Biochemistry

 Volume 25, Number 3 February 11, 1986

Articles

Localization of Segments Essential for Polymerization and for Calcium Binding in the γ-Chain of Human Fibrinogen†

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Received July 9, 1985

ABSTRACT: We have isolated an intermediate plasmic degradation product, D_2, of fibrinogen that does not inhibit the polymerization of fibrin monomer but does bind Ca^{2+}. Fibrinogen was digested to a limited extent with plasmin in the presence of Ca^{2+}, and a "large" fragment D (fragment D_{1A}) was isolated with a γ-chain remnant consisting of residues 63–411. Fragment D_{1A} was digested further in the presence of Ca^{2+}, yielding fragment D_1 (with its γ-chain containing residues 86–411). The digestion of fragment D_1 [in the presence of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to complex Ca^{2+}] led to a gradual shortening of the carboxyl-terminal portion of the γ-chain. Fragment D_2 (with its γ-chain containing residues 86–335/356) was isolated from an intermediate digest in the presence of EGTA. The Lys-338–Cys-339 peptide bond of the γ-chain is intact in this preparation of D_2, even though it is split in the isolated peptide γ303–355 (with an intact disulfide bond at Cys-326–Cys-339). Fragment D_2 does not interfere with the polymerization of fibrin monomer, whereas fragment D_1 is a potent inhibitor of this polymerization. We conclude that the γ-chain segment 356/357–411, present in fragment D_1 but absent from fragment D_2, is essential for maintenance of a polymerization site located in the outer (D) nodule of fibrinogen. This segment (356/357–411) is longer than two shorter ones reported earlier [Olexa, S. A., & Budzynski, A. Z. (1981) *J. Biol. Chem. 256*, 3544–3549; Horwitz, B. H., Váradi, A., & Scheraga, H. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5980–5984]; the data for the earlier reports are reinterpreted here. Finally, fragment D_2 possesses a single Ca^{2+} binding site, as revealed by equilibrium dialysis binding studies. Since fragment D_3 (with its γ-chain containing residues 86–302) fails to bind Ca^{2+}, we conclude that segment γ303–355/356 plays a crucial role in Ca^{2+} binding.

The conversion of fibrinogen to fibrin is started with the thrombin-induced removal of the amino-terminal fibrinopeptides FpA and FpB from the Aα- and Bβ-chains, respectively. The resulting fibrin monomers associate in a half-staggered overlapping manner to form protofibril strands. In a later stage, the protofibrils aggregate laterally. No covalent bonds are formed in this polymerization. However, in vivo, the fibrin network is enforced by covalent cross-links between glutamine and lysine residues. This transamidation is catalyzed by the plasma transglutaminase factor $XIII_a$ [for a recent review, see Doolittle (1984)].

Two different types of polymerization sites participate in the noncovalent association of fibrin monomers (Scheraga & Laskowski, 1957; Kudryk et al., 1974). The first of these is located in the amino-terminal (central) nodule of the molecule (Telford et al., 1980). It is nonfunctional (buried/covered) in fibrinogen and becomes exposed only upon removal of the fibrinopeptide(s). The second type of site, which is complementary to the amino-terminal site, is fully functional in fibrinogen (preexisting polymerization sites), as indicated by the ability of fibrinogen to bind to immobilized fibrin monomer (Heene & Matthias, 1973) and to inhibit the aggregation of fibrin monomers (Donnelly et al., 1955).

Plasmic degradation products, consisting of the amino-terminal nodule (fragment E) or the carboxyl-terminal nodule (fragment D)[1] of fibrinogen, have proven to be excellent

† This work was supported by a research grant (HL-30616) from the National Heart, Lung, and Blood Institute of the National Institutes of Health.

0006-2960/86/0425-0519$01.50/0

materials with which to localize different biological functions of fibrinogen. Affinity chromatography of the plasmic degradation fragments of fibrinogen on fibrin monomer–Sepharose, as well as studies of their inhibitory action on fibrin polymerization, has revealed that the preexisting polymerization sites are localized in fragment D, i.e., in the outer (carboxyl-terminal) nodule of fibrinogen (Matthias et al., 1973; Kudryk et al., 1974; Telford et al., 1980).

The presence of Ca^{2+} during plasmic digestion of fibrinogen influences the covalent structure of fragment D. In the presence of Ca^{2+}, the higher molecular weight fragment, fragment D_{1A} (mixed with D_1) ($\sim M_r$ 100 000), is obtained (Haverkate & Timan, 1977). It contains an active polymerization site. However, when Ca^{2+} is removed by complexation with EDTA, a somewhat lower molecular weight product, fragment D_3, is isolated ($\sim M_r$ 85 000) (Haverkate & Timan, 1977). It neither binds to immobilized fibrin monomer (Collen et al., 1975) nor inhibits fibrin polymerization (Dray-Attali & Larrieu, 1977). Since the only structural difference between fragments D_1 and D_3 is that the latter lacks the C-terminal portion of the γ-chain (residues 303–411) (Henschen, 1981), it is generally accepted that this 109-residue segment plays an essential role in fibrin polymerization.

It became superfluous to try to localize this site further by isolating variants of intermediate fragment D with γ-chains longer than that of fragment D_3 but shorter than that of fragment D_1 when Olexa & Budzynski (1981) demonstrated that a 38-residue peptide, liberated during the proteolytic conversion of D_1 to D_3, binds to the amino-terminal polymerization sites and inhibits fibrin polymerization. This peptide corresponds to the C-terminal region of the γ-chain of fibrinogen, residues 374–411. Therefore, a preexisting polymerization site was localized within this region. We attempted to narrow this site further by isolating shorter peptides, to determine the shortest structure required for the activity of this polymerization site and to identify the essential residues involved in this site. As a first step in this approach, we reported that a 23-residue peptide (γ374–396) also binds to fibrin monomer–Sepharose and inhibits fibrin polymerization (Horwitz et al., 1984).

In this paper, we report the isolation of a variant of fragment D, viz., fragment D_2, with an intermediate-length γ-chain. This fragment does not inhibit fibrin polymerization. We determined the C-terminal boundary of its γ-chain and conclude that the segment missing from fragment D_2 but present in fragment D_1 is essential for fibrin polymerization. This work was necessary because, as will be described herein, we found the following: (i) the binding of peptides γ374–396 and γ374–411 to immobilized fibrin monomer is due to nonspecific (probably electrostatic) interactions with the column matrix, rather than to specific peptide–protein interactions, because these peptides were found to bind to the unsubstituted matrix as well; (ii) contrary to earlier results (Olexa & Budzynski, 1981; Horwitz et al., 1984), the peptides do not inhibit fibrin polymerization. The apparent inhibitory activity of the earlier peptide preparations probably resulted from the presence of EGTA in those samples, since EGTA coelutes with the peptides from the fibrin monomer–Sepharose column.

During the course of the present work, we also found that the segment γ303–355/356 plays a crucial role in Ca^{2+} binding.

Experimental Procedures

Materials. Fibrinogen (human, Kabi grad L), urokinase (Abbokinase, Abbot), Trasylol (Bayer), and atroxin (Bothrops atrox venom, Sigma) were commerical products. Human α-thrombin was a gift from John Fenton II (New York Department of Health, Albany, NY). Human plasminogen was a gift from Laszlo Patthy (Institute of Enzymology, Hungarian Academy of Sciences, Budapest).

Preparation of Variants of Fragment D. Fragment D_{1A} was prepared by the method of Fowler et al. (1980), as described previously (Váradi & Patthy, 1983). Fragment D_1 was prepared by further digestion of D_{1A} (using a higher concentration of protease) as follows: fragment D_{1A} (10 mg/mL) was dissolved in a 0.05 M Tris·HCl and 0.15 M NaCl, pH 7.4, buffer containing Ca^{2+} (5 mM). Plasminogen was added to a final concentration of 100 μg/mL, and the digestion was initiated by addition of urokinase (150 CTA units/mL). The digestion was carried out at 37 °C for 60 min. The proteolysis was arrested by addition of Trasylol (100 KIU/mL), and the digest was dialyzed extensively against 0.1 M NH_4HCO_3 and lyophilized.

To prepare fragment D_2, fragment D_1 (10 mg/mL) was dissolved in 0.05 M Tris·HCl and 0.15 M NaCl, pH 7.4, buffer containing EGTA (5 mM) (to complex Ca^{2+}). Plasminogen was added to a final concentration of 100 μg/mL, and the digestion was initiated (at 37 °C) by addition of urokinase (150 CTA units/mL). After 60 min, the proteolysis was terminated by addition of Trasylol (100 KIU/mL). The digest was applied onto a Sephadex G-25 desalting column (6 × 1.5 cm) equilibrated with 0.02 M Tris·HCl and 0.1 M NaCl, pH 8.0, buffer (i.e., Mono Q/I buffer). Then the desalted digest was applied onto a Mono Q HR 5/5 anion-exchange column (Pharmacia), equilibrated with the Mono Q/I buffer. The separation was carried out with a linear salt gradient between 0.1 M (initial) and 0.2 M (final) concentration of NaCl within 30 min. The pool containing fragment D_2 was stored frozen at −20 °C.

Fragment D_3 was prepared by digesting fragment D_{1A} for 360 min, as described in the next section. Fragment D_3 was isolated from the digest by chromatography on an FPLC gel filtration column (Superose 12, HR 30 × 10, Pharmacia) equilibrated with 0.05 M Tris·HCl and 0.15 M NaCl, pH 7.6, buffer. The concentrations of the variants of fragment D were calculated by using the extinction coefficient, $E_{1\%}^{280nm} = 20.8$, of Marder et al. (1969).

Isolation of Peptides Liberated upon Plasmic Digestion of Variants of Fragment D. To isolate the peptides liberated in the successive plasmic degradation steps, the above digestions were repeated under slightly different conditions (i.e., with repeated additions of urokinase and plasminogen) as follows: fragments D_{1A} and D_2 were dissolved separately in 0.05 M Tris·HCl and 0.15 M NaCl, pH 7.4, buffer, some containing $CaCl_2$ (5 mM) and some containing EGTA (5 mM), and digested as described above. Then, to compensate for autolysis of plasmin, an additional digestion mixture was added in two successive stages; i.e., at 120 and 240 min, the same aliquots of plasminogen and urokinase, that had been used for initiating the proteolysis, were added to the digests. The progress of

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; CTA, Committee on Thrombolytic Agents; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; KIU, kallikrein inhibitory unit(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; NDSK, amino-terminal cyanogen bromide fragment of fibrinogen; fragments D_{1A}, D_1, D_2, and D_3, carboxyl-terminal plasmic fragments of fibrinogen with identical α- and β-chains but with different γ-chains; γ_{1A}, γ_1, γ_2, and γ_3, γ-chains of fragments D_{1A} (residues 63–411), D_1 (residues 86–411), D_2 (residues 86–355/356), and D_3 (residues 86–302), respectively.

degradation was followed by SDS–PAGE.

Peptides liberated during the different plasmic digestions were analyzed by reversed-phase HPLC as described earlier (Horwitz et al., 1984). The amino acid compositions of the isolated peptides were determined by amino acid analysis on a Waters Picotag HPLC system.

Reduction of Disulfide Bonds. To determine whether any disulfide-linked polypeptides had internal proteolytic splits, the disulfide bonds were reduced, and the number of resulting fragments was assessed by isolation. The peptide mixtures or the protein fragments to be analyzed were dissolved in 0.05 M Tris·HCl and 0.15 M NaCl, pH 7.4, buffer previously deaerated with N_2. Dithioerythritol (0.1 M) was added, and the mixture was incubated at 100 °C for 15 min under a N_2 atmosphere.

Ca^{2+} Binding. Equilibrium dialysis was used to characterize the Ca^{2+} binding properties of fragment D_2, following the procedure used by Van Ruijven-Vermeer et al. (1978) for rat fibrinogen fragments. The buffer used, 0.05 M Tris·HCl and 0.15 M NaCl, pH 7.6, was freed from Ca^{2+} by Chelex (Bio-Rad), and the dialysis bags were pretreated according to the method of Marguerie et al. (1977). Variants of fragment D were dissolved in this buffer and dialyzed extensively against the same buffer (containing EGTA, 3 mM) to remove traces of Ca^{2+}. EGTA was then removed by extensive dialysis against the Ca^{2+}-free buffer. Aliquots of fragment D_1 and D_2 (0.5 mL, 1.1×10^{-5} and 1.5×10^{-5} M, respectively) were dialyzed against 20 mL of buffer containing (4–400) $\times 10^{-6}$ M $CaCl_2$ at 22 °C for 48 h. The Ca^{2+} concentration of the solution inside and outside the dialysis bag was determined with an inductively coupled argon–plasma atomic emission spectrometer.

Aliquots of fragment D_3 (0.5 mL, 1.3×10^{-5} M) were dialyzed against 20 mL of buffer containing 2, 20, and 200 $\times 10^{-6}$ M $CaCl_2$, enriched with $^{45}Ca^{2+}$ (Amersham), 15 μCi/μmol. After 48 h of dialysis, 200-μL aliquots of the solution inside and outside the dialysis bag were taken out and mixed with 2.5 mL of scintillation fluid [aqueous counting scintillant (Amersham)] and counted by a liquid scintillation counter (Beckman, LS-230).

Other Procedures. Fibrin monomer–Sepharose chromatography for isolation of peptides, and the experiments on the inhibition of fibrin polymerization, was carried out as in our previous work (Horwitz et al., 1984). Proteins were analyzed by SDS–PAGE, 7% acrylamide gels (Laemmli, 1970) for nonreduced samples or 9% gels (Weber & Osborn, 1969) for reduced samples. The apparent molecular weights of the proteins were calculated by using the "low molecular weight calibration kit" of Pharmacia.

RESULTS

Plasmic Digestion of Fragment D_{1A}: Effect of Ca^{2+}. Fragment D_{1A} was purified from a plasmic digest of fibrinogen. The digestion was carried out in the presence of Ca^{2+}. Under these conditions, the "large" fragment D is obtained (Haverkate & Timan, 1977; Purves et al., 1978); it contains a portion of the alleged triple-helical region as well as the entire outer nodule of fibrinogen. Its β- and γ-chains extend to the C-termini of the original fibrinogen molecule, while its α-chain is relatively short, lacking the long C-terminal "random-coil" region. This large fragment D (fragment D_{1A}) possesses an active polymerization site (Dray-Attali & Larrieu, 1977; Furlan et al., 1983; Knoll et al., 1984).

When fragment D_{1A} was subjected to further plasmic digestion, using a higher concentration of the protease (in the presence of Ca^{2+}), a small reduction of size was observed

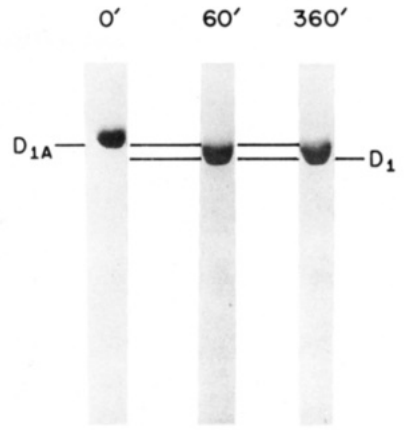

FIGURE 1: Digestion of fragment D_{1A} with plasmin in the presence of Ca^{2+}, as described under Experimental Procedures. The progress of the proteolysis was followed for the indicated times by SDS–PAGE under nonreducing conditions.

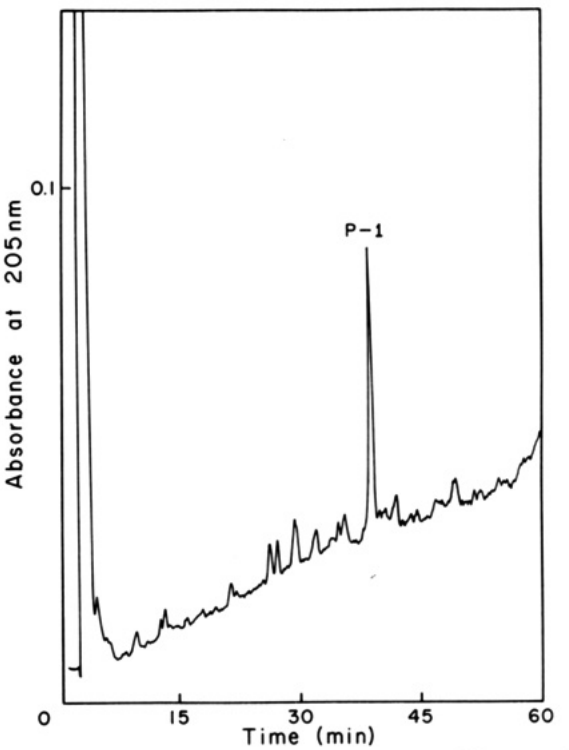

FIGURE 2: Peptides liberated upon plasmic digestion of fragment D_{1A} in the presence of Ca^{2+}. HPLC chromatogram of the peptide fraction of the 360-min digest.

(Figure 1). No further degradation could be achieved upon prolonged incubation and multiple addition of the protease. The peptides liberated during this digestion were analyzed by HPLC. Essentially one peptide, P-1, was found as the major component of the digest in the presence of Ca^{2+} (Figure 2). The peptide was collected, and its amino acid composition was determined (Table I). On the basis of its amino acid composition, and the known sequence of fibrinogen (Doolittle et al., 1979; Henschen et al., 1983), part of which is shown in Figure 3, P-1 is identified as the N-terminal segment of the D_{1A} γ-chain, residues 63–85 (fibrinogen numbering). The segment 86–411 is connected to remnants of both the α- and β-chains by a network of disulfide bonds in the region of residues 86–302. The cleavage of the Lys-85–Ser-86 bond by extensive plasmic digestion is not unexpected (Collen et al., 1975). These authors found that the N-terminal residue of the D_{1A} γ-chain isolated from an "early" plasmic digest is Ala-63, while the N-terminal amino acid of the γ-chain of a

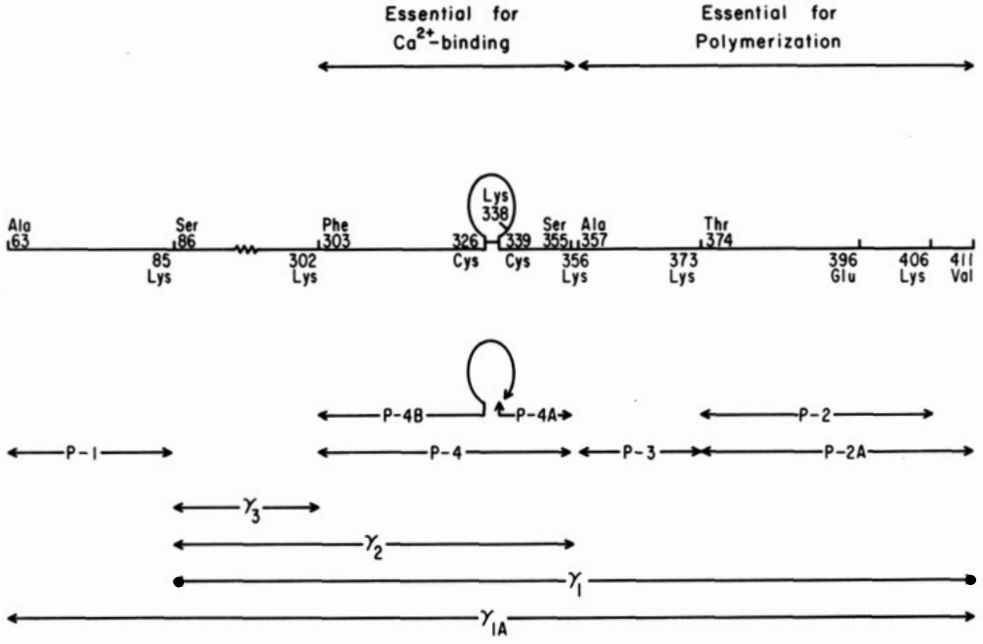

FIGURE 3: Schematic representation of the amino acid sequence of the γ-chain remnants of different variants of fragment D. The regions essential for polymerization and for Ca^{2+} binding, and the locations of peptides P-1, P-2, P-2A, P-3, and P-4 (cleaved by plasmin in the presence of Ca^{2+} or EGTA), are indicated. The residues adjacent to the plasmic cleavage sites are also shown. γ_{1A} (residues 63–411) is the γ-chain of fragment D_{1A}, γ_1 (residues 86–411) is that of fragment D_1, γ_2 (residues 86–355/356) is that of fragment D_2, and γ_3 is that of fragment D_3. The published sequence of the human fibrinogen γ-chain (Henschen et al., 1983) was used for residue numbering. See Doolittle (1984) for the location of fragment D in the fibrinogen molecule, and for the current model of the structure of fibrinogen.

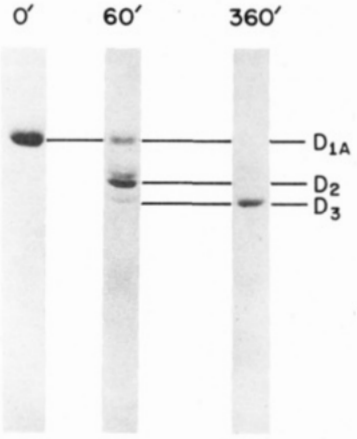

FIGURE 4: Digestion of fragment D_{1A} with plasmin in the presence of EGTA, as described under Experimental Procedures. The progress of proteolysis was followed for the indicated times by SDS–PAGE under nonreducing conditions. The molecular weights of the proteins, as determined by SDS–PAGE, are 100000 (D_{1A}), 91000 (D_2), and 85000 (D_3).

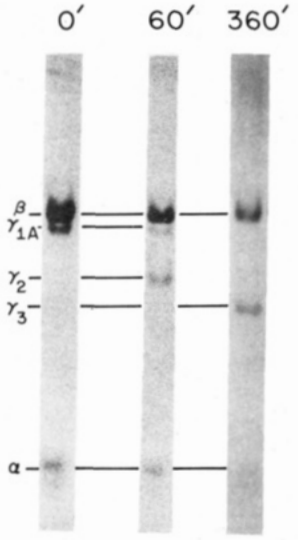

FIGURE 5: Digestion of fragment D_{1A} with plasmin in the presence of EGTA, as described under Experimental Procedures. The progress of proteolysis was followed for the indicated times by SDS–PAGE under reducing conditions. The molecular weights of the isolated chains, as determined by SDS–PAGE, are 44000 (β), 40000 (γ_{1A}), 29000 (γ_2), 25000 (γ_3), and 14000 (α).

"late" digestion product is Ser-86.

Removal of Ca^{2+} from the digestion mixture with the chelating agent EGTA led to a different, more complex degradation pattern. The size of fragment D_{1A} decreased gradually to a final product, fragment D_3, with a molecular weight approximately 15000 smaller than that of D_{1A} (Haverkate & Timan, 1977) (Figure 4). During digestion in the absence of Ca^{2+}, fragment D_1 is not detectable, but D_2 is (at intermediate times; see below). Fragment D_3 is resistant to further plasmic attack, since no further change of its size is apparent between 6- and 24-h digestion (not shown). In agreement with the results of Haverkate & Timan (1977) and Hörmann & Henschen (1979), the observed decrease in size is due to the gradual shortening of the D_{1A} γ-chain from γ_{1A} to γ_2 to γ_3, as revealed by gel electrophoresis of the reduced digest (Figure 5).

The peptides released upon plasmic digestion of fragment D_{1A} in the presence of EGTA were analyzed by HPLC. Figure 6 shows a typical chromatogram of a peptide mixture obtained after 60-min digestion. Three major peptide components of the digest were isolated, and their amino acid compositions are summarized in Table I. P-1 is the N-terminal peptide (Ala-63–Lys-85) which appears in both of the digests, in the presence of Ca^{2+} and of EGTA, respectively. P-2 originates from the C-terminal portion of the γ-chain, corresponding to Thr-374–Lys-406. The last five C-terminal amino acids of the fibrinogen γ-chain are missing from P-2. When a lower protease concentration was used for digestion, peptide P-2A appeared on the HPLC peptide map with a slightly longer

Table I: Amino Acid Composition of Peptides Isolated by HPLC from Various Plasmic Digests of Different Variants of Fragment D

	peptide[a]						
amino acid residue	P-1, 63–85	P-2, 374–406	P-2A, 374–411	P-3, 357–373	P-4, 303–355	P-4A, 339–355	P-4B, 303–338
Asx[b]	4.40 (4)[d]	1.25 (1)	2.20 (2)	3.10 (3)	9.60 (10)	1.10 (1)	8.10 (9)
Glx[c]	1.90 (2)	3.10 (3)	4.00 (4)	0.20 (0)	5.40 (5)	1.10 (1)	3.75 (4)
Ser	2.35 (2)	0.95 (1)	0.85 (1)	0.95 (1)	3.75 (4)	0.90 (1)	3.45 (3)
Gly	0.10 (0)	3.85 (4)	4.70 (5)	2.30 (2)	8.00 (8)	4.10 (4)	4.25 (4)
His	<0.05 (0)	2.40 (2)	2.10 (2)	0.10 (0)	2.65 (3)	1.35 (2)	0.80 (1)
Arg	<0.05 (0)	1.80 (2)	1.70 (2)	<0.05 (0)	0.25 (0)	<0.05 (0)	0.20 (0)
Thr	1.75 (2)	3.90 (4)	3.60 (4)	1.90 (2)	2.75 (3)	1.10 (1)	2.00 (2)
Ala	3.20 (3)	1.25 (1)	2.05 (2)	2.45 (2)	2.15 (2)	1.30 (1)	1.10 (1)
Val	<0.05 (0)	<0.05 (0)	1.30 (1)	<0.05 (0)	0.80 (1)	0.95 (1)	<0.05 (0)
Pro	2.20 (2)	1.05 (1)	1.25 (1)	1.10 (1)	<0.05 (0)	0.25 (0)	0.10 (0)
Tyr	0.80 (1)	1.00 (1)	0.70 (1)	0.70 (1)	2.25 (3)	2.55 (3)	0.35 (0)
Met	0.90 (1)	2.05 (2)	1.75 (2)	<0.05 (0)	1.90 (2)	0.20 (0)	2.20 (2)
Cys	<0.05 (0)	<0.05 (0)	<0.05 (0)	<0.05 (0)	2.25 (2)	nd[e] (1)	0.80 (1)
Ile	1.75 (2)	2.60 (3)	2.60 (3)	1.80 (2)	<0.05 (0)	<0.05 (0)	0.15 (0)
Leu	1.70 (2)	2.00 (2)	2.20 (2)	0.10 (0)	1.15 (1)	1.10 (1)	0.10 (0)
Phe	0.20 (0)	0.90 (1)	1.05 (1)	<0.05 (0)	3.80 (4)	<0.05 (0)	4.25 (4)
Lys	1.70 (2)	4.20 (4)	4.05 (4)	1.10 (1)	2.00 (2)	0.15 (0)	2.10 (2)
Trp	nd (0)	nd (1)	nd (1)	nd (2)	nd (3)	nd (0)	nd (3)

[a] The composition is expressed as number of moles per mole of peptide. The numbers next to the symbols identifying the peptides are the positions in the γ-chain. [b] Asp + Asn. [c] Glu + Gln. [d] The theoretical values (Henschen et al., 1983) are given in parentheses. [e] Not determined.

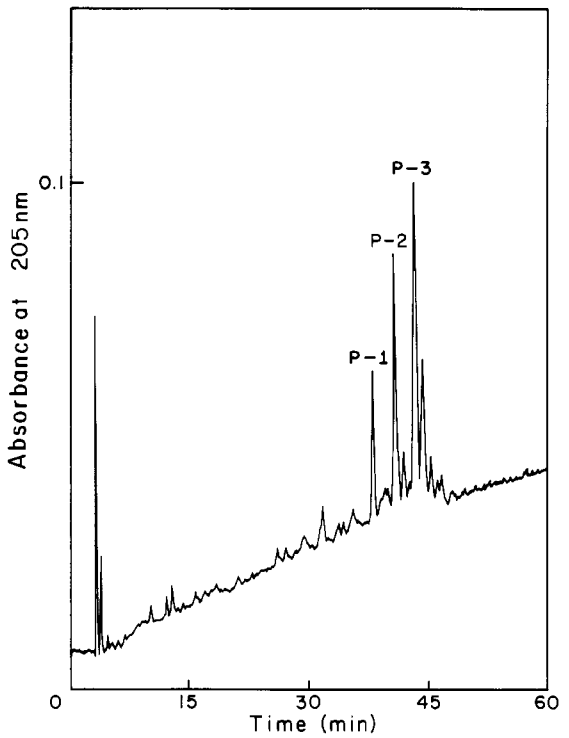

FIGURE 6: Peptides liberated upon plasmic digestion of fragment D_{1A} in the presence of EGTA. HPLC chromatogram of the peptide fraction of the 60-min digest.

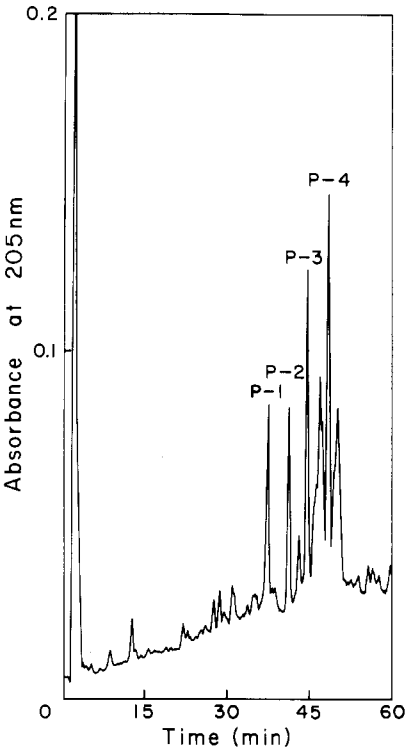

FIGURE 7: Peptides liberated upon plasmic digestion of fragment D_{1A} in the presence of EGTA. HPLC chromatogram of the peptide fraction of the 360-min digest.

retention time than P-2 (not shown). Peptide P-2A is the γ374–411, C-terminal peptide (Table I). The amino acid composition of peptide P-3 corresponds to the γ-chain region 357–373 (Table I). We did not study the relative rates of hydrolysis of the 356–357 vs. 373–374 bonds.

It is worthwhile to note that, after 60-min digestion in the presence of EGTA, when P-1, P-2, and P-3 are present as free peptides in the digest, the majority of fragment D is in the fragment D_2 stage (Figure 4).

In a late digest (after 6 h and after multiple additions of plasmin), a new peptide component, P-4, is observed (Figure 7). The amino acid composition of P-4 suggests that it should correspond to the 303–356 segment of the γ-chain (Table I) but its lysine content is significantly lower than the expected value; i.e., two lysines were found while three would be predicted for the segment 303–356 on the basis of the sequence of fibrinogen and the known specificity of the protease. Furthermore, on the basis of the amino acid composition of the peptide, one cannot answer the question as to whether the Lys-338–Cys-339 peptide bond is intact or cleaved in P-4 because this bond lies within a disulfide loop. If this bond is cleaved, the two halves of the peptide are held together by the 326–339 disulfide bond. To distinguish between these two possibilities, the late digest was reduced, and the resulting peptide mixture was analyzed by HPLC (Figure 8). Peptide P-4 completely disappeared from the peptide map, and its disappearance was accompanied by the appearance of two new peptides, P-4A and P-4B. Their amino acid compositions were also determined (Table I), leading to the conclusion that P-4B corresponds to the Phe-303–Lys-338 segment, while P-4A

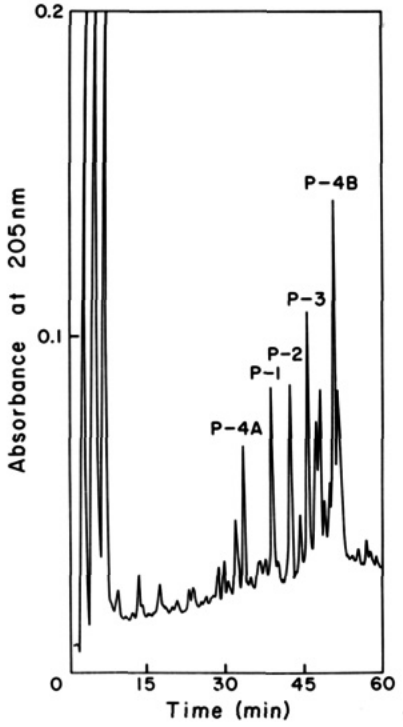

FIGURE 8: Effect of reduction of disulfides on the peptides liberated upon plasmic digestion of fragment D_{1A} in the presence of EGTA. The peptide fraction of the 360-min digest (Figure 7) was incubated under strongly reducing conditions and analyzed by HPLC.

corresponds to the Cys-339–Ser-355 segment. Consequently, the Lys-338–Cys-339 bond is, indeed, cleaved in peptide P-4. It is also clear that Lys-356 is missing from P-4, since P-4A contains no lysine residue but P-4B contains two lysines (at positions 321 and 338, respectively).[2]

Isolation and Structure of Fragment D_2. The intermediate plasmic digest of fragment D_{1A}, obtained in the presence of EGTA (Figure 4, 60 min), provides a good source for the isolation of an intermediate-size fragment (fragment D_2). First, fragment D_{1A} was subjected to plasmic digestion in the presence of Ca^{2+} (as in Figure 1) to remove the N-terminal 63–85 segment of the γ-chain and produce D_1 (see previous discussion in this section). Then Ca^{2+} was removed by dialysis, and fragment D_1 was digested in the presence of EGTA, the latter serving to remove traces of Ca^{2+} (if present) by chelation.

The "EGTA digest" was separated by FPLC on a Mono Q HR 5/5 anion-exchange column (Figure 9). Peak III was found to be a homogeneous intermediate degradation product, fragment D_2. It was homogeneous on the basis of SDS–PAGE under nonreducing and reducing conditions (Figure 10). Its γ-chain is intermediate in size between the γ_1 and γ_3 chains, both of which are absent in the fragment D_2 preparation (Figure 10). Pool I was found to be fragment D_1, while pools II and IV yielded variants of fragment D with heterogeneous γ-chains (not shown).

The N-terminal residues of the α- and β-chains, respectively, of different variants of fragment D are identical (Collen et al., 1975; Takagi & Doolittle, 1975; VanRuijven-Vermeer et al., 1979). Since the size of the Dα- and the Dβ-chains does not change upon extensive plasmic digestion (Pizzo et al., 1973; Haverkate & Timan, 1977; Ferguson, 1980; Figure 5), the structural difference between fragments D_1 and D_2 is restricted

[2] The removal of a C-terminal lysine residue by plasmin (or by a carboxypeptidase-like enzyme present in plasmin preparations) upon extensive digestion was also observed by Olexa et al. (1981) in the case of variants of fibrin fragment E.

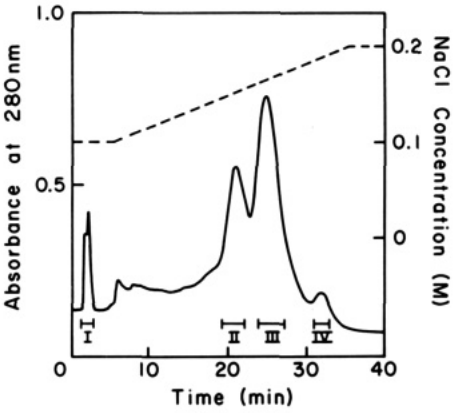

FIGURE 9: Purification of fragment D_2 by anion-exchange chromatography. The 60-min plasmic digest of fragment D_1, obtained in the presence of EGTA, was separated on a Mono Q HR 5/5 (Pharmacia) FPLC anion-exchange column. The column was equilibrated with 0.02 M Tris·HCl and 0.1 M NaCl, pH 8.0, buffer, and the chromatography was carried out with a linear salt gradient between 0.1 M (initial) and 0.2 M (final) concentration of NaCl within 30 min.

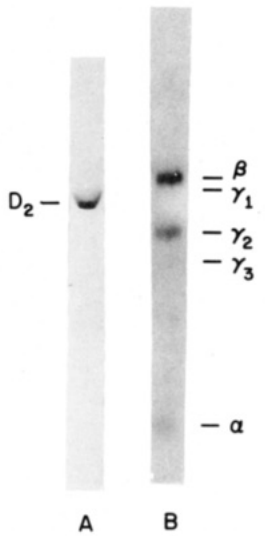

FIGURE 10: SDS electrophoretogram of pool III (fragment D_2) of Figure 9 obtained under nonreducing (A) and reducing (B) conditions.

to the difference between their γ-chains. The N-termini of the γ_1- and γ_2-chains are identical (i.e., Ser-86) since the Ala-63–Lys-85 portion had been completely removed from fragment D_1 by plasmin in the presence of Ca^{2+} prior to digestion in the presence of EGTA. By this procedure, the only heterogeneity of the N-termini of different variants of fragment D (Collen et al., 1975) is eliminated; the C-terminal boundary of the γ_2-chain remains to be determined.

Fragment D_2 was subjected to plasmic digestion in the presence of EGTA, and the peptides released were analyzed by HPLC (Figure 11). The major peptide component of this digest is P-4 (Table I), with an amino acid composition identical with that of P-4 isolated from the late plasmic digest of fragment D_{1A}. The quantity of peptide P-4 liberated from fragment D_2 was determined by amino acid analysis; it was found that 0.81–0.91 mol of peptide was released per mole of fragment D_2 (results of three determinations),[3] indicating that the majority of this preparation of fragment D_2 contains the γ303–355/356 segment. No P-1, P-2, or P-3 could be detected. The peptide pool was incubated under strongly re-

[3] These values are not corrected for possible losses during isolation of the peptides from the HPLC column.

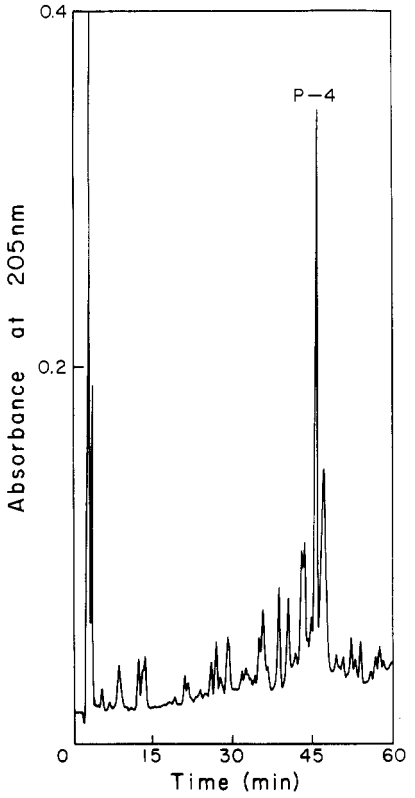

FIGURE 11: Peptides liberated upon plasmic digestion of fragment D_2 in the presence of EGTA. HPLC chromatogram of the peptide fraction of the 360-min digest.

ducing conditions, and the HPLC map of the reduced digest revealed that the Lys-338–Cys-339 peptide bond is cleaved in P-4, as was found in the case of P-4 isolated from the late plasmic digest of fragment D_1 (see Results above). Residue Lys-356 is also missing from this P-4 peptide.

To determine whether the Lys-338–Cys-339 bond had already been cleaved in fragment D_2 or if it is the result of the plasmic digestion of fragment D_2, fragment D_2 was incubated under strongly reducing conditions, and the reaction mixture was assayed for the presence of peptide P-4A (γ339–355). No released peptides were observed upon reduction of fragment D_2, even though its interchain disulfide bonds were reduced (yielding separated α-, β-, and γ-chains); this indicates that the Lys-338–Cys-339 peptide bond is intact in fragment D_2. The absence of Lys-356 from P-4 does not necessarily mean that this residue is not present in fragment D_2 since its removal could very well occur during the digestion of fragment D_2. Whether this residue is present or absent in our preparation of fragment D_2, or whether it retains heterogeneity only in this one residue, was not determined.

In summary, we have isolated an intermediate-sized fragment, fragment D_2, which differs from fragment D_1 only with respect to the C-terminal portion of its γ-chain, which lacks the 356/357–411 segment.

This 356/357–411 C-terminal portion of the γ-chain is rich in positively charged residues; fragment D_2, being more negatively charged than fragment D_1, bound strongly to the anion-exchange resin and could be eluted with a salt gradient (Figure 9).

Inhibition of Fibrin Polymerization with Fragment D_1 and with Fragment D_2. As shown in Figure 12, fragment D_1 strongly inhibits the polymerization of fibrin monomer, in agreement with the fact that a preexisting polymerization site is fully functional in fragment D_1. The inhibitory activity of fragment D_1 has been studied in detailed by many investigators

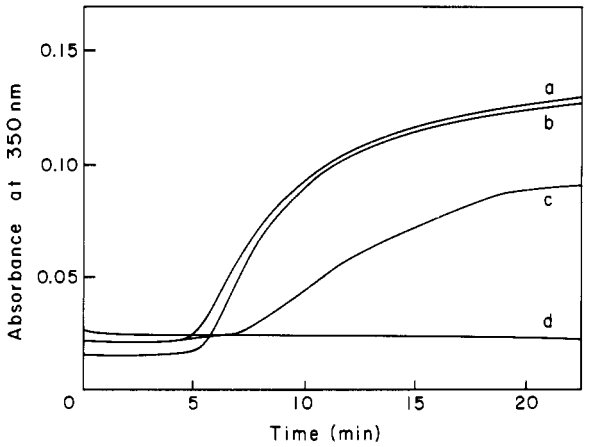

FIGURE 12: Inhibition of the polymerization of fibrin monomer by different variants of fragment D. The aggregation of de-A-fibrinogen (fibrin monomer lacking only fibrinopeptide A) was followed by recording the change of absorbance at 350 nm. The de-A-fibrinogen concentration was 1.2×10^{-6} M containing (a) no fragment D, (b) 6×10^{-6} M fragment D_2, (c) 2.2×10^{-6} M fragment D_1, and (d) 6×10^{-6} M fragment D_1.

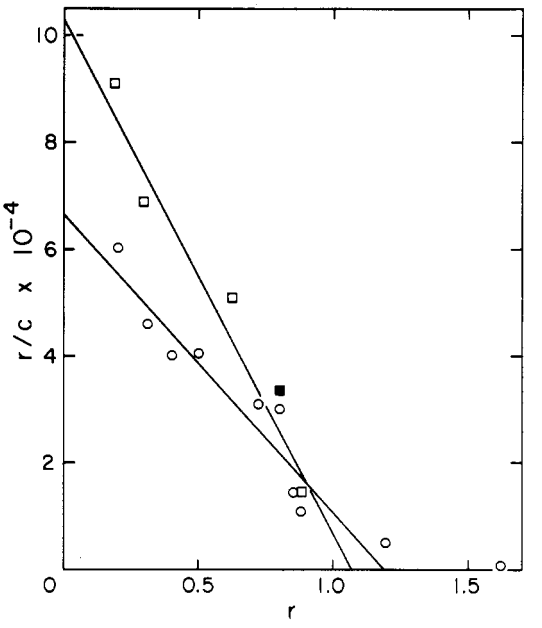

FIGURE 13: Ca^{2+} binding by fragments D_1 and D_2, determined by Scatchard analysis of equilibrium dialysis data. r is the number of moles of Ca^{2+} bound per mole of protein, and c is the concentration of free Ca^{2+} in moles per liter. (□) Fragment D_1; (○) fragment D_2 (data for both D_1 and D_2 were obtained by atomic emission spectroscopy); (■) fragment D_1 (obtained by assay for $^{45}Ca^{2+}$).

(Dray-Attali & Larrieu, 1977; Furlan et al., 1983; Knoll et al., 1984).

In contrast, fragment D_2 does not inhibit the polymerization of fibrin monomer (Figure 12). Since the only structural difference between fragments D_1 and D_2 is that the latter lacks the C-terminal 356/357–411 portion of the γ-chain, we may conclude that this portion plays an essential role in maintaining the fibrin polymerization site present in fragment D_1.

Ca^{2+} Binding by Fragments D_1, D_2, and D_3. Equilibrium dialysis was used to compare the Ca^{2+} binding properties of fragments D_1, D_2, and D_3. A Scatchard analysis of the data (Figure 13) revealed that both fragments D_1 and D_2 bind Ca^{2+}; one binding site was found for each fragment with comparable dissociation constants (0.95×10^{-5} and 1.6×10^{-5} M for fragments D_1 and D_2, respectively). These values lie in the range [$(0.9–1.7) \times 10^{-5}$ M] that had been determined by Nieuwenhuizen et al. (1979) and by Nieuwenhuizen &

Haverkate (1983) for fragment D_1.

No Ca^{2+} binding was detected for fragment D_3 by the equilibrium dialysis method, in agreement with earlier data of Nieuwenhuizen & Haverkate (1983).

Our results show that the structure essential for Ca^{2+} binding by fragment D_1 is preserved in fragment D_2; i.e., removal of the 356/357–411 segment of the γ-chain does not interfere with the Ca^{2+} binding site. On the other hand, removal of γ303–355/356 destroys the Ca^{2+} binding site.

Reinvestigation of the Binding Properties of Peptides Released from the C-Terminal Portion of the γ-Chain. It was previously demonstrated that peptides released upon proteolytic digestion of fragment D_1 interact with the N-terminal polymerization sites. Olexa & Budzynski (1981) isolated the C-terminal peptide of the γ-chain, residues 374–411, and showed that this peptide binds to immobilized fibrin monomer, inhibits fibrin polymerization, and interacts with the N-terminal polymerization site exposed in the N-terminal fragment of fibrinogen (NDSK) after removal of the fibrinopeptides. They also showed that the peptide does not bind to immobilized fibrinogen or to the NDSK before removal of fibrinopeptides. These results led to the conclusion that a polymerization site is present in the γ374–411 region. We isolated a shorter, overlapping peptide, γ374–396, and found that this peptide also binds to immobilized fibrin monomer and inhibits polymerization (Horwitz et al., 1984).

However, when we later assayed our γ374–396 peptide in an equilibrium binding experiment using Sephadex G-25 (Hirose & Kano, 1971), the same method used by Olexa & Budzynski (1981) for the γ374–411 peptide, we found that our peptide binds to the Sephadex matrix and can be desorbed by lowering the pH to 2.5. We obtained the same result with the γ374–411 peptide. Furthermore, the same, nonspecific effect was observed with Sepharose 4B, the matrix to which the fibrin monomer had been coupled and used as an affinity resin in both studies[4] (Olexa & Budzynski, 1981; Horwitz et al., 1984). This observed nonspecific binding of the peptides can be rationalized on the basis of their high net positive charge at neutral pH (approximately 4+ for both peptides) since both Sephadex and Sepharose gels can act as cation exchangers because of the free sulfate and carboxyl groups present at low concentration in both gel types (Pharmacia, 1980). It seems that the binding of these peptides to the immobilized fibrin monomer can be accounted for by nonspecific (electrostatic) binding. Presumably, the desorption of the peptides at low pH is due to the protonation of the negatively charged carboxyl groups of the resin. In addition, we found that fibrinogen–Sepharose also binds both peptides, which is contradictory to the results of Olexa & Budzynski (1981).

We also reinvestigated the experiments designed to determine the inhibitory activity of these peptides on fibrin polymerization. We measured the inhibition of the polymerization of fibrin monomer with both peptides that had been isolated by fibrin monomer–Sepharose chromatography, eluting the bound peptide fraction with a pH 2.5 buffer, as in our earlier experiments (Horwitz et al., 1984). However, when we used a different buffer (pH 3.3) for elution of the fibrin monomer–Sepharose-bound peptide fraction, we found that neither of the peptides (γ374–411 or γ374–396) inhibited the polymerization of fibrin monomer [this pH 3.3 buffer eluted both peptides from the column equally well, and the concentration of the pH 3.3 eluted peptides was 10 times higher (2.5 μM) in the inhibition assay than the pH 2.5 eluted peptides, the latter having reduced the rate of fibrin association to 50%]. To resolve this apparent paradox, we carried out the following control experiment: a "blank" digest was applied onto the fibrin monomer–Sepharose column, which contained all the constituents of the digests used in the previous experiments (buffer, salt, EGTA, protease), but fragment D_{1A} was omitted; i.e., no γ374–411 or γ374–396 peptide was present in these samples. The "bound" fractions of these blank digests were eluted with the pH 2.5 buffer. These pools caused strong inhibition of fibrin polymerization; however, it is apparent that they contained no peptide material, as was proved by HPLC. The use of the pH 3.3 buffer as an eluant led to a preparation which had no inhibitory effect in the fibrin polymerization assay. We conclude that some constituent of the digest, which coeluted with the peptide(s) when the pH 2.5 eluant was used, is responsible for the inhibitory properties of these samples. This "effector" was not eluted when the peptides were isolated by using the pH 3.3 eluant.

We found that the effector is EGTA since, when the same blank digest (but with EGTA omitted) was applied to and eluted from the fibrin monomer–Sepharose column under identical condition as above (i.e., with the pH 2.5 buffer),[5] we observed no inhibition of fibrin polymerization with this sample. EGTA is known to be a strong inhibitor of fibrin polymerization (Blombäck et al., 1966). In fact, we found that the rate of polymerization is reduced to 50% in the presence of 125 μM EGTA in our assay system. When we examined the pH 2.5 eluates, the EGTA concentration was even higher (10^{-3}–10^{-4} M) in the polymerization assay.

In summary, we found that peptides γ374–411 and γ374–396 do not interfere with fibrin polymerization and their binding to immobilized fibrin monomer can be accounted for by nonspecific interaction with the negatively charged polysaccharide matrix.

Discussion

Two different experimental approaches have been used for the localization of the polymerization site present in the outer nodule of fibrinogen. The first one is an "indirect" method, involving the isolation of large fragments of fibrinogen and then their assay for the presence of the polymerization site. The active fragment(s) was (were) then subjected to further degradation and examined to determine whether the binding site is preserved or not upon the removal of peptide segments. Using this type of method, it was established that a polym-

[4] The following experiments were carried out to compare the binding activities of peptides γ374–411 and γ374–396 to fibrin-substituted Sepharose and to unsubstituted Sepharose, respectively. An aliquot of 0.5 nmol of the peptide under study was applied to the column (2.2 cm × 1.2 cm), which was then washed with different volumes of buffer (0.1 M sodium phosphate, pH 7.6). After each washing, the remaining bound peptide was eluted with the pH 2.5 buffer, as described earlier (Horwitz et al., 1984), and its concentration was determined by HPLC. The amount of peptide that remained bound to the column decreased with each washing by 0.15% and 0.39% per milliliter for γ374–411 and γ374–396, respectively. These decreases were linear with volume of washing liquid, and the same decreases were found for *both* the fibrin-substituted and the unsubstituted Sepharose columns; i.e., both peptides showed the same affinity for both columns, indicating that the "binding" of these peptides to immobilized fibrin monomer can be accounted for by nonspecific (electrostatic) binding.

[5] Fibrinogen binds tri- and tetracarboxylic acids (Nieuwenhuizen et al., 1981). The elution of EGTA from immobilized fibrin monomer with the pH 2.5 buffer may be due to partial protonation of its second carboxyl group ($pK_2 \sim 2.0$–2.5); EGTA (with a single negative charge) *may* not bind to fibrin monomer. At pH 3.3, the second carboxyl group of EGTA is partially ionized, and this more highly negatively charged EGTA (possibly enhanced by the special geometric arrangement of the carboxyl groups of this compound) may bind to fibrin monomer.

erization site is present in fragment D_1 and lost when fragment D_1 is converted to fragment D_3 (Kudryk et al., 1974; Olexa & Budzynski, 1980); i.e., the γ303–411 segment that is removed plays an essential role in maintaining the polymerization site.

The direct method involves the isolation of the peptides removed from the active fragment and their assay for the presence of the polymerization site. By this approach, Olexa & Budzynski (1981) found that a peptide corresponding to the γ374–411 region of the γ-chain contains an active polymerization site. This result was very much in agreement with the results obtained by the indirect method and allowed a more precise localization of this polymerization site to be made. In our previous report, we narrowed this region further to the γ374–396 segment (Horwitz et al., 1984) by isolating and characterizing the corresponding peptide.

However, as shown under Results, the binding properties of these peptides to fibrin monomer and their inhibitory activities on fibrin polymerization may be artifacts. Although we found that both the γ374–411 and γ374–396 peptides do bind to immobilized fibrin monomer, this binding is not specific, since they bind to the Sepharose matrix as well, probably by electrostatic interactions. Both peptides contain approximately a 4+ net charge at neutral pH and can bind to the small number of sulfate and carboxyl groups of the Sepharose gel matrix (Pharmacia, 1980).

Olexa & Budzynski (1981) found that the γ374–411 peptide does not bind to immobilized fibrinogen, and this control experiment supported the specificity of this peptide for the site(s) exposed upon conversion of fibrinogen to fibrin. In contrast, we found that both the γ374–411 and γ374–396 peptides bind to fibrinogen–Sepharose probably because of the same nonspecific interactions that we observed between these peptides and the unsubstituted Sepharose matrix.

We also found that neither the γ374–411 nor the γ374–396 peptide is an inhibitor of fibrin polymerization. By eluting the peptides from fibrin monomer–Sepharose at pH 3.3 instead of pH 2.5, it was possible to prepare samples containing either of the two peptides, with no resulting inhibition of fibrin polymerization. Furthermore, we found that a "fibrin monomer–Sepharose-bound" fraction, prepared under identical conditions (i.e., pH 2.5) as for our earlier "active" peptide preparations but containing neither the γ374–411 nor the γ374–396 peptide, strongly inhibits fibrin polymerization. These results suggest that these peptides cannot interfere with fibrin polymerization. We found that the inhibitory action associated with the earlier peptide preparations is due to the presence in these samples of EGTA, which coeluted with the peptides from the fibrin monomer–Sepharose column when the pH 2.5 eluant was used. EGTA is a potent inhibitor of fibrin polymerization (Blombäck et al., 1966).

All of the above-mentioned artifacts/problems in the binding and inhibition experiments using the peptides corresponding to the C-terminal portion of the γ-chain of fibrinogen make the localization of the polymerization site (Olexa & Budzynski, 1981; Horwitz et al., 1984) ambiguous. Therefore, in this work, we have used the indirect approach instead for the localization of this polymerization site.

We isolated an intermediate fragment, fragment D_2, whose covalent structure differs from that of fragment D_1 in that the former lacks the C-terminal γ356/357–411 segment. In this fragment, the γ-chain polymerization site is not preserved, since the fragment does not inhibit polymerization (Figure 12). Therefore, we conclude that the γ356/357–411 region is essential for the γ-chain polymerization site.

There are two tyrosine residues in γ356/357–411, at positions 363 and 377, and two histidine residues at positions 400 and 401. A polymerization site consisting of two D fragments and one E fragment would contain twice this number of tyrosine and histidine residues. Even though the corresponding polymerization site in the E fragment has not yet been identified, the presence of these tyrosine and histidine residues in γ356/357–411 is compatible with the suggestion of Sturtevant et al. (1955) that the formation of tyrosyl–histidine hydrogen bonds during polymerization can account for the observed pH range of polymerization, the number of protons released or absorbed as a function of the initial pH, and the observed pH-dependent heat of polymerization.

Fragment D_2, like fragment D_1, possesses a strong Ca^{2+} binding site (Figure 13). This means that the 356/357–411 region, not present in fragment D_2, does not play an important role in the Ca^{2+} binding activity of the outer nodule of fibrinogen. Since fragment D_3 does not bind Ca^{2+} (Nieuwenhuizen & Haverkate, 1983; this paper), the region γ303–355/356, present in fragment D_2 but absent in fragment D_3 (Henschen, 1981), is essential for Ca^{2+} binding. Our result, that the intermediate fragment (fragment D_2) does not inhibit polymerization but binds Ca^{2+}, is in harmony with the observations of Nieuwenhuizen et al. (1982) and those of Niewenhuizen & Haverkate (1983), who also found that an intermediate fragment has no anticlotting properties and binds Ca^{2+}. Since they did not purify their intermediate but used a mixture of fragments D_2 and D_3, they could not determine the C-terminal boundary of the γ-chain of their intermediate and could not localize the Ca^{2+} binding segment as well as the polymerization site in the outer nodule of fibrinogen.

It is of interest to point out that Ca^{2+} is known to bind to both fibrinogen and fibrin monomer and to increase the rate of polymerization of fibrin monomer, as measured by the increase in the rate of proton release and the reduction in clotting time during polymerization (Endres & Scheraga, 1972); Mg^{2+} exhibits no such effect (Endres & Scheraga, 1972). Conceivably, segment γ303–355/356, which contains aspartic acid residues at positions 316, 318, and 320 and serine and threonine residues at positions 313 and 314, respectively, may constitute a specific binding site for Ca^{2+}, and such binding may enhance the reactivity of the adjacent polymerization site in segment γ356/357–411.

Added in Proof

After this paper was submitted for publication, a conference abstract by Southan et al. (1985a), dealing with the problem of the localization of the polymerization site in the γ-chain of fibrinogen, appeared. This work has subsequently been published (Southan et al., 1985b). These authors isolated and studied peptides corresponding to P-2, P-3, and P-4 of this paper, with agreement with our results on the identity of the plasmic cleavage sites in the C-terminal portion of the γ-chain. Their results and ours are also in agreement in the finding that these peptides do not inhibit fibrin polymerization.

Also, after submission of this paper, the paper of Dang et al. (1985) appeared. It reported the localization of a fibrinogen Ca^{2+} binding site between γ-chain residues 311 and 336 by terbium fluorescence.

Acknowledgments

We thank M. Rutzke and G. W. Feigenson for help with the Ca^{2+} determinations by atomic emission and by radioassay, respectively, and T. W. Thannhauser for performing the amino acid analyses. We gratefully acknowledge valuable discussions with B. H. Horwitz.

Registry No. Ca, 7440-70-2.

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