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Plasminogen Tochigi: Inactive plasmin resulting from replacement of alanine-600 by threonine in the active site

(serine proteinase/molecular abnormality/thrombosis)

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ABSTRACT **Structural studies on a hereditarily abnormal plasminogen, plasminogen Tochigi, have been performed to identify the difference responsible for its lack of proteolytic activity. The plasminogen sample used was from a heterozygote and thus consisted of apparently equal amounts of normal and defective plasminogen molecules. Amino acid sequence analysis of a tryptic peptide isolated from the abnormal plasminogen indicated that Ala-600 (equivalent to Ala-55 in the chymotrypsin numbering system) had been replaced by Thr. No other substitutions in the active-site residues—namely, His-57, Asp-102, and Ser-195—were found. Molecular models for chymotrypsin and the bovine trypsin–pancreatic trypsin inhibitor complex indicate that Ala-55 is very near the active-site His. The Thr at position 55 in plasminogen (plasmin) Tochigi may perturb His-57 such that the proton transfers associated with the normal catalytic process cannot occur in the abnormal plasmin.**

Human plasminogen is a single-chain glycoprotein that consists of 790 amino acid residues (1). Upon activation by urokinase, plasminogen is cleaved at the Arg-Val bond between residues 560 and 561 (2), resulting in the formation of a two-chain plasmin molecule held together by two disulfide linkages (3). These chains contain, respectively, 560 amino acid residues in the heavy chain (1) and 230 residues in the light chain (3). Like trypsin, plasmin belongs to the so-called family of serine proteinases, in which the active site catalytic triad, His-57, Asp-102, and Ser-195 (chymotrypsin numbering), is situated in the light chain (1, 3, 4).

In 1978, Aoki *et al.* (5) identifed a patient with thrombosis recurring over the last 15 years, who possesses an abnormally low plasminogen activity but normal levels of immunoreactive plasminogen. This antigen has been characterized to be a mixture of normal and abnormal molecules of plasminogen in approximately equal amounts. Further studies of the patient's family (6) have suggested that the molecular abnormality is inherited on an autosomal gene. One of the family members appears to be a homozygote and possesses practically no plasminogen activity but possesses a normal concentration of plasminogen antigen.

The properties of the abnormal plasminogen established by prior studies are as follows (5, 6): (*i*) Plasminogen Tochigi forms a complex with streptokinase as normal plasminogen does, but the complex is insensitive to diisopropyl phosphorofluoridate, in contrast to the normal plasminogen–streptokinase complex. (*ii*) Urokinase-catalyzed hydrolysis of plasminogen Tochigi yields a two-chain plasmin but little plasmin activity, and no incorporation of diisopropyl phosphorofluoridate is observed, again in contrast to normal plasmin. (*iii*) Plasminogen Tochigi is adsorbed on a lysine-Sepharose column, suggesting that its lysine-binding sites are intact and functional. These results suggest that the abnormality resides at the active site of the molecule. The present work was undertaken to elucidate the structural abnormality of plasminogen Tochigi that explains the functional impairment of the molecule. The results indicate that the absence of proteolytic activity is due to replacement of Ala-600 by Thr in the abnormal plasminogen molecule. We know of no other example of a molecular defect in a serine proteinase due to an amino acid substitution near the active site.

MATERIALS AND METHODS

Preparation of Heterozygote Plasminogen and Its Derivatives. Plasminogen was purified according to published methods (5) from plasma samples of the heterozygous individual and from a normal individual that served as a control. The normal and the heterozygote-derived mixture of normal and defective plasminogens were activated by urokinase and the plasmins were reduced and S-carboxymethylated (7, 8). The resulting S-alkylated proteins were separated into heavy and light chains, according to the method of Wiman and Wallén (8). The cyanogen bromide fragments (CN-1 and CN-2) derived from the light chains from plasminogen Tochigi and the normal control were prepared by the method of Wiman and Wallén (9).

Tryptic Peptide Mapping by HPLC. To the fragment CN-1 (110 μg), suspended in 20 μl of 0.1 M ammonium bicarbonate, 2 μl of trypsin (1 mg/ml, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) was added. After incubation at 37°C for 4 hr, the digest was chromatographed on a reverse-phase C_{18} column (0.4 × 30 cm, TSK LS-410 ODS SIL; Toyo Soda, Tokyo, Japan), using two Waters model M-45 pumps and a Waters model 660 solvent programmer. The solvents used for HPLC were prepared by using acetonitrile for liquid chromatography (Katayama Chemicals, Osaka, Japan), analytical grade ammonium formate, and glass-distilled water. The solvents were passed through a Millipore HA 0.45-μm-pore diameter filter and degassed prior to use. The peptides were detected by absorbance at 210 nm by using a model II (UV-8) variable-wavelength detector from Toyo Soda. Optimal separation of the digest was obtained at room temperature with a linear gradient from 5% (vol/vol) acetonitrile/10 mM ammonium formate, pH 4.0, to 50% acetonitrile/10 mM ammonium formate, pH 4.0, at a flow rate of 1 ml/min. The isolated peptides were pooled, lyophilized, and used for amino acid and sequence analyses.

Amino Acid Analysis and Sequence Determination. Samples were hydrolyzed in 5.7 M HCl or 4 M methanesulfonic acid, in evacuated sealed tubes at 110°C for 24 hr. After evaporation, the amino acids were analyzed on a Hitachi 835 automatic ana-

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Abbreviation: CmCys, carboxymethylcysteine.

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lyzer (10). The sequences of the peptides were determined by the dansyl-Edman method (11). The details of the manual technique, including the identification of the dansyl amino acids, were as described previously (12). Phenylthiohydantoin derivatives of the amino acids were identified by HPLC according to the method of Zimmerman *et al.* (13), with the same column used for peptide mapping.

RESULTS

Isolation of Cyanogen Bromide Fragments CN-1 and CN-2 from Normal and Abnormal Plasminogens. Prior to cyanogen bromide hydrolysis of plasminogen, the plasmin heavy and light chains were prepared from the *S*-alkylated abnormal plasminogen and their amino acid compositions were compared with those of the normal control. No significant differences were detected. Moreover, the NH_2-terminal sequence of the isolated plasmin Tochigi light chain was established to have Val-Val-Gly-Gly-CmCys-Val-Ala, which was identical to the sequence determined for the normal control (CmCys indicates carboxymethylcysteine). We therefore made cyanogen bromide fragments from the intact plasminogen molecule. As shown in Fig. 1, this methionyl bond cleavage provides two large fragments, CN-1 (203 residues) and CN-2 (122 residues), in addition to the fragments derived from the heavy chain. CN-1 contains the active site catalytic triad and the substrate binding site (2), and CN-2 contains the NH_2-terminal portion of the plasminogen light chain (14). Both fragments were isolated from plasminogen

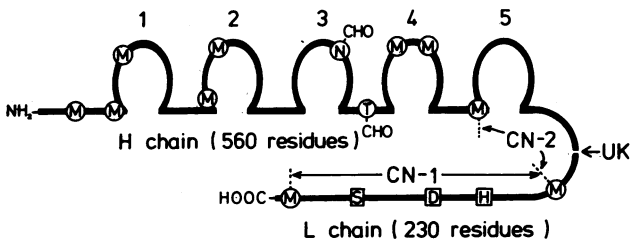

FIG. 1. Schematic representation of the gross structure of human plasminogen. The so-called kringle regions (1) are numbered, and the positions of Met residues (M) and catalytically important amino acid residues (S, Ser; D, Asp; H, His) along the polypeptide chain are indicated. N, Asn; T, Thr; CHO, carbohydrate units; H and L chains, heavy and light chains, respectively. The urokinase (UK)-sensitive bond is shown by an arrow.

from the normal control and from the heterozygote and were homogeneous on $NaDodSO_4$ gel electrophoresis. The mobilities of fragments CN-1 and CN-2 from abnormal plasminogen were identical to those of the fragments from the normal control. The amino acid compositions of the two CN-1s were also indistinguishable from each other, as were those of the two CN-2s (data not shown).

Tryptic Peptide Mapping of the Two Isolated CN-1 Fragments. To elucidate the structural difference, the CN-1 fragments derived from abnormal and normal plasminogens were each digested with trypsin and the peptides were separated with

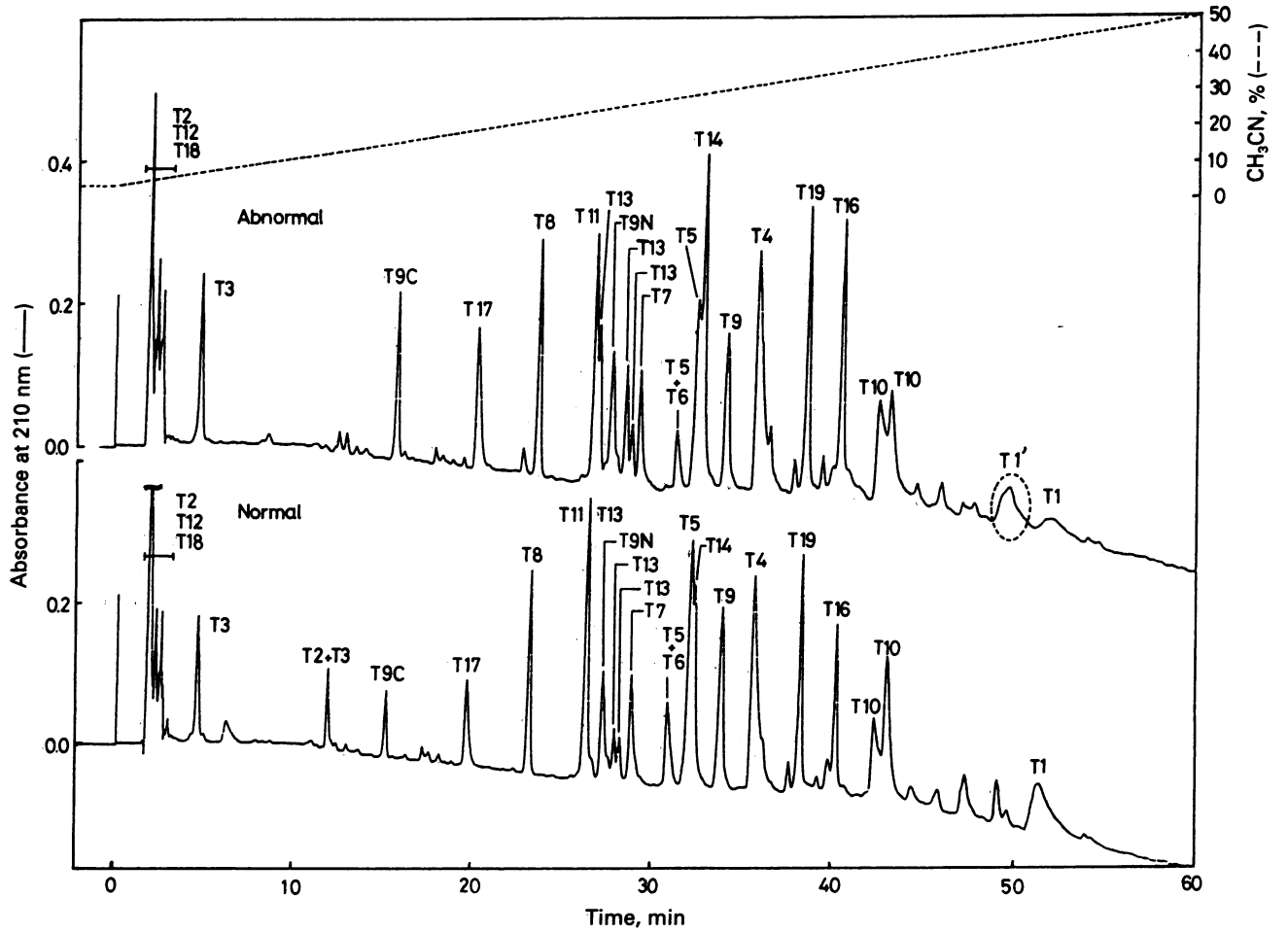

FIG. 2. HPLC of tryptic (T) peptides derived from the CN-1 fragments of abnormal and normal plasminogens. The tryptic digests (110 μg each) were separately subjected to HPLC on a 0.4 × 30 cm TSK LS-410 SIL column. T9N and T9C on the peptide peaks are abbreviations used for NH_2- and COOH-terminal peptides derived from peptide T9. The peptide T1′ found only in the abnormal plasminogen is indicated by a broken circle.

a reverse-phase HPLC column. The results are shown in Fig. 2. Except for the peptides T1 and T1′, the chromatogram of the CN-1 digest from abnormal plasminogen was not significantly different from that obtained from the normal control. A slight but reproducible difference associated with peptide T1 was observed between the two chromatograms. The area of the peptide peak, T1, derived from abnormal plasminogen was smaller than that of T1 from the normal control, and a new peptide peak, named T1′, indicated by a broken circle, appeared in the chromatogram. All of the other peptides from both samples were separated as single peaks, except for the first peak, which contained three peptides, T2, T12, and T18. The peptides eluted in the first peak were further purified by the same column as used for the whole digest, with a different linear gradient system from 0% to 1.25% acetonitrile in 10 mM ammonium formate, pH 4.0.

Amino Acid Compositions of Tryptic Peptides Derived from the CN-1 Fragment of Abnormal Plasminogen. Table 1 shows the amino acid compositions of all the tryptic peptides thus isolated. The data indicated a high purity for all the peptides, except for peptide T-10, and made it possible to unambiguously identify their positions in the known amino acid sequence for the CN-1 region in the plasmin light chain. Among these peptides, the peptide T10 gave double peaks with identical amino acid compositions, most likely due to oxidation of the Trp residue present in the peptide. The peptide T13 gave three peaks with different retention times, although the amino acid compositions of the three peptides were indistinguishable. This microheterogeneity of the peptide T13 must be related to that of the CN-1 fragment, which has been reported previously by Wiman and Wallén (9). Peptide T13 appears to be especially prone to rearrangement of the Asn-Gly linkage in the sequence to the β-peptide form, giving rise to isopeptides.

Peptides T1 and T1′ each consisted of 22 amino acid residues. Peptide T1 had the same amino acid composition as that isolated from the normal control. However, T1′ differed from T1 in the amount of Thr and Ala. To conclusively establish the existence of this difference, the peptides T1 and T1′ were digested with staphylococcal protease V8 (Miles) and the digests were separated by HPLC under the same conditions as used for the tryptic peptides. As shown in Fig. 3, three major peaks were obtained;

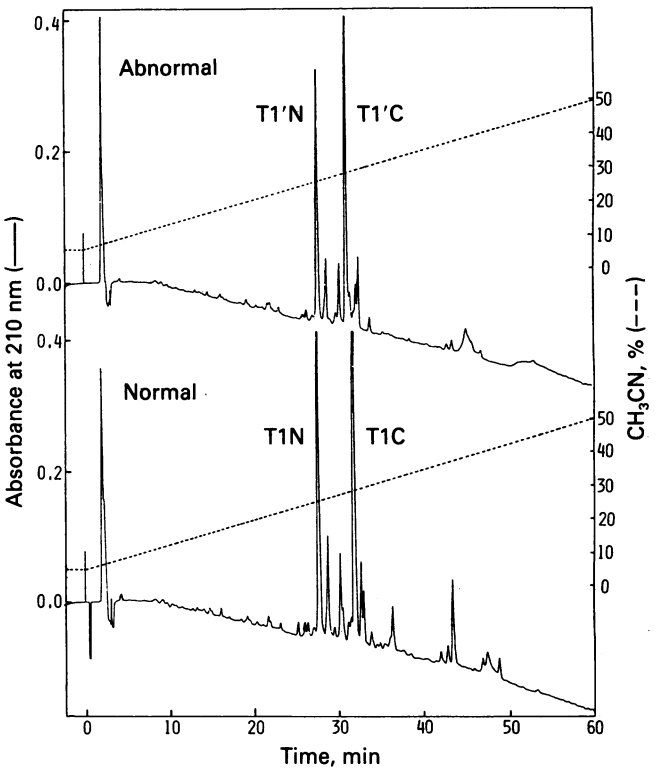

FIG. 3. HPLC of the staphylococcal protease digest of peptides T1′ and T1 derived from abnormal and normal plasminogens. The digests were subjected, separately, to HPLC under the conditions described in the text.

Table 1. Amino acid compositions (residues per mole) of tryptic peptides obtained from the CN-1 fragment of abnormal plasminogen

Residue	T1	T1′	T2	T3	T4	T5	T6	T7	T8	T9N	T9C	T10*	T10	T11	T12	T13	T13	T13	T14	T15	T16*	T17	T18	T19*
CmCys	1.4	1.3								0.5		1.5	1.0		0.8				2.3		1.1			
Asp					1.3			1.0	1.1	1.1	0.9	0.6	0.6	1.1	0.9	0.9	0.9	0.8	2.3	(1)		1.0		
Thr	1.8	2.3				1.1			1.0			3.3	2.6						2.0		0.9			0.9
Ser	1.0	0.8	0.9	2.1	1.2				1.9	1.1		0.6	0.7						2.8		1.0		0.8	
Hse																								0.6
Glu	1.8	1.8			5.8	1.1						2.8	2.4	3.0		1.1	0.9	0.9	3.8		1.1			1.0
Pro	1.4	1.2	1.0	1.0	1.0	1.0			1.0	2.8				0.9					1.4			1.9		
Gly	2.1	2.1			1.4							4.8	4.6			1.0	1.0	1.0	5.6		2.6	1.1		1.2
Ala	1.5	0.9			1.1			1.0	1.1	1.0	0.9	1.1	1.1	1.0					1.9		0.9			
Val	1.2	1.1			3.7				0.9	1.1	1.7	0.7	1.5	0.9	0.9				2.3		1.0	1.9	0.9	1.7
Ile	1.1	1.0			1.8			1.0	0.8	0.7		1.2	1.3	0.8							0.7			1.0
Leu	2.6	2.5			2.2	2.1		2.0	1.1	1.2		2.4	2.5	1.1		1.2	1.3	1.4	3.2		1.6	0.7		
Tyr				1.1							1.3	0.6	1.0			1.0	0.9	1.2			0.8	1.2		
Phe	1.2	1.3				1.0						2.4	1.6			1.0	1.3	1.4	1.3					1.1
Lys	1.0	1.0		1.0			1.0	0.9	1.0			0.9	1.0	1.0					0.9	(1)		0.8		
His	1.6	1.8			2.2														1.4					
Trp	(1)	(1)										1.3	(1)								0.7			0.9
Arg			1.0		1.1	0.9					0.8		0.9		1.0	0.8	0.7	0.8			0.6	0.9	1.0	
Total	22		3	5	22	7	1	6	10	10	6	21		10	4	7	7	7	31	2	15	9	3	9
Position	1		23	26	31	53		61	67	77	87	93	93	114	124	128	128	128	135	166	168	183	192	195
	|		|	|	|	|	60	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	22		25	30	52	59		66	76	86	92	113	113	123	127	134	134	134	165	167	182	191	194	203

* Hydrolysis for 24 hr with 4 M methanesulfonic acid. The positions of each tryptic peptide derived from the CN-1 fragment correspond to those taken from the amino acid sequence (3).

Table 2. Amino acid compositions (residues per mole) of T1 and T1′ and their fragments

	Normal			Abnormal		
Residue	T1	T1N	T1C*	T1′	T1′N	T1′C
CmCys	1.2 (2)	0.9 (1)	0.9 (1)	1.3 (2)	0.5 (1)	0.5 (1)
Thr	2.0 (2)	1.0 (1)	0.9 (1)	2.3 (3)	1.0 (1)	1.9 (2)
Ser	1.0 (1)	0.8 (1)		0.8 (1)	1.0 (1)	
Glu	1.9 (2)	1.0 (1)	0.9 (1)	1.8 (2)	1.0 (1)	1.1 (1)
Pro	1.4 (1)	1.2 (1)		1.2 (1)	1.1 (1)	
Gly	2.2 (2)	2.0 (2)		2.1 (2)	2.0 (2)	
Ala	1.6 (2)		1.6 (2)	0.9 (1)		1.0 (1)
Val	1.2 (1)		1.4 (1)	1.1 (1)		1.1 (1)
Ile	1.1 (1)	1.0 (1)		1.0 (1)	0.9 (1)	
Leu	2.7 (3)	1.0 (1)	1.8 (2)	2.5 (3)	1.0 (1)	2.1 (2)
Phe	1.4 (1)	1.0 (1)		1.3 (1)	0.9 (1)	
Lys	1.0 (1)		1.0 (1)	1.0 (1)		1.0 (1)
His	2.0 (2)	1.0 (1)	1.2 (1)	1.8 (2)	1.0 (1)	0.8 (1)
Trp	(1)†		0.6 (1)	(1)†		(1)†
Total	22	11	11	22	11	11

Numbers in parentheses are integral numbers of residues per mole.
* Hydrolysis for 24 hr with 4 M methanesulfonic acid.
† Taken from the sequence data of Fig. 3.

their amino acid compositions are listed in Table 2. The first peak from each digest contained the ammonium bicarbonate used as the buffer for digestion. The second peaks, which eluted with the same retention time, were named T1′N and T1N and consisted of 11-residue peptides. These were derived from the NH_2-terminal regions of peptides T1′ and T1, respectively. The third peaks, named T1′C and T1C, also contained 11 residues and were derived from the COOH-terminal regions of T1 and T1′. However, the retention time of T1C was 50 sec longer than that of T1′C. A clear difference between peptide T1′C and peptide T1C was found also in their amino acid compositions: T1′C contained one more Thr and one less Ala than did T1C.

Amino Acid Sequence of Peptide T1′N and T1′C. Fig. 4 shows the amino acid sequences of peptides T1′N and T1′C, in addition to the sequence of peptide T1C derived from the CN-1 fragment of the normal control. The sequence of T1′N was the same as that of T1N, as expected from its amino acid composition (Table 2). On the other hand, the peptide T1′C had the sequence Trp-Val-Leu-Thr-Thr-Ala-His-CmCys-Leu-Glu-Lys and differed from peptide T1C from normal plasminogen, which had Ala at the fifth residue from the NH_2 terminus. These results establish that the peptide T1′C, with the replacement of an Ala for a Thr residue, is a variant of the normal plasminogen peptide, T1C.

Tryptic Peptide Mapping of the Two Isolated CN-2 Fragments. Fragment CN-2, which is derived from the NH_2-terminal portion of the plasmin light chain (Fig. 1), was digested with trypsin and the resulting peptides were separated by HPLC under the same conditions used for the separation shown in Fig. 1. The chromatogram of the tryptic digest obtained from the CN-2 fragment of abnormal plasminogen was identical to that of the normal control (data not shown), indicating that no amino acid replacements are evident in the CN-2 region.

DISCUSSION

In the present work, we have used cyanogen bromide fragmentation of heterozygote plasminogen to elucidate its structural abnormality. This procedure provides two large fragments, CN-1 and CN-2, which include the entire sequence of the plasmin light chain, except for the COOH-terminal tripeptide, Arg-Asn-Asn (Fig. 1). Fragment CN-1 contains the active site catalytic triad and the substrate binding site at positions corresponding to Asp-102, His-57, Ser-195, and Ser-189 of α-chymotrypsin (15, 16). On the other hand, fragment CN-2 contains the NH_2-terminal Val corresponding to Ile-16 of α-chymotrypsin, which participates in formation of an ion pair with Asp-194. Among the tryptic peptides isolated from CN-1 from abnormal plasminogen, the three peptides T7, T1, and T14 contained the

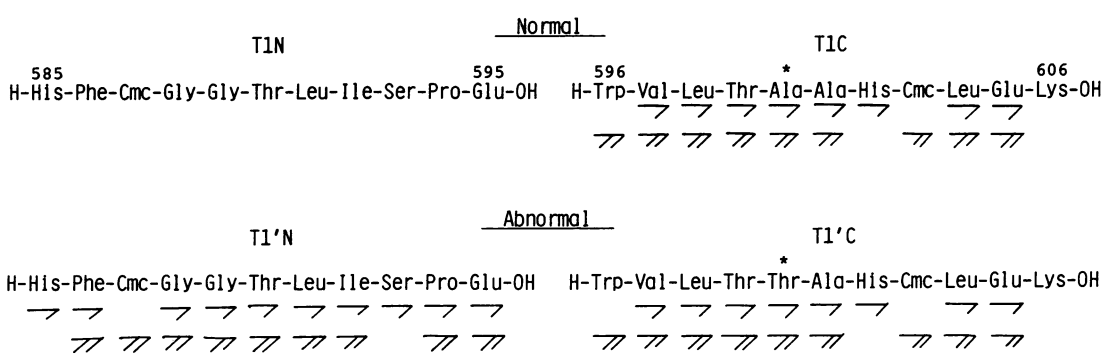

FIG. 4. Amino acid sequences of the peptides, T1 and T1′, derived from normal and abnormal plasminogens, respectively. The sequence of T1N was taken from the sequence data of the plasmin light chain (3). The His-602 in the peptides T1C and T1′C corresponds to the catalytically important His-57 of α-chymotrypsin. Dansyl amino acids (single-barb arrows) and phenylthiohydantoin amino acids (double-barb arrows) were identified by TLC (12) and HPLC (13), respectively. Cmc, carboxymethylcysteine.

functionally important residues Asp-102, His-57, Ser-195, Asp-189, and Asp-194. Of these residues, the aspartate residue (Asp-102) in peptide T7 was identified by the direct Edman degradation method. Peptide T14, the peptide containing the active site serine residue (Ser-195) and consisting of 31 amino acid residues, was digested further with staphylococcal protease V8, and the resulting four peptides were separated by HPLC. One of these peptides contained the NH_2-terminal serine residue corresponding to the active site Ser-195 of α-chymotrypsin (data not shown). This result, together with the amino acid composition of peptide T14 (Table 2), demonstrated the presence of the active-site Ser in the abnormal molecule. Peptide T14 also contains the side-chain-binding site, Asp-189 (in trypsin-like proteases) and the NH_2-terminal ion-pair-interacting residue, Asp-194. Because the retention times of T14 and the four peptides derived from it were identical to those of the corresponding peptides isolated from the normal control (Fig. 2), we conclude that all active site residues are intact. Collectively the data described above make it very unlikely that the molecular abnormality of plasminogen Tochigi is due to replacement of any amino acid residue associated directly with the active site catalytic triad or the side-chain-binding site. The replacement of Ala by Thr at position 600 from the NH_2-terminus of abnormal plasminogen appears, therefore, to be responsible for the loss in catalytic activity. The minimal genetic change responsible for this substitution is a G-to-A transition in the 5′-terminal position of the Ala codon. Ala-600 (equivalent to Ala-55 in chymotrypsin) is located very close to the active site residues Asp-102 and His-57, as inferred from making the reasonable assumption of a polypeptide chain conformation similar to that of trypsin (17) or chymotrypsin. It can be seen from the crystallographic data for a bovine trypsin–pancreatic trypsin inhibitor complex (18) that the side-chain CH_3 group of the Ala in the trypsin molecule is located just behind the imidazole ring of His-57. It is situated within 3.9 Å from the $N^{\delta 1}$ and $C^{\epsilon 1}$ atoms of the imidazole ring of His-57 and from the $O^{\delta 1}$ atom of the β-carboxyl group of Asp-102. Therefore, we propose that the Ala → Thr replacement found in the plasmin light chain variant alters the properties of the side chain of Asp-645 or the side chain of His-602 (corresponding to Asp-102 or His-57 of α-chymotrypsin), perhaps inducing a change in charge distribution in the His residue, and thus leads to the loss of the enzyme activity.

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