Prothrombin Salakta: Substitution of Glutamic Acid-466 by Alanine Reduces the Fibrinogen Clotting Activity and the Esterase Activity†

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ABSTRACT: Structural studies on a hereditary abnormal prothrombin, prothrombin Salakta, have been performed to identify the difference responsible for its reduced fibrinogen clotting activity and its reduced esterase activity. Amino acid composition and sequence analyses of a peptide isolated from a lysylendopeptidase digest of the abnormal thrombin indicated that Glu-466 had been replaced by Ala. This amino acid substitution can result from a single nucleotide change in the codon for Glu-466 (GAG → GCG). The model building and the molecular dynamics simulation of thrombin Salakta suggest that the Glu-466 → Ala substitution would change the proper conformation around the substrate binding site containing Trp-468, which is a unique surface loop on the thrombin molecule. This is the experimental and theoretical evidence supporting the role of the surface loop containing Trp-468 for the proper conformation of the substrate binding site.

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). It is the zymogen of the serine protease thrombin, and factor Xa cleaves two peptide bonds, resulting in the formation of α-thrombin (Downing et al., 1975), composed of a small A chain (residues 285–320) and a large B chain (residues 321–579) held together by a disulfide bond.

Five prothrombin variants have been characterized, and a single amino acid mutation was identified in each case. The molecular defects of prothrombin Barcelona (Rabiet et al., 1986) and prothrombin Madrid (Diuguid et al., 1989) have been shown to be a specific impairment of one of the two peptide bond cleavages catalyzed by factor Xa due to the amino acid exchange of Arg-271 to Cys. In the cases of prothrombin Tokushima (Miyata et al., 1987), prothrombin Quick I (Henriksen & Mann, 1988), and prothrombin Quick II (Henriksen & Mann, 1989), the molecular defect has been reported to be in the thrombin portion, namely, Arg-418 → Trp, Arg-382 → Cys, and Gly-558 → Val, respectively. Characterizations of these mutants provided valuable information for the correlation between structure and function of α-thrombin.

We identified a female originating from Tunisia having an abnormal prothrombin, designated "prothrombin Salakta" (Bezeaud et al., 1984). Prothrombin activity of the patient's plasma was 15–18% that of the normal, when measured using either the classical one-stage and two-stage assays or assays with *Echis carinatus* venom or staphylocoagulase, whereas prothrombin antigen was 100%. There was no history of excessive bleeding. The prothrombin Salakta purified from the propositus plasma exhibited the following properties (Bezeaud et al., 1988). (i) Prothrombin Salakta had the same molecular weight as that of normal prothrombin and factor Xa-catalyzed proteolysis was normal. (ii) Thrombin derived from prothrombin Salakta exhibited 17% clotting activity relative to normal thrombin and showed a 44-fold slower activation of factor V and a 3-fold slower activation of protein C either in the absence or in the presence of thrombomodulin. (iii) The apparent Michaelis constant of thrombin Salakta toward S-2238 was 4.4-fold larger than that of normal thrombin, and the catalytic rate constant (k_{cat}) was the same as that of the normal enzyme. (iv) Its active site was fully titrable with *p*-nitrophenyl *p*-guanidinobenzoate. (v) The rate constant for thrombin Salakta inhibition by antithrombin III, diisopropylfluorophosphate, and *N*-tosyl-L-lysyl chloromethyl ketone was 17.5-fold, 2.5-fold, and 3.2-fold lower than that observed for normal thrombin. (vi) The interaction of thrombin Salakta with benzamidine was altered as evidenced by a 12.5-fold increase of inhibition constant. These results strongly suggest that the abnormality in this enzyme resides at or near the primary substrate binding site and not in the active site of the molecule.

The crystallographic structure of a complex formed between α-thrombin and D-Phe-Pro-Arg chloromethyl ketone has been solved at 1.9-Å resolution (Bode et al., 1989). It showed structural similarity to trypsin-like proteases but with two extended insertions on the surface, including Tyr-367(60A)-Pro-368(60B)-Pro-369(60C)-Trp-370(60D)[1] and the enlarged loop around Trp-468(148). These two loops were estimated to be responsible for the strict substrate specificity of α-thrombin. The present study demonstrates that prothrombin Salakta has a substitution of Glu-466(146) by Ala in the thrombin portion. We speculate that the Glu-to-Ala substitution affects the proper conformation of the surface loop

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[1] For comparison, the prothrombin amino acid numbering system has been used. The numbers in parentheses are based on the chymotrypsinogen numbering system shown in Table I of Bode et al. (1989).

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containing Trp-468(148), resulting in the reduced affinity for fibrinogen, antithrombin III, and the chromogenic substrate S-2238.

MATERIALS AND METHODS

Preparation of Abnormal Thrombin Salakta. Prothrombin Salakta was purified from 230 mL of plasma from the propositus, with a yield of 24% (Bezeaud et al., 1984). We also purified prothrombin from normal pooled plasma that served as a control. The purified normal and the abnormal prothrombins were each activated by *E. carinatus* venom (Sigma, St. Louis, MO). The resulting thrombins were then isolated by heparin–agarose chromatography (Pharmacia Fine Chemicals AB, Uppsala, Sweden) (Bezeaud et al., 1984).

Lysylendopeptidase Peptide Mapping by HPLC.[2] Normal thrombin and thrombin Salakta were each reduced and S-pyridylethylated (Hermodson et al., 1973). The S-pyridylethylated protein was dialyzed against water in a Spectrapor 6 dialysis tube (molecular weight cutoff 2000; Spectrum Medical Industries Inc.) and lyophilized. Five nanomoles each of the S-pyridylethylated thrombin and mutant thrombin was dissolved in 75 μL of 50 mM Tris-HCl, pH 9.0, containing 8 M urea. After dilution with 75 μL of 50 mM Tris-HCl, pH 9.0, lysylendopeptidase (Masaki et al., 1981) from *Achromobacter lyticus* M497-1 (Wako Pure Chemical Industries, Ltd., Osaka) was added to an enzyme-to-substrate ratio of 1:100, and the digestion was performed at 37 °C for 17 h (Miyata et al., 1987). Each digest was chromatographed on a reversed-phase C8 column (2.1 × 150 mm, μBondasphere S5, C8; Waters) using a Hitachi Model L-6200 HPLC system equipped with a Hitachi Model L-4000 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 40 min and then increased linearly to 80% acetonitrile in 10 min at a flow rate of 0.2 mL/min. The effluent was monitored by measuring the absorbance at 210 nm. Those fractions containing several peptides were rechromatographed using the same column equilibrated with 10 mM ammonium formate buffer, pH 6.5. A linear gradient of 0–80% acetonitrile in 40 min at a flow rate of 0.2 mL/min was used to separate the peptides (Miyata et al., 1982). One of the fractions was further rechromatographed on a reversed-phase C18 column (2.1 × 150 mm, Chemcosorb 7-ODS-H; Chemco Scientific Co., Ltd., Osaka) with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid for 40 min at a flow rate of 0.2 mL/min.

Amino Acid Analysis and Sequence Determination. Samples were hydrolyzed in 5.7 M HCl containing 2% phenol in tubes sealed under reduced pressure for 20 h at 110 °C. Amino acid analysis of the peptides was performed by reversed-phase HPLC of phenylthiocarbamoyl derivatives using the Pico Tag system (Waters, Millipore Corp., Milford, MA) (Bidlingmeyer et al., 1984). Sequence analyses were performed with an Applied Biosystems Model 477A gas-phase sequencer (Hewick et al., 1981). The PTH derivatives were identified on-line with an Applied Biosystems Model 120A PTH analyzer.

Structural Modeling. The modeling procedure used to predict the tertiary structure of α-thrombin and thrombin Salakta has been described previously (Miyata et al., 1991). The coordinates of a complex formed between human α-thrombin and D-Phe-Pro-Arg chloromethyl ketone at 1.9-Å resolution (Bode et al., 1989) were kindly supplied by Dr. Wolfram Bode, Max Planck Institute. It was used as the basis for the modeling of α-thrombin and thrombin Salakta. To obtain the best overall structural model, the full set of α-thrombin coordinates was subjected to structural energy minimization until the root mean square value of the energy gradients was below 0.1 kcal/(mol Å), using the BIOCES system, version 1.4 (NEC Co., Ltd., Tokyo), and AMBER3.0, Revision A (Singh et al., 1986). The tertiary structure of the mutant thrombin Salakta with Glu → Ala exchange was obtained after structural energy minimization.

The movements of a surface loop containing Glu-466/Ala-466 were calculated by using the molecular dynamics method in the program of AMBER3.0, Revision A. The simulations were performed in the approximation of the united atom force field. Nonbonded interaction energies were included within 10 Å, and the 1.4-nonbonded interaction energies were scaled by 0.5. Although the solvent effect was not explicitly included in the calculation, it was considered implicitly by using a distance-dependent dielectric constant (Kollman et al., 1981) which weakens the electrostatic interaction with the increase of the atomic distance. Each simulation consists of a 6-ps heating process from 100 to 310 K and a 20-ps equilibration process at 310 K. The data collection was done for 20 ps. The molecular dynamics calculations were carried out at a constant temperature of 310 K, and a time step of 2 fs was adapted in the SHAKE procedure (Ryckaert et al., 1977). The coordinates were sampled at 0.2-ps intervals, and the structure was expressed with the wire model in the computer graphics of the BIOCES system. Two peptide regions [Trp-461(141) to Val-479(154) and Cys-551(220) to Gly-555(223)] were chosen to be calculated.

The movements of the loop encompassing Trp-461(141) to Val-479(154) in the normal and in the mutant thrombins simulated by the molecular dynamics were also expressed as the distance between the center of gravity of the indole ring of Trp-468(148) and the α-carbon of Trp-547(215). We have chosen Trp-547(215) to simulate the movement of Trp-468-(148), because it locates at the opposite site over the proposed S1 pocket and it is one of the highly conserved amino acids among serine proteases. Thus, the distance between two Trp residues would reflect the width of the substrate binding site.

RESULTS

Lysylendopeptidase Peptide Mapping of Abnormal and Normal S-Pyridylethylated Thrombin. The pyridylethylated thrombins derived from abnormal and normal prothrombins were digested separately with lysylendopeptidase, and the resulting peptides were separated on a reversed-phase HPLC column. The results of the HPLC are shown in Figure 1. The chromatogram (A) from the mutant thrombin was almost the same as that from the normal control (N), except for one peak that shifted from a retention time of 20 min (B11) in the normal to 19.5 min (B11′) in the abnormal. The aberrant peptide B11′ eluted 30 s faster than the normal peptide B11. All the other peaks from both samples were separated as pure peptides, except for the peaks eluting at 20.5, 29, and 33 min. These peaks were purified further by rechromatography as described under Materials and Methods, and the elution profiles of peptides B5, A1, A4, B8–9, B12, and B16 were indistinguishable from both normal and abnormal thrombins. Peptide B11′ was also rechromatographed using the ammonium formate buffer to confirm its displacement from the normal peptide, B11. It eluted 30 s later than the normal peptide (data not shown).

Amino Acid Compositions of Lysylendopeptidase Peptides Derived from Abnormal Thrombin. Table I shows the

[2] Abbreviations; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

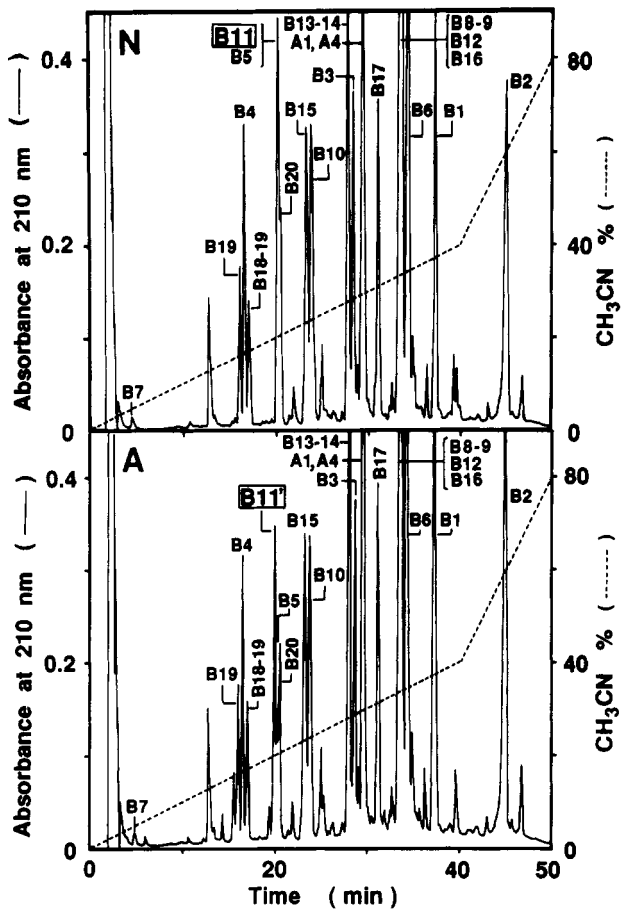

FIGURE 1: HPLC of peptides derived from normal (N) and abnormal (A) thrombin after lysylendopeptidase digestion. Approximately 2.5 nmol was applied to a 0.21 × 15 cm μ-Bondasphere S5C8 column. Peptides derived from thrombin A and B chains were designated A and B, respectively.

amino acid compositions of all the lysylendopeptidase-digested peptides. The data indicated a high purity for all the peptides. This made it possible to unambiguously assign their positions to the known amino acid sequence for the A and B chains of human α-thrombin. The peptides B8–9, B13–14, and B18–19 contained respectively two lysine residues per mole of peptide, and this is probably due to incomplete hydrolysis of the Lys-427–Pro-428, Lys-511–Pro-512, and Lys-568–Trp-569 peptide bonds. Peptides A2 (Lys) and A3 (Ser-Leu-Glu-Asp-Lys) were not identified. Peptide B11 isolated from normal α-thrombin consisted of 9 amino acid residues containing one glutamic acid and one alanine, as expected from the sequence data (Butokowski et al., 1977; Degen et al., 1983). However, the amino acid composition of peptide B11′ isolated from the abnormal thrombin lacked glutamic acid and contained one more alanine than that of peptide B11. To determine the basis for this difference, sequence analysis of peptide B11′ was performed.

Amino Acid Sequence of Aberrant Peptide B11′ Isolated from Thrombin Salakta. The sequence of peptide B11′ was determined by automated Edman degradation. As shown in Table II, peptide B11′ differed by a single amino acid at position 466, where glutamic acid in the normal was replaced by alanine in the abnormal.

DISCUSSION

In this work, we have identified the amino acid substitution of Glu-466 by Ala in the thrombin portion of prothrombin Salakta. Amino acid analyses of peptides generated from lysylendopeptidase digestion of normal and abnormal throm-

Table I: Amino Acid Compositions (Residues per Molecule) of Lysylendopeptidase Peptides Derived from S-Pyridylethylated Thrombin Salakta[a]

amino acid	A chain		B chain																				
	A1	A4	B1[b]	B2	B3	B4	B5	B6	B7	B8–9	B10	B11′	B12[b]	B13–14	B15	B16[b]	B17	B18–19	B19	B20[b]
Asp	1.3 (1)	1.3 (1)	0.9 (1)	2.2 (2)	3.0 (3)	1.1 (1)		4.1 (4)		2.4 (2)	1.2 (1)	1.3 (1)	1.1 (1)	4.3 (4)	2.0 (2)	4.3 (4)				1.1 (1)
Glu	2.1 (2)	3.2 (3)	2.7 (3)	2.1 (2)	1.1 (1)	2.1 (2)	1.1 (1)	1.0 (1)		2.0 (2)		0.0 (0)	3.1 (3)	1.0 (1)	1.0 (1)	2.1 (2)		1.4 (1)	1.1 (1)	2.3 (2)
Ser	0.9 (1)	0.9 (1)	1.7 (2)	2.8 (3)		0.9 (1)	1.0 (1)			1.9 (2)			1.0 (1)	0.9 (1)	0.9 (1)	1.7 (2)				
Gly	2.7 (3)	0.9 (1)	2.1 (2)	1.4 (1)	1.3 (1)					1.0 (1)	3.3 (3)	1.0 (1)	0.9 (1)	1.8 (2)	4.3 (4)	3.6 (4)	1.4 (1)			1.3 (1)
His				1.1 (1)				0.7 (1)		0.8 (1)							1.0 (1)			
Arg	0.9 (1)	1.6 (2)	1.0 (1)	1.0 (1)	1.0 (1)	2.5 (3)		3.0 (3)		1.0 (1)	0.9 (1)		0.9 (1)	2.1 (2)	1.2 (1)	2.0 (2)	1.1 (1)			
Thr	1.1 (1)	1.1 (1)		1.2 (1)	1.1 (1)	1.0 (1)				1.1 (1)	0.9 (1)	2.2 (2)		2.0 (2)			1.1 (1)			
Ala	0.9 (1)		0.9 (1)	2.8 (3)				0.9 (1)		3.7 (4)		1.9 (2)		0.9 (1)	0.9 (1)					
Pro	1.1 (1)		1.0 (1)	3.5 (3)				1.2 (1)		3.3 (3)			3.1 (3)	1.3 (1)	1.2 (1)	1.0 (1)				
Tyr		1.0 (1)		1.1 (1)		1.0 (1)		1.9 (2)		2.0 (2)				1.0 (1)		1.2 (1)	2.0 (2)			
Val			1.8 (2)	1.0 (1)	0.8 (1)					1.8 (2)	0.9 (1)	1.0 (1)	4.6 (5)		0.9 (1)	1.1 (1)	0.9 (1)			1.0 (1)
Met			2.3 (2)				0.9 (1)	0.6 (1)						1.2 (1)	1.1 (1)	0.8 (1)				
PeCys[c]	1.0 (1)			1.9 (2)						0.6 (1)			0.9 (1)	0.6 (1)	1.0 (1)	1.2 (1)				
Ile		1.0 (1)	1.8 (2)	0.8 (1)	0.9 (1)	0.9 (1)	0.9 (1)	2.5 (3)		0.8 (1)			0.9 (1)	1.7 (2)		0.9 (1)		1.0 (1)	0.9 (1)	1.0 (1)
Leu	2.3 (2)	2.3 (2)	0.9 (1)	5.5 (6)	1.8 (2)		1.0 (1)	2.0 (2)	1.0 (1)	2.9 (3)	0.8 (1)		2.4 (2)				1.1 (1)			
Phe	2.1 (2)		0.8 (1)		0.9 (1)					1.0 (1)				1.0 (1)	0.9 (1)	1.1 (1)	1.9 (2)			1.0 (1)
Trp			nd[d] (1)	nd (2)				nd (1)			nd (1)	nd (1)				nd (2)		nd (1)	nd (1)	
Lys	1.0 (1)		1.0 (1)	1.2 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)	1.9 (2)	1.0 (1)	1.0 (1)	1.0 (1)	1.9 (2)	1.0 (1)	1.0 (1)	1.0 (1)	2.0 (2)	1.0 (1)	
Glc NH_2[e]					+															
total	17	13	21	31	13	12	6	21	2	29	10	9	20	22	16	24	11	5	4	7
position	285–301	308–320	321–341	342–372	373–385	386–397	398–403	404–424	425–426	427–455	456–465	466–474	475–494	495–516	517–532	533–556	557–567	568–572	569–572	573–579

[a] The positions of each lysylendopeptidase peptide derived from thrombin correspond to those from the amino acid sequence (Degen et al., 1983). [b] Val and Ile contents were obtained from the 72-h hydrolysates. [c] S-(Pyridylethyl)cysteine. [d] Not determined. [e] Glucosamine.

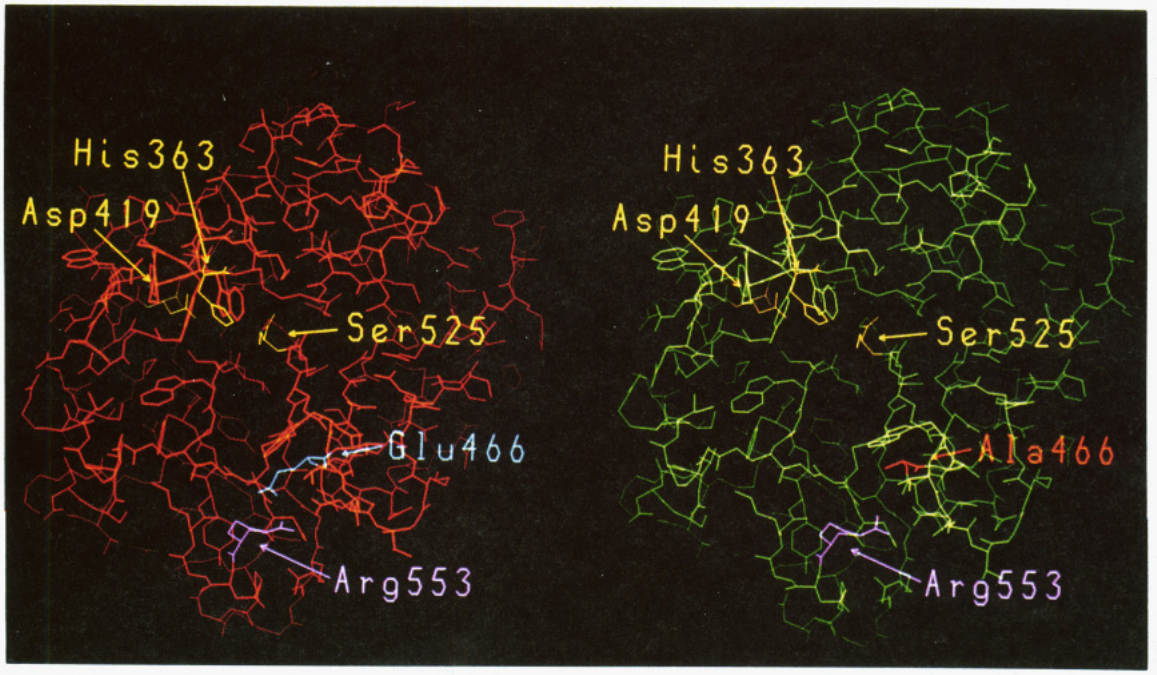

FIGURE 2: Model views showing normal thrombin and thrombin Salakta. (A, left) Model of α-thrombin. Glu-466(146) and Arg-553(221A) must be hydrogen bonded. The active site triad, His-363(57), Asp-419(102), and Ser-525(195), is shown in yellow. (B, right) Putative model of thrombin Salakta. Ala-466(146) and Arg-553(221A) do not form a hydrogen bond.

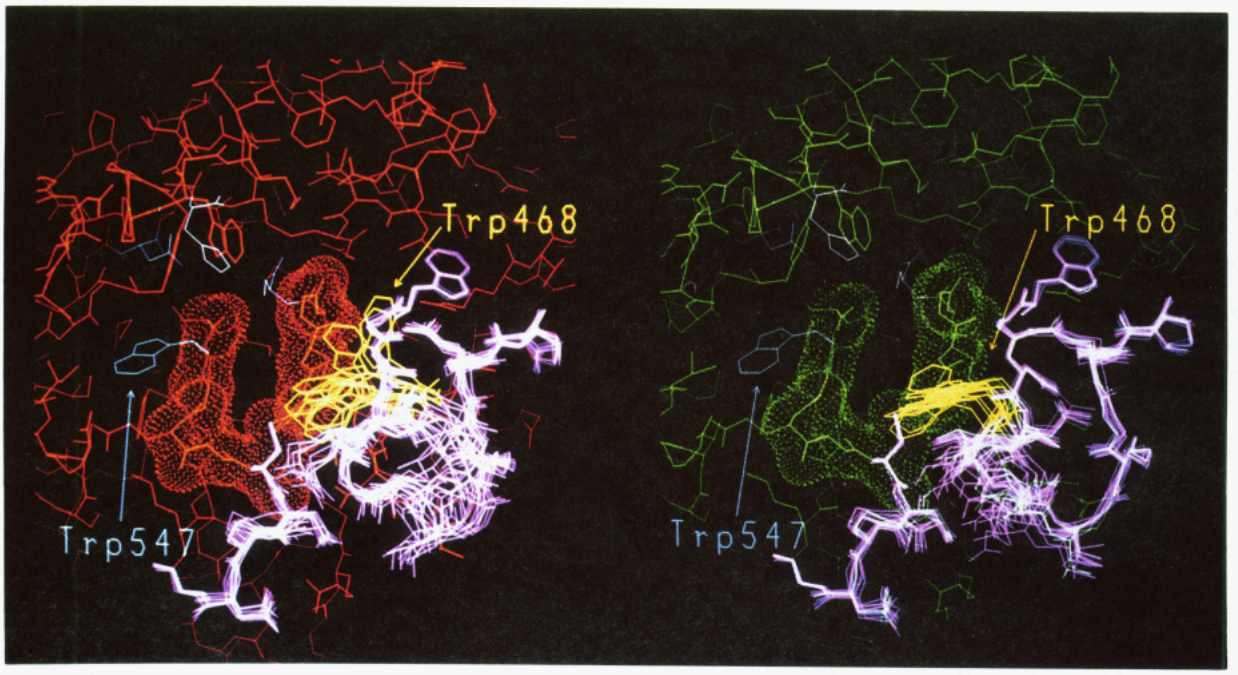

FIGURE 3: Movements of the polypeptide backbone and side chain of Trp-468(148) calculated by molecular dynamics. The movements of polypeptide backbones from Trp-461(141) to Val-479(154) and from Cys-551(220) to Gly-555(223) are shown in pink, and those of the side chain of Trp-468(148) are shown in yellow. The analysis covered a time of 20 ps, and the coordinates were sampled at 0.2-ps intervals. The residues around the proposed S1 pocket [from Cys-521(191) to Asp-524(194) and from Ser-546(214) to Cys-551(220)] are shown with a dot surface of van der Waals radius. Trp-547(215), located at the opposite site of Trp-468(148) over the proposed S1 pocket, is shown in blue. (A, left) α-Thrombin; (B, right) thrombin Salakta.

bins covered 98% of the sequence of the thrombin molecule. The small peptide Ser-Leu-Glu-Asp-Lys (residue 303–307) and Lys-302 in the A chain were not identified. However, these residues were confirmed by sequence analysis of the S-pyridylethylated A chain derived from thrombin Salakta and showed no difference when compared to the sequence of normal thrombin (data not shown). The entire region of the B chain was covered by peptides from the lysylendopeptidase digest. When the peptides derived from the abnormal and normal thrombins were compared to each other, no difference in their retention time on HPLC was observed and their amino acid compositions were consistent with the known protein sequence, except for one peptide (B11/B11′). Sequence analysis revealed that peptides B2, B6, and B15 from thrombin Salakta contain the active site catalytic triad at positions corresponding to His-57, Asp-102, and Ser-195 of α-chymotrypsin, and peptide B15 also contained the aspartate residue corresponding to Ser-189 in the α-chymotrypsin substrate binding site. Peptide B1 was found to contain the NH_2-terminal Ile corresponding to Ile-16 of α-chymotrypsin, which participates in the formation of an ion pair with Asp-524-(194). These results indicate that there is no difference in the

Table II: Amino Acid Sequence of Aberrant Lysylendopeptidase Peptide B11′ Derived from S-Pyridylethylated Thrombin Salakta

cycle	position[a]	PTH amino acid	pmol	normal peptide[a]
1	466	Ala	424	Glu
2	467	Thr	146	Thr
3	468	Trp	135	Trp
4	469	Thr	91	Thr
5	470	Ala	140	Ala
6	471	Asn	91	Asn
7	472	Val	136	Val
8	473	Gly	84	Gly
9	474	Lys	90	Lys

[a] The amino acid sequence from Glu-466 to Lys-474 of normal prothrombin was referred (Degen et al., 1983).

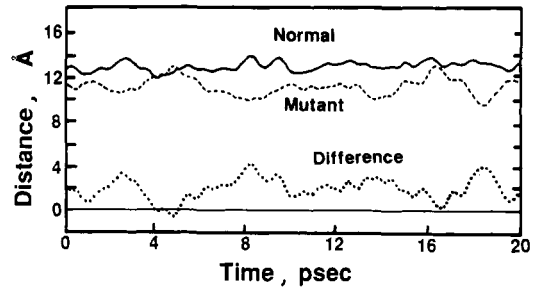

FIGURE 4: Distance between Trp-468(148) and Trp-547(215). The distances between the center of gravity of the indole ring of Trp-468(148) and the α-carbon of Trp-547(215) were calculated by molecular dynamics and sampled at 0.2 ps.

catalytic site residues of the abnormal molecule with those of normal thrombin. Collectively, the data described thus far make it very unlikely that the molecular abnormality of prothrombin Salakta is due to replacement of any amino acid residues associated directly with the active site catalytic triad or the primary substrate binding site. Therefore, the replacement of Glu by Ala at position 466 from the NH_2 terminus of abnormal prothrombin (equivalent to 146 in α-chymotrypsin) appears to be responsible for the observed decrease of its functional activity. Since the genetic codon for Glu-466 has been determined as GAG (Degen et al., 1983), we conclude that the patient's genomic DNA encoding prothrombin has a GCG nucleotide sequence; i.e., the single base mutation of adenine to cytosine in the middle position of the codon for Glu-466 is responsible for this hereditary disorder.

Glu-466(146) in α-thrombin is not a highly conserved residue in serine proteases including trypsin, chymotrypsin, elastase, the blood coagulation factors, and the complement factors, except for factor X, factor XII, prekallikrein, and C1r. Recently, the crystallographic structure of a complex formed between human α-thrombin and D-Phe-Pro-Arg chloromethyl ketone has been solved at 1.9-Å resolution (Bode et al., 1989). It showed structural similarity to trypsin-like proteases but with two extended insertions, including Tyr-367-(60A)-Pro-368(60B)-Pro-369(60C)-Trp-370(60D) and the enlarged loop around Trp-468(148). These two loops, especially the Tyr-Pro-Pro-Trp loop, were estimated to be responsible for the strict substrate specificity.

On the basis of the coordinates of a complex formed between human α-thrombin and D-Phe-Pro-Arg chloromethyl ketone, we have constructed structural models of α-thrombin and thrombin Salakta to estimate the effects of the Glu-466-to-Ala mutation. Figure 2A shows a model view of α-thrombin. Examination of the three-dimensional structure of α-thrombin showed that Glu-466 is present in the surface loop containing Trp-468(148) and is located far away from the active site triad. Therefore, the Glu-466-to-Ala exchange would not physically displace the catalytic site, as was expected from the full titrability of thrombin Salakta with *p*-nitrophenyl *p*-guanidinobenzoate (Bezeaud et al., 1988). The three-dimensional structure also showed that a hydrogen bond between the γ-carboxyl group of the side chain of Glu-466-(146) and the guanidine group of the side chain of Arg-553-(221A) was buried within the normal thrombin molecule and seemed to fix the surface loop around Trp-468(148). However, the hydrogen bond did not form once Glu-466(146) was substituted with Ala in the mutant protein (Figure 2B). So, we speculated at this moment that the movements of the surface loop which contains the mutation site in thrombin Salakta become wider than in the normal due to the lack of the hydrogen bond. However, this assumption turned out to be wrong by means of the molecular dynamics simulation. To examine the effects of the Glu-466-to-Ala substitution on the enlarged surface loop from Trp-461(141) to Val-479(154), the movements of this loop were calculated by molecular dynamics and expressed as the wire model at every 0.2 ps, as shown in Figure 3. The conformations of the main chain calculated in normal thrombin moved widely, but those in the mutant did not move so much. Furthermore, the indole ring of Trp-468(148) moved widely in normal thrombin and at moments looked likely to turn away from the substrate binding site located just next to Trp-468(148). In the case of the mutant, the side chain of Trp-468(148) stayed toward the substrate binding site and did not move to turn away from the substrate binding site. We have performed the molecular dynamics calculation three times and obtained the same conformational state in each case. To know the relative movements of the side chain of Trp-468(148), we calculated the distance between the center of gravity of the indole ring of Trp-468(148) and the α-carbon of Trp-547(215). We have chosen Trp-547(215) to measure the relative movement of Trp-468(148), because it locates at the opposite site over the proposed S1 pocket (Figure 3) and is one of the highly conserved amino acids among serine proteases. The distance between the two Trp residues would reflect the width of the substrate binding site. As shown in Figure 4, the distance calculated between Trp-468(148) and Trp-547(215) in normal thrombin would be approximately 2 Å wider than that of thrombin Salakta. So, we speculated that the width of the substrate binding site of the mutant would be 2 Å narrower. These data would account for the decreased affinity for fibrinogen, antithrombin III, and the chromogenic substrate S-2238.

To explain the conformational change of Trp-468(148) in the mutant, we looked at the microenvironment around the mutation site Ala-466(146) in the thrombin Salakta in detail. As we have pointed out, the normal α-thrombin had the hydrogen bond between the γ-carboxyl group of the side chain of Glu-466(146) and the guanidine group of the side chain of Arg-553(221A), but the mutant did not. Models suggested that thrombin Salakta has a hydrogen bond between the carbonyl group of the main chain of Lys-465(145) and the side chain of Arg-553(221A), but α-thrombin does not (data not shown). The new hydrogen bond could result in the conformational change including the indole ring of Trp-468(148) to disturb the proper conformation of the substrate binding site.

It would be noteworthy to point out that thrombin Salakta shows a 3-fold slower activation of protein C either in the absence or in the presence of thrombomodulin (Bezeaud et al., 1988), which indicates that it correctly binds to thrombomodulin. It is reported that the binding site on α-thrombin for thrombomodulin is located within the sequence between Thr-467(147) and Ser-478(153) of the molecule (Suzuki et al., 1990). Although the mutation site found in thrombin

Salakta is close to the proposed thrombomodulin binding site, the predicted conformational changes that occurred around the substrate binding site in thrombin Salakta would not disturb the binding to thrombomodulin.

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