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Antithrombin III Toyama: Replacement of arginine-47 by cysteine in hereditary abnormal antithrombin III that lacks heparin-binding ability

(protease inhibitor/molecular abnormality/recurrent thrombophlebitis/HPLC)

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ABSTRACT **Structural analyses of a hereditary abnormal antithrombin III, antithrombin III Toyama, which has normal progressive antithrombin activity but no heparin cofactor activity, have been carried out to elucidate the molecular abnormality causing recurrent thrombophlebitis of a patient and to identify an amino acid residue essential for the binding with heparin. Abnormal antithrombin III was reduced, *S*-pyridylethylated, and treated with cyanogen bromide. Eleven fragments were isolated by the combination of Sephadex G-50 gel filtration and reversed-phase HPLC and compared with those from normal antithrombin III. One large fragment (CN-III) that appeared to have a different amino acid composition from that of the corresponding fragment from normal antithrombin III was digested with trypsin, and the digests were separated by HPLC. The abnormal peptide was identified by comparing the peptide map with that from normal antithrombin III. Amino acid sequence analysis of the abnormal peptide indicated that the arginine-47 of normal antithrombin III had been replaced by cysteine in antithrombin III Toyama. One base mutation, C → T, in the 5′ terminal position of the arginine-47 genetic codon (CGT) is probably responsible for this substitution. These results also suggest that arginine-47 is an essential amino acid residue for the binding with heparin.**

Human antithrombin III is a single-chain α_2-glycoprotein in plasma that is composed of 432 amino acid residues (1, 2). The glycoprotein forms a stoichiometric enzyme–inhibitor complex with a wide variety of serine proteases involved in the coagulation and the fibrinolysis systems and plays a principal role in regulation of the hemostatic mechanism (3, 4). The primary contact site (reactive site) of antithrombin III with proteases in the complex formation has been identified as Arg-Ser near the carboxyl terminus of the inhibitor (5, 6). Antithrombin III strongly binds heparin, which greatly accelerates the reaction rate of the inhibitor with proteases (3, 4). One tryptophan (4) and one or more lysine (7) residues (or both) of antithrombin III have been suggested to be involved in the binding with heparin, although their locations in the amino acid sequence have not been assigned.

In 1965, Egeberg (8) described a thrombophilic family having a hereditary antithrombin III deficiency and, in 1974, Sas *et al.* (9) described a family with a thrombolism due to abnormal antithrombin III. Since then, several reports have been presented on the familial antithrombin III abnormality associated with thromboembolic disorder (10–15).

Recently, we identified a Japanese family having the abnormal antithrombin III "antithrombin III Toyama" (16). The propositus of this family was a 23-year-old female suffering from recurrent thrombophlebitis. She was a homozygote transmitted from heterozygous parents. The antithrombin III antigen concentration in her plasma was as high as 54 mg/dl and the progressive antithrombin activity assayed in the absence of heparin was of normal level, but the heparin cofactor activity of her plasma was as low as 26% of the normal (15). This value of the heparin cofactor activity in her plasma is probably due to heparin cofactor II (17), since crossed immunoelectrophoresis in the presence of heparin in the first-dimension agarose gel showed that her antithrombin III apparently has no heparin-binding ability (16). These results suggest that the abnormality in the patient's antithrombin III is occurring at the heparin-binding site of the inhibitor, whereas the reactive site is intact and functional.

Abnormal antithrombin III has been purified from the patient's plasma and the following properties have been established (18). (*i*) Antithrombin III Toyama, like normal antithrombin III, is a single-chain glycoprotein; it has the same molecular weight, amino-terminal amino acid sequence, and carboxyl-terminal amino acid as normal antithrombin III; and the two proteins are immunologically identical. These results indicate that the functional abnormality of antithrombin III Toyama is not caused by loss of the terminal part of the polypeptide chain. (*ii*) Antithrombin III Toyama has the same UV absorption spectrum as normal antithrombin III, suggesting that neither tryptophan nor tyrosine residues are replaced by other amino acids. (*iii*) Antithrombin III Toyama migrates to the anode faster than normal antithrombin III on agarose gel electrophoresis (pH 8.6), and this difference in mobility is unchanged after a sialidase digestion. These results suggest that, in antithrombin III Toyama, an amino acid residue at the heparin-binding site has been replaced by a less-basic or more-acidic one that has no ability to interact with heparin.

In this paper, we present the evidence that arginine-47 has been replaced by cysteine in antithrombin III Toyama, resulting in the total loss of heparin-binding ability.

MATERIALS AND METHODS

Preparation of Homozygous Abnormal Antithrombin III. Antithrombin III Toyama was purified by immunoadsorption chromatography on anti-antithrombin III-Sepharose followed by DEAE-Sephadex A-50 chromatography (18). Normal antithrombin III was purified as previously described (19).

Fragmentation of Antithrombin III by Treatment with Cyanogen Bromide and Isolation of Fragment CN-III. Normal and abnormal antithrombin III were reduced and *S*-pyridylethylated (19). The *S*-pyridylethylated proteins were degraded with cyanogen bromide and the resulting fragments were fractionated by gel filtration on Sephadex G-50 followed by HPLC. The HPLC conditions were the same as described

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Abbreviation: PeCys, pyridylethylcysteine.

below for the peptide mapping except that the linear gradient of 5–60% acetonitrile was run in 20 min instead of in 40 min.

Tryptic Digestion and Peptide Mapping by HPLC. The fragment (normal or abnormal CN-III; 3.6 or 3.0 mg, respectively) was dissolved in 300 μl of 0.05 M Tris·HCl/0.15 M NaCl/10 mM $CaCl_2$, pH 8.0, and 100 μl of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin-Sepharose CL-4B suspension (1 mg of trypsin/ml of suspension) was added. After incubation at 25°C with constant vibration for 24 hr, the digestion mixture was centrifuged and the supernatant was chromatographed on a reversed-phase C_{18} column (0.4 × 30 cm; TSK LS-410K ODS SIL; Toyo Soda, Tokyo, Japan) using a Hitachi model 638 liquid chromatograph. HPLC was carried out at 25°C with a linear gradient of 5–60% acetonitrile in 10 mM ammonium formate (pH 4.0) in 40 min. The flow rate was 1 ml/min and the eluate was monitored by measuring absorbance at 220 nm. Peptides that passed through the column were rechromatographed on the same column after equilibrating it with 10 mM ammonium formate (pH 4.0). A gradient of 0–5% acetonitrile in the equilibration buffer was run in 20 min at a flow rate of 1 ml/min. The isolated peptides were lyophilized and used for amino acid and sequence analyses.

Amino Acid Analysis and Sequence Determination. Amino acid analyses were performed by the method of Moore and Stein (20) on a JEOL-6AS amino acid analyzer. Performic acid oxidation was carried out as described by Hirs (21) and free sulfhydryl group was determined by Ellman's method (22). Amino acid sequences were determined by manual Edman degradation (23). Phenylthiohydantoin derivatives were identified as described (18) and also by HPLC on a 30-cm Waters μBondapak C_{18} column using stepwise elution with a system of methanol-containing buffers (Waters Associates).

RESULTS

Separation of Cyanogen Bromide Fragments of Normal and Abnormal Antithrombin III and Isolation of the CN-III Fragments. The elution profiles of cyanogen bromide fragments of *S*-pyridylethylated abnormal and normal antithrombin III from a Sephadex G-50 column are shown in Fig. 1. Pooled fractions II, III, IV, V, and VIII contained mostly fragments CN-II, CN-III, CN-IV, CN-V, and CN-VIII, respectively, with small amounts of contaminating fragments, which were removed by HPLC. Pooled fractions VI, VII, and IX were further separated by HPLC into two components each, fragments CN-VI-1 and -2, CN-VII-1 and -2, and CN-IX-1 and -2. Eleven cyanogen bromide fragments were finally obtained from abnormal antithrombin III as well as from normal antithrombin III. Amino acid compositions of the purified cyanogen bromide fragments of abnormal antithrombin III are shown in Table 1. The total amino acid composition of the 11 cyanogen bromide fragments closely accounts for that of intact antithrombin III. Identities between the smaller fragments, CN-V, CN-VI-1 and -2, CN-VII-1 and -2, CN-VIII, and CN-IX-1 and -2, from abnormal antithrombin III and the corresponding fragments from normal antithrombin III were determined from their similar amino acid compositions and HPLC retention times. However, it was difficult to determine the identity or difference between the larger fragments, CN-II, CN-III, and CN-IV, from abnormal antithrombin III and the corresponding normal antithrombin III fragments by comparing only the amino acid compositions and HPLC retention times.

Tryptic Peptide Mapping of Large Cyanogen Bromide Fragments. To further investigate the structural difference between abnormal and normal antithrombin III, the three large cyanogen bromide fragments from abnormal antithrombin III were digested with trypsin and each digest was subjected to HPLC to compare the peptide map with that of the corresponding fragment from normal antithrombin III. There was

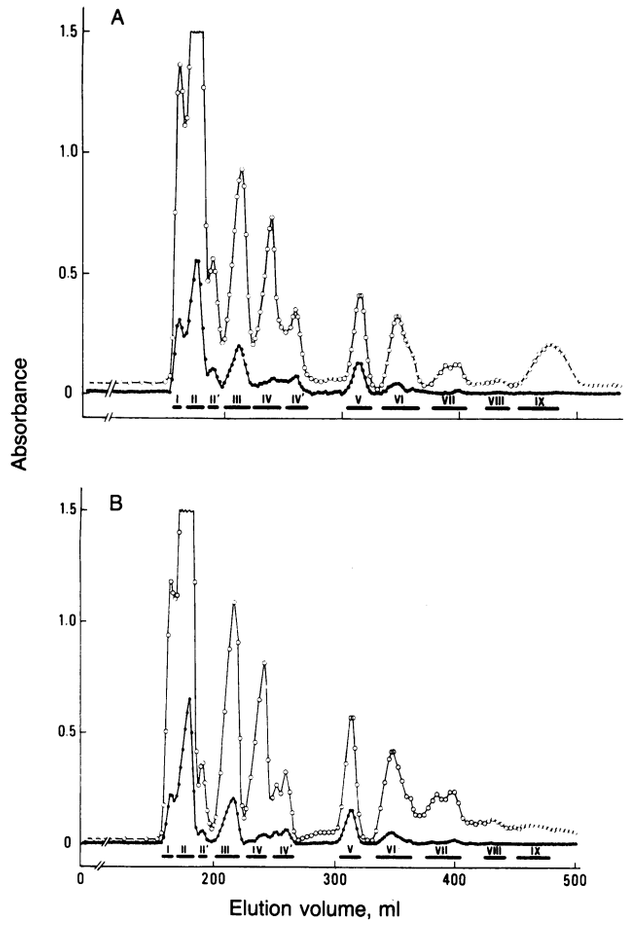

FIG. 1. Separation of cyanogen bromide fragments of *S*-pyridylethylated abnormal (*A*) and normal (*B*) antithrombin III by gel filtration on Sephadex G-50. *S*-Pyridylethylated abnormal or normal antithrombin III (26 or 30 mg, respectively) was treated with 60 mg of cyanogen bromide (100-fold molar excess over methionine residues) in 2.5 ml of 70% formic acid. The reaction mixture was incubated for 24 hr at 4°C, then diluted with 9 vol of distilled water and lyophilized. A column (2.6 × 95 cm) of Sephadex G-50 was equilibrated with 0.1 M formic acid. (*A*) Cyanogen bromide-treated abnormal antithrombin III (26 mg) was applied to the column and eluted at a flow rate of 6 ml/hr; 2-ml fractions were collected. (*B*) Cyanogen bromide-treated normal antithrombin III (30 mg) was applied to the column and eluted at a flow rate of 5.4 ml/hr; 1.8-ml fractions were collected. Eluates were monitored by measuring the absorbance at 230 (○) and 280 (●) nm. Fractions indicated by bars and roman numerals were pooled. The last broad peak detected at 230 nm in *A* was mainly due to the more concentrated formic acid added to dissolve the abnormal antithrombin III sample.

no significant difference in the peptide maps of fragments CN-II and CN-IV from abnormal and normal antithrombin III. However, two distinctive features were observed when the chromatograms of the CN-III fragments from abnormal and normal antithrombin III were compared (Fig. 2*A*). (*i*) The chromatogram of the tryptic digest of CN-III from normal antithrombin III had the largest peptide T-7‡-containing peak whereas that of the tryptic digest of CN-III from abnormal antithrombin III had the largest peptide T-X-containing peak, and that peak was completely absent from the former chromatogram. (*ii*) The retention time of peptide T-Xa was slightly but reproducibly different from that of peptide T-7a.

‡In addition to peptide T-7, this peak contained an unidentifiable peptide that was identical to the component of the corresponding peak in the chromatogram of the tryptic digest of CN-III from abnormal antithrombin III.

Table 1. Amino acid compositions (residues per mol) of cyanogen bromide fragments from abnormal antithrombin III

Residue	VI-1	IX-2	III	II	IX-1*	VI-2	V	IX-1*	VIII	VII-2	IV	VII-1
Asp	2.1 (2)	1.0 (1)	11.3 (11)	14.8 (17)		2.0 (2)	1.4 (1)			2.1 (2)	8.8 (9)	1.0 (1)
Thr	0.9 (1)		6.8 (7)	7.7 (9)		1.9 (2)	1.0 (1)				3.0 (3)	
Ser	0.8 (1)		7.7 (8)	11.2 (15)			1.2 (1)			0.8 (1)	5.6 (6)	
Hse	1.1 (1)	0.8 (1)	2† (2)	1† (1)	1 (1)	1† (1)	1† (1)	1 (1)	0.7 (1)	1† (1)	1† (1)	
Glu			10.6 (10)	19.0 (16)		5.5 (5)	9.3 (9)			4.7 (4)	7.9 (7)	
Pro	3.0 (3)	0.9 (1)	2.8 (3)	3.0 (3)		1.3 (1)	3.0 (3)			1.2 (1)	5.1 (5)	0.9 (1)
Gly	1.2 (1)		2.2 (2)	5.6 (5)		3.0 (3)				1.3 (3)	5.1 (5)	1.0 (1)
Ala	0.9 (1)		7.9 (8)	9.5 (10)		1.2 (1)	1.1 (1)				8.5 (9)	1.0 (1)
Val	1.0 (1)		1.2 (1)	9.1 (8)		2.0 (2)	2.8 (3)		2.0 (2)		8.6 (9)	1.9 (2)
Ile	1.8 (2)		3.6 (4)	7.6 (8)		0.9 (1)	1.0 (1)			0.9 (1)	5.0 (5)	
Leu			6.7 (7)	14.5 (13)		2.0 (2)	6.5 (7)		1.0 (1)	2.0 (2)	8.1 (8)	
Tyr			1.9 (2)	4.8 (5)		1.7 (2)					1.1 (1)	
Phe			4.0 (4)	9.9 (11)		2.0 (2)				2.0 (2)	6.7 (7)	
Trp‡			1 (1)	2 (2)			1 (1)					
Lys	1.1 (1)		6.0 (6)	16.0 (16)		2.0 (2)	4.4 (4)			1.1 (1)	4.1 (4)	1.0 (1)
His	1.0 (1)		1.2 (1)	1.1 (1)					1.0 (1)		0.9 (1)	
PeCys	1.3 (1)		3.0 (2)	1.9 (2)								0.8 (1)
Arg	1.1 (1)		3.2 (4)	6.3 (6)		2.9 (3)				2.0 (2)	4.9 (5)	1.0 (1)
Total	(17)	(3)	(83)	(148)	(1)	(29)	(33)	(1)	(5)	(18)	(85)	(9)
Position	1–17	18–20	21–103	104–251	252	253–281	282–314	315	316–320	321–338	339–423	424–432

Values in parentheses and positions of each fragment are those from normal antithrombin III (1). PeCys, pyridylethylcysteine.
*Fragment IX-1 (homoserine) was aligned at positions 252 and 315 according to the known sequence (1).
†Values for homoserine are included in those for glutamate due to insufficient separation.
‡Determined from UV absorption spectrum.

The first peak in either chromatogram was a mixture of several peptides, which were further separated as described in *Materials and Methods*. Four peaks were obtained in each chromatogram (Fig. 2*B*). Each of the latter three peaks was found to be identical between the two chromatograms. However, the first peak of either chromatogram, which was mostly due to the Tris buffer used for the tryptic digestion, gave different results from each other on amino acid analyses, both with and without acid hydrolysis of the samples. The first peak obtained from normal antithrombin III gave lysine (T-3) and arginine (T-6) in a 1:1 molar ratio, while that from abnormal antithrombin III gave only lysine (T-3), indicating

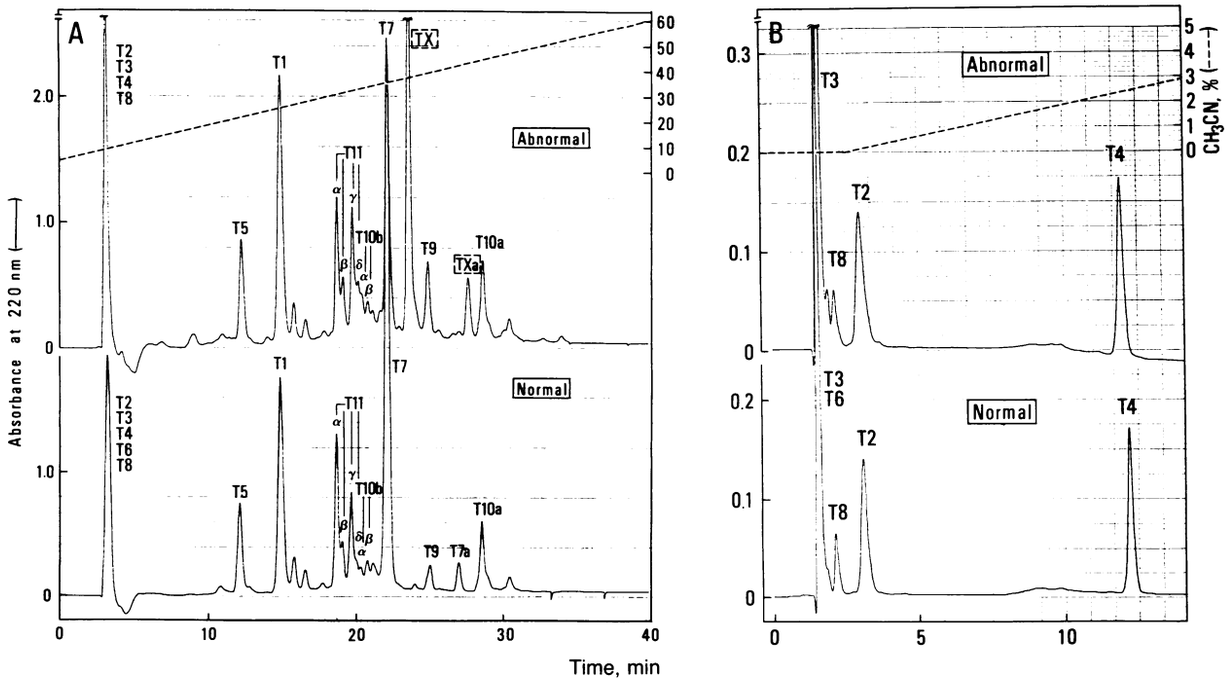

FIG. 2. HPLC analysis of the tryptic peptides derived from the CN-III fragments of abnormal and normal antithrombin III. (*A*) Comparison of the tryptic peptides (1.5 mg each, two runs) from the CN-III fragment of abnormal antithrombin III (upper trace) with those (1.2 mg each, three runs) from the CN-III fragment of normal antithrombin III (lower trace). (*B*) Rechromatography of the first peaks of the traces shown in *A*. Arabic numerals of the tryptic (T) peptides are numbered on the basis of their occurrence in the sequence of the CN-III fragment starting with T-1 at the amino-terminal end. Letters a and b following the peptide number refer to the amino- and the carboxyl-terminal peptides, respectively, derived from the numbered peptide. Greek letters under the same peptide number refer to those peptides having the same amino acid composition but different retention times on HPLC.

Table 2. Amino acid compositions (residues per mol) of tryptic peptides obtained from the CN-III fragment of abnormal antithrombin III

Residue	T-1	T-2	T-3	T-4	T-5	T-6*	T-7a*	T-Xa†	T-7*†	T-X†	T-8	T-9	T-10a	T-10b α	T-10b β	T-11 α	T-11 β	T-11 γ	T-11 δ
Asp				1.0	1.1						1.0	1.1	4.8			2.0	2.0	2.0	2.0
Thr				1.0	1.0							2.1		1.9	1.6	0.9	1.0	1.0	0.9
Ser		1.0		1.1					1.1	1.2	0.8	1.1	1.0	2.0	2.0				
Hse														(1)	(1)	(1)	(1)	(1)	(1)
Glu		1.0		4.0	1.1		1.0	1.1	0.9	1.0		1.1				2.0	2.1	1.9	2.0
Pro		1.0			1.1								1.0						
Gly				1.0												1.0	1.0	1.0	1.1
Ala				1.0	1.0						1.0	1.7		1.7	1.7	1.0	1.0	1.0	0.9
Val							1.1	1.0	1.0	1.0									
Ile	1.0				0.9								1.0	1.2	1.0				
Leu							0.9	1.2	1.1	1.2		1.1	2.0			2.9	2.9	2.8	2.9
Tyr	0.9											0.9							
Phe												2.0	1.1	0.8	1.0				
Trp							(1)	0.8	0.8	0.6									
Lys		1.0	1.0	1.1					1.1	0.9		1.0		1.1	1.0				
His												0.8							
PeCys	0.9							0.9		0.9						0.9	0.8	1.0	0.7
Arg	1.1				1.0	1.0					1.2								
Total	4	4	1	10	7	1	4	5	6	7	4	13	11	10	10	12	12	12	12
Yield, nmol	72	64	71	53	71			15		46	37	21	45	6	15	32	14	30	9
Position	21–24	25–28	29	30–39	40–46	47	48–51		48–53		54–57	58–70	71–81	82–91	82–91	92–103	92–103	92–103	92–103

The positions of the tryptic peptides derived from fragment CN-III correspond to those taken from amino acid sequence (1, 2).
*Tryptic peptides obtained from fragment CN-III of normal antithrombin III.
†Hydrolysis for 24 hr with 3 M mercaptoethanesulfonic acid.

that free arginine (T-6) was absent from the tryptic peptides of fragment CN-III from abnormal antithrombin III.

Amino Acid Compositions of the Tryptic Peptides Obtained from Fragment CN-III of Abnormal Antithrombin III. The amino acid compositions of the tryptic peptides purified by HPLC are shown in Table 2. HPLC of peptide T-10b gave two peaks, α and β, with the same amino acid compositions, although the reason for this separation is not known. Peptide T-11 gave four peaks, α, β, γ, and δ, on HPLC, having indistinguishable amino acid compositions. The microheterogeneity of the carbohydrate structure involved in this peptide (24) and the two forms of its carboxyl-terminal amino acid (homoserine and its lactone) are most likely as the causes of these multiple peaks for a single peptide. All of these peptides but two could be unambiguously aligned in the known amino acid sequence of the CN-III fragment from normal antithrombin III (Fig. 3). The amino acid compositions of the two unique peptides, T-X and T-Xa, isolated only from abnormal antithrombin III differed from those of peptides T-7 and T-7a, respectively, from normal antithrombin III only in having an additional PeCys residue.

Amino Acid Sequences of Peptides T-X and T-Xa. Purified peptides T-X and T-Xa were subjected to sequence analyses. Five steps of Edman degradation with an average repetitive yield of 82% established the sequence of peptide T-X as PeCys-Val-Trp-Glu-Leu-Ser-Lys. Lysine was placed at the carboxyl terminal from the specificity of trypsin. Four steps of Edman degradation with an average repetitive yield of 70% established the sequence of T-Xa as PeCys-Val-Trp-Glu-Leu, indicating that peptide T-Xa is derived from peptide T-X by the further digestion of Leu-Ser bond with trypsin. There is no such sequence in normal antithrombin III as

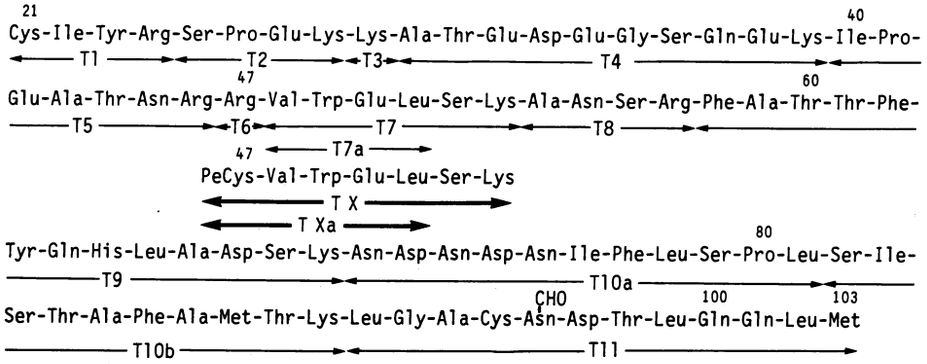

FIG. 3. Alignment of the tryptic peptides in the known amino acid sequence of the CN-III fragment from normal antithrombin III (1, 2). Peptide nomenclature is as Fig. 2. Abnormal peptides are indicated by bold arrows. Residue numbers are those from the amino terminus of normal antithrombin III (1, 2). CHO, carbohydrate attaching to asparagine-96.

that of peptide T-X. However, the sequence Val-Trp-Glu-Leu-Ser-Lys is the same as that of peptide T-7, which corresponds to the valine-48 to lysine-53 sequence in normal antithrombin III. The preceding residue (no. 47) in normal antithrombin III is arginine, which was obtained as peptide T-6 only in the tryptic peptides of CN-III from normal antithrombin III. These results suggest that peptide T-X from abnormal antithrombin III corresponds to peptides T-6 *and* T-7 from normal antithrombin III, thus identifying the replacement of arginine-47 by cysteine in abnormal antithrombin III.

Form of Cysteine-47 in Abnormal Antithrombin III. No free sulfhydryl groups could be detected in abnormal antithrombin III by Ellman's method but a total of 8.2 mol of cysteic acid per mol of protein was found by amino acid analysis when performic acid-oxidized abnormal antithrombin III was directly hydrolyzed in 6 M HCl whereas a total of 7 mol of PeCys was recovered in the cyanogen bromide fragments of abnormal antithrombin III (Table 1). These results suggest that the sulfhydryl group of cysteine-47 in abnormal antithrombin III forms a disulfide bond with 1 mol of free cysteine, since normal antithrombin III contains 6 mol of half-cystine residues as three disulfide bonds. A previous paper from this laboratory reported the same disulfide bond form of a cysteine residue in α_1-antitrypsin (25).

DISCUSSION

In this paper, we present structural evidence for the abnormality of antithrombin III Toyama that caused a Japanese patient to suffer from recurrent thrombophlebitis. In the proposed classification of antithrombin III deficiencies (12), antithrombin III Toyama, as described (15), belongs to type III, in which the heparin cofactor activity of antithrombin III is reduced but the progressive antithrombin activity and the plasma level of the antithrombin III antigen are normal. Type III deficiency, therefore, shows the physiological importance of the heparin-binding ability of antithrombin III.

In the present study, we identified the amino acid substitution in antithrombin III Toyama as cysteine for arginine-47 in normal antithrombin III. Since the genetic codon for arginine-47 has been determined as CGT (1), we conclude that the patient's genomic DNA coding antithrombin III has a TGT nucleotide sequence; i.e., the single base mutation of cytosine to thymidine in the 5'-terminal position of the codon for arginine-47 in the patient's genomic DNA is responsible for this hereditary disorder.

The amino acid residues involved in the primary contact site (reactive site) of antithrombin III with proteases in the complex formation have been established as arginine-393 and serine-394 (5, 6), but the amino acid residue(s) involved in the heparin-binding site have not been identified. Antithrombin III Toyama, which has completely lost its heparin-binding ability because of the change of a single amino acid residue in the polypeptide, gives us integral evidence for identification of the amino acid residue that is essential for the binding of antithrombin III with heparin. The present study suggests that arginine-47 in normal antithrombin III is the putative amino acid residue that interacts directly with heparin. Loss of one positive charge at the heparin-binding site because of the arginine-to-cysteine substitution prevents antithrombin III Toyama from ionic binding with heparin through the sulfate or the carboxyl group of the mucopolysaccharide. As shown in the sequence study of peptide T-X and by the known sequence of antithrombin III (Fig. 3), a tryptophan residue is located at position 49, only two residues from arginine-47. This tryptophan-49 may be the one that has been suggested by chemical modification (4) to be involved in the heparin-binding site in antithrombin III.

Recently, we determined the amino-terminal sequence of a plasma histidine-rich glycoprotein that has a heparin-binding ability comparable with that of antithrombin III and showed that the sequence is homologous to the sequence in the amino-terminal region (arginine-24 to arginine-46) of antithrombin III, for which no other homologous proteins could be found in the data base of protein sequences (26). From these observations, we suggested that the heparin-binding sites of the two plasma proteins are located in the amino-terminal portion where the sequences of the two proteins are homologous. The present finding of the arginine-to-cysteine substitution at position 47 from the amino terminus of antithrombin III Toyama, which lacks heparin-binding ability, supports our proposal.

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