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Analysis of Fibrinogen Aα-fusion Proteins

MUTANTS WHICH INHIBIT THROMBIN EQUIVALENTLY ARE NOT EQUALLY GOOD SUBSTRATES*

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We have examined the interaction of thrombin with fibrinogen Aα chain residues 7–16. Using genetically engineered constructions, we have synthesized in *Escherichia coli* a fibrinogen Aα 1–50 fusion protein and seven mutant proteins with single amino acid substitutions. These are: $Asp^{7} \rightarrow Ala$, $Phe^{8} \rightarrow Tyr$, $Glu^{11} \rightarrow Ala$, $Gly^{12} \rightarrow Val$, $Gly^{13} \rightarrow Val$, $Gly^{14} \rightarrow Val$, and $Arg^{16} \rightarrow Leu$. Competitive immunoassay of cell lysates showed that all the mutations but one, $Arg^{16} \rightarrow Leu$, altered the structure of the protein such that cross-reactivity with the Aα-specific monoclonal antibody, Y18, was significantly reduced. The fusion proteins were purified and analyzed as thrombin inhibitors and substrates. All the fusion proteins are competitive inhibitors of the amidolytic hydrolysis of Spectrozyme TH, a thrombin-specific chromogenic substrate, with inhibition constants corresponding to that for fibrinogen. We conclude that these 7 amino acid substitutions do not alter thrombin binding to the fusion proteins. The fusion proteins were tested as substrates by monitoring thrombin-dependent peptide release. The natural sequence and three mutants, $Asp^{7} \rightarrow Ala$, $Glu^{11} \rightarrow Ala$, and $Gly^{14} \rightarrow Val$, are good substrates. The other mutants are either poor substrates or are not cleaved by thrombin within Aα 1–50. These results indicate that residues between Asp^{7} and Arg^{16} are critical to efficient peptide hydrolysis, whereas residues outside this region are critical to thrombin binding.

The conversion of soluble fibrinogen to an insoluble fibrin clot is catalyzed by the serine protease thrombin. Thrombin cleaves four peptide bonds in fibrinogen, the Arg^{16}-Gly^{17} bonds in each of the two Aα chains, releasing FpA,[1] and the Arg^{14}-Gly^{15} bonds in each of the two Bβ chains, releasing FpB. Previous investigations demonstrated that the CNBr fragment Aα 1–51 contained all the residues required for thrombin recognition and cleavage of the Aα chain (1, 2). Using an active site mapping approach, Scheraga and his colleagues have shown that, in particular, residues between Asp^{7} and Val^{20} are important (3–5). The specificity constant, k_{cat}/K_m, for the peptide Aα Asp^{7}-Val^{20} was only 10-fold lower than that for Aα 1–51 (5). Recently, NMR techniques have been used to study the structures of synthetic Aα peptides free in solution and bound to bovine thrombin (6, 7). Results from these experiments indicated that thrombin recognizes a peptide structure which includes a tight turn between Asp^{7} and Arg^{16} and that several residues within this region are directly involved in the peptide-thrombin complex (7). Based on these and additional NMR studies, model structures were proposed for the decapeptide Asp^{7}-Arg^{16} bound to thrombin (8).

We have initiated experiments to examine the significance of these and other residues in Aα 1–50 to the thrombin-fibrinogen interaction. We approached this problem using genetic engineering to synthesize a model thrombin substrate in *Escherichia coli* (9). This model substrate is a tripartite protein consisting of Aα residues 1–50 linked by a 59-residue segment of collagen to *E. coli* β-galactosidase. Previously, we reported that thrombin cleavage of the recombinant protein in crude cell lysates approximated thrombin cleavage of fibrinogen (9). The data presented here demonstrates that the purified recombinant protein is remarkably similar to native fibrinogen in its interaction with thrombin. Using directed mutagenesis, we have synthesized seven analogous proteins with single amino acid substitutions between residues 7 and 16. Analysis of the mutated proteins indicates that this region of Aα is critical to efficient peptide hydrolysis, but is less important to thrombin binding. A preliminary report of these results has been presented (10).

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. Isopropyl-β-D-thiogalactopyranoside was obtained from United States Biochemical Corp. Phenylmethanesulfonyl fluoride, *o*-nitrophenyl-β-D-galactopyranoside, *p*-aminophenyl-1-thio-β-D-galactopyranoside, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 6-aminohexanoic acid-Sepharose 4B, and chemicals for buffers and media were purchased from Sigma. Spectrozyme®TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide) was purchased from American Diagnostica, Inc., human fibrinogen, Grade L, from KabiVitrum, the Vydac C18 cartridge column from Alltech Applied Science Labs, and human fibrinopeptide A (FpA) from Bachem Bioscience, Inc. Human α-thrombin was the generous gift of Dr. Roger Lundblad (Department of Pathology, University of North Carolina).

Mutant Plasmid Construction—The DNA segment encoding amino acids 1–50 of the Aα chain of human fibrinogen was assembled from seven oligonucleotides and cloned as previously described (9). Two of the seven oligonucleotides, presented in Fig. 1*A*, each contained equimolar mixtures of 2 bases at three positions. DNA from cloned isolates, each containing a single coding sequence, was prepared and sequenced as described (9). *E. coli* strain JM101 (supE, thi, Δ(lac-pro), [F′, traD36, proAB, $lac^{q}ZΔM15$) was used for all cloning and expression experiments. Cells harboring plasmid were grown in medium containing ampicillin (100 μg/ml).

Tribrid Protein Purification—Cells harboring the plasmid expres-

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[1] The abbreviations used are: FpA and FpB, fibrinopeptides A and B, respectively; Spectrozyme®TH, H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; Y18/HRP, horseradish peroxidase-conjugated monoclonal antibody Y18.

sion vector were grown in 10 liters of 2 × YT fermentation medium (11) in a MagnaFerm Bench Top Fermentor Model MA-100 (New Brunswick Scientific Co.). Cultures were started the previous day from a single colony and grown in 5 ml of M9 (11) throughout the day. A series of 10-fold dilutions from 1:10 to 1:10,000 were prepared from the 5-ml culture and grown overnight at 37 °C in M9. The overnight culture with $OD_{590} \sim 0.5$ was used to inoculate 500 ml of 2 × YT. This culture was grown for 2 h and used to inoculate the 10-liter fermentor vessel. Cells were grown to $OD_{590} = 8$ (usually 2 h) at which time isopropyl-β-D-thiogalactopyranoside was added to 1 mM and lactose to 5 mM. During fermentation the pH was maintained at 7, and the β-galactosidase activity was monitored by a colorimetric assay with *o*-nitrophenyl-β-D-galactopyranoside as substrate (11). When the β-galactosidase activity plateaued, the cells were harvested by centrifugation and stored at −20 °C.

The tribrid was purified by a combination of previously published procedures (12, 13). Frozen cell paste was resuspended in 0.05 M Tris-HCl, pH 8.0, 15% sucrose, 0.05 M EDTA (1 g of cell paste/5 ml of buffer) to which 1 mg/ml lysozyme had been added. The cells were incubated on ice 30 min and then frozen at −70 °C. After a quick thaw, the cells were sonicated briefly, phenylmethanesulfonyl fluoride added to 1 mM, and the suspension centrifuged at 27,000 × *g* for 30 min at 4 °C. Nucleic acids were precipitated by dropwise addition of 30% streptomycin sulfate (12). The supernatant was adjusted to 1.6 M NaCl and applied to a *p*-aminophenyl-1-thio-β-D-galactopyranoside-Sepharose column (13). A 3 × 7-cm column was routinely used for 24 g of frozen cell paste. The tribrid was eluted with 0.1 M sodium borate, pH 10.5, and fractions were promptly precipitated with 40% ammonium sulfate (12). The fractions were assayed for β-galactosidase activity and the active fractions pooled and centrifuged at 12,000 × *g* for 20 min at 4 °C. The tribrid precipitate was dissolved in and dialyzed overnight against 0.05 M Tris-HCl, pH 8.3, 0.15 M NaCl, 0.02% sodium azide, 0.1% polyethylene glycol 8000 (TSAP) at 4 °C. The purity of the tribrid was monitored as units of β-galactosidase/mg of protein, measured by the Bradford Assay (Bio-Rad). SDS-PAGE (14) and protein blot analysis were as described previously (9).

Competitive Enzyme-linked Immunosorbent Assay—A competitive cross-reactivity assay similar to that described by Koppert *et al.* (15) was used to quantitate the interaction between the recombinant proteins and the monoclonal antibody Y18. A working dilution of peroxidase-conjugated Y18 (Y18/HRP, kindly provided by W. Nieuwenhuizen) was determined by serial dilution, selecting a concentration within the linear range. *E. coli* cells were grown and lysates prepared (9) and diluted with 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween-20. Tribrid concentration was quantitated as β-galactosidase activity based on the ratio of FpA/β-galactosidase activity for the Aα tribrid (9). Y18/HRP was incubated with cell lysates overnight at 4 °C and cross-reactivity was quantitated on fibrinogen-coated microtiter plates, using hydrogen peroxide and 2,2′-azino-di-(3-ethylbenzthiazopine sulfonate) (Kirkegaard and Perry Laboratories) as substrates. Results were determined on a Molecular Devices kinetic microplate reader with a 405–490-nm filter. Fibrinogen was assayed in the presence of an *E. coli* lysate not expressing the tribrid. Percent inhibition was determined as [1-(absorbance with competitor/absorbance without competitor)] × 100%.

Inhibition of Synthetic Substrate Hydrolysis—Various concentrations of Spectrozyme TH, from 1 to 10 × K_m, were incubated with human α-thrombin (0.02 unit/ml) either alone, with fibrinogen, or with purified tribrid. The concentration of enzymatically hydrolyzable Spectrozyme TH was determined after incubating for 2 h with 200 units/ml thrombin, using an extinction coefficient $E_{405} = 9920$ M^{-1} cm^{-1} for *p*-nitroaniline (17). Aliquots of α-thrombin were stored at −70 °C, and a fresh sample was thawed and diluted in TSAP immediately before use. Fibrinogen was dialyzed against TSAP at 4 °C and the concentration determined with the extinction coefficient $E^{1\%}_{280} = 15$ (18). Tribrid concentrations were determined from total protein and densitometry of Coomassie-stained gels using an LKB Ultroscan XL laser densitometer. Results from multiple scans were averaged to give the percent purity. Where possible, the concentration of tribrid was also determined by HPLC (see below), quantitating FpA release after incubating the tribrid with 200 units/ml α-thrombin at 37 °C for 2 or more h. Since no FpA-like peptide was released from the F8Y tribrid, we relied solely on the densitometry scan for this mutant. Thus, the particularly low value for F8Y ($K_i = 1.5$ μM) may be misleading.

Inhibition assays were performed in microtiter plates with a Molecular Devices kinetic reader as described (16). Reactions were initiated by the addition of thrombin and incubated at ambient temperature with constant mixing. Initial velocity measurements were determined at or below 20% conversion of substrate to product. Michaelis-Menten parameters were obtained by using the nonlinear regression analysis program Enzfitter (Biosoft, Inc.).

HPLC Analysis of Tribrid Hydrolysis—Fibrinopeptides released by thrombin digestion were analyzed by an LKB HPLC system which includes a 2157 Autosampler, a 2152 HPLC Controller, a 2150 HPLC pump, a 2138 Uvicord S detector with a 206-nm filter, and a 2211 Superrac Fraction Collector. An isocratic solvent system (0.1% trifluoroacetic acid in 20% acetonitrile) (19) was used with a flow rate of 0.5 ml/min on a Vydac C18 reversed-phase column (250 × 4.6 mm, particle size 10 μm).

Samples were prepared essentially as described (20). Recombinant tribrid was incubated with α-thrombin which was diluted in TSAP buffer (0.02–20 units/ml) immediately before use. The reaction proceeded at ambient temperature and was terminated by heating for two min at 90 °C. Precipitate was removed by centrifugation at 4 °C for ten min at 13,600 × *g*. The supernatant was filtered with a 0.2-μm low protein binding filter (Gelman Sciences) before HPLC analysis. Peptide concentrations were quantitated by comparing the peak area to a calibration curve with a standard human fibrinopeptide A solution.

Fractions containing tribrid peptides were collected and analyzed for amino acid content. Peptide solutions were evaporated to dryness and hydrolyzed by gas-phase hydrolysis at 165 °C for 1 h. Samples were analyzed with Applied Biosystems Inc. equipment including a Model 470 Derivatizer, a Model 120 phenylthiohydantoin Analyzer, and a Model 920 Data Analysis Module.

RESULTS

Construction of Mutant Genes—Plasmid expression vectors encoding substitutions in fibrinogen Aα 7–16 were constructed by oligonucleotide assembly as described previously (9) using the oligonucleotides listed in Fig. 1*A*. Cloned isolates were characterized by DNA sequence analysis. We sequenced 70 clones, identifying six mutants with single, seven mutants with double, and three mutants with triple amino acid substitutions. We have analyzed the six recombinant proteins with single substitutions, $Asp^7 \rightarrow$ Ala (*D7A*), $Phe^8 \rightarrow$ Tyr (*F8Y*), $Glu^{11} \rightarrow$ Ala (*E11A*), $Gly^{12} \rightarrow$ Val (*G12V*), $Gly^{13} \rightarrow$ Val (*G13V*), and $Arg^{16} \rightarrow$ Leu (*R16L*), along with a mutant, $Gly^{14} \rightarrow$ Val (*G14V*), identified previously (9). The mutants are listed in Fig. 1*B*.

Cross-reactivity Assays—Protein blot analysis of lysates harboring the tribrid plasmids using a monoclonal antibody to β-galactosidase (data not shown) demonstrated that all the

A. Mutagenic oligonucleotides used in this Aα assembly:

```
              T T         T
1. 5′ GCTGAAGGAGGAGGCGTGCGTGGCCCAAGGGTTGTGGAAAGACATCAA
      A E G G G V R G P R V V E R H Q
              V V         L

                          G T         G
2. 3′ CGTCTATCACCACTTCCACTGAAAGATCGACTTCCTCCTCCGCACGCACCG
      A D S G E G D F L A E G G G V R G
                          A Y         A
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B. Substitutions used in this study:
D7A--position 7 substitutes Ala for Asp
F8Y--position 8 substitutes Tyr for Phe
E11A--position 11 substitutes Ala for Glu
G12V--position 12 substitutes Val for Gly
G13V--position 13 substitutes Val for Gly
G14V--position 14 substitutes Val for Gly
R16L--position 16 substitutes Leu for Arg

FIG. 1. **Nucleotide and peptide sequences of tribrid clones.** *A*, oligonucleotides used to assemble the vector for expressing natural and mutated tribrids. Below the DNA sequences are the amino acids (single letters) encoded by these oligonucleotides. The mixed bases were equimolar for the two bases noted. Oligonucleotides 1 and 2 here correspond to numbers 3 and 2, respectively, in Fig. 1 of Lord and Fowlkes (9). *B*, single amino acid substitutions in the Aα chain segment of human fibrinogen. The plasmids encoding these mutant proteins are similarly denoted; for example the plasmid pD7A encodes the tribrid where position 7 is Ala.

mutated tribrid proteins migrated on SDS-PAGE with the predicted mobility, 127 kDa, and all the mutants were synthesized at the same level as the tribrid carrying the natural Aα sequence. Western blot analysis using a monoclonal antibody, Y18, whose epitope maps within Aα 1–51 (15) demonstrated that only some of the mutated proteins cross-react with this antibody. To quantitate this cross-reactivity, we analyzed the recombinant proteins in a competitive enzyme-linked immunosorbent assay where the test antigen was preincubated in solution with Y18/HRP and then assayed on microtiter plates coated with fibrinogen. The results are presented in Fig. 2. By comparing the quantity of antigen which gives 50% inhibition, it was found that both the natural recombinant protein and the R16L mutant reacted strongly with Y18, within an order of magnitude of fibrinogen. Compared to the Aα tribrid, the D7A substitution reduced cross-reactivity more than 10-fold, whereas the F8Y substitution reduced cross-reactivity more than 100-fold. The other mutants, E11A, G12V, G13V, and G14V, did not cross-react with Y18.

Tribrid Protein Purification—The tribrid proteins were purified by affinity chromatography based on their β-galactosidase activity. As shown by SDS-PAGE, Fig. 3, this single chromatography step resulted in a virtually pure preparation of the tribrid. A major contaminant of ~110 kDa was seen in most preparations. This protein cross-reacted with the monoclonal antibody to β-galactosidase (9) and therefore is probably the truncated protein (predicted mass = 113 kDa) (22) encoded by the lacZΔM15 gene in JM101. Although this truncated protein is not catalytically active, it retains the substrate binding site (22) and, therefore, bound to the substrate analog matrix. Control experiments indicated that this protein did not interfere with the tribrid assays. Other minor contaminants, ~50 kDa, were reproducibly present; these proteins did not cross-react with the β-galactosidase monoclonal antibody. The concentration of tribrid protein, quantitated by scanning laser densitometry of the Coomassie-stained gels, varied from 30 to 65% of the total purified protein. As shown in Table I, purification resulted in about a 250-fold increase in specific activity, indicating that the tribrid represented 0.1–0.3% of total cellular protein.

Thrombin Inhibition—The tribrid proteins were analyzed as inhibitors of thrombin-catalyzed cleavage of the chromogenic substrate Spectrozyme TH. As described under "Experimental Procedures," initial velocity measurements were determined at or below 20% substrate hydrolysis. The kinetic data are summarized in Table II and a representative Lineweaver-Burk plot is shown in Fig. 4. The K_m determined for Spectrozyme TH was 5.8 μM, a value slightly larger than that reported by the supplier, 3.2 μM. The mutant tribrids all inhibited Spectrozyme TH hydrolysis equally and to the same

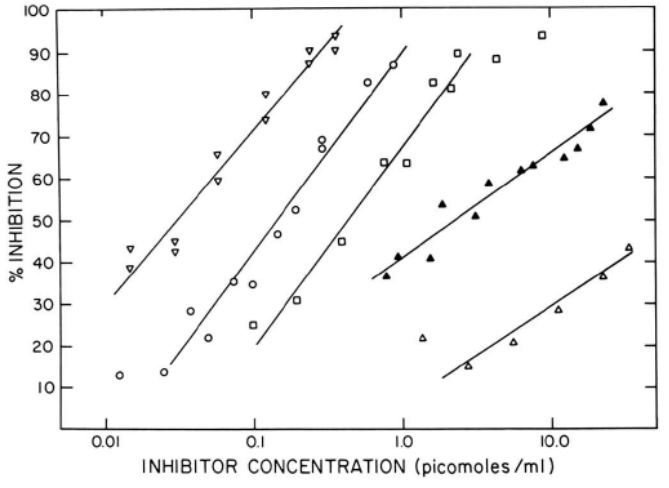

FIG. 2. **Competitive enzyme-linked immunosorbent assays with mAb Y18.** Competitor and antibody were incubated for 18 h at 4 °C, and cross-reactivity was quantitated on fibrinogen-coated microtiter plates. Tribrid concentrations were determined from β-galactosidase activity as described under "Experimental Procedures." ▽, fibrinogen; ○, Aα tribrid; □, R16L; ▲, D7A, △, F8Y.

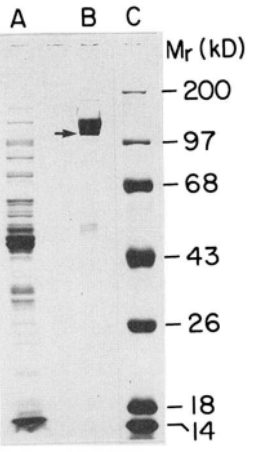

FIG. 3. **SDS-PAGE analysis of tribrid purification.** An 8% polyacrylamide gel stained with Coomassie Blue. *A*, approximately 15 μg of protein sampled before affinity chromatography. *B*, 2 μg of purified tribrid. The band marked with an *arrow* is the lacZΔM15 protein synthesized by the host cell. *C*, molecular mass standards: (200 kDa) myosin, (97 kDa) phosphorylase *b*, (68 kDa) bovine serum albumin, (43 kDa) ovalbumin, (26 kDa) α-chymotrypsinogen, (18 kDa) β-lactoglobulin, (14 kDa) lysozyme.

TABLE I

Purification of tribrid protein

Assays were performed as described under "Experimental Procedures." This preparation was from 24 g of frozen cell pellet.

	Protein	β-Gal activity	Specific activity	Purification	Recovery
	mg	*× 10⁻⁵ units/ml*	*units/mg*	*-fold*	*%*
Total cell lysate	3.2×10^3	27	0.8×10^3		100
Strep-sulfate supernatant	1.8×10^3	22	1.2×10^3	1.5	81
Affinity column eluate	3.2	6.5	2.0×10^5	250	24

TABLE II

Summary of kinetic analysis

Substrate	Spectrozyme inhibition K_i[a]	Substrate hydrolysis[b]		
		50% cleavage time	Thrombin concentration	Cleavage site
	μM	*min*	*units/ml*	
Fibrinogen	5.7 ± 3.5 (7)	25 ± 2	0.02	Arg^{16}
Aα tribrid	3.5 ± 3.0 (6)	44 ± 2	0.02	Arg^{16}
D7A	4.5 ± 2.1 (9)	61 ± 17	0.02	Arg^{16}
F8Y	1.5 ± 0.7 (3)	—[c]	—	—
E11A	5.8 ± 2.2 (3)	33 ± 23	0.02	Arg^{16}
G12V	8.9 ± 6.3 (4)	109 ± 24	2.0	Arg^{16}
G13V	6.9 ± 6.3 (2)	51 ± 6	2.0	Arg^{16}
G14V	5.4 ± 3.7 (4)	28 ± 2	0.02	Arg^{16}
R16L	5.0 ± 0.5 (3)	69 ± 25	2.0	Arg^{19}

[a] All inhibition assays were with 0.02 unit/ml α-thrombin. Results are expressed as mean ± S.D. The numbers in parentheses indicate the number of experiments.

[b] Substrate concentrations were all 5 mM. Results are expressed as mean ± S.D. ($n = 3$).

[c] No peptide was isolated from F8Y.

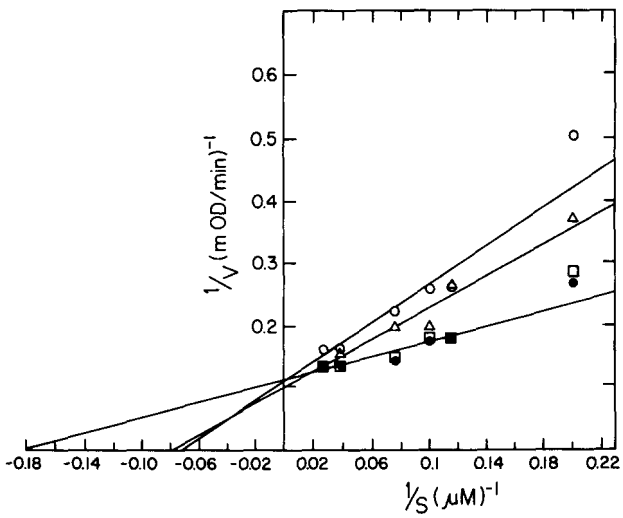

FIG. 4. **Lineweaver-Burk plot of representative inhibition data.** As described under "Experimental Procedures," Spectrozyme TH was incubated with 0.02 unit/ml thrombin either alone, with fibrinogen, or with purified tribrid. The plots are directly from the Enzfitter analysis. Similar analysis was used to determine the K_i values listed in Table II. ●, no additions; □, +10 μM control tribrid; △, +6 μM G12V; ○, +9 μM fibrinogen.

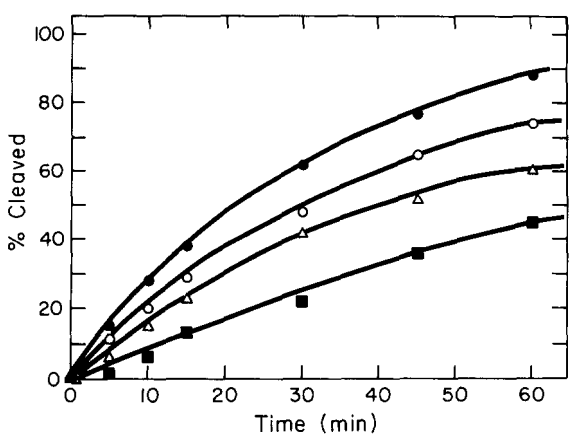

FIG. 5. **Progress curves of thrombin catalysis.** Rates of release of FpA and FpA-like peptides were determined by HPLC as described under "Experimental Procedures." All substrates were 5 μM. The concentration of thrombin was 0.02 unit/ml except for substrate G12V where thrombin was 2 unit/ml. ●, fibrinogen; ○, G14V; △, Aα tribrid; ■, G12V.

extent as the natural tribrid. Furthermore, within experimental error, the tribrids all inhibited as well as human fibrinogen. To insure that this inhibition was due to the presence of Aα 1–50 in the tribrid, we tested an analogous protein in which these 50 residues were replaced by an unrelated 121-residue segment. This protein, purified as described for the Aα tribrids, did not inhibit Spectrozyme TH hydrolysis (Fig. 4).

Thrombin Substrates—Purified fusion proteins were tested as thrombin substrates using HPLC to follow peptide release. Typical progress curves are presented in Fig. 5 and the data are summarized in Table II. Because thrombin cleaves Aα 1–51 after both Arg^{16} and Arg^{19}, although the latter at a significantly slower rate (3), we performed amino acid analysis on the released peptide to determine where cleavage occurred. The data, summarized in Table III, show that all the tribrid substrates were cleaved after Arg^{16} except R16L, which was cleaved after Arg^{19}.

Several of the tribrid proteins were good thrombin substrates as compared with fibrinogen. Three mutant tribrids,

TABLE III

Amino acid composition of thrombin-released peptides

	Aα-tribrid	D7A	E11A	G12V	G13V	G14V	R16L
Ala	2.0 (2)[a]	3.0	3.0	2.0	2.0	2.0	2.0
Arg	1.0 (1)	0.8	1.1	1.0	0.9	1.3	1.1
Asp	1.8 (2)	1.0	2.0	1.6	1.9	2.0	2.0
Glu	2.1 (2)	2.5	1.3	2.2	2.0	2.1	2.2
Gly	4.7 (5)	5.6	4.9	4.6	4.0	3.9	6.2
Leu	1.2 (1)	1.0	1.3	1.2	1.1	1.4	2.1
Phe	1.0 (1)	0.9	0.9	1.0	1.0	1.0	0.9
Pro	(0)						1.1
Ser	1.0 (1)	1.7	1.1	1.0	1.0	1.0	1.1
Val	0.9 (1)	0.7	0.9	1.8	1.8	1.4	0.9

[a] Theoretical values for FpA are in parentheses (25).

D7A, E11A, and G14V, were cleaved as readily as the natural tribrid. Interestingly, the mutant G14V was apparently cleaved more rapidly than the natural sequence. Three other mutants, G12V, G13V, and R16L, were cleaved much more slowly, requiring 100-fold more thrombin for cleavage to occur within the same time frame as the good substrates. Using a gradient elution for HPLC, we isolated another peptide from the digestion of G12V with 2 units/ml thrombin. The amino acid composition of this peptide was consistent with a peptide starting at Val^{20} and ending at Arg^{67}, the 17th residue of the collagen linker. This result (data not shown) indicates that at this higher enzyme concentration, thrombin also cleaved all the tribrids after Arg^{19} in the α sequence and after an arginine in collagen.

The mutant F8Y tribrid has been extensively analyzed with no definitive result. At 20 units/ml of thrombin no FpA-like peptide was found by isocratic HPLC. However, Western blot analysis with mAb Y18 of thrombin-treated F8Y demonstrated that the cross-reacting band at 127 kDa was lost after incubation with 20 units/ml of thrombin. Accordingly, it is likely that thrombin cleaved this tribrid in the collagen linker, removing the Y18 reactive domain. Although no definitive data were obtained to indicate such a specific cleavage, it appears that thrombin did not cleave F8Y after either Arg^{16} or Arg^{19}.

DISCUSSION

The experiments described here were designed to examine the significance of Aα residues Asp^7–Arg^{16} to thrombin-catalyzed hydrolysis of fibrinogen. We constructed a recombinant model substrate and modified substrates with altered residues in this region. Analysis of crude lysates demonstrated that all the recombinant proteins were synthesized similarly; that is, mutation *per se* did not alter the level of tribrid synthesis. Because the recombinants included active β-galactosidase, these proteins were readily purified by affinity chromatography on a substrate analog matrix.

The reduced cross-reactivity with mAb Y18 readily demonstrated that most of our mutant tribrids have altered structures. Only the tribrid with the R16L substitution cross-reacted strongly, indicating that Arg^{16} is not a critical part of the epitope. This is in contrast to the loss of reactivity between Y18 and dysfibrinogen Metz, where Arg^{16} is replaced by Cys (15). Together these data suggest that the Cys substitution causes a change in the conformation of this important amino-terminal domain. This change may simply make the mAb Y18 epitope inaccessible.

The results from the Spectrozyme TH experiments demonstrate that all the Aα-tribrids are good competitive inhibitors of amidolytic activity. The values for K_i are the same and importantly are the same as that for fibrinogen. Our data lack precision because of difficulties in determining the tribrid

concentration, due mostly to contamination by the lacZΔM15 gene product. However, the results indicate that among all these inhibitors there is at most a 5-fold difference in affinity for thrombin.

Although all the tribrids are good inhibitors, several mutant tribrids are not good substrates. The reduced reactivity found with R16L and G12V is not surprising in light of previously reported data. The specificity of thrombin *per se* leads one to expect that the Leu^{16}-Gly^{17} bond would not be cleaved at all and that cleavage at the Arg^{19}-Val^{20} bond would be slow (3). The G12V mutant in dysfibrinogen Rouen is a poor thrombin substrate (23), so the reduced reactivity of this mutant is expected. The reduced reactivity of G13V is consistent with the thrombin-bound peptide model structure (8), where G13 is the third residue of a β-turn, a position where glycine is strongly preferred (24). This residue is further constrained by backbone hydrogen bonds via its α-amino to the α-carbonyl of Leu^9 and via its α-carbonyl to the α-amino of Arg^{16}. Consequently, alteration of this residue is likely to disrupt the structure of the bound peptide and reduce the rate of catalysis. The observation that the F8Y mutant appears not to be cleaved in Aα is consistent with the preservation of this residue throughout evolution (25) and with the kinetic data for synthetic peptides (4).

The three mutant tribrids which are good substrates are more difficult to rationalize. The loss of cross-reactivity with Y18 for E11A and G14V indicates that these substitutions do alter the local surface structure of these peptides in solution. In the thrombin-bound peptide structure (8) Glu^{11} is the first residue of a β-turn, a position which is compatable with alanine, and Gly^{14} is the last residue of a β-turn, a rather unrestricted position (24). Thus these changes may not significantly disrupt the structure of the bound peptide. Perhaps more importantly for G14V, the hydrophobic side-chain substitution at position 14 can be readily juxtaposed to Val^{15}, Phe^8, and Leu^9 and therefore may contribute positively to the hydrophobic interaction between thrombin and Aα. The findings with the D7A substitution are most difficult to explain. In dysfibrinogenemia Lille Asp^7 is replaced by Asn, and this protein has a significantly prolonged thrombin time (26). The importance of Asp^7 has also been indicated by peptide studies (5), where the addition of this residue resulted in a 1.5-fold increase in k_{cat}. The progress curves indicate that the natural sequence is cleaved about 1.5-fold more rapidly than D7A, but these two values are within experimental error in our data. Given that dysfibrinogen Lille is a poor substrate for thrombin, one would expect D7A to also be a poor substrate. However, this was not observed. Perhaps the mutation to Asn alters the structure differently than the mutation to Ala.

Together the studies of these altered proteins as inhibitors and substrates indicate that the features critical to hydrolysis reside in a domain different from those critical to binding. Although our data are not sufficiently accurate to detect small differences in K_i, they do demonstrate that changes which alter catalysis by 100-fold or more do not similarly alter binding. Thus, our results indicate that residues critical to thrombin binding lie outside the region Asp^7-Arg^{16}.

This result is consistent with the existence of a second domain in Aα 1–50 which interacts with a thrombin site outside the catalytic domain. The presence of a second site was first proposed when it was found that thrombin binds to fibrin (27) and a secondary binding site in fibrinogen has been characterized by several groups (28–30). This site is removed from the thrombin catalytic site, as demonstrated by the finding that thrombin bound to fibrin retains the ability to hydrolyze short synthetic substrates and can react with active site inhibitors (31). In addition, affinity chromatography using immobilized catalytic site-inhibited thrombin demonstrates that fragment E from fibrinogen and fragment E from fibrin bind equally well to this matrix (30). That is, the loss of FpA does not substantially change the affinity of fibrin(ogen) for catalytic site-inhibited thrombin, which supports the hypothesis that residues 7–16 do not contribute significantly to binding of thrombin to fibrinogen.

This additional site is thought to have important ionic contributions (28–30) and therefore may involve the multiple negative side chains found in Aα 30–40 (5 of 11 residues). We are in the process of constructing mutant tribrids with substitutions in this region. If these mutants have reduced binding but unaltered rates of catalysis, this would strongly support our hypothesis that binding of the Aα chain of fibrinogen to thrombin is dominated by the second site, whereas catalysis is controlled by the structure surrounding the cleavage site.

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REFERENCES

1. Hogg, D. H., and Blombäck, B. (1974) *Thrombosis Res.* **5,** 685–693
2. Hageman, T. C., and Scheraga, H. A. (1974) *Arch. Biochem. Biophys.* **164,** 707–715
3. Meinwald, Y. C., Martinelli, R. A., van Nispen, J. W., and Scheraga, H. A. (1980) *Biochemistry* **19,** 3820–3825
4. Marsh, H. C., Jr., Meinwald, Y. C., Lee, S., and Scheraga, H. A. (1982) *Biochemistry* **21,** 6167–6171
5. Marsh, H. C., Jr., Meinwald, Y. C., Thannhauser, T. W., and Scheraga, H. A. (1983) *Biochemistry* **22,** 4170–4174
6. Ni, F., Scheraga, H. A., and Lord, S. T. (1988) *Biochemistry* **27,** 4481–4491
7. Ni, F., Konishi, Y., Frazier, R. B., Scheraga, H. A., and Lord, S. T. (1989) *Biochemistry* **28,** 3082–3094
8. Ni, F., Meinwald, Y. C., Vasquez, M., and Scheraga, H. A. (1989) *Biochemistry* **28,** 3094–3105
9. Lord, S. T., and Fowlkes, D. M. (1989) *Blood* **73,** 166–171
10. Lord, S. T., Hede, K. L., Byrd, P. A., and Wei, C. (1988) in *Fibrinogen 3, Biochemistry, Biological Function, Gene Regulation and Expression* (Mosesson, M. W., Amrani, D., Siebenlist, K. R., and DiOrio, J. P., eds) pp. 41–44, Elsevier Science Publishers B. V., Amsterdam
11. Miller, J. H. (1972) *Experiments in Molecular Genetics*, p. 432, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Germino, J., Gray, J. G., Charbonneau, H., Vanaman, T., and Bastia, D. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6848–6852
13. Ullman, A. (1984) *Gene (Amst.)* **29,** 27–31
14. Laemmli, U. K. (1970) *Nature* **227,** 680–685
15. Koppert, P. W., Huijsmans, C. M. G., and Nieuwenhuizen, W. (1985) *Blood* **66,** 503–507
16. Humphries, G. M. K. (1988) *Biotechniques* **6,** 354–360
17. Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., and Jackson, C. M. (1983) *Biochim. Biophys. Acta* **742,** 539
18. Doolittle, R. F. (1975) in *Plasma Proteins* (Putnam, F. W., ed) Vol. 2, p. 109, Academic Press, New York
19. Ebert, R. F., and Bell, W. R. (1985) *Anal. Biochem.* **148,** 70–78
20. Hanna, L. S., Scheraga, H. A., Francis, C. W., and Marder, V. J. (1984) *Biochemistry* **23,** 4681–4687
21. Deleted in proof
22. Langley, K. E., Villarejo, M. R., Fowler, A. V., Zamenhof, P. J., and Zabin, I. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 1254–1257
23. Soria, J., Soria, C., Samama, M., and Caen, J. (1985) in *Fibrinogen, Structural Variants and Interactions* (Henschen, A., Hessel, B., McDonagh, J., and Saldeen, T., eds) pp. 165–183, Walter de Gruyter, Berlin

24. Richardson, J. S. (1981) *Adv. Protein Chem.* **34,** 203–216
25. Blombäck, B. (1967) in *Blood Clotting Enzymology* (Seegers, W. H., ed) pp. 143–215, Academic Press, New York
26. Morris, S., Denninger, M. H., Finlayson, J. S., and Menache, D. (1981) *Thromb. Haemostasis* **46,** 104 (Abstr. 315)
27. Lui, C. Y., Nossel, H. L., and Kaplan, K. L. (1979) *J. Biol. Chem.* **254,** 10421–10425
28. Fenton, J. W., II, Olson, T. A., Zabinski, M. P., and Wilner, G. D. (1988) *Biochemistry* **27,** 7106–7112
29. Kaczmarek, E., and McDonagh, J. (1988) *J. Biol. Chem.* **263,** 13896–13900
30. Vali, Z., and Scheraga, H. A. (1988) *Biochemistry* **27,** 1956–1963
31. Kaminski, M., and McDonagh, J. (1987) *Biochem. J.* **242,** 881–887