preparation of the manuscript.

Registry No. ATPase, 9000-83-3; Ca, 7440-70-2.

References

Adelstein, R. S., & Esienberg, E. (1980) *Annu. Rev. Biochem. 49*, 921–956.
Aksoy, M. O., Mras, S., Kamm, K. E., & Murphy, R. A. (1983) *Am. J. Physiol. 245*, C255–C270.
Bennett, V., Davis, T., & Fowler, W. E. (1982) *Nature (London) 299*, 126–131.
Bretscher, A. (1984) *J. Biol. Chem. 259*, 12873–12880.
Burridge, K., Kelly, T., & Mangeat, P. (1982) *J. Cell Biol. 95*, 478–485.
Chacko, S., & Rosenfeld, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 292–296.
Chatterjee, M., & Murphy, R. A. (1983) *Science (Washington, D.C.) 221*, 464–466.
Dabrowska, R., Sherry, J. M. F., Aromatorio, D., & Hartshorne, D. J. (1978) *Biochemistry 17*, 253–258.
Davies, P. J. A., & Klee, C. B. (1981) *Biochem. Int. 3*, 203–212.
Glenney, J. R., Jr., Glenney, P., & Weber, K. (1982) *J. Biol. Chem. 257*, 9781–9787.
Goodman, S. R., & Zagon, I. S. (1984) *Res. Bull. 13*, 813–832.
Goodman, S. R., Zagon, I. S., & Kulikowshi, R. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7570–7574.
Hoar, P. E., Kerrick, W. G. L., & Cassidy, P. S. (1979) *Science (Washington, D.C.) 204*, 503–506.
Ikebe, M., & Hartshorne, D. J. (1985) *Biochemistry 24*, 2380–2387.
Kakiuchi, S., Sobue, K., Morimoto, K., & Kanda, K. (1982) *Biochem. Int. 5*, 755–762.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Levine, J., & Willard, M. (1981) *J. Cell Biol. 90*, 631–643.
Levine, J., & Willard, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 191–195.
Nelson, W. J., Colaco, C. A. L. S., & Lazarides, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1626–1630.
Ngai, P. K., Carruthers, C. A., & Walsh, M. P. (1984) *Biochem. J. 218*, 863–870.
Nishikawa, M., Hidaka, H., & Aelstein, R. S. (1983) *J. Biol. Chem. 258*, 14069–14072.
Nonomura, Y., & Ebashi, S. (1980) *Biomed. Res. 1*, 1–14.
Palfrey, H. C., Schiebler, W., & Greengard, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3780–3784.
Persechini, A. M., & Hartshorne, D. J. (1981) *Science (Washington, D.C.) 213*, 1383–1385.
Sharma, R. K., Wang, T. H., Wirch, E., & Wang, J. H. (1979) *Adv. Cyclic Nucleotide Res. 10*, 187–198.
Sharma, R. K., Wang, T. H., Wirch, E., & Wang, J. H. (1980) *J. Biol. Chem. 255*, 5916–5923.
Shimo-Oka, T., & Watanabe, Y. (1981) *J. Biochem. (Tokyo) 90*, 1297–1307.
Sobiezek, A., & Small, J. V. (1977) *J. Mol. Biol. 112*, 559–576.
Sobue, K., Kanda, K., Inui, M., Morimoto, K., & Kadiuchi, S. (1982) *FEBS Lett. 148*, 221–225.
Wagner, P. D. (1984) *J. Biol. Chem. 259*, 6306–6310.
Walsh, M. P., Daabrowska, R., Hinkins, S., & Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279–288.
Zot, H. G., & Potter, J. D. (1981) *Prep. Biochem. 11*, 381–395.

Prothrombin Tokushima, a Replacement of Arginine-418 by Tryptophan That Impairs the Fibrinogen Clotting Activity of Derived Thrombin Tokushima†

Toshiyuki Miyata,‡ Takashi Morita,‡ Takashi Inomoto,§ Shigenori Kawauchi,§ Akira Shirakami,§ and Sadaaki Iwanaga*,‡

Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812, Japan, and First Department of Internal Medicine, School of Medicine, The University of Tokushima, Tokushima 770, Japan

Received July 10, 1986; Revised Manuscript Received October 15, 1986

ABSTRACT: Structural studies on a hereditarily abnormal prothrombin, prothrombin Tokushima, have been performed to identify the difference responsible for its reduced fibrinogen clotting activity upon conversion to thrombin. The prothrombin sample used was from a heterozygote but contained exclusively a defective prothrombin molecule, since the patient was heterozygous for both dysprothrombinemia and hypoprothrombinemia. Amino acid sequence analysis of a peptide isolated from a lysyl endopeptidase digest of the abnormal thrombin indicated that Arg-418 (equivalent to Asn-101 in the chymotrypsin numbering system) had been replaced by Trp. This amino acid substitution can result from a single nucleotide change in the codon for Arg-418 (CGG → TGG). The Arg → Trp replacement found in the thrombin portion of prothrombin Tokushima appears to reduce its interaction with various substrates including fibrinogen and platelet receptors and accounts for the recurrent bleeding episode observed in the propositus.

Human prothrombin is a single-chain glycoprotein composed of 579 amino acid residues (Walz et al., 1977; Butokowski et al., 1977; Thompson et al., 1977; Magnusson et al., 1975; Degen et al., 1983). This protein can be divided into three segments, fragment 1 (residues 1–155), fragment 2 (residues 156–271), and prethrombin 2 (residues 272–579). Upon activation by factor Xa, prethrombin 2 is cleaved at an Arg–Ile linkage between residues 320 and 321, resulting in the formation of two-chain α-thrombin (Downing et al., 1975). Human α-thrombin is composed of a small A chain (residues 285–320) and a large B chain (residues 321–579), and these

† This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.
* Address correspondence to this author.
‡ Kyushu University 33.
§ The University of Tokushima.

0006-2960/87/0426-1117$01.50/0

chains are held together by a disulfide bridge. Thrombin belongs to the family of serine proteases, in which the active site catalytic triad and the substrate binding pocket are located in the B chain.

Recently, we identified a Japanese family having an abnormal prothrombin, designated "prothrombin Tokushima" (Shirakami & Kawauchi, 1984). The propositus of this family is a 10-year-old female suffering from a bleeding tendency. She is a double heterozygote transmitted from both the maternal dysprothrombinemia and the paternal hypoprothrombinemia. Subsequently, the prothrombin Tokushima has been purified from the propositus plasma in our laboratory and exhibited the following properties (Inomoto et al., 1986). (i) Prothrombin Tokushima has the same molecular weight as that of normal prothrombin. (ii) Factor Xa catalyzed proteolysis of the abnormal prothrombin in the presence of factor Va, phospholipids, and Ca^{2+} yields a two-chain thrombin having normal-sized fragment 1 and fragment 2. (iii) Thrombin derived from prothrombin Tokushima exhibited 21.5% clotting activity relative to normal thrombin and reduced platelet aggregation activity. (iv) The apparent Michaelis constant of thrombin Tokushima toward Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide is 8-fold larger than that of normal thrombin, and the catalytic rate constant (k_{cat}) is approximately half of the corresponding value of the normal enzyme. (v) Its active site is fully titrable with *p*-nitrophenyl *p'*-guanidinobenzoate. These results strongly suggest that the abnormality in this enzyme resides at a putative substrate binding site and not in the active site of the molecule.

Five of the prothrombin variants have so far been fully purified and characterized. In the case of prothrombin Barcelona (Rabiet et al., 1979) and prothrombin Madrid (Guillin & Bezeaud, 1981), the molecular defect has been shown to be a specific impairment of one of the two peptide bond cleavages catalyzed by factor Xa. Moreover, in the case of prothrombin Quick (Henriksen et al., 1980) prothrombin Metz (Rabiet et al., 1984), and prothrombin Salakta (Bezeaud et al., 1984), the defect has been reported to be in the thrombin portion of the molecule, although none of them has been characterized by the amino acid substitution.

The present work was undertaken to elucidate the structural abnormality of prothrombin Tokushima that would account for the functional impairment of the molecule. The results of our studies indicate that the reduced clotting activity is due to replacement of Arg-418 by Trp in the abnormal prothrombin molecule. We know of no other example of a molecular defect in prothrombin due to an amino acid substitution at a substrate binding site.

Materials and Methods

Preparation of Abnormal Thrombin Tokushima. Prothrombin was purified according to a published method (Morrison & Esnouf, 1973) from plasma samples of the heterozygous individual and from normal pooled plasma that served as a control. The normal and the heterozygote-derived defective prothrombins were each activated by bovine factor Xa in the presence of phospholipids, Ca^{2+}, and bovine factor Va. The resulting thrombins were isolated by CM-Sepharose CL-6B chromatography as described previously (Kawabata et al., 1985).

Lysyl Endopeptidase Peptide Mapping by HPLC.[1] Normal thrombin and thrombin Tokushima were each reduced and S-pyridylethylated (Hermodson et al., 1973). Abnormal thrombin (approximately 5 nmol) was dissolved in 280 μL of 0.5 M Tris-HCl (pH 8.5) containing 6 M guanidine hydrochloride and 10 mM EDTA. Dithiothreitol was added to give a 200-fold molar excess over the concentration of protein disulfide groups. The mixture was then treated with 3 mol of 4-vinylpyridine/mol of dithiothreitol for 90 min at room temperature, dialyzed against water in a Spectrapor 6 dialysis tube (molecular weight cut-off 2000, Spectrum Medical Industries Inc.), and lyophilized.

The S-pyridylethylated abnormal thrombin was suspended in 56 μL of 50 mM Tris-HCl, pH 9.0, containing 8 M urea. After dilution with 56 μL of 50 mM Tris-HCl (pH 9.0), 2.8 μL of lysyl endopeptidase (1 mg/mL) from *Achromobacter lyticus* M497-1 (Wako Pure Chemical Industries, Ltd., Osaka) was added (Masaki et al., 1981) and the digestion performed at 37 °C for 4 h (Srimal et al., 1985). At that point, an equal amount of lysyl endopeptidase was added and incubation continued for an additional 2.5 h. the S-pyridylethylated normal thrombin (19 nmol) was also digested with lysyl endopeptidase at an enzyme:substrate weight ratio of 1:100 for 6.5 h at 37 °C. The digest was stored at −20 °C until use. Each digest was chromatographed on a reverse-phase C18 column (0.4 × 30 cm, Cosmosil 5C18-P; Nakarai Chemicals Ltd., Kyoto), on a Beckman Model 344 HPLC system equipped with a Hitachi Model 655A variable-wavelength detector. HPLC separation of the digest was performed at room temperature with a linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid (Mahoney & Hermodson, 1980) for 80 min and 40–80% acetonitrile in 0.1% trifluoroacetic acid for 20 min at a flow rate of 0.5 mL/min. The effluent was monitored by measuring absorbance at 214 and 280 nm. Those fractions containing several peptides were rechromatographed on the same column with a linear gradient of 0–50% acetonitrile in 10 mM ammonium formate (pH 6.5) for 30 min at a flow rate of 0.5 mL/min (Miyata et al., 1982). The isolated peptides were used for amino acid composition and sequence analyses. Tryptic peptides were fractionated by a different reverse-phase C18 column (0.21 × 15 cm, Chemcosorb 7-ODS-H; Chemco Scientific Co., Ltd., Osaka) with the acetonitrile–trifluoroacetic acid system.

Amino Acid Analysis and Sequence Determination. Samples were hydrolyzed in 5.7 M HCl containing 0.1% phenol or 3 M mercaptoethanesulfonic acid (Penke et al., 1974) in evacuated sealed tubes at 110 °C for either 24 or 36 h. Amino acids were quantitated on a Hitachi 835 automatic analyzer (Spackman et al., 1958). Sequence analysis of the peptide was performed with an Applied Biosystems Model 470 A gas-phase sequencer (Hunkapiller et al., 1983). PTH-amino acids were identified and quantitated by a Spectra-Physics HPLC system with a reverse-phase column, Aquasil SEQ-4(K) (0.46 × 30 cm, Senshu Science Co., Tokyo), and a ternary gradient system at a flow rate of 0.9 mL/min at 48 °C. Buffer A was acetonitrile containing 5% 2-propanol and 2% butyl chloride by volume, buffer B was 40 mM sodium acetate containing 0.1% acetic acid and 2% tetrahydrofuran by volume, and buffer C was 2% tetrahydrofuran–water (v/v). The gradient was composed of a complex linear gradient of 32% of buffer A and 30% of buffer B at 0 min, 49% of buffer A and 30% of buffer B at 8 min, 60% of buffer A and 5% of buffer B at 12 min, 65% of buffer A and 5% of buffer B at 12.1 min, 65% of buffer A and 5% of buffer B at 13 min, and 95% of buffer A and 5% of buffer B at 13.1 min. The 19 PTH-amino acids and byproducts generated during Edman degradation were well resolved under the above conditions. The recovery at each

[1] Abbreviations: PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

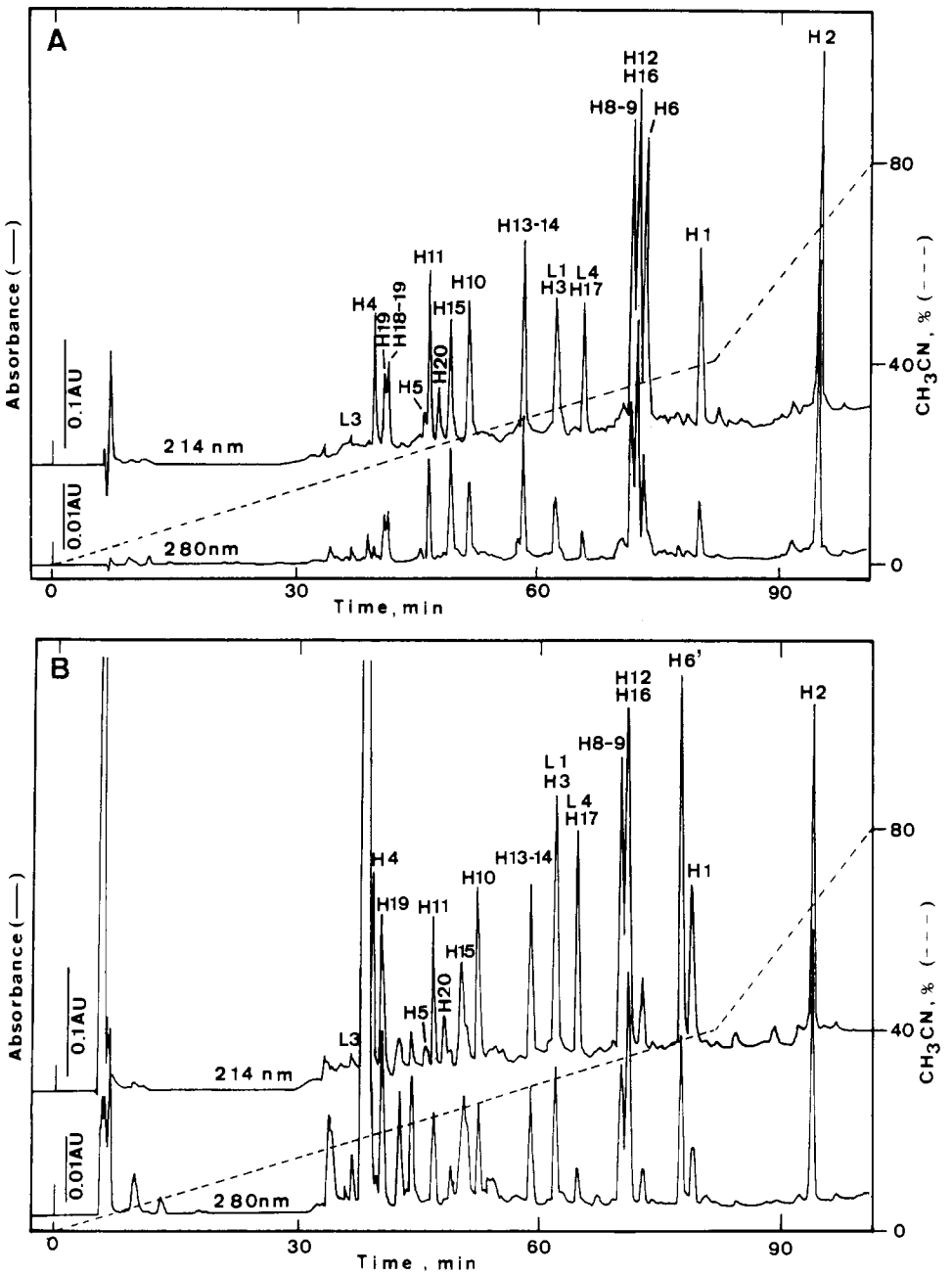

FIGURE 1: HPLC of peptides derived from normal (A) and abnormal (B) thrombin after lysyl endopeptidase digestion. Two-fifth volume each of the lysyl endopeptidase digests (approximately 5 nmol) was applied to a 0.4 × 30 cm Cosmosil 5C18-P column. Peptides derived from thrombin A and B chains were designated L and H, respectively.

stage was quantitated by its peak height with detection at 269 nm, ethyl *p*-hydroxybenzoate being used as an internal standard. PTH-amino acids were also identified by an Applied Biosystems Model 120A on-line PTH-amino acid analyzer.

RESULTS

Lysyl Endopeptidase Peptide Mapping of Abnormal and Normal Pyridylethylated Thrombin. The pyridylethylated thrombins derived from abnormal and normal prothrombins were digested separately with lysyl endopeptidase, and the resulting peptides were fractionated on a reverse-phase HPLC column. The results of the HPLC are shown in Figure 1. The HPLC chromatograms of each digest were very reproducible, and the same elution patterns were obtained for several analyses of the normal thrombin-derived digests. Except for three peaks with retention times of 38, 73 (H6), and 78 min (H6′), the chromatograms of the digests from both normal and abnormal thrombins were not significantly different. The extra peak that eluted in 38 min (Figure 1B) was tentatively identified as 4-vinylpyridine used in the S-alkylation of thrombin, since this peak did not contain any amino acids in the HCl hydrolysate and showed a UV spectrum identical with that of 4-vinylpyridine. On the other hand, peptide H6′ derived from abnormal thrombin showed a 5 min longer retention time than that of the comparable peptide H6 derived from normal thrombin. Moreover, the absorbance intensity ratio of the peptide H6′ at 280 and 214 nm, calculated on the basis of its peak height, was estimated to be 0.0753, whereas that of peptide H6 was 0.0588. This suggested that peptide H6′ contained an additional aromatic amino acid, i.e., tryptophan, tyrosine, or *S*-(pyridylethyl)cysteine. All of the other peptides from both samples were separated as a single peak, except for the peaks eluted in 62, 65, and 71 min. The peptides eluted in these peaks were purified further by rechromatography as described under Materials and Methods. The isolated peptides L1, H3, L4, H17, H12, and H16 from both normal and ab-

Table I: Amino Acid Compositions (Residues per Mole) of Peptides Derived from Pyridylethylated Thrombin Tokushima after Digestion with Lysyl Endopeptidase[a]

amino acid	A chain			B chain														
	L1	L3	L4	H1[b]	H2[b]	H3	H4	H5	H6′	H8-9	H10[b]	H11	H12[b]	H13-14	H15	H16	H17	H19	H20
Asp	1.1	1.0	1.0	1.3	2.3	2.6	1.0		3.6	2.1	1.2	1.0	1.2	3.5	1.9	3.7			1.0
Thr	1.0		0.9		1.0	0.8	0.9			1.0	0.9	1.8		1.6			1.0		
Ser	1.0	1.0	0.9	2.1	2.8		0.9	0.9		1.9			1.0	1.3	1.0	2.2	0.4		
Glu	2.1	1.1	2.7	2.9	2.4	1.1	1.9	1.0	1.2	2.2		1.0	2.9	1.4	1.1	2.4	0.7	0.8	1.8
Pro	1.1			1.3	2.6				1.3	2.6			2.5	1.1	1.0	1.6			
Gly	3.1		1.1	2.2	1.6	1.4	1.4			1.4	3.4	1.1	1.4	2.7	3.9	4.0	1.4		1.1
Ala	1.0			1.2	2.8				1.2	3.5		1.0		1.0	1.0				
Val				0.9[c]	1.0	0.8	0.7			2.1	0.7	0.9	2.0[c]		0.9	1.0	0.8		0.8
Met				1.9				0.4	0.9					0.7	0.8	0.6			
Ile			0.8	1.0[c]	1.1	0.7	0.9	0.9	2.9	0.9			0.4[c]	1.7		0.8		0.7	0.7
Leu	2.0	0.9	1.7	1.2	5.7	1.6		1.0	2.2	2.9	0.9		1.5				1.1		
Tyr			0.9		1.0		0.9		2.0	1.8				1.0		0.9	1.7		
Phe	2.0			0.9		1.1	1.0			1.0				1.1	1.0	1.0	1.6		1.0
Lys	1.0	1.0		0.9	1.0	0.8	0.7	1.0	1.1	1.9	0.9	1.0	1.0	1.7	1.0	0.9	0.8	1.0	
His					0.9				1.0	0.8							0.8		
Trp				1.1	1.6				(2)[d]		1.1	0.4				(2)[d]		0.3	
Pe-Cys	0.8				1.5					0.8			0.5	0.5	0.8	0.7			
Arg	1.0		1.7	1.0	1.1	0.7	2.6		2.1	1.0	0.7		1.0	1.8	1.0	1.9	1.0		
total	17	5	13	21	31	13	13	6	21	29	10	9	20	22	16	24	11	4	7
position	285–301	303–307	308–320	321–341	342–372	373–385	386–397	398–403	404–424	427–455	456–465	466–474	475–494	495–516	517–532	533–556	557–567	569–572	573–579

[a] The positions of each lysyl endopeptidase peptide derived from thrombin correspond to those taken from the amino acid sequence. Pe-Cys is *S*-(pyridylethyl)cysteine. [b] Hydrolysis for 24 h with 3 M mercaptoethanesulfonic acid. [c] Val and Ile contents in the peptides H1 and H12 gave a low value, because of the hydrolysis at 110 °C only for 24 h with 3 M mercaptoethanesulfonic acid. [d] Taken from sequence data.

Table II: Amino Acid Sequence of Peptide H6′ from Thrombin Tokushima

		peptide H6′[a]				
		700 pmol of peptide		300 pmol of peptide		
cycle	position[b]	PTH-amino acid	pmol	PTH-amino acid	pmol	normal peptide[b]
1	404	Ile	226	Ile	71.9	Ile
2	405	Tyr	122	Tyr	39.5	Tyr
3	406	Ile	171	Ile	68.0	Ile
4	407	His	15	X[d]		His
5	408	Pro	102	Pro	45.9	Pro
6	409	Arg	NQ[c]	X		Arg
7	410	Tyr	67	Tyr	40.4	Tyr
8	411	Asn	76	Asn	28.4	Asn
9	412	Trp	14	Trp	8.4	Trp
10	413	Arg	NQ	X		Arg
11	414	Glu	63	Glu	27.7	Glu
12	415	Asn	57	Asn	17.6	Asn
13	416	Leu	77	Leu	25.1	Leu
14	417	Asp	20	Asp	5.7	Asp
15	418	Trp	8	Trp	3.3	Arg
16	419	Asp	16	Asp	8.0	Asp
17	420	Ile	17	Ile	9.2	Ile
18	421	Ala	18	Ala	9.4	Ala
19	422	Leu	17	Leu	8.6	Leu
20	423	Met	12	Met	4.9	Met
21	424	X		X		Lys

[a] Amino acid sequences were analyzed in duplicate. [b] Amino acid sequence from Ile-404 to Lys-424 of normal prothrombin was referred (Butokowski et al., 1977; Degen et al., 1983). [c] NQ, not quantitated. [d] X, unidentified amino acid.

normal thrombins were found respectively to have the same amino acid compositions.

Amino Acid Compositions of Lysyl Endopeptidase Peptides Derived from Abnormal Thrombin. Table I shows the amino acid compositions of all the lysyl endopeptidase derived peptides. The data indicated a high purity for all the peptides and thus made it possible to unambiguously assign their positions in the known amino acid sequence for the A and B chains of human α-thrombin. Among these peptides, peptides H3 from both abnormal and normal thrombins were obtained as two separate peaks in the rechromatograms, although their amino acid compositions were indistinguishable. This microheterogeneity appeared to be due to the carbohydrate structure attached to the Asn-373. The peptides H8-9 and H13-14 contained respectively two lysine residues per mole of peptide, and this is probably due to incomplete hydrolysis of the Lys-427–Pro-428 and Lys-511–Pro-512 peptide bonds. Peptides L2 (Lys), H7 (Leu-Lys), and H18 (Lys) were not identified, as they might be coeluted in the break-through fraction with salts. Peptide H6 isolated from normal α-thrombin consisted of 21 amino acid residues containing one tryptophan and three arginines, as expected from the sequence data (Butokowski et al., 1977; Degen et al., 1983). However, the amino acid composition of peptide H6′ isolated from the abnormal thrombin showed one less arginine than that of peptide H6. To determine the basis for this difference, sequence analysis of the peptide H6′ was performed.

Amino Acid Sequence of Aberrant Peptide H6′ Isolated from Thrombin Tokushima. The sequence of peptide H6′ was determined with duplicate samples by automated Edman degradation. As shown in Table II, peptide H6′ differed by a single amino acid at position 418, an arginine in the normal and a tryptophan in the abnormal. This result indicates that an Arg-to-Trp substitution at position 418 has shifted the retention time from 73 min for the normal peptide H6 to 78 min for the abnormal peptide H6′. To further confirm an

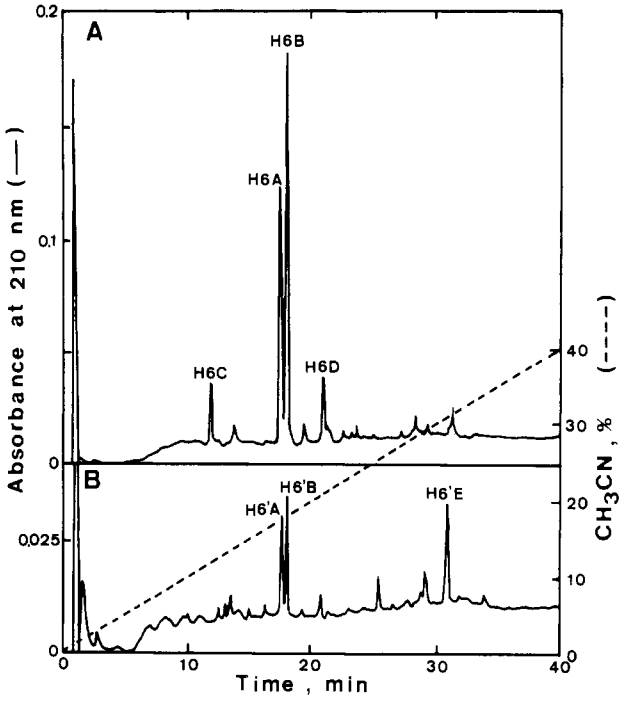

FIGURE 2: HPLC of tryptic peptides derived from the peptides H6 (A) and H6′ (B). The tryptic digests were separately subjected to HPLC on a 0.21 × 15 cm Chemcosorb 7-ODS-H column.

Table III: Amino Acid Sequence of Peptide H6′E from Thrombin Tokushima

cycle	PTH-amino acid	pmol	cycle	PTH-amino acid	pmol
1	Glu	85.1	7	Ile	14.4
2	Asn	69.0	8	Ala	14.3
3	Leu	43.2	9	Leu	10.5
4	Asp	46.8	10	Met	1.5
5	Trp	28.9	11	Lys	1.3
6	Asp	30.0			

Arg-to-Trp substitution at position 418, peptides H6 and H6′ were digested separately with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin and the digests subsequently separated by HPLC. As shown in Figure 2, four major peaks were obtained from the normal peptide H6, designated peptides H6A, H6B, H6C, and H6D. From the amino acid compositions, these peptides were unambiguously assigned respectively to the positions of 404–409 (H6A), 410–413 (H6B), 414–418 (H6C), and 419–424 (H6D) (data not shown). A clear difference in the digest of the aberrant peptide H6′ with that of peptide H6 was the absence of peptides H6C and H6D and the appearance of a new peak with retention time at 31 min, designated H6′E. The entire sequence of this peptide H6′E was established and corroborated our initial findings demonstrating an Arg-to-Trp substitution at position 418, as shown in Table III.

Discussion

In this work, we have successfully used the lysyl endopeptidase digestion of S-pyridylethylated thrombin Tokushima to elucidate its structural abnormality. This enzyme is known to have a strict specificity toward Lys residues at pH 9.0 and is very stable even in the presence of 4 M urea. This strategy made it possible to digest exhaustively the normal and abnormal thrombin molecules and to obtain reproducible HPLC chromatograms for comparison. Peptide mappings and subsequent amino acid analyses for various peptides derived from the normal and abnormal thrombins have identified 98.6% of the entire amino acid residues of thrombin molecule, missing only Lys and Leu-Lys. Some earlier discrepancies in the amino acid sequence of human prothrombin, which was determined respectively by amino acid (Walz et al., 1977; Butkowski et al., 1977; Thompson et al., 1977) and nucleotide sequencing techniques (Degen et al., 1983), have now been resolved. Our present data based on the amino acid composition of lysyl endopeptidase derived peptides from normal and abnormal thrombins are in good agreement with the amino acid sequence predicted from the nucleotide sequence with the exception of the amide assignment. Peptides H2, H6, and H15 contain the active site catalytic triad at positions corresponding to His-57, Asp-102, and Ser-195 of α-chymotrypsin, and peptide H15 contains the aspartate residue corresponding to Ser-189 in the α-chymotrypsin substrate binding site. Peptide H1 contains the NH_2-terminal Ile corresponding to Ile-16 of α-chymotrypsin, which participates in the formation of an ion pair with Asp-194. Of these peptides, the retention times of peptides H2, H15, and H1 derived from abnormal thrombin on HPLC are indistinguishable from those of normal thrombin, and their amino acid compositions are consistent with the known amino acid sequence. These results, together with the identification of Asp-419 (equivalent to Asp-102 in α-chymotrypsin), indicate that all of the active site residues in the abnormal molecule are intact. Collectively, the data described thus far make it very unlikely that the molecular abnormality of prothrombin Tokushima is due to replacement of any amino acid residue associated directly with the active site catalytic triad or the substrate binding pocket. The identity of the COOH-terminal peptide, H20, in the normal and abnormal thrombins also rules out any mutation that modifies the structure of the COOH terminus, such as a frameshift or premature chain termination mutation. Therefore, the replacement of Arg by Trp at position 418 from the NH_2 terminus of abnormal prothrombin appears to be responsible for the observed decrease of its functional activity.

Since the genetic codon for Arg-418 has been determined as CGG (Degen et al., 1983), we conclude that the patient's genomic DNA encoding prothrombin has a TGG nucleotide sequence; i.e., the single base mutation of cytosine to thymidine in the 5′-terminal position of the codon for Arg-418 is responsible for this hereditary disorder. This type of nucleotide mutation is not surprising, since the CpG dimer is the major site of methylation of human DNA. The CpG dimer seems to be a mutational "hot spot", due to subsequent deamination of methylcytosine to thymidine, leading to a C to T transition (Barker et al., 1984). In fact, there are some examples in inherited disorders of blood coagulation factors explained by this transition in the genetic loci associated with the defective molecule, and these include antithrombin III (Koide et al., 1984), some cases of factor VIII (Gitshier et al., 1985), and abnormal fibrinogen (Henschen et al., 1984).

Arg-418 (equivalent to Asn-101 in α-chymotrypsin) in the thrombin molecule is not conserved in other serine proteases including the blood coagulation factors. On the basis of the sequence comparisons of trypsin, α-chymotrypsin, thrombin, factor Xa, and factor IXa, Furie et al. (1982) have recently reported that there are seven well-preserved regions and six variable regions predicted from the sequence comparisons of these five enzymes. They have also postulated that one of the variable regions, variable region 2, is prominent and substantial in terms of surface area and probably represents an area of the thrombin surface that is involved in protein substrate recognition. The variable region 2 corresponds to the residues from 365 to 418 in human thrombin. This part of the molecule

contains not only Asn-373 covalently attached to the carbohydrate but also Arg-382 and Arg-393, which are autocatalytically cleaved in the conversion of α-thrombin to β-thrombin (Boissel et al., 1984). As is well-known, β-thrombin is unable to transform fibrinogen to fibrin although it retains full hydrolytic activity toward synthetic ester substrates, such as $N\alpha$-tosylarginine methyl ester. The site of the amino acid substitution identified in prothrombin Tokushima, Arg-418, corresponds to the carboxyl-terminal residue of the variable region 2, suggesting that it is one of the sites for substrate recognition. The side-chain group of Arg-418 is also assumed to be located at the external surface of the normal molecule, as inferred from making the reasonable assumption of a polypeptide chain conformation similar to that of α-chymotrypsin (Birktoft & Blow, 1972). Although thrombin Tokushima contains an active site fully titrated with *p*-nitrophenyl *p'*-guanidinobenzoate, it shows reduced fibrinogen clotting activity as described above (Inomoto et al., 1986). These properties are well explained by the fact that the site for interacting with fibrinogen in thrombin Tokushima is impaired by the Arg-418 → Trp replacement, resulting in the partial loss of the clotting activity. This suggests that Arg-418 in the thrombin molecule is important for binding to fibrinogen.

ACKNOWLEDGMENTS

We thank Dr. C. T. Esmon (Thrombosis/Hematology Research Program, Oklahoma Medical Research Foundation, OK) for his kind gift of bovine factor Va and Dr. Y. Sakaki (Kyushu University) for permission to use a gas-phase sequencer. We also thank Chizuko Sueyoshi for amino acid analyses and Mizumo Akiyoshi for her expert secretarial assistance. We greatly appreciate the help of Dr. Walter Kisiel (Deptartment of Pathology and Biochemistry, The University of New Mexico School of Medicine, Albuquerque) in the preparation of the English version of the manuscript.

REFERENCES

Barker, D., Schafer, M., & White, R. (1984) *Cell (Cambridge, Mass.) 36*, 131–138.

Bezeaud, A., Drouet, L., Soria, C., & Guillin, M. C. (1984) *Thromb. Res. 34*, 507–518.

Birktoft, J. J., & Blow, D. M. (1972) *J. Mol. Biol. 68*, 187–240.

Boissel, J. P., Bonniec, B. L., Rabiet, M. J., Labie, D., & Elion, J. (1984) *J. Biol. Chem. 259*, 5691–5697.

Butkowski, R. J., Elion, J., Downing, M. R., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 4942–4957.

Degen, S. J. F., MacGillivray, R. T. A., & Davie, E. W. (1983) *Biochemistry 22*, 2087–2097.

Downing, M. R., Butkowski, R. J., Clark, M. M., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 8897–8906.

Furie, B., Bing, D. H., Feldmann, R. J., Robison, D. J., Burnier, J. P., & Furie, B. C. (1982) *J. Biol. Chem. 257*, 3875–3882.

Gitshier, J., Wood, W. I., Tuddenham, E. G. D., Shuman, M. A., Goralka, T. M., Chen, E. Y., & Lawn, R. M. (1985) *Nature (London) 315*, 427–430.

Guillin, M. C., & Bezeaud, A. (1981) *Ann. N.Y. Acad. Sci. 370*, 414–425.

Henriksen, R. A., Owen, W. G., Neshheim, M. E., & Mann, K. G. (1980) *J. Clin. Invest. 66*, 934–940.

Henschen, A., Kehl, M., & Southan, C. (1984) in *Variants of Human Fibrinogen* (Beck, E. A., & Furlan, M., Eds.) pp 273–320, Hans Huber, Berne.

Hermodson, M. A., Ericsson, L. H., Neurath, H., & Walsh, K. A. (1973) *Biochemistry 12*, 3146–3153.

Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., & Hood, L. E. (1983) *Methods Enzymol. 91*, 399–413.

Inomoto, T., Kawauchi, S., Shigekiyo, T., Hirai, Y., Azuma, H., Shirakami, A., Morita, T., & Iwanaga, S. (1986) *Acta Haematol. Jpn. 49*, 391.

Kawabata, S., Morita, T., Iwanaga, S., & Igarashi, H. (1985) *J. Biochem. (Tokyo) 97*, 1073–1078.

Koide, T., Odani, S., Takahashi, K., Ono, T., & Sakuragawa, N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 289–293.

Magnusson, S., Petersen, T. E., Sottrup-Jensen, L., & Claeys, H. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B., & Shaw, E., Eds.) pp 123–149, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mahoney, W. C., & Hermodson, M. A. (1980) *J. Biol. Chem. 255*, 11199–11203.

Masaki, T., Fujihashi, T., Nakamura, K., & Soejima, M. (1981) *Biochim. Biophys. Acta 660*, 51–55.

Miyata, T., Iwanaga, S., Sakata, Y., & Aoki, N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6132–6136.

Morrison, S. A., & Esnouf, M. P. (1973) *Nature (London), New Biol. 242*, 92–94.

Penke, B., Ferenczi, R., & Kovács, K. (1974) *Anal. Biochem. 60*, 45–50.

Rabiet, M. J., Elion, J. Benarous, R., Labie, D., & Josso, F. (1979) *Biochim. Biophys. Acta 584*, 66–75.

Rabiet, M. J., Jandrot-Perrus, M., Boissel, J. P., Elion, J., & Josso, F. (1984) *Blood 63*, 927–934.

Shirakami, A., & Kawauchi, S. (1984) *Acta Haematol. Jpn. 47*, 1697–1704.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.

Srimal, S., Miyata, T., Kawabata, S., Miyata, T., & Iwanaga, S. (1985) *J. Biochem. (Tokyo) 98*, 305–318.

Thompson, A. R., Enfield, D. L., Ericsson, L. H., Legaz, M. E., & Fenton, J. W., II (1977) *Arch. Biochem. Biophys. 178*, 356–367.

Walz, D. A., Hewett-Emmett, D., & Seegers, W. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1969–1972.