and the water molecules were allowed to move during the course of MD simulation. The residues that construct the active site were all allowed to move. The whole system was thermalized from 0 to 300 K for 10 ps, and a 190 ps simulation was performed. The system was kept at 300 K during the productive simulation by applying a temperature coupling $\tau=0.1$ ps. All calculations were carried out using APRICOT with AMBER united-atom force field, a non-bonded cut-off of 12 Å and a constant dielectric of 1. The SHAKE algorithm was applied to all bonds involving hydrogen atoms. A time step of 0.002 ps was used. Data were collected every 0.5 ps.

3. Results and discussion

3.1. HUPL and A55T HUPL models

First, in order to evaluate the accuracy of the model derived from CHIMERA, we constructed a model of bovine trypsin (BOTR) whose X-ray structure had been solved. The root mean square deviations for superposition between the main chain atoms in the model and in the corresponding X-ray structure are 0.90 Å (all residues) and 0.50 Å (residues lying within 15 Å of His⁵⁷ Ce1). The model was very similar to the X-ray structure, especially in the catalytic site region.

HUPL showed high sequence identities to the reference proteins, among which the sequence identity to BOTR was the highest (41.3%; Fig. 2). The HUPL model is shown in Fig. 3. The X-ray structure of BOTR is also shown in Fig. 3 for comparison. The orientation of the catalytic triad and the hydrogen bonding pattern in the catalytic site are conserved in the HUPL model. No unfavorable contacts between

Table 1
Hydrogen bonds in the catalytic sites^a of HUPL and A55T HUPL models

Hydrogen bond partners	HUPL model		A55T HUPL model					Time (%)				
	Distance (Å) ^b		Distance (Å)					Time (%)				
			0° ^d	30°	60°	180°	−60°	0°	30°	60°	180°	−60°
A56 N–D102 Oδ1 ^e	2.89	43.7	2.83	2.87	2.77	2.85	2.80	45.1	86.7	34.2	70.9	44.7
H57 N–D102 Oδ1 ^e	2.83	49.2	2.88	2.84	2.85	2.85	2.87	51.8	95.7	37.0	80.4	36.7
A56 N–D102 Oδ2 ^e	2.90	46.5	2.88	2.96	2.87	2.84	3.15	46.1	2.2	58.0	20.3	0.7
H57 N–D102 Oδ2 ^e	2.83	49.5	2.87	3.00	2.84	2.91	3.01	51.8	4.2	63.7	22.5	1.5
H57 Nδ1–D102 Oδ1 ^e	2.93	93.5	2.89	2.96	2.90	2.93	2.94	68.1	72.5	90.5	79.4	34.2
H57 Nδ1–D102 Oδ2 ^e	2.95	95.0	2.96	2.91	2.96	2.89	2.95	69.4	77.5	88.5	79.6	34.2
D102 Oδ1 ^e –S214 Oγ	3.04	7.2	—	—	3.03	3.00	3.23	0	0	2.5	2.7	0.2
D102 Oδ2 ^e –S214 Oγ	2.76	26.0	2.76	2.75	2.73	2.75	2.78	21.3	33.7	30.2	28.5	14.5
H57 Ne2–S195 Oγ	3.10	0.7	3.18	3.18	3.07	3.14	3.15	1.7	4.0	1.2	5.7	1.0
T54 N–A104 O	2.86	99.5	2.89	2.91	2.93	2.89	2.92	98.7	99.7	99.0	100	99.5
T54 O–A104 N	2.85	99.7	2.85	2.86	2.88	2.85	2.88	98.9	99.5	99.2	99.4	99.7
G43 O–T54 Oγ1	2.87	99.0	2.91	2.91	2.93	2.90	2.91	94.7	96.5	94.2	96.2	97.7
G43 N–S195 O	2.92	98.2	2.88	2.88	2.91	2.91	2.88	98.4	98.7	97.2	98.7	99.2
G196 N–T213 O	2.85	97.7	2.86	2.89	2.89	2.84	2.83	99.7	97.7	99.2	99.7	99.7
G197 O–T213 N	3.00	90.5	2.98	3.08	3.00	2.99	2.99	94.2	86.5	88.5	92.7	95.2

^aThe hydrogen bonds in the catalytic site are schematically shown in Fig. 1, in which positions 54, 104 and 213 are Thr, Ala and Thr, respectively, in HUPL.

^bThe distances are average distances of snapshots where the interactions are characterized as hydrogen bonds (2.4–3.4 Å) during MD simulations.

^cTimes (%) are percentages of snapshots where the interactions are characterized as hydrogen bonds during MD simulations.

^dThe starting χ_1 values of Thr⁵⁵ in MD simulations of A55T HUPL.

^eWhen there are rotations of the χ_2 angle of Asp¹⁰², Asp¹⁰² Oδ1 and Oδ2 alternate in accepting the hydrogen bonds from Ala⁵⁶ N, His⁵⁷ N, His⁵⁷ Nδ1 and Ser²¹⁴ Oγ.

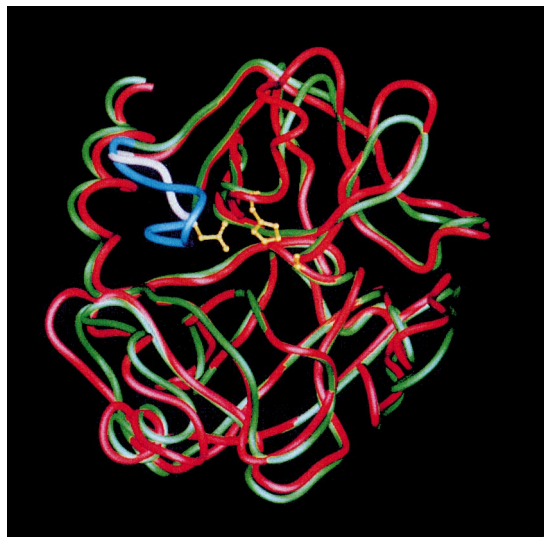


Fig. 3. Tube diagram of the superposition of the main chains of the HUPL model (red) and the X-ray structure of BOTR (green; PDB code 4PTP). The side chains of the catalytic triad of the HUPL model are shown in yellow. Loop 5-6 of BOTR is shown in blue and that of HUPL is shown in white.

the atoms and no unnatural chiral centers are observed. In the Ramachandran plot of the main-chain ϕ - ψ angles, all of the non-glycine residues are in the most favored or allowed re-

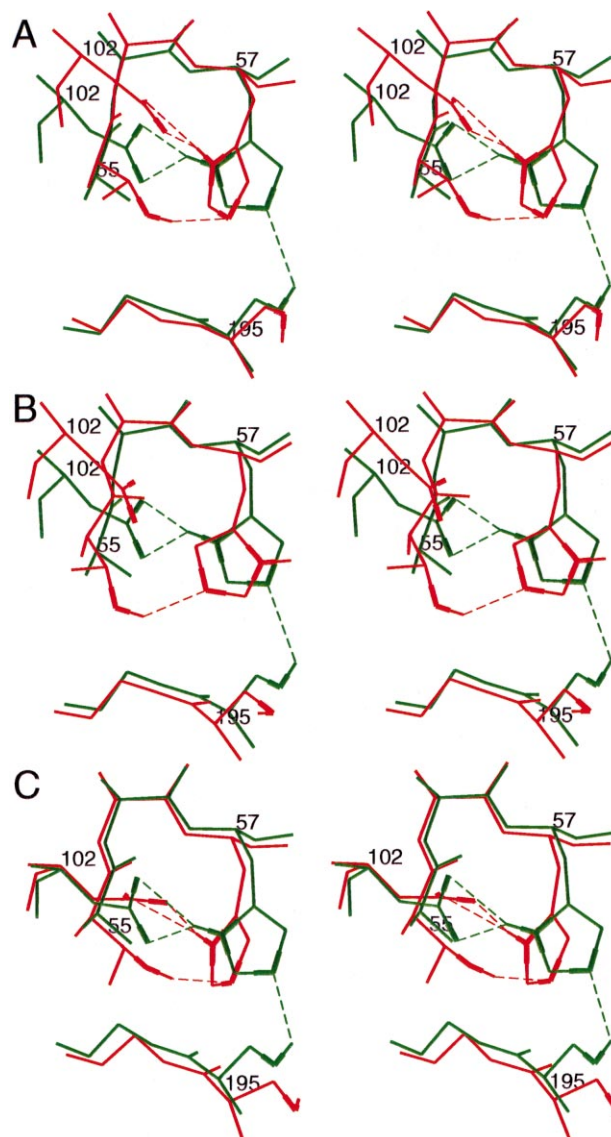
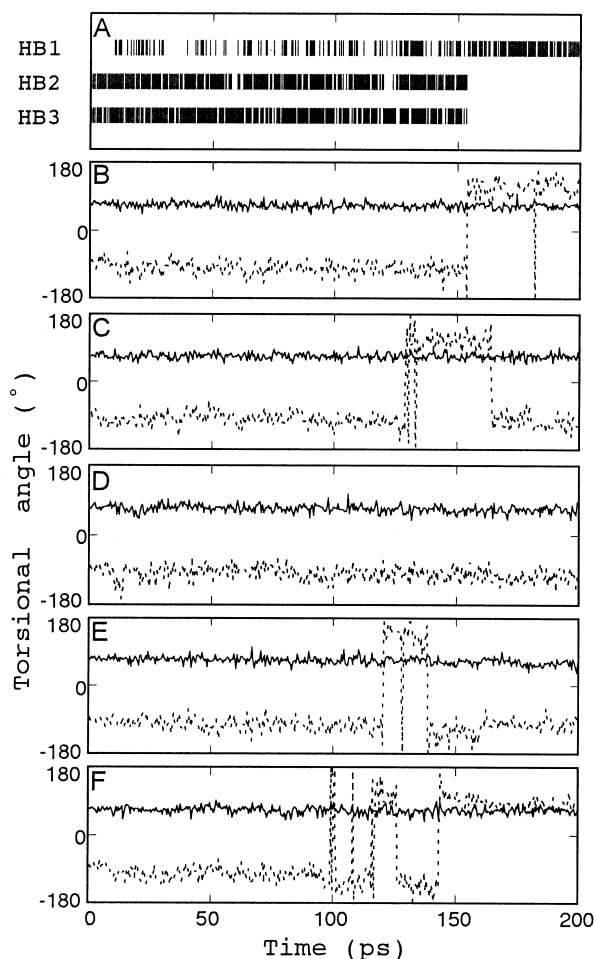


Fig. 5. Stereo views of the catalytic triad and Ala/Thr⁵⁵, Thr⁵⁵ O γ 1, His⁵⁷ N δ 1, His⁵⁷ Ne2, Asp¹⁰² O δ atoms and Ser¹⁹⁵ O γ are drawn with thicker lines. The hydrogen bonds are drawn with dotted lines. The hydrogen atoms (Thr⁵⁵ HO γ 1, His⁵⁷ HN δ 1 and Ser¹⁹⁵ HO γ) are shown. Green: Active conformation of HUPL (A and B) and BOTR (C). Red: Conformations A and B of A55T HUPL (A and B, respectively) and conformation A of A55T BOTR (C).

gions. The main-chain ω angles are all trans-planar. In general serine proteases, Asp¹⁰² is fully shielded from the solvent by seven highly conserved residues [24], Ala⁵⁵, Ala⁵⁶, His⁵⁷, Cys⁵⁸, Tyr⁹⁴, Leu⁹⁹ and Ser²¹⁴ in BOTR for example, but is partially buried in the HUPL model. Two of the seven residues (position 94 and 99) are included in the loop between 5 and 6 β -strands in the N-terminal domain (loop 5-6), but HUPL lacks these two residues because loop 5-6 is small

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Fig. 4. Dynamic properties of the catalytic site of A55T HUPL in MD simulations. The starting χ 1 value of Thr⁵⁵ were 0° (A and B), 30° (C), 60° (D), 180° (E) and -60° (F). A: Small bars denote the formation of the hydrogen bonds between Thr⁵⁵ O γ 1 and His⁵⁷ Ne2 (HB1), between His⁵⁷ N δ 1 and Asp¹⁰² O δ 1 (HB2) and between His⁵⁷ N δ 1 and Asp¹⁰² O δ 2 (HB3). B-F: The χ 1 (solid lines) and χ 2 (dotted lines) values of His⁵⁷.

(Figs. 2 and 3). Therefore Asp¹⁰² in the HUPL model is not fully buried. Experimental results demonstrate that plasmin has considerable room in the loop 5–6 region [25,26].

In the A55T HUPL model refined by energy minimization, the A55T substitution appears to have no bad effect on the catalytic site in spite of its reduced activity. The hydrogen bonding pattern in the catalytic site is similar to the normal one, and the catalytic triad is in the active conformation. However, further evaluation of the model is required as shown in the next section, because while refinement by energy minimization is able to remove short contacts among atoms, it is not able to correct any serious errors in the model.

3.2. Evaluation of the models by molecular dynamics simulations

We performed MD simulations of the models to investigate whether the hydrogen bonding pattern in the catalytic site and the active conformation of the catalytic residues are maintained or not. The dynamic properties of the hydrogen bonds in the catalytic site are presented in Table 1. In the simulation of the HUPL model, the orientation of the His⁵⁷ side chain was stably maintained. Due to 180° rotations of the side-chain torsional angle χ_2 of Asp¹⁰² (C α -C β -C γ -O δ 1), Asp¹⁰² O δ 1 and O δ 2 alternated in accepting the hydrogen bonds from Ala⁵⁶ N, His⁵⁷ N and Ser²¹⁴ O γ . All but two of the hydrogen bonds in the catalytic site were stably maintained during the simulation. The exceptions were the hydrogen bond between His⁵⁷ Ne2 and Ser¹⁹⁵ O γ , and the hydrogen bond between Asp¹⁰² O δ 1/O δ 2 and Ser²¹⁴ O γ . The former was lost. This hydrogen bond is thought to be formed after substrate is bound [27]. The latter was effective 7.2% (between Asp¹⁰² O δ 1 and Ser²¹⁴ O γ) and 26.0% (between Asp¹⁰² O δ 2 and Ser²¹⁴ O γ) of the simulation time. This hydrogen bond may not always be necessary for catalysis, because S214A rat anionic trypsin is more active than native ones [28]. The result of our simulation was in good agreement with those of whole unconstrained MD simulations of the X-ray structures of BOTR [29] and porcine pancreatic elastase [30], suggesting that the catalytic sites of normal serine proteases show similar dynamic properties and that the catalytic region of the HUPL model is accurate enough.

For A55T HUPL, we performed five MD simulations to investigate the preferred side-chain conformation of Thr⁵⁵; the starting χ_1 values of Thr⁵⁵ (N-C α -C β -O γ 1) were 0°, 30°, 60°, 180° and -60°, respectively. Independent of the starting conformation, Thr⁵⁵ was only in the gauche⁻ conformation (mean χ_1 value = 28.8°). The backbone-dependent rotamer library [31] also shows that the suitable conformation for Thr⁵⁵ is gauche⁻. The most remarkable feature of A55T HUPL is the formation of an unusual hydrogen bond between Thr⁵⁵ O γ 1 and His⁵⁷ Ne2 (HB1 in Fig. 4A). It is never formed in normal serine proteases, because the side chain of Ala⁵⁵ includes no hetero atoms that can be hydrogen bond donor to His⁵⁷ Ne2. Although this unusual hydrogen bond was not found by energy minimization because Thr⁵⁵ O γ 1 and His⁵⁷ Ne2 were not close to each other at first, it was found in all five MD simulations. It was not stable at first but gradually became stable. The catalytic site of A55T HUPL had two conformations A and B. Fig. 4A,B shows the results of one of five simulations. As shown in Fig. 4A, the normal hydrogen bonds between His⁵⁷ N δ 1 and Asp¹⁰² O δ atoms were maintained together with the unusual hydrogen bond at first (con-

formation A; Fig. 5A). Next, the hydrogen bonds between His⁵⁷ N δ 1 and Asp¹⁰² O δ atoms were lost (HB2 and HB3 in Fig. 4A) and there was a rotation of the χ_2 angle of His⁵⁷ (Fig. 4B), which produced another conformation B (Fig. 5B). The form of the unusual hydrogen bond in conformation B is different from that in conformation A. Also in the other three simulations, both conformations A and B were found due to the rotation of the χ_2 angle of His⁵⁷ (Fig. 4C,E,F). In some simulations, conformation A is found again due to the second rotation of the χ_2 angle of His⁵⁷ (Fig. 4C,E). Conformation B was not found in one of five simulations, in which the catalytic site is only in conformation A (Fig. 4D). A55T HUPL has a slightly higher isoelectric point (pI) than normal [15]. Our results indicate that the different pI value of A55T HUPL results from the conformational changes of the catalytic residues that probably accompany the changes of the charge distribution.

3.3. The cause of reduced activity of A55T HUPL

The unusual hydrogen bond between Thr⁵⁵ O γ 1 and His⁵⁷ Ne2 due to the A55T substitution alters His⁵⁷ into the unusual conformation. Fig. 5A,B shows conformations A and B of A55T HUPL, together with the corresponding active conformations of HUPL. To act as the catalytic base normally, His⁵⁷ Ne2 has to be able to accept a proton from Ser¹⁹⁵ O γ as drawn in green in Fig. 5A,B (schematically shown in Fig. 1). In conformation A, however, it is difficult for His⁵⁷ to accept the proton from Ser¹⁹⁵, because His⁵⁷ Ne2 retreats from Ser¹⁹⁵ O γ toward Thr⁵⁵ O γ 1 to accept the hydrogen bond from Thr⁵⁵ O γ 1 (drawn in red in Fig. 5A). Also in conformation B, His⁵⁷ is clearly unable to accept the serine hydroxyl proton because the His⁵⁷ conformation is quite different from normal one (drawn in red in Fig. 5B). This indicates that His⁵⁷ in A55T HUPL is unable to act as the catalytic base and enhance the nucleophilicity of Ser¹⁹⁵ effectively. The studies of D102N rat trypsin demonstrate that the inability of His⁵⁷ to act as the catalytic base reduces protease activity [1,2]. Also in the case of A55T HUPL, the reduced activity probably results from the inability of His⁵⁷ to act as the catalytic base, although the immediate cause of it is different from that of D102N trypsin.

In A55T HUPL, Asp¹⁰² moved from the normal position as shown in Fig. 5A,B. Asp¹⁰² in A55T HUPL is not stabilized by the surrounding residues because loop 5–6 is unusually small as described above, so that Asp¹⁰² may be greatly influenced by the steric hindrance caused by the A55T substitution, and the hydrogen bonds between His⁵⁷ N δ 1 and Asp¹⁰² O δ atoms tend to become lost, which produces conformation B. We constructed the A55T BOTR model for comparison. In the case of A55T BOTR, Asp¹⁰² itself and the hydrogen bonds between His⁵⁷ N δ 1 and Asp¹⁰² O δ atoms are stabilized by the neighboring residues. In the resulting model of A55T BOTR, Asp¹⁰² and the hydrogen bonds between His⁵⁷ N δ 1 and Asp¹⁰² O δ atoms are not disordered by the A55T substitution as expected, and A55T BOTR is only in conformation A (Fig. 5C). Both A55T HUPL and A55T BOTR models show that conformation B is not found when Asp¹⁰² and the hydrogen bonds between His⁵⁷ N δ 1 and Asp¹⁰² O δ atoms are stable. A55T HUPL is probably in conformation A over conformation B when substrate binds, because substrate may fill the space provided by the unusual small loop 5–6 of A55T HUPL and the environment of Asp¹⁰² becomes similar to that of

A55T BOTR. This suggestion is reasonable because conformation A seems to have slight activity, which agrees with the experimental data that A55T HUPL retains slight activity (k_{cat} is 10 times lower than normal [16]). The A55T BOTR model indicates that the activity of A55T mutants of serine proteases other than A55T HUPL is reduced.

The X-ray structures of normal serine proteases show that the region where the proton transfer from Ser¹⁹⁵ to His⁵⁷ occurs is completely conserved in both the primary and tertiary structures throughout this family. Moreover, this region is stabilized by the common hydrogen bonding network in this family. In this region, there are no hetero atom candidates for the hydrogen bond donor to His⁵⁷ Ne2 except Ser¹⁹⁵ O γ , which probably ensures the proton transfer from Ser¹⁹⁵ O γ to His⁵⁷ Ne2 after the substrate binds. These completely conserved structural features indicate that the steric requirement of the proton transfer is very strict, which means that the activity can be reduced by a slight change of this region. In the case of A55T mutants, the unusual hydrogen bond between His⁵⁷ Ne2 and Thr⁵⁵ O γ 1 disturbs the proton transfer from Ser¹⁹⁵ to His⁵⁷, which reduces the protease activity.

4. Conclusions

In conclusion, the present study demonstrates that the residue at position 55 provides an important contribution to the active conformation of the catalytic residues. In the case of A55T HUPL, since His⁵⁷ Ne2 unusually accepts the hydrogen bond from Thr⁵⁵ O γ 1 and retreats from Ser¹⁹⁵ O γ , His⁵⁷ is unable to accept the serine hydroxyl proton and enhance the nucleophilicity of Ser¹⁹⁵ effectively. The reduced activity of A55T HUPL is probably caused by the inability of His⁵⁷ to act as the catalytic base. On the other hand, in the case of normal serine proteases, Ala⁵⁵ has no hetero atoms that can be hydrogen bond donor to His⁵⁷ Ne2, and, therefore, His⁵⁷ is in the active conformation and is able to accept a proton from Ser¹⁹⁵ as the catalytic base after substrate is bound. The present study will shed new light on the essential environment for the catalysis of serine proteases.

Acknowledgements: We thank Dr. Shigetaka Yoneda for helpful discussions. This work was supported by a grant-in-aid for special project research from the Ministry of Education, Science, Sports and Culture in Japan.

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