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The Structure of Residues 7–16 of the Aα-Chain of Human Fibrinogen Bound to Bovine Thrombin at 2.3-Å Resolution*

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The tetradecapeptide Ac-D-F-L-A-E-G-G-G-V-R-G-P-R-V-OMe, which mimics residues 7f–20f of the Aα-chain of human fibrinogen, has been co-crystallized with bovine thrombin from ammonium sulfate solutions in space group $P2_1$ with unit cell dimensions of a = 83.0 Å, b = 89.4 Å, c = 99.3 Å, and β = 106.6°. Three crystallographically independent complexes were located in the asymmetric unit by molecular replacement using the native bovine thrombin structure as a model. The standard crystallographic R-factor is 0.167 at 2.3-Å resolution. Excellent electron density could be traced for the decapeptide, beginning with Asp-7f and ending with Arg-16f in the active site of thrombin; the remaining 4 residues, which have been cleaved from the tetradecapeptide at the Arg-16f/Gly-17f bond, are not seen. Residues 7f–11f at the NH_2 terminus of the peptide form a single turn of α-helix that is connected by Gly-12f, which has a positive ϕ angle, to an extended chain containing residues 13f–16f. The major specific interactions between the peptide and thrombin are 1) a hydrophobic cage formed by residues Tyr-60A, Trp-60D, Leu-99, Ile-174, Trp-215, Leu-9f, Gly-13f, and Val-15f that surrounds Phe-8f; 2) a hydrogen bond linking Phe-8f NH to Lys-97 O; 3) a salt link between Glu-11f and Arg-173; 4) two antiparallel β-sheet hydrogen bonds between Gly-14f and Gly-216; and 5) the insertion of Arg-16f into the specificity pocket. Binding of the peptide is accompanied by a considerable shift in two of the loops near the active site relative to human D-phenyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK)-thrombin.

α-Thrombin (EC 3.4.21.5), is the product of prothrombin cleavage by factor Xa in the final step of the blood clotting cascade. It is a very specific serine protease that cleaves after a limited number of arginine (rarely lysine) bonds in proteins. During clotting, thrombin cuts the fibrinogen Aα-chain and Bβ-chain at Arg-16f[1] and Arg-14f, respectively. Except for a slow cleavage at Arg-19f in the Aα-chain (Blombäck *et al.*, 1967), the other arginine and lysine bonds in the two chains of fibrinogen are unaffected. By contrast, trypsin, which has a similar preference for lysyl and arginyl peptide bonds, cleaves fibrinogen into 100–150 fragments (Blombäck *et al.*, 1967). Thrombin also has a secondary binding site for fibrinogen that is remote from the active site (Noe *et al.*, 1988). The bovine enzyme contains an A-chain of 49 residues (M_r = 5721), which is not required for activity or specificity (Pirkle *et al.*, 1989), linked via a disulfide bond to a B-chain of 259 residues (M_r = 29,683) at residues 1 and 122, respectively (Magnusson *et al.*, 1975; MacGillivray and Davie, 1984). The B-chain contains a carbohydrate chain at Asn-60G and the active site residues His-57, Asp-102, and Ser-195. The thrombin residue numbers in this paper, which are assigned by homology with chymotrypsin (Bode *et al.*, 1989), use uppercase letters for inserted residues. Bovine α-thrombin has 32 residues identical with human α-thrombin in the A-chain and 225 identical residues in the B-chain. Three-dimensional structures of human thrombin complexed with PPACK[2] (Bode *et al.*, 1989) and with hirudin (Rydel *et al.*, 1990; Grütter *et al.*, 1990) have been published recently. A preliminary report of the fibrinopeptide structure discussed in this paper has appeared as an abstract (Martin *et al.*, 1991).

When thrombin reacts with fibrinogen, the first 16 residues of the Aα-chain of fibrinogen are released rapidly as fibrinopeptide A, followed more slowly by fibrinopeptide B, which contains residues 1–14 of the Bβ-chain. The specific interactions that govern the binding of fibrinogen to the active site of thrombin have been explored in considerable detail by determining the conserved sequences in fibrinogen (reviewed by Henschen *et al.*, 1983; Ménaché, 1983) and by active site mapping with synthetic analogs of fibrinopeptide A (reviewed by Blombäck, 1986; Scheraga, 1986). These studies have shown that the first 6 residues of fibrinopeptide A do not interact with thrombin, whereas Asp-7f, Phe-8f, Gly-12f, and Arg-16f are required for maximum binding of the peptide to thrombin.

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[1] Sequence numbers for fibrinopeptide residues are distinguished from those for thrombin residues by the suffix "f." The notation of Schechter and Berger (1967) has been used for the substrate residues and their subsites on thrombin, namely P_n for substrate residues and S_n for their corresponding enzyme subsites on the amino-terminal side of the scissile bond and P'_n and S'_n for the substrate residues and enzyme subsites, respectively, on the carboxyl side of the scissile bond. The scissile bond lies between the P_1 and P'_1 residues. The three separate complexes in the asymmetric unit are numbered I, II, and III and their components as fibrinopeptide I, II, and III and thrombin I, II, and III.

[2] The abbreviations used are: PPACK, D-phenyl-L-prolyl-L-arginyl chloromethyl ketone; Ac, acetyl; OMe, methoxy.

Since Asp-7f and Phe-8f are relatively far in sequence from the scissile bond at Arg-16f, several investigators have proposed that the amino-terminal end of fibrinopeptide A binds to thrombin in a bent configuration with Phe-8f close to Arg-16f (Blombäck *et al.*, 1969; Rae and Scheraga, 1979). Marsh *et al.* (1983) proposed that this hairpin-like structure was stabilized by a salt link between Asp-7f and Arg-19f. More recent studies by Ni *et al.* (1989a, 1989b), using high resolution NMR techniques, have suggested that residues Asp-7f through Glu-11f are in an α-helical structure that is stabilized by a backbone hydrogen bond between Asp-7f and Glu-11f and possibly also by another hydrogen bond between the side chain of Asp-7f and the NH proton of Leu-9f. Ni *et al.* (1989b) also proposed that this turn of helix, together with a multiple-turn structure for residues Glu-11f to Val-15f, group the side chains of Phe-8f, Leu-9f, and Val-15f into a hydrophobic cluster that is a key determinant of the interaction with thrombin.

The crystallographic structure presented in this paper of the complex between bovine thrombin and the decapeptide Ac-D-F-L-A-E-G-G-G-V-R, which contains residues 7f–16f of the fibrinogen Aα chain, has completely defined the structure of the bound peptide and delineated subsites S_9 to S_1 on thrombin. The peptide has the α-helical turn and hydrophobic cluster observed by Ni *et al.* (1989b) but differs significantly in the backbone structure for residues Gly-12f to Arg-16f, which are poorly defined by the NMR data.

MATERIALS AND METHODS

Preparation of Bovine Thrombin—The enzyme was prepared as described previously (Martin *et al.*, 1983) and had approximately 2000 NIH clotting units/mg of protein. It was stored at −20 °C as a precipitate in ammonium sulfate before being set up for crystallization. The synthetic peptide Ac-D-F-L-A-E-G-G-G-V-R-G-P-R-V-OMe (peptide F8 of Ni *et al.*, 1989a), containing residues 7f–20f of the Aα-chain of human fibrinogen or residues P_9 to P'_4 in the notation of Schechter and Berger (1967), was a gift from Harold Scheraga Department of Chemistry, Cornell University, Ithaca, NY.

Crystallization—Crystals of the thrombin/peptide complex were grown by mixing a 5:1 molar ratio of peptide to protein at pH 8.0 and setting the resulting mixture up for crystallization in 20 μl of hanging drops on silanized coverslips inverted over wells containing 40% ammonium sulfate as the precipitant. The crystals belong to space group $P2_1$ with unit cell dimensions a = 83.02 Å, b = 89.41 Å, c = 99.30 Å, β = 106.64°, with three separate complexes of bovine thrombin and the fibrinopeptide in the asymmetric unit. Polyacrylamide electrophoresis (Laemmli, 1970) showed that approximately one-half of the α-thrombin had converted to the β-, ϵ-, and γ-proteolytic derivatives during crystallization (Fig. 1; Edwards *et al.*, 1986). Data were collected on two crystals taken from the same crystallization well using a Siemens area detector with a Rigaku RU200H rotating anode x-ray source and Supper graphite monochromator operated at 40 kV and 70 mA. Crystals were mounted in quartz capillaries without regard to orientation. Three "sweeps," each of which covered 110° in ω with 440 frames of data, were collected for each crystal; the ϕ angle was incremented by 70° between sweeps. Each data frame was collected for 3–4 min to ensure that it contained at least 2 million total counts. The data were merged and scaled with XENGEN (Howard *et al.*, 1987) yielding 176,974 reflections with $I/\sigma > 1.0$ of which 47,459 were unique between 7.0 and 2.3 Å. These data, which represent 78.9% of the possible reflections, had an overall unweighted absolute R_{sym} of 0.075. The fraction of observed reflections for the shells ∞–7.0, 7.0–3.0, 3.0–2.5, 2.5–2.4, and 2.4–2.3 Å were, respectively, 95, 95, 77, 59, and 39%.

Molecular Replacement—The structure was solved by the molecular replacement method (Rossman and Blow, 1962) using the MERLOT programs (Fitzgerald, 1988) with a previously determined structure of free bovine thrombin as the model. Details of the model structure, which was solved in space group $P2_12_12$ using the structure of human thrombin (Bode *et al.*, 1989), will be presented elsewhere. A molecular transform was computed by placing the model structure in a 120-Å P1 orthogonal unit cell. Rotation functions were first calculated with the fast rotation function program CROSUM (Crowther, 1972), using an increment of 2.5° for α, an increment of 5° for β and γ, 4σ data between 7 and 3 Å, and a radius of integration of 17 Å. The calculations were repeated around the major peaks with LATSUM (Lattman and Love, 1970) using an increment of 1° for all angles and 40% of the highest data between 7 and 4 Å. Translation functions (Crowther and Blow, 1967; Tollin *et al.*, 1966) were calculated at 7.0–3.5 Å for the entire unit cell in fractions of 0.02 of a unit cell. The three solutions from the translation function were refined by rigid body R-factor search with RMINIM (Ward *et al.*, 1975) at 7.0–3.5 Å.

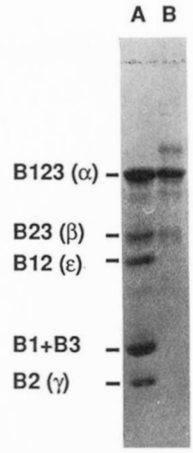

FIG. 1. **Polyacrylamide gel electrophoresis of the thrombin crystal.** A crystal of the fibrinopeptide complex used for data collection was dissolved in 1% sodium dodecyl sulfate sample solution and analyzed in *lane A* on a 17% acrylamide gel under reducing conditions (Laemmili, 1970). *Lane B* shows a crystal of α-thrombin complexed with hirudin. A proteolytic cut at Arg-77A converts the intact B-chain (I16–S247, 259 residues), labeled as *B123* in the figure, which is unique to α-thrombin, into fragment B1 (I16–R77A, 73 residues) and fragment B23 (K78–S247, 186 residues), which is unique to β-thrombin; a proteolytic cut at Thr-149A converts the intact B-chain into fragment B12 (I16–T149A, 150 residues), which is unique to ϵ-thrombin, and fragment B3 (S149B–S247, 109 residues), and cuts at both Arg-77A and Thr-149A convert the intact B-chain into fragment B1, fragment B2 (K78-T149A, 77 residues), which is unique to γ-thrombin, and fragment B3.[3] Fragment B1 exhibits an anomalously high molecular weight, because it carries the polysaccharide chain at Asn-60G.

Structure Analysis—Positions for hydrogens were determined with CHARMM21 and were used to estimate hydrogen bond angles. Our criteria for hydrogen bonds were a distance of 3.35 Å or less between the non-hydrogen donor and acceptor atoms for a standard hydrogen bond, a distance of 3.35–3.5 Å or less for a "long" hydrogen bond and an angle of 110° or greater for the donor-H. . .acceptor atoms (Vinogradov, 1980; Wlodawer and Sjölin, 1983). Surface areas were calculated according to Connolly (1983) in QUANTA using a surface point density of 5.0 and a probe radius of 1.7 Å.

RESULTS

Structure Solution and Refinement—The rotation and translation solutions are listed in Tables I and II. Numerous other rotation function peaks, all of which failed to give a result in the translation function, appeared at 60–70% of the maximum peak height when 7 Å-5 Å or 7 Å-4 Å data were used. Translation functions were also calculated for several peaks that were smaller than those listed in Table I in an attempt to find a putative fourth molecule, but only noise peaks appeared in the maps. The rigid body refinement with RMINIM reduced the R-factor from 0.464 to 0.398.

The first 14 cycles of restrained, least squares refinement with PROFFT (Hendrickson and Konnert, 1979; Agarwal 1978; Finzel 1987) dropped the R-factor from 0.378 at 3.0 Å to 0.294 at 2.3 Å. A $2F_o - F_c$ electron density map revealed

[3] R. Kunjummen, V. Kumar, W. D. Robertson, P. D. Martin, and B. F. P. Edwards, manuscript in preparation.

TABLE I

Rotation function angles

Molecule	Program	Angles			Root mean square units	
		α	β	γ	Height	Above noise
I	CROSUM	153.75	92.20	166.72	6.4	2.2
I	LATSUM	153.31	92.86	164.03		
I	RMINIM	154.31	91.61	165.83		
II	CROSUM	29.25	95.16	274.95	6.3	2.1
II	LATSUM	29.97	95.30	275.70		
II	RMINIM	29.71	95.70	276.40		
III	CROSUM	29.14	86.11	167.53	5.2	1.0
III	LATSUM	31.17	86.05	167.67		
III	RMINIM	27.92	87.75	168.68		

TABLE II

Translation function results

Calculated with 4σ data in the range 7–3.5 Å.

Molecule	U	V	W	Height[a]	Next[b]
1[c]	0.90	0.50	0.12	12.6	5.9
2[c]	0.08	0.50	0.98	21.3	4.9
3[c]	0.92	0.50	0.82	10.9	5.9
1 *vs* 2[d]	0.42	0.72	0.08	16.0	5.5
1 *vs* 3[d]	0.50	0.90	0.66	11.2	4.4
2 *vs* 3[d]	0.08	0.18	0.58	13.9	4.6
1[e]	0.047	0.250	0.436		
2[e]	0.460	0.973	0.510		
3[e]	0.541	0.149	0.090		

[a] Peak height expressed as the number of σ above the mean. In every case, the peaks in this column were the highest peaks in the map.

[b] The next highest peak expressed as the number of σ above the mean.

[c] Self vector calculated by TRNSUM in MERLOT.

[d] Cross-vector calculated by TRNSUM in MERLOT.

[e] Calculated with RMINIM; values given are *x*, *y*, *z*.

density in the active sites of all three complexes in the asymmetric unit. Alternate cycles of refinement followed by graphics intervention allowed us to fit the entire sequence Ac-D-F-L-A-E-G-G-G-V-R-COO in all three molecules. After a total of 48 cycles, the R-factor was 0.246 with root mean square deviations for bonds and ω angles of 0.022 Å and 1.7°, respectively. Water molecules were then added at positions that were within 2.5–3.5 Å of a hydrogen-bonding donor or acceptor and had electron density in both $F_o - F_c$ and $2F_o - F_c$ maps. A total of 97 cycles of PROFFT refinement dropped the R-factor to 0.199 at 7.0–2.3-Å resolution. At this point, the three thrombin molecules were refit manually and further refined with EREF (Jack and Levitt, 1978) to an R-factor of 0.174 with a root mean square of 0.016 on bonds and 9.2° on ω angles, which are not restrained in EREF. Electron density maps phased with this model revealed a new trace for the 145–150 loop in thrombin I that placed Thr-149A and Ser-149B 15 Å apart. Continuing the refinement with PROFFT to cycle 188 reduced the R-factor to 0.172 (Tables III and IV). Additionally, the final 15 cycles of refinement used the values for AFSIG and BFSIG suggested by the program. This produced a σ_A plot (Read, 1986) that placed a least squares line through the origin, giving an estimate for the mean error of the structure of 0.25 Å. Other weighting schemes gave slightly lower R values, but produced σ_A plots that did not go through the origin and had worse estimates for the mean error.

Throughout the refinement, fibrinopeptide I had a higher average temperature factor than either peptide II or III (50 Å^2 *versus* 27 Å^2 and 19 Å^2, respectively). Moreover, Arg-16f in peptide I had a higher average temperature factor than the same residue in fibrinopeptides II or III (40 Å^2 *versus* 13 and 16 Å^2, respectively), although it was tightly constrained in the specificity pocket and pinned by the same H bonds and salt links. Consequently, we used the XPLOR program (Brünger, 1988) to refine only the B values of the three complexes while successively lowering the occupancy of fibrinopeptide I from 1.0 to 0.4 in 0.1 increments. Fifteen cycles of temperature factor optimization were done at each occupancy value using target σs of 1.5 (backbone) and 2.0 (side chain) for 1-2 B factor pairs and 2.0 (backbone) and 2.5 (side chain) for 1-3 angle B-factor pairs. At occupancy values of 0.7, 0.6, and 0.5, the average temperature factor of Arg-16f in peptide I was 21, 16, and 13 Å^2, respectively. An occupancy of 0.6, which made the average temperature factor of Arg-16f in fibrinopeptide I similar to that in fibrinopeptide III, was used for the final model, which had an R-factor of 0.167. At lower occupancies, some of the atoms in Arg-16f of fibrinopeptide I had anomalously low temperature factors.

TABLE III

Refinement statistics from PROFFT

Total number of atoms	8,084
Number of solvent atoms	706
Number of observations	47,459
Mean isotropic B (Å^2)	33.5
Root mean square deviations	
Bond distances (Å)	0.020
Angle distances (Å)	0.037
1–4 distances (Å)	0.037
Bond angles (degrees)	1.1
915 peptide planes (Å)	0.0107
111 aromatic planes (Å)	0.0087
Chiral volume (Å^3)	0.141
Single torsion contacts (Å)	0.213
Multiple torsion contacts (Å)	0.313
Possible (X . . . Y) H bond contacts (Å)	0.305
Planar (ω) torsion angles (degrees)	3.3
Staggered torsion angles (degrees)	21.6
Orthonormal torsion angles (degrees)	27.4
Final R-factor from PROFFT	0.172
R-factor from XPLOR (B refinement only)	0.167

TABLE IV

Final R-factors from PROFFT as a function of resolution

D_{min} Å	Reflections	Shell	Sphere
6.0	1,306	0.234	0.234
4.0	8,320	0.148	0.157
3.5	5,693	0.143	0.152
3.0	9,656	0.170	0.157
2.5	15,792	0.200	0.168
2.4	3,730	0.211	0.170
2.3	2,962	0.220	0.172

Structure of Bovine Thrombin—The three models of bovine thrombin include residues 1H through 247; there is no electron density for the first 13 residues at the amino terminus of the A-chain. The single carbohydrate chain at Asn-60G, which is completely open to solvent in all three structures, also lacks convincing density and has been left out. When human PPACK-thrombin (Bode *et al.*, 1989) and bovine thrombin molecule III are superimposed by a least squares calculation (Rossman and Argos, 1975), the root mean square difference between C_α atoms is 0.41 for 245 coincident residues. The 51 residues which are structurally different include the regions that are not well defined in the electron density maps such as the termini of the A chain and the carboxyl terminus of the B chain. There were some movements of well defined parts of the structure that were greater than 1 Å relative to human PPACK-thrombin, but in all cases they are explained by intermolecular contacts between symmetry related molecules

or by interactions with the fibrinopeptide, or both.

Two chain displacements relative to human thrombin are particularly cogent. The YPPW loop centered at Trp-60D has moved by 0.6 Å in molecule I and 1.4 Å in molecules II and III to more fully cover the Val-15f side chain. Part of these perturbations are due to intermolecular contacts in this region, but we believe that the presence of the entire fibrinopeptide in the active site exerts an additional force to more fully enclose the active site with this loop. The C_α atom of Lys-97 moves 1.5 Å in all three thrombin molecules to make the backbone hydrogen bond between Lys-97 and Phe-10f. This portion of all three molecules is open to solvent and not near any symmetry related molecules.

There are 43 differences between the human and bovine thrombin sequences, most of which are on the surface of the molecule. Some of these sites differ by more than the overall root mean square deviation between the two structures but they all fall in mobile poorly defined sections of the map or are involved in intermolecular or fibrinopeptide contacts. The only residue in direct contact with the fibrinopeptide that differs between bovine and human thrombin is Lys-97, which is arginine in human thrombin.

Structure of the Bound Peptides—Our electron density maps, which show no density following Arg-16f in the specificity pocket for any of the three crystallographically independent peptide structures, confirm that thrombin cleaves the substrate peptide at the Arg-16f/Gly-17f bond (Ni *et al.*, 1989a). The structure of the 10 residues 7f–16f bound to thrombin can be described as one turn of helix, composed of residues Asp-7f to Glu-11f (Fig. 2*A*), linked via Gly-12f and a type I β bend involving residues 9f–12f, to an extended chain composed of Gly-13f to Arg-16f (Fig. 2*B*). The backbone hydrogen bonds (Fig. 3) and main chain torsion angles (Fig. 4) are similar in all three fibrinopeptides. The root mean square deviation in C_α positions are 0.37, 0.44, and 0.29 Å, respectively, between fibrinopeptides I and II, I and III, and II and III.

Thrombin Subsites—The five principal interactions that bind the peptide to thrombin (Fig. 5) are (1) a hydrophobic cage surrounding Phe-8f (Fig. 6), (2) a hydrogen bond linking Phe-8f NH to Lys-97 O, (3) a salt link between Glu-11f and Arg-173 (Table V), (4) two antiparallel β-sheet hydrogen

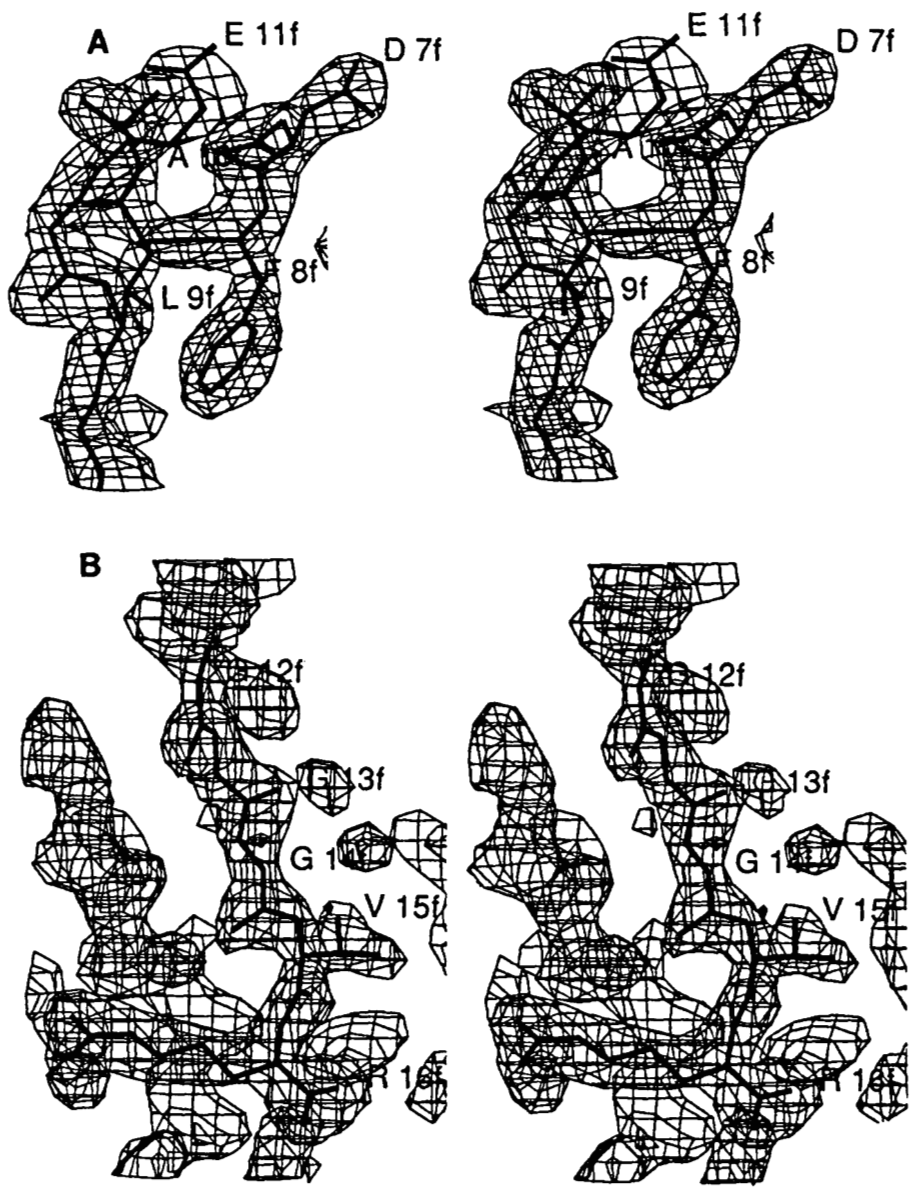

FIG. 2. **Electron density and model for the fibrinopeptide.** The electron density in a $2F_o - F_c$ map contoured at 1.5 σ by the program CHAIN is shown in stereo for residues 7f–11f (*A*) and residues 12f–16f (*B*) of the fibrinopeptide bound to thrombin molecule III. The final refined model is shown in *thick lines*. Thrombin residues have been omitted for clarity.

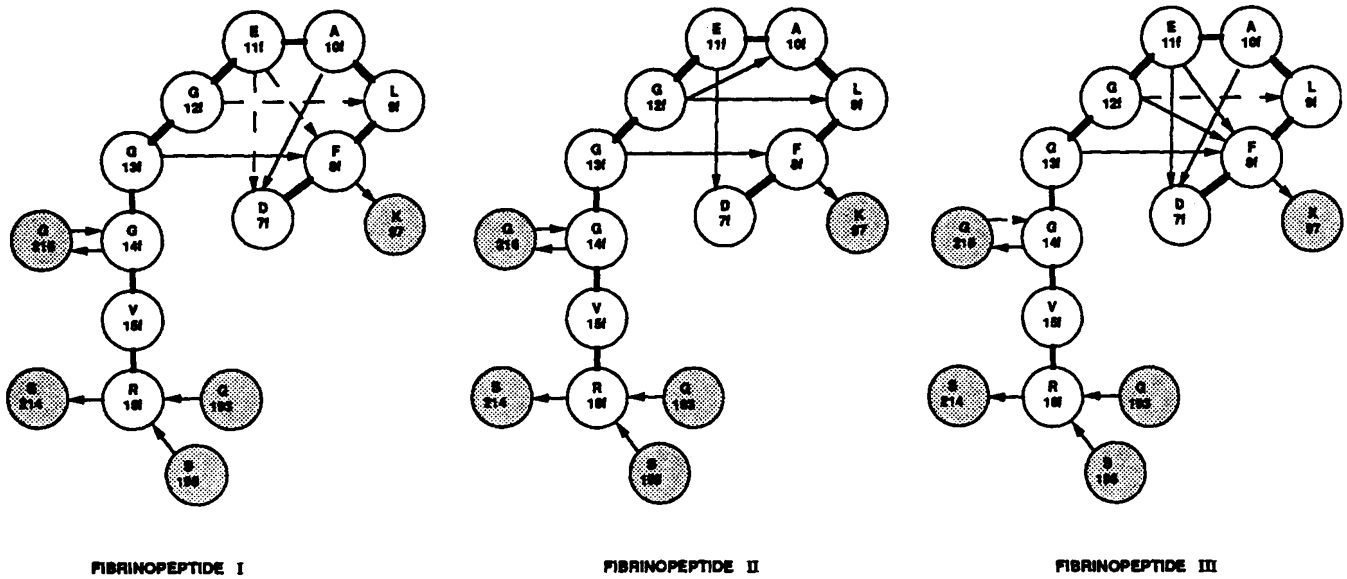

FIG. 3. **Intrapeptide backbone hydrogen bonds.** The backbone hydrogen bonding is slightly different among the three fibrinopeptides (molecule I is ϵ-thrombin, molecules II and III are α-thrombin). The *solid lines* are hydrogen bonds between 2.5 and 3.35 Å long; the *dashed lines* represent bonds that are 3.35–3.5 Å long.

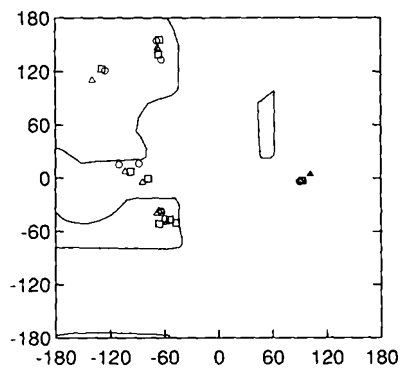

FIG. 4. **Main-chain torsion angles for the three fibrinopeptide molecules.** The ϕ (*abscissa*) and ψ (*ordinate*) angles for fibrinopeptides I (○), II (□), and III (△) are shown relative to the allowed regions. The markers for Gly-12f are *shaded*.

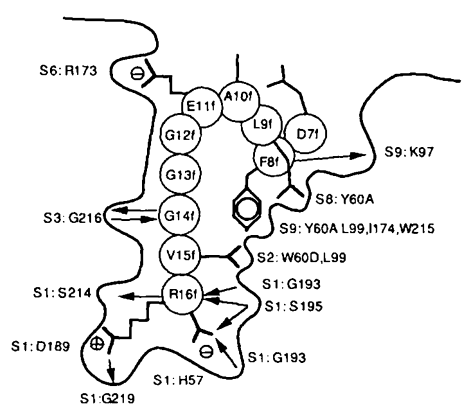

FIG. 5. **Thrombin-fibrinopeptide interactions.** The interactions are shown schematically for subsites S1–S9 on thrombin. *Arrows* indicate hydrogen bonds from donor to acceptor atom.

bonds between Gly-14f and Gly-216, and (5) the insertion of Arg-16f into the specificity pocket (Table V). The subsites may be cataloged as follows.

S1/Arg-16f—The side chain of Arg-16f is bound in the specificity pocket via a salt bridge to the carboxyl group of Asp-189 and a hydrogen bond to the carbonyl oxygen of Gly-219. The mainchain NH atom of Arg-16f makes a hydrogen bond with the carbonyl oxygen of Ser-214, whereas the carboxyl-terminal oxygen atoms of Arg-16f interact with the main chain NH atoms of Gly-193, and Ser-195, the imidazole ring of His-57, and the OG atom of Ser-195.

S2/Val-15f—This residue, which is part of the hydrophobic cage surrounding Phe-8, is sandwiched between Trp-60D and Leu-99.

S3/Gly-14f—This subsite involves two antiparallel β-sheet hydrogen bonds between Gly 14f and Gly-216.

S4/Gly-13f—There is no S4 subsite on bovine thrombin. Gly-13f interacts only with the carbonyl oxygen of Phe-8f and solvent molecules.

S5/Gly-12f—There is no S5 subsite on bovine thrombin. Gly-12f interacts only with solvent.

S6/Glu-11f—There is a salt bridge formed between the side chain carboxyl group of Glu-11f and the guanidinium group of Arg-173.

S7/Ala10f—This residue does not interact with thrombin.

S8/Leu-9f—Leu-9f, which is part of the hydrophobic cage around Phe-8f, is close to Tyr-60A, but is only partially buried, unlike the other hydrophobic groups of the peptide.

S9/Phe-8f—The side chain of Phe-8f is enclosed in a hydrophobic cage (Fig. 6) formed by 4 thrombin residues, namely Tyr-60A, Leu-99, Ile-174, and Trp-215, which form the "apolar binding site" of thrombin (Berliner and Shen, 1977; Bing *et al.*, 1981; Sonder and Fenton, 1984) and 3 fibrinopeptide residues, namely Leu-9f, Gly-13f, and Val-15f. The aromatic ring of Phe-8f is perpendicular to that of Trp-215, an orientation which has been observed in other proteins (Burley and Petsko, 1985).

S10/Asp-7f—There is no interaction between Asp-7f and thrombin in any of the three complexes in the asymmetric unit. In fibrinopeptides II and III, the side chain carboxyl group makes a hydrogen bond with the NH of Ala-10f.

The surface area that is buried when a substrate or inhibitor binds an enzyme is an approximate measure of the contribution of Van der Waals enthalpy and the entropy from released solvent molecules to the binding constant (Chothia, 1974). The surface area buried by the interaction between thrombin

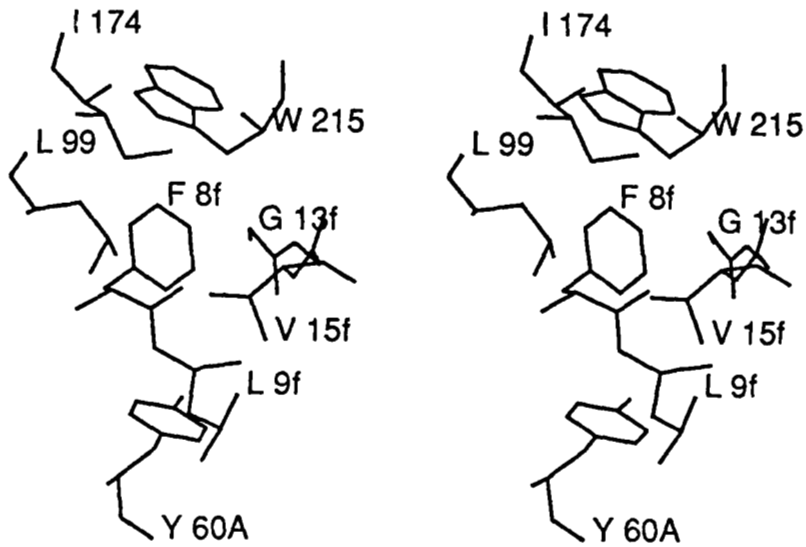

FIG. 6. **The hydrophobic cage for Phe-8f.** The orientation of the 7 hydrophobic residues that surround Phe-8f are shown in stereo for thrombin molecule III.

TABLE V

Peptide/Thrombin ionic interactions

Peptide residue	Atom	Thrombin residue	Atom	Distance		
				Molecule 1	Molecule 2	Molecule 3
					Å	
Glu-11f	OE1	Arg-173	NE	2.85	3.44	
Glu-11f	OE2	Arg-173	NH2		3.48	
Glu-11f	OE2	Lys-109[a]	NZ			2.82
Arg-16f	NH1	Asp-189	OD2	3.27	2.89	3.19
Arg-16f	NH2	Asp-189	OD1	3.17	2.52	2.86
Arg-16f	NH2	Gly-219	O	3.13	3.07	3.08
Arg-16f	O	Ser-195	N	3.14	3.12	3.20
Arg-16f	O	Gly-193	N	2.78	2.99	2.73
Arg-16f	OT	His-57	NE2	3.10	2.78	2.51
Arg-16f	OT	Ser-195	OG	2.82	2.40[b]	

[a] On a symmetry-related molecule.

[b] This short distance is due to the inability of PROFFT to handle the situation in which multiple positioning of a pair of adjacent atoms produces essentially constant electron density between them.

and the 10 residues of the fibrinopeptide is 893 Å^2, which is comparable with the 1022 Å^2 buried in the interaction between trypsin and the 14 residues of bovine pancreatic trypsin inhibitor that actually contact trypsin (Ruehlmann *et al.*, 1973; Janin and Chothia, 1976; Huber and Bode, 1978).

DISCUSSION

Structure of the Thrombin Molecules—Although the three thrombin molecules in the asymmetric unit are crystallographically independent, they have very similar structures, as evidenced by the small root mean square deviations in C_α positions, which are 0.29, 0.30, and 0.32, respectively, between molecules I and II, I and III, and II and III, as determined by program ALIGN (Cohen *et al.*, 1981) for 270 pairs of residues. Molecules I and II are related to one another by a rotation of 116° on an axis almost parallel to the crystallographic *z* axis and molecules I and III by a rotation of 126° on an axis almost coincident with the *y* axis. Molecules II and III, however, are related by a local 2-fold (178°) which is roughly in the *xy* plane. Local differences larger than 0.5 Å occur only for C_α atoms of residues close to intermolecular contacts.

Residues in the flexible insertion loop 145–150, which have high temperature factors and noncontinuous density, exhibit the largest differences between thrombin molecules. In thrombin molecule I, the electron density has been interpreted such that the C_α atoms of Thr-149A and Ser-149B are 15 Å apart, which identifies molecule I as predominantly ϵ-thrombin. Trp-148 in molecule I, which has strong well defined density, is tightly packed between the same loop in molecule II and the region about residues Ile-16 and Gln-239 in a symmetry mate of molecule II. In thrombin molecules II and III, the electron density for the mainchain atoms is well-defined for all the loop residues except 149A, 149B, and 149C in molecule II and 148 through 149C in molecule III. We have assumed that molecules II and III are primarily α-thrombin and fit this region with a closed loop, because polyacrylamide gel electrophoresis indicates that the crystals are approximately one-half α-thrombin (Fig. 1), and because, in contrast to thrombin molecule I, the gap in the electron density can be spanned by the indeterminate residues. The similarity of thrombin I to thrombin molecules II and III everywhere but at the insertion loop 145–150 agrees with the observation that ϵ-thrombin, which has been specifically generated by elastase digestion of α-thrombin (Kawabata *et al.*, 1985), has approximately 60% of the clotting activity and essentially 100% of the esterase and amidase activity of α-thrombin (Brower *et al.*, 1987; Hofsteenge *et al.*, 1988; Brezniak *et al.*, 1990).

Some β- and γ-thrombin, which have a chain break between Arg-77A and Lys-78, are also present in the crystals, albeit at lower concentrations than α- or ϵ-thrombin (Fig. 1). There is no evidence for a chain break at this position in the electron density of thrombin molecules I and II. The main chain electron density for these 2 residues is weaker in thrombin III but still continuous at the peptide bond, although there is a gap at the amide nitrogen of Arg-77A. We have fit this region in thrombin III as an intact loop, that is, as α-thrombin, because it gave the best fit for the existing density.

Structure of the Fibrinopeptide Molecules—Like the thrombin molecules, the three bound peptides in the asymmetric unit are very similar in their overall structure, but do exhibit some differences. All three peptides have the hydrogen bond linking the amide nitrogen of Glu-11f to the carbonyl oxygen of Asp-7f as reported for the structure determined by NMR (Ni *et al.*, 1989a, 1989b), but we do not see the backbone hydrogen bond from Gly-13f to Ala-10f which defines a type II β-turn in the NMR structure. Instead, we see a hydrogen bond from Gly-12f to Leu-9f in a type I β-turn. Also, the side chain carboxyl group of Asp-7f is hydrogen-bonded to the amide of Ala-10f, not Leu-9f as proposed by Ni *et al.* (1989b). We also see five other intrapeptide hydrogen bonds in one or

more of the three fibrinopeptide structures (Fig. 3). Two of these hydrogen bonds, namely A10f → D7f and E11f → F8f also define type I β-turns. Residues Gly-14f and Val-15f are in an extended conformation in the x-ray structures, not a type II′ β-turn as reported in the NMR structure (Ni *et al.*, 1989b; Fig. 7). In the NMR structure calculations, the backbone structure of these residues varied considerably due to limited data and a resonance overlap between Gly-13 and Gly-14 (Ni *et al.*, 1989b).

The temperature factors for the main chain atoms of the three peptides indicated that peptide I, which had an average temperature factor of 50 Å^2, was either disordered or only partially bound relative to peptides II and III. The latter explanation was supported by the fact that the electron density for peptide I was well defined, albeit somewhat weaker than for the other peptides. The discrepancy between the density map and the calculated temperature factors was most striking for Arg-16f in peptide I, which had an average temperature factor of 40 Å^2 when an occupancy of 1.0 was used. An occupancy of 0.6 reduced the average temperature factor for the entire peptide I and its Arg-16f residue to values comparable with those of the other two peptides. In the final refinement, the average temperature factors for all atoms in peptides I, II, and III were 30, 27, and 19 Å^2, respectively. The average temperature factors for the main chain atoms of thrombin molecules I, II, and III were 32, 26, and 39 Å^2, respectively. In the discussion that follows, we have taken complex III as the model because it has the lowest temperature factors for the fibrinopeptide residues.

Thrombin/Fibrinopeptide Interactions—As seen in Fig. 8, the P1–P3 residues of the fibrinopeptide overlap closely with the three residues of PPACK in the structure of human α-thrombin (Bode *et al.*, 1989) and consequently have many of the same interactions with subsites S1–S3. In both structures the guanidino group forms a salt link with Asp-186 and a hydrogen bond with the carbonyl oxygen of Gly-219. Arg-16f has additional interactions with Gly-193, Ser-195, and His-57, thanks to its free carboxyl group, whereas the arginine in PPACK is covalently linked to His-57 and Ser-195. The P2 residue in both complexes, valine in the fibrinopeptide and proline in PPACK, form part of the hydrophobic cluster that surrounds their respective phenylalanine groups. The P3 residue in both complexes forms an antiparallel β-sheet structure with Gly-216. In the fibrinopeptide, these two hydrogen bonds pin one end of the 3 extended glycine residues which have no other specific interactions with thrombin. In the PPACK structure the two hydrogen bonds are augmented by the hydrophobic interactions of the D-phenylalanine side chain. Antiparallel β-sheet hydrogen bonds are often involved in the interaction of serine proteases with peptide ligands or inhibitors (Polgar, 1989 and references therein; Bode and Huber, 1991).

The fact that Gly-12f has torsion angles that are energetically costly for amino acids with a C_β atom explains why glycine is highly conserved in the P5 position. Fibrinogen Rouen, which has a valine in the P5 position, is cleaved more slowly by thrombin (Henschen *et al.*, 1983; Ménaché, 1983, Ni *et al.*, 1989b). Using NMR data, Ni *et al.* (1989a) reported that Gly-12f is involved in a type II β-turn and, along with Gly-13f, has a positive φ torsion angle when the fibrinopeptide was bound to thrombin. They further showed that the G12fV substitution changed the intrapeptide hydrogen bonds and displaced Gly-14f relative to Phe-8f (Ni *et al.*, 1989c). Our work shows that Gly-12f is actually part of a type I β-turn but does confirm a positive φ torsion angle for Gly-12f. However, Gly-13f does not have the positive φ torsion angle as reported by Ni *et al.* (1989b).

The salt bridge between the side chain of Glu-11f in position P6 and the guanidinium group of Arg-173 on thrombin is a previously unsuspected interaction. When Marsh *et al.* (1983) added the 3 residues A10f-E11f-G12f to a synthetic fibrinopeptide, the binding constant with thrombin was actually reduced. In our structure, the alanine in P7 and glycine in P5 have no specific repulsive or attractive interactions with thrombin other than Van der Waals contacts. Evidently, the favorable interaction between Glu-11f and Arg-173 is not strong enough to overcome the entropic effects of adding 3 additional residues which must be part of a turn to bind optimally but lack the intrapeptide interactions needed to stabilize that turn. When the turn is completed by adding the

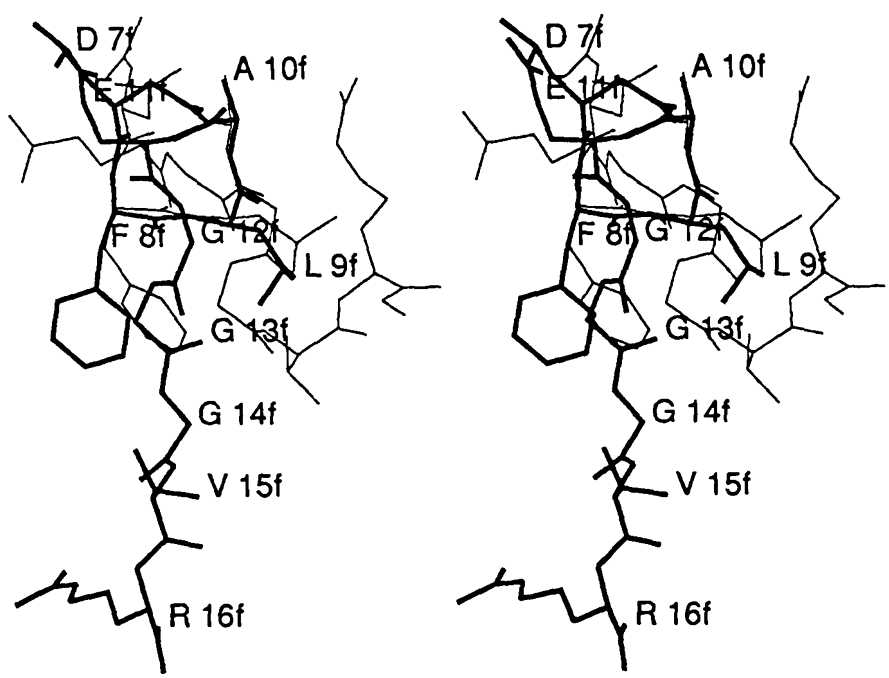

FIG. 7. **Comparison of the fibrinopeptide structures determined by NMR and x-ray crystallography.** The structure of the fibrinopeptide bound to thrombin molecule III is drawn in stereo with *thick lines*. The structure of the fibrinopeptide bound to thrombin as determined by NMR methods (Ni *et al.*, 1989) is shown in *thin lines*. The two structures were overlapped using the main chain coordinates for residues 7f-11f.

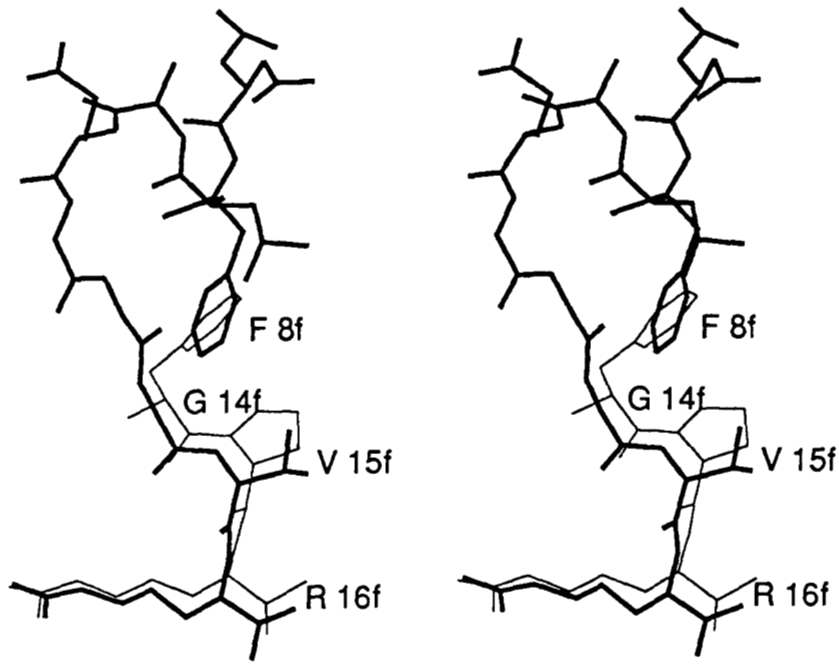

FIG. 8. **Comparison of the fibrinopeptide and PPACK structures.** The structure of the fibrinopeptide bound to thrombin molecule III is drawn in stereo with *thick lines*. The structure of the PPACK residue bound to human thrombin is shown by *thin lines*. The coordinates of PPACK-inhibited human thrombin were overlapped with those of bovine thrombin molecule III.

residues in positions P8 (Leu-9f) and P9 (Phe-8f), the binding constant improves by a factor of 2.3, the catalytic rate by a factor of 36, and as a consequence, the specificity constant by a factor of 83 (Marsh *et al.*, 1983). The fact that Glu-11f is strongly conserved among mammalian fibrinogens, (Henschen *et al.*, 1983; Southern, 1988; Table VI) suggests that this salt link is an important factor in the productive binding of fibrinogen to thrombin in most species but it is not an absolute requirement in the same way that Gly-12 is.

Phe-8f at position P9, which is widely conserved in nature (Blombäck, 1967; Henschen *et al.*, 1983), is responsible for most of the increased binding (Marsh *et al.*, 1983). Its main chain carbonyl atom makes the key hydrogen bond that stabilizes the α-helical loop at the amino-terminal end of the bound fibrinopeptide (Fig. 3), its amide NH makes a strong hydrogen bond to the carbonyl of Lys-97 (Fig. 5), and its phenyl ring anchors a large hydrophobic cluster that includes residues from both the peptide and from thrombin (Fig. 6). This hydrophobic cage, which completely encloses Phe-8f, contributes greatly to thrombin's specificity toward fibrinogen, since it is not accessible in the native structure[4] without a considerable movement of the insertion loop containing Tyr-60A. In human PPACK-thrombin, the phenyl ring of D-Phe in position P3, which occupies the same site as Phe-8f in the fibrinopeptide complex, but enters the site from the opposite direction (Fig. 8), is partially covered by the insertion loop (43 Å^2 accessible to solvent). The binding of Phe-8f also pulls the exposed loop containing Trp-96 and Lys-97 toward the fibrinopeptide-Trp-96 clusters with Pro-60B and Pro-60C, whereas the carbonyl of Lys-97 makes a hydrogen bond with the NH of Phe-8f.

The role of the Asp-7f side chain at position P10 is not as obvious as that of Phe-8f at P9, although fibrinogen Lille, which has asparagine at the P10 position, clots slowly (Morris *et al.*, 1981), and adding aspartic acid to a synthetic fibrinopeptide increases the specificity constant by a factor of 3 (Marsh *et al.*, 1983). Asp-7f has no direct interactions with thrombin in any of the three complexes, although rotation of the side chain of Lys-97, which is an arginine in human thrombin, would allow a salt link with the Asp-7f carboxyl group. Instead, the side chain of Asp-7f hydrogen bonds to the amide of Ala-10f, whereas the main chain carbonyl makes a hydrogen bond with the amide nitrogen of Glu-11f. Together, these two interactions stabilize the helical turn and thus facilitate binding of the fibrinopeptide to thrombin.

Almost all of the known sequences that are homologous to human fibrinopeptide A (Table VI) can be accommodated by our structure without invoking any species-specific changes in the thrombin molecule. The most common substitution at position P10 is glutamic acid, which can salt-link with Lys-97 as easily as the aspartic acid in the human sequence. The position is also exposed enough to accept threonine and serine. Phenylalanine at P9 is conserved in all species but the pronghorn antelope. Position P8 is occupied by a hydrophobic residue in all species but the guinea pig. Position P7, which has the most exposure to solvent, also has the most substitutions. Glutamic acid at P6, which binds with Arg-173 in our structure, is strongly conserved, with alanine appearing in three species and aspartic acid in one. Position P5, which can only accept glycine in our structure, has glycine present in every species but the pronghorn antelope and the lizard. Glycine at P4, which has torsion angles available to other amino acids but no exposure to solvent, is strongly conserved, with substitution by alanine in only three species. An alanine C_β atom at this position in our structure is 2.50 Å from a ring atom of Phe-8f. These three species all have a complementary replacement of Val-15f by glycine that provides the space for the phenylalanine ring to relieve the close contact with Ala-13f. The glycine at P3, which has torsion angles available to other amino acids and significant exposure to solvent, is still strongly conserved but is replaced by larger amino acids in four species. The valine at P2, which is part of the hydrophobic cage around Phe-8f in our structure, is replaced only by isoleucine or smaller hydrophic residues.

Of all the sequences listed in Table VI, only those for the pronghorn, lizard, and lamprey are incompatible with our structure. With a serine at position P9 and a threonine at position P5 as listed in the Swiss Protein Data Bank (entry FIBA$ANTAM), the pronghorn fibrinopeptide A cannot form the hydrophobic cage nor bend its amino terminus back

[4] P. D. Martin, R. Kunjummen, V. Kumar, W. Bode, R. Huber, and B. F. P. Edwards, manuscript in preparation.

TABLE VI

Fibrinopeptide A sequences for positions P10 through P1

Sequences taken from Swiss Protein Data Bank, release 18, Protein Identification Resource Data Bank, release 28, Furlan (1988) and Ebert (1991). Bullets (•) indicate positions that are the same as the human sequence.

Variant or species	No. of cases or species	P10 7f	P9 8f	P8 9f	P7 10f	P6 11f	P5 12f	P4 13f	P3 14f	P2 15f	P1 16f
Human-Lille I	1	N	•	•	•	•	•	•	•	•	•
Human-Rouen I	1	•	•	•	•	•	V	•	•	•	•
Human-Zürich I[a]	14	•	•	•	•	•	•	•	•	•	C
Human-Manchester I[b]	26	•	•	•	•	•	•	•	•	•	H
Human[c]	11	D	F	L	A	E	G	G	G	V	R
Gibbon[d]	15	E	•	•	•	•	•	•	•	•	•
Dog[e]	5	E	•	I	•	•	•	•	•	•	•
Wombat	1	S	•	•	•	•	•	•	•	•	•
White rhinoceros	1	•	•	I	•	•	•	•	•	•	•
Cow	1	•	•	•	T	•	•	•	•	•	•
Horse	1	E	•	•	H	•	•	•	•	•	•
Muntjak	1	E	•	•	T	•	•	•	•	•	•
Kangaroo	1	T	•	I	•	•	•	•	•	•	•
Drill	1	•	•	I	T	•	•	•	•	•	•
Guinea pig	1	E	•	E	•	A	•	•	•	•	•
Reindeer	1	E	•	•	E	A	•	•	•	•	•
Donkey	1	E	•	I	S	•	•	•	•	•	•
Eurasian badger	1	E	•	I	•	•	•	A	V	G	•
American mink	1	E	•	I	•	•	•	A	A	G	•
Rabbit	1	T	•	I	D	•	•	A	T	G	•
Rat	1	E	•	I	E	A	•	•	D	I	•
Pronghorn	1	E	S	•	P	D	T	•	•	A	•
Domestic duck	1	S	•	Q	K	•	•	•	•	•	•
Chicken	1	T	•	E	K	•	•	•	•	G	•
Beaded lizard	1	T	•	E	E	G	H	•	•	•	•
Sea lamprey	1					D	D	I	S	L	•

[a] Also Bergamo I, Geneva I, Hershey II, Homberg II and III, Kawaguchi I, Ledyard I, Leogan I, Metz I, Osaka I, Schwarzach I, Stonybrook I, and Torino I.

[b] Also Amiens I and II, Barcelona II, Bergamo III, Bern II, Bicetre I, Birmingham I, Chapel Hill II, Clermont-Ferrand I, Giessen I, Kendal I, Leitchfield I, Long Beach I, Louisville I, Milan VI, New Albany I, Paris VI, Petoskey I, Sapporo I, Seattle II, Sheffield I, Stonybrook II, Sydney II, White Marsh I.

[c] Also Japanese macaque, Rhesus monkey, red guenon, olive baboon, hamadryas baboon, gelada baboon, baboon, European bison, American elk, gray seal.

[d] Also sheep, goat, barbary sheep, domestic water buffalo, water buffalo, cape buffalo, Arabian camel, sika deer, European moose, llama, mule deer, gazelle, pig, tapir.

[e] Also lion, cat, brown bear, fox.

toward the active site. The serine residue was specifically assigned by Edman sequencing and amino acid analysis. However, positions P8 to P2 were assigned by amino acid composition and homology with other fibrinopeptides (Mross and Doolittle, 1967). Consequently, glycine could be at P5 and the threonine at P3 in the true sequence. The same argument pertains to the histidine at P5 in the lizard sequence which was also assigned only by amino acid compositions and homology (Blombäck and Blombäck, 1968; Furlan, 1988). The sea lamprey fibrinogen A-chain, which has no residues after the P6 position, lacks the interactions with thrombin contributed by the residues in the helical turn of fibrinopeptide A, most especially the hydrophobic interactions of Phe-8f. These drastic differences explain why bovine thrombin cannot release lamprey fibrinopeptide A (Doolittle, 1965).

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REFERENCES

Agarwal, R. C. (1978) *Acta Crystallogr.* **A34,** 791–809.
Berliner, L. J., and Shen, Y. Y. L. (1977) *Biochemistry* **16,** 4622–4626
Bing, D. H., Laura, R., Robison, D. J., Furie, B., Furie, B. C., and Feldman, R. J. (1981) *Ann. N. Y. Acad. Sci.* **370,** 496–510
Blombäck, B. (1967) in *Blood Clotting Enzymology* (Seegers, W. H., ed) pp. 143–215, Academic Press, New York
Blombäck, B. (1986) *Ann. N. Y. Acad. Sci.* **484,** 120–123
Blombäck, B., and Blombäck, M. (1968) in *Chemotaxonomy and Serotaxonomy,* (Hawkes, J. G., ed) pp. 3–20, Academic Press, London
Blombäck, B., Blombäck, M., Hessel, B., and Iwanaga, S. (1967) *Nature* **215,** 1445–1448
Blombäck, B., Blombäck, M., Olsson, P., Svendsen, L., and Åberg, G. (1969) *Scand. J. Clin. Lab. Invest.* **24,** (Suppl. 107) 59–64
Bode, W., and Huber, R. (1991) *Curr. Opin. Struct. Biol.* **1,** 45–52
Bode, W., Mayr, I., Baumann, U., Huber, R., and Stone, S. R. (1989) *EMBO J.* **8,** 3467–3475
Brezniak, D. V., Brower, M. S., Witting, J. I., Walz, D. A., & Fenton, J. W., II (1990) *Biochemistry* **29,** 3536–3542
Brower, M. S., Walz, D. A., Garry, K. E., and Fenton, J. W., II (1987) *Blood* **69,** 813–819
Brünger, A. T. (1988) *J. Mol. Biol.* **203,** 803–816
Burley, S. K., and Petsko, G. A. (1985) *Science* **229,** 23–28
Chothia, C. (1974) *Nature* **248,** 338–339
Cohen, G. H., Silverton, E. W., and Davies, D. R. (1981) *J. Mol. Biol.* **148,** 449–479
Connolly, M. L. (1983) *Science* **221,** 709–713
Crowther, R. A. (1972) in *The Molecular Replacement Method* (Rossmann, M. G., ed) pp. 173–178, Gordon and Breach, New York
Crowther, R. A., and Blow, D. M. (1967) *Acta Crystallogr.* **23,** 544–548
Doolittle, R. F. (1965) *Biochem. J.* **94,** 735–741
Ebert, R. F. (1991) *Index of Variant Human Fibrinogens,* CRC Press, Boca Raton, FL
Edwards, B. F. P., Kumar, V., Bedford, B. A., Martin, P. D., and Kunjummen, R. D. (1986) *Ann. N. Y. Acad. Sci.* **485,** 411–413
Finzel, B. C. (1987) *J. Appl. Crystallogr.* **20,** 53–55
Fitzgerald, P. M. D. (1988) *J. Appl. Crystallogr.* **23,** 544–548
Furlan, M. (1988) in *Fibrinogen, Fibrin Stabilisation, and Fibrinolysis,* (Francis, J. L., ed) pp. 17–64, Ellis Horwood Ltd., Chichester, Great Britain
Grütter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., and Stone, S. R. (1990) *EMBO J.* **9,** 2361–2365
Hendrickson, W. A., and Konnert, J. H. (1979) in *Biomolecular Structure, Conformation, Function and Evolution* (Srinivasan, R., ed) Vol. I, pp. 43–57, Pergamon Press, New York
Henschen, A., Lottspeich, F., Kehl, M., and Southan, C. (1983) *Ann. N. Y. Acad. Sci.* **408,** 28–43
Hofsteenge, J., Braun, P. J., and Stone, S. R. (1988) *Biochemistry* **27,** 2144–2151
Howard, A. J., Gilliland, G. L., Finzel, B. C., Poulos, T. L., Ohlendorf, D. H., and Salemme, F. R. (1987) *J. Appl. Crystallogr.* **20,** 383–387
Huber, R., and Bode, W. (1978) *Accts. Chem. Res.* **11,** 114–122
Jack, A., and Levitt, M. (1978) *Acta Crystallogr.* **A345,** 931–935
Janin, J., and Chothia, C. (1976) *J. Mol. Biol.* **100,** 197–211
Kawabata, S., Morita, T., Iwanaga, S., and Igarashi, H. (1985) *J. Biochem. (Tokyo)* **97,** 325–331
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Lattman, E. E., and Love, W. E. (1970) *Acta Crystallogr.* **26,** 1854–1857
MacGillivray, R. T. A., and Davie, E. W. (1984) *Biochemistry* **23,** 1626–1634
Magnusson, S., Petersen, T. E., Sottrup-Jensen, L., Claeys, H. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B., and Shaw, E., eds) pp. 123–149, Laboratory, Cold Spring Harbor, New York
Marsh, H. C., Jr., Meinwald, Y. C., Thannhauser, T. W., and Scheraga, H. A. (1983) *Biochemistry* **22,** 4170–4174
Martin, P. D., Kumar, V. K., Tsernoglou, D., and Edwards, B. F. P. (1983) *Fed. Proc.* **42,** 1861
Martin, P. D., Robertson, W., and Edwards, B. F. P. (1991) *FASEB J.* **5,** A515
Ménaché, D. (1983) *Ann. N. Y. Acad. Sci.* **408,** 121–129
Morris, S., Denninger, M.-H., Finlayson, J. S., and Menache, D. (1981) *Thromb. Haemostasis* **46,** 104, Abstr. 315

Mross, G. A., and Doolittle, R. F. (1967) *Arch. Biochem. Biophys.* **122,** 674–684
Ni, F., Konishi, Y., Frazier, R. B., Scheraga, H. A., and Lord, S. T. (1989a) *Biochemistry* **28,** 3082–3094
Ni, F., Meinwald, Y. C., Vásquez, and Scheraga, H. A. (1989b) *Biochemistry* **28,** 3094–3105
Ni, F., Konishi, Y., Bullock, L. D., Rivetna, M. N., and Scheraga, H. A. (1989c) *Biochemistry* **28,** 3106–3119
Noe, G., Hofsteenge, J., Rovelli, G., and Stone, S. R. (1988) *J. Biol. Chem.* **263,** 11729–11735
Pirkle, H., Theodor, I., Christofferson, M., Vukasin, P., and Miyada, D. (1989) *Thromb. Res.* **55,** 737–746
Polgar, L. (1989) in *Mechanisms of Protease Action* (Polgar, L., ed) pp. 87–122, CRC Press, Boca Raton, FL
Rae, I. D., and Scheraga, H. A. (1979) *Int. J. Pept. Protein Res.* **13,** 304–314
Read, R. J. (1986) *Acta Crystallogr.* **A42,** 140–149
Ruehlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973) *J. Mol. Biol.* **77,** 417–436
Rossman, M. G., and Argos, P. (1975) *J. Biol. Chem.* **250,** 7525–7532
Rossman, M. G., and Blow, D. M. (1962) *Acta Crystallogr.* **15,** 24–31
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., and Roitsch, C. (1990) *Science* **249,** 277–283
Schechter, J., and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27,** 157–162
Sheraga, H. A. (1986) *Ann. N. Y. Acad. Sci.* **484,** 124–133
Sonder, S. A., and Fenton, J. W., II (1984) *Biochemistry* **23,** 1818–1823
Southern, C. (1988) in *Fibrinogen, Fibrin Stabilization, and Fibrinolysis,* (Francis, J. L., ed) pp. 65–99, Ellis Horwood Ltd., Chichester, Great Britain
Tollin, P., Main, P., and Rossmann, M. G. (1966) *Acta Crystallogr.* **20,** 404–407
Vinogradov, S. N. (1980) in *Molecular Interactions* (Ratajczak, H., and Orville-Thomas, W. J., eds) pp. 179–229, Vol. 2, John Wiley & Sons, New York
Ward, K. B., Wishner, B. C., Lattman, E. E., and Love, W. E. (1975) *J. Mol. Biol.* **98,** 161–177
Wlodawer, A., and Sjölin, L. (1983) *Biochemistry* **22,** 2720–2728