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Amino Acid Sequence Studies on the α Chain of Human Fibrinogen. Location of Four Plasmin Attack Points and a Covalent Cross-Linking Site†

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ABSTRACT: The amino acid sequence of a 38-residue midsection piece of the α chain of human fibrinogen has been determined using a combination of plasmin-derived peptides and cyanogen bromide fragments. The segment contains several important features, including four early plasmin attack points, one of the two α-chain cross-linking acceptor sites, and a peptide homologous to one isolated from plasmin digests of bovine fibrinogen, and reported to have anticoagulant activity. The segment is sequentially adjacent to and overlapping with a large molecular weight (20000–25000) fragment released during plasminolysis. This latter material is very rich in glycine and serine and deficient in nonpolar amino acids. It also contains the other α-chain cross-linking acceptor site.

During the course of structural studies aimed toward elucidating the entire covalent structure of human fibrinogen, we have deduced the amino acid sequence of a 38-residue segment from the central portion of the α chain. As it happens, this region of the molecule contains a number of important features—including four early plasmin cleavage points, one of the two α-chain cross-linking acceptor sites, and a peptide homologous to one isolated from plasmin digests of bovine fibrinogen and reported to have anticoagulant activity. The segment occupies a position in the α chain corresponding approximately to residues 200–250.

The sequence was determined using data from several different starting materials. First, the progressive plasminolysis of human fibrinogen was studied over a time course ranging from 10 min to 15 hr, the digestion stages being terminated by heat precipitating the incubation mixtures. This report deals with the material which remains in solution upon such treatment; we have previously reported observations dealing with the precipitated material (Takagi and Doolittle, 1975). Secondly, we have purified the same peptides from plasmin digests of fibrin containing an incorporated substitute cross-linking donor. Finally, we isolated the corresponding fragments from cyanogen bromide digestions of purified α chains. The combination of plasmin-derived peptides and cyanogen bromide fragments allowed us to find the necessary overlaps for proving the peptide ar-

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rangement. As a part of this study, we have also characterized the major large molecular weight fragment (fragment A) which is released during the initial stages of plasmin digestion of fibrinogen. This material is sequentially adjacent to the three small peptides released at this stage of plasminolysis and contains the other α-chain cross-linking acceptor site. It has an unusual amino acid composition, being very rich in glycine and serine and deficient in nonpolar amino acids.

Experimental Section

Materials

Human fibrinogen was prepared from blood bank plasma by a cold ethanol fractionation procedure (Doolittle et al., 1967). U.S. Red Cross human plasmin in 50% glycerol (10 C.T.A. U/ml) was kindly supplied by Dr. Alan Johnson, New York University Medical Center. Soybean trypsin inhibitor, Tos-PheCH_2Cl-trypsin,[1] α-chymotrypsin, carboxypeptidase A, and carboxypeptidase B were all obtained from Worthington Biochemicals. [^{14}C]Glycine ethyl ester hydrochloride was purchased from New England Nuclear; it contained approximately 10 Ci/mol. Cyanogen bromide was obtained from Matheson Coleman and Bell. Thioacetylthioglycolic acid (TATG) was synthesized according to the procedure of Jensen and Pedersen (1961). Phenyl isothiocyanate (PhNCS) and dimethylallylamine were purchased from Eastman and distilled before use. 5-Dimethylaminonaphthalene-1-sulfonyl (Dns) chloride was obtained from Pierce Chemicals.

Methods

Amino acid analysis was performed on a Spinco Model 117 analyzer employing a single-column, three-buffer system similar to that recommended by the manufacturer. Peptides were hydrolyzed with 5.7 *N* HCl at 105° for 24 hr.

Sequence Determinations. The methods employed for sequence determination have all been described and/or referenced in detail in two earlier articles (Takagi and Doolittle, 1974, 1975). In essence, the major approaches include application of the TATG stepwise degradation procedure (Mross and Doolittle, 1971) for larger fragments and the use of the Dns-Cl-PhNCS procedure (Hartley, 1970) for smaller peptides. Amides were established on the basis of peptide mobilities on paper electrophoresis. Fragmentation of the assorted materials was achieved with the CNBr procedure (Gross, 1967), or traditional enzymic digestion with trypsin, chymotrypsin, and/or thermolysin. The purification of relevant peptides is described fully in subsequent sections.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was conducted along the lines described by Weber and Osborn (1969). Samples were dissolved in a solution of 4 *M* urea–1% sodium dodecyl sulfate and incubated at 37° in the presence or absence of mercaptoethanol (10 μl/ml) for 2–24 hr. The acrylamide concentration of the gels was varied between 5 and 15% depending on specific requirements; in all cases the running buffer was 0.1 *M* phosphate (pH 7.2) containing 0.1% sodium dodecyl sulfate. The gels, which measured 6 × 140 mm, were run at a current of 8–10 mA/gel. A variety of proteins was used as reference substances for mol wt determination, including lysozyme, trypsinogen, ovalbumin, and the light and heavy chains of rabbit immunoglobulin G.

Preparation of α Chains from Human Fibrinogen. Human fibrinogen was subjected to sulfitolysis in 8 *M* urea and separated into its constituent chains by carboxymethylcellulose chromatography using urea-containing buffers (Henschen, 1964). Because the β and α chains are not always completely resolved by this procedure, the α-chain pools were rechromatographed on carboxymethylcellulose. Since *S*-sulfocysteine is not readily recovered upon acid hydrolysis, the α chains were subsequently reduced (mercaptoethanol) in the presence of 6 *M* guanidinium chloride and alkylated (iodoacetamide). After extensive dialysis against distilled water they were freeze-dried.

Cyanogen bromide fragmentation was carried out essentially as described by Gross (1967). Samples were dissolved in 70% formic acid at concentrations in the range of 5–7 mg/ml, cyanogen bromide was added (10 mg/ml), and the reaction was allowed to take place for 16–24 hr. At the end of that time, samples were diluted tenfold with ice-cold water and freeze-dried over NaOH pellets. The dried samples were subsequently dissolved in 10% acetic acid and gel filtered on an appropriate Sephadex column for separation of the fragments.

Peptide Nomenclature. All the peptides discussed in this paper were derived from human fibrinogen or fibrin, so no species-identifying letter appears in the designations. Beyond that, however, a peptide's history can generally be derived from its label as follows: P at the beginning of a name indicates that the material originated in a plasmic digestion of fibrinogen. Names with an "α" at the beginning indicate that the peptides were derived from α chains, and those with an "X" at the start denote origin in human fibrin formed under cross-linking conditions in the presence of a substitute donor. CN indicates that the material is the product of a cyanogen bromide fragmentation, whereas T, C, and Th refer to digestion with trypsin, chymotrypsin, and thermolysin, respectively. The progressive subdivision of pools during purification generally follows a sequence of a Roman numeral, a capital letter, an Arabic numeral, and finally a small letter. An * indicates a peptide containing radioactive glycine ethyl ester.

Results

Peptides Released during the Progressive Plasminolysis of Fibrinogen. The exact conditions used for the plasmin digestion of fibrinogen have been recorded in our earlier article dealing with the heat-precipitable fractions (Takagi and Doolittle, 1975). Simply put, plasmin–fibrinogen mixtures were incubated for varying times and the digestion was terminated by the addition of soybean trypsin inhibitor and immediate placement at 60° to precipitate the larger molecular weight fragments. The present study deals with material which is *not* heat precipitated and which remains in the supernatant after centrifugation. Because the products released earliest in the plasmin digestion course were the ones most used in determining the sequence, the complete workup of the 10-min digest is described. Similar procedures were used for the larger fragments derived from some longer digestions.

A 10-min digest containing 250 mg of starting fibrinogen was heat precipitated at 60° for 20 min and then centrifuged in a Sorvall RC-2B centrifuge at 12000*g* for 10 min.

[1] Abbreviations used are: Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TATG, thioacetylthioglycolic acid; Dns, 5-dimethylaminonaphthalene-1-sulfonyl; PhNCS, phenyl isothiocyanate; CNBr, cyanogen bromide; GlyOEt, glycine ethyl ester.

Table I: Amino Acid Compositions of Fragment A Obtained at Different Stages of Plasmin Digestion.[a]

	A 10-Min Digest	B 90-Min Digest	C 15-Hr Digest	D 20-Hr Digest
Aspartic acid	9.1	10.0	9.4	8.3
Threonine	9.0	8.7	8.5	10.6
Serine	18.2	19.4	19.0	21.7
Glutamic acid	9.1	9.2	7.8	4.6
Proline	10.0	9.3	12.5	12.0
Glycine	18.5	19.7	22.4	28.0
Alanine	3.4	3.1	3.0	4.4
Cysteine	0.4	0.6		
Valine	2.7	2.6	2.0	0.7
Methionine	1.3	0.3	0.4	
Isoleucine	1.3	1.0	0.6	0.8
Leucine	3.1	2.3	1.4	1.2
Tyrosine	1.7	1.8	1.6	1.2
Phenylalanine	2.2	1.6	1.6	0.2
Histidine	1.0	0.9	4.4[b]	
Lysine	3.7	3.3		1.5
Arginine	5.4	6.0	5.2	4.4

[a] Values are given as molar ratios. Preparations A, B, and C were isolated by gel filtration on G-200 of the material remaining soluble after heat denaturation of the plasmin digests (peak III in Figure 1). Preparation D, on the other hand, is the first material to emerge from DEAE chromatography of a complete plasmin digest of fibrinogen, corresponding to the peak designated "A" by Nussenzweig et al. (1961). [b] Histidine and lysine did not separate well in this run.

Table II: Amino Acids Recovered from TATG Degradation of Pools IIIA and IIIB.[a]

	Cycle				
Pool	1	2	3	4	5
IIIA	Ala (15)	Leu (15)			
	(Gly)[b]	(Gly)			
IIIB	Ala (32)	Leu (34)	Leu (26)	Glu (34)	Met (17)
	Met (10)	Glu (19)		Asp (33)	Arg (16)
					Lys (15)
		(Gly)	(Gly)	(Gly)	(Gly)

[a] The nanomoles of amino acid recovered at each step are given in parentheses; readings are taken directly from Autolab Integrator tape and are uncorrected for yield. [b] α chain material always yields a significant glycine background, presumably because the carboxy-terminal half is so rich in glycine and serine and small amounts of chain breakage occurs during the cleavage steps.

The supernatant (ca. 20 ml) was passed over a Sephadex G-200 column (3 × 90 cm) equilibrated with 0.2 *M* ammonium bicarbonate (Figure 1). The four major peaks were divided into six pools (I, II, IIIA, IIIB, IIIC, and IV) and freeze-dried. Pools I, II, IIIA, and IIIB were subjected to amino acid analysis and amino-terminal sequence identification, the latter by the TATG procedure. Pools I and II, which together accounted for less than a quarter of the supernatant material, did not yield clearly defined amino-terminal sequences and were not used further in this study. Pools IIIA and IIIB both had unusual amino acid compositions, being especially rich in glycine, serine, and proline and spectacularly deficient in hydrophobic amino acids (Table I). The only amino-terminal sequence found in fraction IIIA began with Ala-Leu. Fraction IIIB also had a major component beginning with the sequence Ala-Leu, but in addition it contained material beginning with the sequence Met-Glu-Leu-Glu-Arg (Table II). In later plasmin

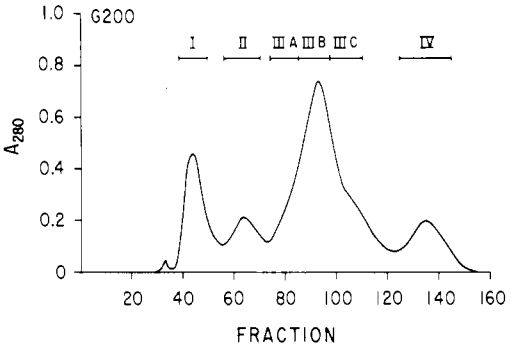

FIGURE 1: Gel filtration of supernatant from heat-precipitated 10-min plasmin digest of human fibrinogen on Sephadex G-200 (3 × 90 cm) equilibrated and eluted with 0.2 *M* ammonium bicarbonate. Fraction size = 4 ml; flow rate = 24 ml/hr.

digests, the component beginning with the amino terminal sequence Ala-Leu was absent, being replaced by the Met-Glu type.[2] The fourth and fifth cycles of the TATG degradation on pool IIIB also revealed the presence of aspartic acid and methionine (Table II), residues which were ultimately positioned by ancillary information derived from other fragments.

Pool IIIB was reduced and alkylated and its amino acid composition determined (preparation A in Table I). A relatively broad band was observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis, the molecular weight of which was in the range 20000–25000. The only amino acid released from this material upon digestion with carboxypeptidase B was arginine; no amino acids were released upon digestion with carboxypeptidase A. The average molecular weight of this material became progressively smaller and its dispersity less marked when isolated from plasmin digests of longer duration, the final core fragment having a molecular weight of about 20000 ± 2000. It must be remarked that these polypeptides stain poorly with Coomassie Blue, and gels had to be heavily loaded.

The reduced and alkylated pool IIIB material was subjected to cyanogen bromide fragmentation and passed over a Sephadex G-50 column (3 × 85 cm) equilibrated with 0.1 *M* ammonium bicarbonate. Although the bulk of the digest eluted with the void volume, later-eluting fractions were also pooled and concentrated; these latter were subjected to paper electrophoresis at pH 5. Three small homoserine-containing peptides were isolated, including a pentapeptide with amino-terminal alanine. It was subsequently shown (below) that the CNBr digestion had removed all three of these peptides from the amino-terminus of a fragment beginning with the sequence Ala-Leu. The large mol wt CNBr fragment which eluted early on the G-50 column had a mol wt of about 20000 as determined by sodium dodecyl sulfate gel electrophoresis. Three cycles of the Dns-PhNCS procedure revealed an amino-terminal sequence of Glu-Leu-Glu, in agreement with the pre-CNBr sequence obtained for the plasmin-generated component in pool IIIB beginning Met-Glu-Leu-Glu- (Table II).

The G-200 pool designated IIIC was gel filtered on G-50 and found to be free of small peptides; this material was set aside for further sequence studies of the carboxy-terminal

[2] Harfenist and Canfield have also isolated the corresponding fragment (what they call fragment H) from later-stage digests and found the principal component to begin with the sequence Met-Glu-Leu-Glu . . ., etc. (E. Harfenist and R. Canfield, personal communication).

Table III: Amino Acid Compositions of Three α-Chain Peptides Released during Early Stages of Plasmin Digestion of Human Fibrinogen.[a]

	PIVB2B[b]	PIVC3[b]	PIVC2[c]
Aspartic acid	2.0 (2)		0.9 (1)
Threonine			1.2 (1)
Serine		1.2 (1)	
Glutamic acid		2.9 (3)	1.3 (1)
Proline	3.1 (3)	2.2 (2)	1.2 (1)
Glycine	1.0 (1)		
Alanine			0.9 (1)
Valine	1.9 (2)	0.9 (1)	
Methionine	0.9 (1)		1.5 (2)
Isoleucine			
Leucine	0.9 (1)	1.2 (1)	1.2 (1)
Tyrosine			
Phenylalanine	1.0 (1)		
Histidine			
Lysine	2.2 (2)	1.8 (2)	
Arginine			1.1 (1)
Tryptophan[d]		+(1)	
Total residues	(13)	(11)	(9)
Position in midpiece	1–13	14–24	25–33
Approximate yield (%)	23	25	17

[a] Presented as molar ratios. [b] From 10-min plasmin digest. [c] From 60-min plasmin digest. [d] Determined by Ehrlich's stain on paper electrophoresis.

half of the α chain. Pool IV from the G-200 column (Figure 1) was concentrated and subjected to gel filtration on Sephadex G-25. Three pools, designated A, B, and C, were concentrated by freeze-drying and purified further by paper electrophoresis at pH 2 and 5. Pool IVA contained several intermediate-sized peptides, most of which are evidently from the carboxy-terminal section of the α chain. However, one peptide from this pool had an amino acid composition identical with β chain residues 22–42 (Blombäck and Blombäck, 1972), and most assuredly represents degradation at the amino-terminus of the β chain as reported by others (Kierulf, 1972; Lahiri and Shainoff, 1973; Budzynski et al., 1974) and confirmed and pinpointed by our degradation studies on the heat-precipitated material from this same digest (Takagi and Doolittle, 1975). Similarly, the fibrinopeptide B was isolated in pure form and high yield after electrophoresis of pool IVB at pH 2.

Several other peptides were isolated from pool IVB, including a 13-residue peptide with amino-terminal methionine (Table III). As will be shown below, this latter peptide (PIVB2b) represents the amino-terminal segment of the 38-residue portion which is the subject of the present report. It was subjected to the Dns-PhNCS procedure and the first eight residues were identified. Paper electrophoresis of the G-25 pool designated IVC also revealed a variety of small peptides. One of these (PIVC7) had an amino acid composition corresponding to residues 15–21 of the β chain. In addition, two very important α chain peptides were also isolated from this pool. One was a tryptophan-containing undecapeptide (PIVC3), the entire sequence of which was readily deduced by a combination of the Dns-PhNCS approach and enzymatic digestions. The other (PIVC2) was a nonapeptide beginning with the amino-terminal sequence Ala-Leu. Because it was present in very small amounts in the 10-min digest, the 60-min digest was used as a source for further characterization. Its sequence was determined by the Dns-PhNCS procedure and CNBr degradation followed by paper electrophoresis to isolate the products. As will be shown below, the two peptides PIVC3 and PIVC2 lie next to each other in the central segment.

Summing to this point, three key peptides (PIVB2b, PIVC3, PIVC2) were isolated from early plasmin digests of human fibrinogen. In addition to these peptides—which will subsequently be shown to originate in the middle section of the α chain—three other peptides were identified whose amino acid compositions correspond (in toto) to the first 42 residues in the β chain. Several other small peptides were also found, and these are presumed to be derived from the extreme carboxy-terminal region of the α chain.

In addition to these small peptides, a closely related family of large fragments was isolated. These large polypeptides have molecular weights of the order of 20000–25000 and have carboxy-terminal arginine. They differ in their amino-terminal sequences in a manner consistent with the progressive removal of the three small α-chain peptides (PIVB2b, PIVC3, and PIVC2) during the course of plasmin digestion. That this is indeed the case is manifestly demonstrated by the following results obtained by cyanogen bromide digestion of purified α chains.

Cyanogen Bromide Fragmentation of Purified α Chains. Fifty milligrams of α chain prepared from human fibrinogen was subjected to cyanogen bromide digestion. After freeze-drying, the material was dissolved in 10% acetic acid and fractionated on Sephadex G-50; seven pools were promptly concentrated by freeze-drying. Pool I was a large molecular weight fragment whose amino acid composition resembled the large molecular weight material isolated from the soluble portion of heat-precipitated plasmin digests of fibrinogen in that it is relatively rich in glycine and serine and deficient in nonpolar amino acids. The second and third pools contained material which is only tangentially involved in the present report, and a full characterization will appear elsewhere.[3] It should be noted, however, that a variety of considerations leads us to position the material in these pools between the amino-terminal fragment and the midsection piece, and it is on this basis that we have tentatively positioned the midsection peptide in the span of residues numbering 200–250 from the α chain amino terminus. A fourth pool contains the amino-terminal 51-residue fragment originally reported by Blombäck et al. (1972) as part of the "disulfide knot" released by the cyanogen bromide digestion of fibrinogen.

It is the material isolated from smaller molecular weight pools which most concerns us here, however. In this regard, pool V was rechromatographed on the same G-50 column and then purified further by electrophoresis at pH 2. It was found to be a tryptophan-containing peptide with amino-terminal lysine (Table IV). Amino acid analysis revealed a composition consistent with a portion of the midsection sequence reconstructed from the plasmic digestion of fibrinogen. It was digested with trypsin and electrophoresed at pH 5.0; four peptides were recovered in good yield (Table IV). It is clear that this peptide (and its subpeptides) provides overlap between the plasmin-derived peptides PIVB2b and PIVC3, on the one hand, and PIVC3 and PIVC2, on the other (Figure 2).

Pools VI and VII were subjected to paper electrophoresis at pH 3.6. Two very significant peptides were isolated from the pool VII run, one (αCNVIIA) a tripeptide with a com-

[3] Doolittle, Cassman, Cottrell, Friezner, Hucko, and Takagi, in preparation.

Table IV: Amino Acid Compositions of Relevant α-Chain Cyanogen Bromide Fragments and Tryptic Subpeptides.[a]

	αCNVA	αCNVAT4	αCNVAT5	αCNVAT3	αCNVAT1	αCNVIIA	αCNVIIB
Aspartic acid	3.2 (3)	1.8 (2)			1.3 (1)		
Threonine	0.7 (1)				0.9 (1)		
Serine	1.3 (1)		0.8 (1)				
Glutamic acid	3.3 (3)		2.1 (2)	1.0 (1)		1.2 (1)	
Proline	5.0 (5)	3.2 (3)		2.2 (2)		0.9 (1)	
Glycine	1.6 (1)	1.1 (1)					
Alanine	0.9 (1)				1.1 (1)		
Valine	2.6 (3)	1.9 (2)		0.9 (1)			
Isoleucine							
Leucine	2.7 (3)	1.2 (1)	1.1 (1)		1.0 (1)		
Tyrosine							
Phenylalanine	0.9 (1)	0.9 (1)					
Histidine							
Lysine	4.0 (4)	1.9 (2)	1.0 (1)	0.8 (1)			
Arginine							1.0 (1)
Tryptophan	+ (1)			0.7 (1)[b]			
Homoserine[c]	0.6 (1)				0.7 (1)	0.5 (1)	0.4 (1)
Total residues	(28)	(12)	(5)	(6)	(5)	(3)	(2)
Position in midpiece	2–29	2–13	14–18	19–24	25–29	30–32	33–34
Approximate yield (%) (uncorrected)	19	14	14	15	16	11	11

[a] Presented as molar ratios. [b] Determined by quantitative *p*-dimethylaminobenzaldehyde test at 590 mμ. [c] Includes homoserine lactone.

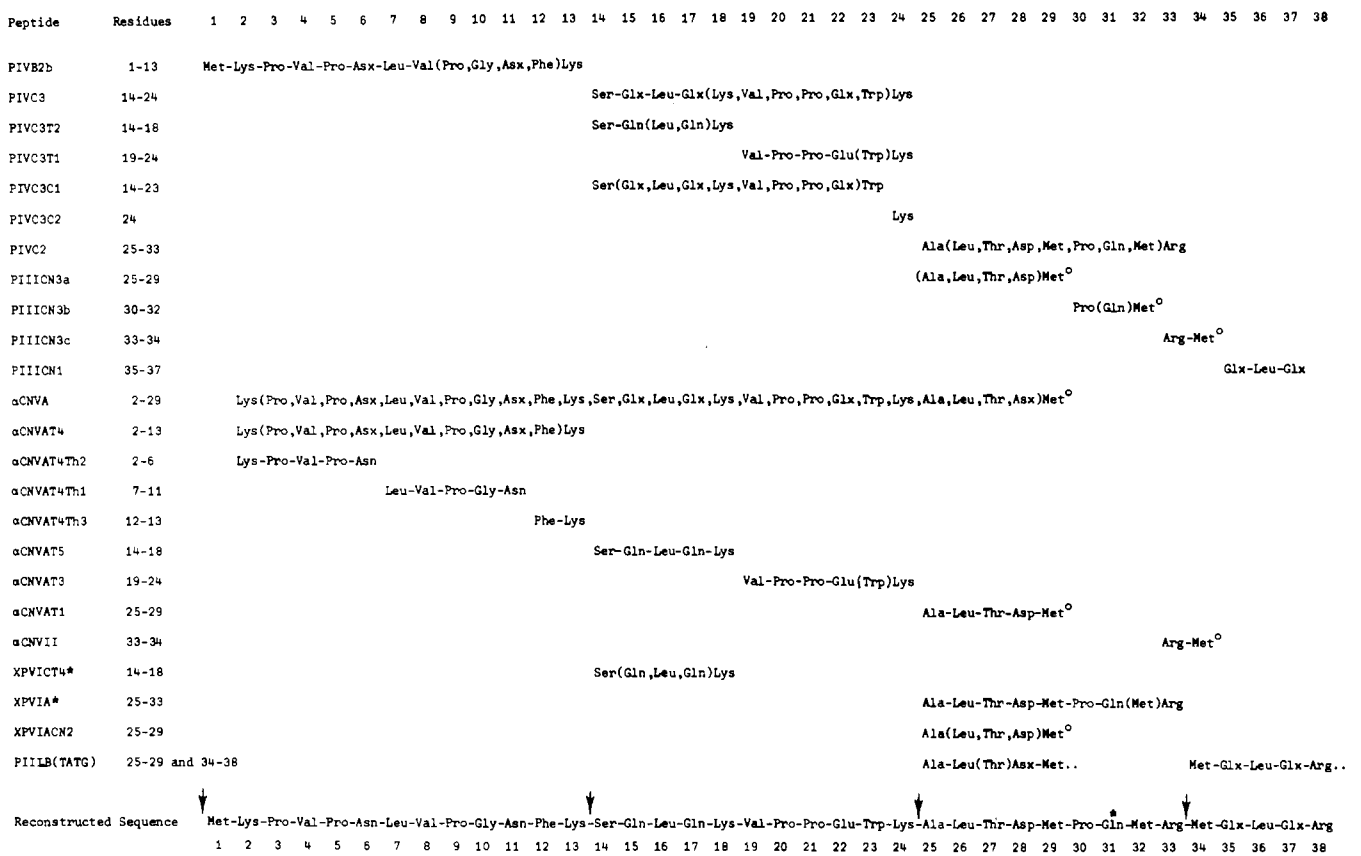

FIGURE 2: Summary of the peptides isolated and characterized in order to deduce the amino acid sequence of a 38-residue midsection piece of the human fibrinogen α chain. The glutaminyl residue marked with an * is an acceptor site for substitute donors incorporated by activated factor XIII. Arrows indicate the four plasmin attack points. Met^0 indicates methionine determined as homoserine.

position identical with that of PIIICN3b, and the other (αCNVIID) a dipeptide, Arg-Hser, which is the same as peptide PIIICN3c (Figure 2).

Plasminolysis of [^{14}C]Glycine Ethyl Ester Labeled Fibrin. Human fibrin with an incorporated radioactive substitute cross-linking donor, [^{14}C]GlyOEt, was prepared according to previously published procedures (Chen and Doolittle, 1969, 1971). In this regard, human fibrinogen solutions (in 0.25 *M* NaCl) were clotted by the addition of thrombin solutions containing [^{14}C]GlyOEt, cysteine, and calcium ions; the clots were let stand for 3–4 hr to ensure full incorporation of the substitute donor into α chains. At that point the excess [^{14}C]GlyOEt was removed by repeatedly washing the fibrin with 0.15 *M* NaCl. The washed fi-

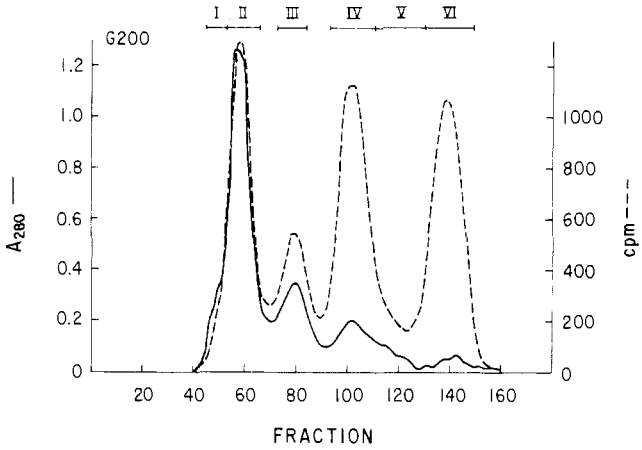

FIGURE 3: Gel filtration on Sephadex G-200 (3 × 95 cm) of the soluble portion of a 210-min plasmin digest of [^{14}C]glycine ethyl ether derivatized human fibrin. Fraction size = 3.3 ml; flow rate = 20 ml/hr. Radioactivity determined by counting 0.2-ml aliquots. Equilibration and elution with 0.2 *M* ammonium bicarbonate.

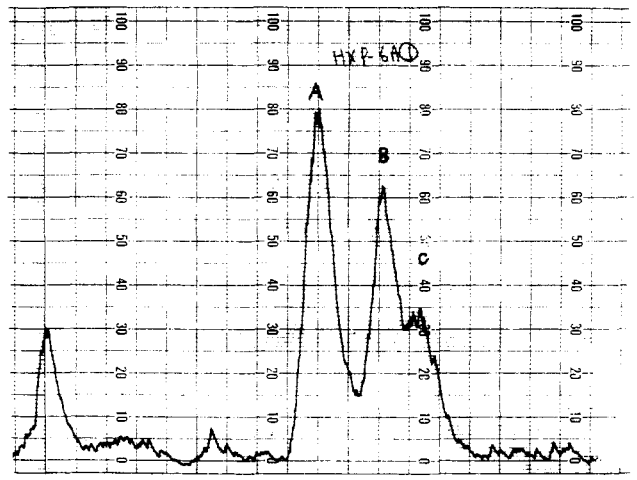

FIGURE 4: Radioactive scan (Packard Model 7201 strip counter) of a paper electrophoresis run at pH 2 of peptide material XPVI*. The origin is indicated by the marker peak at left. The peaks marked A, B, and C were eluted and purified further. Peptide XPVIA* contains the major labeling site, the glutamine residue at position 31 (Figure 5).

brin was resuspended in 0.15 *M* NaCl–0.05 *M* Tris-chloride buffer (pH 7.5) and plasmin (in 50% glycerol) added in a ratio of 1:14 by volume. The release of radioactive peptides into the liquid phase was monitored over the course of several hours. In the isolation experiments described below, soybean trypsin inhibitor was added after 210-min digestion at room temperature, by which time the clot had been rendered partially soluble and about 75% of the total radioactivity released into the supernatant. Insoluble material was removed by centrifugation and the supernatant passed over a Sephadex G-200 column (3 × 95 cm) equilibrated with 0.2 *M* ammonium bicarbonate. Four major radioactive peaks were observed (Figure 3), the first two of which are attributable to the incorporation of the radioactive substitute donor incorporated in the carboxy-terminal segment of γ chains, a region which remains a part of fragment D during plasminolysis, and a portion of which themselves become cross-linked even in the presence of a substitute donor. Both of these peaks (designated pools II and III in Fig. 3) were found to have chains beginning with the amino-terminal sequence Asp-Asp-Glu, a finding characteristic of fragment D (Takagi and Doolittle, 1975). The other two radioactive peaks (pools IV and VI in Figure 3) were shown to be α chain acceptor sites. The larger of these (pool IV) corresponds to fragment A. This material was subjected to tryptic digestion and a 32-residue peptide containing [^{14}C]GlyOEt isolated, the amino acid composition of which has been previously determined (Chen, 1970; Doolittle et al., 1972). The other radioactive peak (pool VI) was concentrated and then passed over a Sephadex G-25 column (2.5 × 70 cm), equilibrated, and eluted with 0.1 *M* ammonium bicarbonate. The radioactive pool was freeze-dried and purified further by paper electrophoresis at pH 2. Three radioactive peaks were observed upon scanning of the strips (Figure 4). These were eluted and analyzed and their amino-terminal sequences determined by the Dns-PhNCS procedure. The major radioactive peptide (XPVIA*) had an amino acid composition indistinguishable from the nonapeptide PIVC2. Seven degradative steps revealed the sequence Ala-Leu-Thr-Asp-Met-Pro-Glx, in complete harmony with this peptide being identical with PIVC2. Finally, a pentapeptide of the appropriate composition was released by CNBr digestion. A complete characterization of the other two radioactive peptides, both of which were present in lesser amounts, has not been without its difficulties. Indications are, however, that the second most radioactive peptide corresponds to residues 14–33 (Figure 2) and is just an earlier digestion product of the same acceptor site. Interestingly enough, however, a small but significant amount of radioactivity (5–10% of the total at this stage) was found to be associated with a tryptic fragment of the peptide corresponding to PIVC3. Thus, a peptide corresponding to the sequence . . .Ser-Gln-Leu-Gln-Lys . . . was radioactive (C in Figure 4). Not enough material was available to position the [^{14}C]GlyOEt on one or the other glutamine residues, however. It is doubtful that this small amount of incorporation has any physiological significance.

Discussion

Human fibrinogen is composed of three pairs of disulfide-linked nonidentical chains ($\alpha_2\beta_2\gamma_2$). The α chains have the largest molecular weights of the three chain types, being of the order of 64000 compared with an accepted value of 56000 for β chains and 47000 for γ chains (McKee et al., 1966). The amino-terminal segment of the α chain is released by thrombin during the transformation of fibrinogen into fibrin, under which circumstances it is referred to as the fibrinopeptide A. In contrast, plasmin initiates its attack in the middle region of α chains, releasing several small peptides and a large fragment which is relatively plasmin resistant. In this article we have reported the amino acid sequence of a central segment of the α chain which is a primary target for the action of plasmin. As a result of four exactly located cleavages, three small peptides are released from this section. Although the order of these four cleavages may not be absolutely fixed, at least one digestion route occurs whereby the large fragment unleashed is progressively shortened from its amino-terminal.

Since the advent of sodium dodecyl sulfate polyacrylamide gel electrophoresis, scores of laboratories have studied virtually every aspect of the plasminolytic breakdown of fibrinogen. From the outset (e.g., Mills and Karpatkin, 1970) there has been almost universal agreement that the carboxy-terminal "half" of the α chain is among the first moieties to be lost from the parent molecule during the di-

gestion. The observation that these regions of the molecule are removed by a broad variety of proteases, as well as the fact that these same parts of the molecule are the most readily accessible for chemical cross-linking, gave rise to the notion that the carboxy-terminal halves of α chains are "free swimming appendages" (Doolittle, 1973). This portion of the chain has also been shown to be involved in the factor XIII-induced cross-linking of α chains, since its proteolytic removal prevents multimer formation (e.g., Mattock and Esnouf, 1971; Finlayson and Mosesson, 1973). It also contains the acceptor sites for the incorporation of substitute donors in the cross-linking process, which in α chains reaches a plateau level of about 2 mol per mol (Pisano et al., 1971). Ferguson et al. (1974) have localized these sites to the central region of α chains, in substantive agreement with the estimated location of the cross-linking sites themselves determined by Finlayson and Mosesson (1973).

What we can add to these findings are the relative locations of the two different α-chain acceptor sites and the sequential arrangement of peptides around one of them. The other is located somewhat more distally (toward the α chain carboxy-terminal) in the large molecular weight fragment. We have designated this latter "fragment A" since it has an unusual amino acid composition resembling that part of a plasmin digest which elutes earliest on DEAE (Nussenzweig et al., 1961). The amino acid composition deserves special comment (Table I). Forty percent of the residues are glycine or serine, with proline and threonine contributing another 20%. Nonpolar residues make up fewer than 15% of the approximately 200 residues which constitute this section of the chain. Circular dichroism studies[4] on purified fragment A prepared under nondenaturing conditions indicate that the material is almost entirely a random coil under simulated physiological conditions.

It ought to be remarked that the midsection segment described in this article includes two peptides which are very similar to peptides characterized by other workers. Funatsu and his co-workers (Takaki et al., 1972a,b) isolated a decapeptide from plasminolyzed *bovine* fibrinogen, the sequence of which is very similar to peptide PIVC3 isolated from our human digests (Figure 5). Those authors reported that the bovine peptide had anticoagulant activity in that it inhibited the partial thromboplastin time in vitro. In our laboratory, we have synthesized what we believe to be the corresponding human sequence and found it to be inactive in that regard, however (Hucko and Doolittle, 1975). On a similar note, Lorand (1972) has reported that one of his students partially characterized an α chain (cross-linking) acceptor site peptide which began with the sequence Ala-Leu-Thr-Asp-Lys-Pro-Gln. . . . The species of origin was not mentioned in that report, however, and it remains conjectural as to whether the single amino acid difference compared with the corresponding sequence reported in this article is due to a species difference (Figure 5). In either case, there is no significant resemblance between this cross-linking acceptor site and the intermolecular cross-linking site which exists near the carboxy-terminus of γ chains (Chen and Doolittle, 1971).

In summary, the removal of a large portion of the fibrinogen α chain from the parent molecule is effected by a series of four closely spaced plasmin clips in the region of the chain approximately delineated by residues 200–250. As a consequence, three small peptides (9–14 residues in length) are released very early in the digestion process. These peptides are small enough that they are readily synthesized for use in radio-immune assays, and as such they may be useful indicators of plasminolytic activity, since their immunologic character after release ought to be significantly different from the intact molecule. In contrast, the large molecular weight, free-swimming appendage (fragment A) is likely to be a random coil both before and after its release from the parent molecule. As a result, its main immunologic features ought not to change significantly in the process, thereby limiting its usefulness in that sense. The function of these highly exposed and easily removed polar appendages remains entirely mysterious.

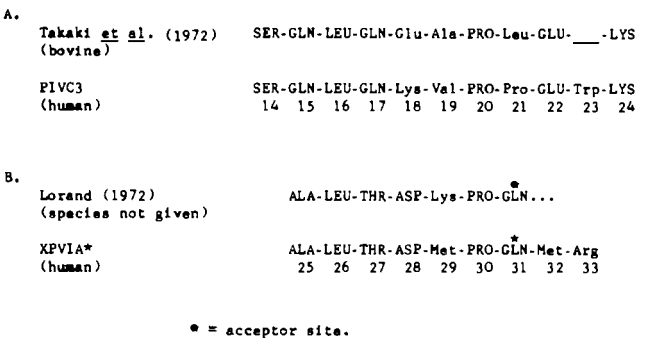

FIGURE 5: Comparison of amino acid sequences of two peptides studied by other workers with portions of the polypeptide segment reported in this article. (A) A peptide isolated from plasmin digests of bovine fibrinogen by Funatsu and co-workers (Takaki et al., 1972) and reported to have anticoagulant activity, compared with peptide PIVC3 from plasminolyzed human fibrinogen. (B) An acceptor peptide fragment described by Lorand (1972) (species not reported), compared with acceptor peptide XPVIA* from plasminolyzed human fibrinogen.

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[4] The circular dichroism spectra were determined with the cooperation of Dr. Wilson Radding, Department of Chemistry, University of California, San Diego.

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The Covalent Modification of Myosin's Proteolytic Fragments by a Purine Disulfide Analog of Adenosine Triphosphate. Reaction at a Binding Site Other than the Active Site†

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ABSTRACT: A purine disulfide analog of ATP, 6,6′-dithiobis(inosinyl imidodiphosphate), forms mixed disulfide bonds between the 6 thiol group on the purine ring and certain key cysteines on myosin, heavy meromyosin, and subfragment one. The EDTA ATPase activities of myosin and heavy meromyosin were completely inactivated when 4 mol of thiopurine nucleotide was bound. When similarly inactivated, subfragment one, depending on its method of preparation, incorporated either 1 or 2 mol of thiopurine nucleotide. Modification of a single cysteine on subfragment one resulted in an inhibition of both the Ca^{2+} and the EDTA ATPase activities, but the latter always to a greater extent. Modification of two cysteines per head of heavy meromyosin had the same effect suggesting that the active sites were not blocked by the thiopurine nucleotides. Direct evidence for this suggestion was provided by equilibrium dialysis experiments. Heavy meromyosin and subfragment one bound 1.9 and 0.8 mol of [8-^{3}H]adenylyl imidodiphosphate per mol of enzyme, respectively, with an average dissociation constant of 5×10^{-7} *M*. Heavy meromyosin with four thiopurine nucleotides bound or subfragment one with two thiopurine nucleotides bound retained 65–80% of these tight adenylyl imidodiphosphate binding sites confirming the above suggestion. Thus previous work assuming reaction of thiopurine nucleotide analogs at the active site of myosin must be reevaluated. Ultracentrifugation studies showed that heavy meromyosin which had incorporated four thiopurine nucleotides did not bind to F-actin while subfragment one with one thiopurine nucleotide bound interacted only very weakly with F-actin. Thus reaction of 6,6′-dithiobis(inosinyl imidodiphosphate) at nucleotide binding sites other than the active sites reduces the rate of ATP hydrolysis and inhibits actin binding. It is suggested that these second sites may function as regulatory sites on myosin.

The role of ATP in contraction and relaxation of muscle remains an area of active investigation despite literally hundreds of papers on this subject. ATP appears to play a dual role in these reactions. In the presence of micromolar concentrations of Ca^{2+}, its hydrolysis is the immediate source of energy for movement. In the absence of Ca^{2+}, ATP acts to dissociate actin and myosin allowing myofibrils to relax. The ability to label ATP binding sites covalently with an ATP-like molecule should allow one to better define these two functions of ATP.

Recently we have reported on the stoichiometry and sub-

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