Role of the αC Domains of Fibrin in Clot Formation†

Oleg V. Gorkun,‡,§ Yuri I. Veklich,‡,§ Leonid V. Medved',§ Agnes H. Henschen,‖ and John W. Weisel*,‡

Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, Institute of Biochemistry, Academy of Sciences of Ukraine, Kiev 252030, Ukraine, and Department of Molecular Biology and Biochemistry, School of Biological Sciences, University of California, Irvine, California 92717

Received November 3, 1993; Revised Manuscript Received February 3, 1994®

ABSTRACT: The role of the carboxyl-terminal portion of the α chains of fibrin (αC domains) in clot formation was investigated by transmission and scanning electron microscopy and turbidity studies of clots made from preparations of molecules missing one or both of these domains. Highly purified and entirely clottable preparations of bovine fragment X monomer, one containing primarily molecules missing a single αC domain (fragment X_1) and the other consisting of molecules missing both αC domains (fragment X_2), were used for these experiments. These preparations were characterized by various methods, including the complete determination of the amino- and carboxyl-termini of all peptides and fragments. These preparations for ed clots on dilution to neutral pH. In all cases, clots observed by either scanning or transmission ele. .ron microscopy were made up of a branched network of fibers, similar to those formed by thrombin treatment of intact fibrinogen, suggesting that the αC domains are not necessary for protofibril and fiber formation or branching. However, both the fiber and clot structure varied with the different fractions, indicating that the αC domains do participate in polymerization. The rate of assembly, as indicated by the lag period and maximum rate of turbidity increase, as well as the final turbidity, was decreased with removal of the αC domains, suggesting that they accelerate polymerization. Preparations of isolated αC fragment added to fibrin monomer have striking effects on the turbidity curves, showing a decrease in the rate of polymerization in a dose-dependent manner but not complete inhibition. Electron microscopy of fibrin monomer desA molecules at neutral pH showed that most of the αC domains, like those in fibrinogen, remain associated with the central region. Thus, it appears that normally with thrombin cleavage of fibrinogen the effects of the interactions of αC domains observed here will be most significant for lateral aggregation.

Fibrinogen is a polyfunctional, multidomain protein involved in several different aspects of hemostasis and wound healing. Its main function is the formation of the three-dimensional network of fibrin fibers which is the structural basis of the blood clot (Mosesson & Doolittle, 1983; Henschen & McDonagh, 1986). Fibrinogen, with a molecular mass of 340 kDa, consists of three pairs of nonidentical polypeptide chains, Aα, Bβ, and γ. All of these chains are linked together by 29 disulfide bonds and assemble to form a number of independently folded domains (Weisel et al., 1981; Privalov & Medved, 1982; Weisel et al., 1985; Medved', 1990); distinctive functions for several of these domains have been elucidated. Some of the domains are involved in the fibrin assembly process. Among these are the central domains, each of which comprises the amino termini of all three chains and contains two active sites (called A and B) exposed upon removal of fibrinopeptides A and B by the proteolytic enzyme thrombin (Mosesson & Doolittle, 1983). Complementary binding sites (called a and b) that interact with the A and B sites of a neighboring molecule to yield fibrin polymers are situated in the largely symmetric carboxyl-terminal domains of the molecule. In the early stages of the fibrin assembly process, the interaction between these polymerization sites leads to the half-staggering of molecules to form two-stranded protofibrils (Weisel et al., 1983; Henschen & McDonagh, 1986). Protofibrils then aggregate laterally to form fibers, which branch to produce a three-dimensional network. Aspects of network formation, including branching, can occur early in the assembly process (Blombäck et al., 1984; Mosesson et al., 1989).

Each fibrinogen molecule also contains two αC domains made up of the interacting carboxyl-terminal parts of the Aα chains (Erickson & Fowler, 1983; Medved' et al., 1983; Weisel et al., 1985). These αC domains are cleaved from fibrinogen, along with the connecting polypeptide chain, by the enzyme plasmin very early in the process of fibrinolysis, yielding a derivative of fibrinogen called fragment X. Many studies have been carried out using such preparations (Marder et al., 1969; Pizzo et al., 1972; Budzynski et al., 1974; Shen et al., 1977). These and other early investigations with proteolytically modified derivatives of fibrinogen isolated from the blood (Mosesson et al., 1967) indicated that cleavage of the carboxyl-terminal portion of the Aα chain affected the turbidity curves that characterize polymerization of such modified molecules. Although it has been shown that the absence of αC domains influences fibrin polymerization, it is not yet clear, however, how they are involved in the assembly process. On the one hand, by using soluble fibrin or fragment X_1 monomers (missing one αC domain), it was shown that the αC domains accelerate the formation of protofibrils, fibers, and the fibrin network, especially in dilute solution, but do not substantially influence the structures of intermediates or the final clot (Medved' et al., 1985). On the other hand, it was observed that addition of thrombin to early fragment X preparations yields clots that are mechanically less stable and appear to consist of fibers that are substantially less branched than those of normal clots (Weisel & Papsun, 1987). The lack of stable

† Part of this work was supported by National Institutes of Health Grants HL30954 to J.W.W. and HL42412 to A.H.H.

* Address correspondence to this author at the following address: Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058. Tel.: 215-898-3573; Fax: 215-898-9871.

‡ University of Pennsylvania.

§ Academy of Sciences of Ukraine.

‖ University of California, Irvine.

® Abstract published in *Advance ACS Abstracts*, May 1, 1994.

0006-2960/94/0433-6986$04.50/0

branch points was accounted for by the absence of αC domains on fragment X, and it was proposed that the αC domains are necessary for the branching of fibers to form a stable three-dimensional gel. Other studies have compared the polymerization of normally circulating lower molecular mass fractions of fibrinogen with intact fibrinogen (Holm et al., 1985). Clots formed from such derivatives are made up of thinner fiber bundles and clot more slowly with lower final turbidity (Hasegawa & Sasaki, 1990). In all of these studies, the preparations of X fragments used as a model for fibrinogen lacking intact αC domains were only partially characterized. To clarify the obvious contradictions of this research, highly purified and well-characterized proteins are required.

We performed a detailed study of clots formed from highly purified fibrin monomer and well-characterized monomeric early X fragments lacking one or both αC domains, as well as αC fragments. While fragment X preparations are invariably heterogeneous, here nonclottable and less soluble molecules were removed by clotting preparations with thrombin and then dissolving the molecules forming the clot in dilute acetic acid. When such preparations are carried out carefully, the resulting fibrin or fragment X are uniquely in the monomeric form and their main domains are intact [Marguerie et al., 1973; Privalov & Medved, 1982 (and L. V. Medved' and P. L. Privalov, unpublished observations); Veklich et al., 1993]. The polymerization process can be studied by reclotting the fibrin or fragment X monomer through dilution into neutral pH buffer. Such fibrin monomer clots, the formation of which constitutes a standard procedure to characterize dysfibrinogenemias or fibrinogen derivatives (Henschen & McDonagh, 1986), are similar to those formed by adding thrombin to fibrinogen, although there are some differences (Weisel et al., 1993). The results of these experiments with fragment X and the αC fragments clearly indicate that the αC domains accelerate the fibrin polymerization process and influence the final clot structure but are not necessary for clot formation.

MATERIALS AND METHODS

Fibrinogen. Bovine fibrinogen, with clottability of 98%, was prepared from oxalated plasma by salting out with sodium sulfate (Varetskaya, 1960) and subsequent purification to remove fractions with partially degraded Aα chains, as described previously (Phillips, 1981; Medved' et al., 1985).

Plasmin Digestion of Fibrinogen. Fibrinogen (20 mg/mL) in 100 mM Tris–HCl, pH 7.5, 150 mM NaCl buffer solution was incubated with plasmin (0.01 caseinolytic units/mL) at 4 °C. The reaction was stopped after 9–11 h by addition of diisopropyl fluoro phosphate to a final concentration of 5–10 mM. The digest was passed through a 2.5 × 30-cm column with lysine–Sepharose 4B equilibrated with 100 mM Tris–HCl, pH 7.5, 400 mM NaCl buffer to remove plasmin.

Fragment X Isolation. Fragment X was prepared by stepwise salting out of the plasmin digest with ammonium sulfate according to Phillips (Phillips, 1981) with several modifications. The digest was dialyzed thoroughly against 20 mM 2-*N*-morpholinoethanesulfonic acid, pH 5.9, 400 mM NaCl and 5% sucrose buffer at 4 °C. The digest was then diluted with the same buffer to a concentration of 0.9–1.5 mg/mL and salted out stepwise with a saturated solution of $(NH_4)_2SO_4$. The salting out procedure was performed by the addition of small portions of the saturated $(NH_4)_2SO_4$ to a final concentration of 20% to the digest with continual stirring, followed by incubation at 4 °C for 6–12 h. The precipitated protein was centrifuged for 20 min at 1500*g* at 4 °C and then dissolved in 100 mM Tris–HCl, pH 7.5, 150 mM NaCl buffer. The supernatant containing the rest of the protein was subjected to further stepwise salting out in the same way. Eight fractions, 0–20%, 20–22%, 22–24%, 24–26%, 26–28%, 28–30%, 30–32%, and 32–34% of saturation, were prepared and dialyzed against 100 mM Tris–HCl, pH 7.5, 150 mM NaCl. Experiments were carried out using all of these fractions, but most of the research described here concerned fractions 28–30% and 32–34%, which will be designated fragments X_1 and X_2, although there was some small variations from one preparation to another; the characterization of these fragments is described below. Note that these fragments are not necessarily the same as similarly named fragments prepared by other investigators.

Preparation of Fibrin Monomer and X_1 and X_2 Monomers. Fibrin monomer desAB was prepared by incubation of fibrinogen (2 mg/mL) in 100 mM Tris–HCl, pH 7.5, 150 mM NaCl buffer with thrombin (0.1 NIH unit/mL) for 60 min at 37 °C in a glass tube (Belitser et al., 1968). The clot which formed was twisted around a glass rod, washed with 150 mM NaCl and dissolved to a final concentration of 15 mg/mL in 0.125% acetic acid at 4 °C. Fibrin monomer desA was prepared in the same manner, but instead of thrombin, reptilase was used to selectively remove the A fibrinopeptides. X_1 and X_2 monomers were prepared in the same way as fibrin monomer by incubation of fractions 28–30% and 32–34%, respectively, with the same concentration of thrombin and subsequent dissolution of the resulting clot in 0.125% acetic acid to the same concentration as described for fibrin monomer. All monomer preparations were repolymerized by 7× dilution in 400 mM Tris–HCl, pH 7.5, 50 mM NaCl buffer, which shifts the pH to neutrality, allowing the monomeric molecules to repolymerize. The polymers were dissolved again in acetic acid and the entire procedure was repeated two or three times. Fibrin monomer and X_1 and X_2 monomer solutions can be stored in a refrigerator for 1–2 weeks at 4 °C with no significant changes in their clotting properties.

Characterization of Fibrin and Fragment X_1 and X_2 Monomers. The molecular masses of the proteins were determined by SDS–polyacrylamide gel electrophoresis, using protein standards. Protein concentrations were determined spectrophotometrically using extinction coefficients ($E^{1\%}_{280}$) of 15.1, 15.7, and 17.1 for fibrin monomer, X_1, and X_2, respectively (Medved' et al., 1986). Protein clottabilities were determined by measurement of the absorption at 280 nm after removal of the clot according to standard procedures.

The amino-terminal protein sequences were determined from isolated, unfragmented polypeptide chains. For this purpose, 1 mg of each preparation was dissolved in 100 μL of 6 M guanidine–0.1 M Tris pH 8.6, 5 mL of β-mercaptoethanol was added, and the sample was incubated at 40 °C overnight. The peptide chains were separated by reversed-phase high-performance liquid chromatography (HPLC)[1] using a Vydac C_{18} column (0.47 × 25 cm) and a linear 60-min gradient between 30 and 45% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min and monitoring at 206 nm (Kehl et al., 1982). Manually-collected fractions were then subjected to amino-terminal sequence analysis in an Applied Biosystems 477A protein/peptide sequencer equipped with a 120 (phenylthio)hydantoin analyzer or a Hewlett-Packard G1005A protein sequencing system.

The carboxyl-terminal ends of the individual polypeptide chain components were identified by isolation of short carboxyl-terminal cyanogen bromide fragments which were then

[1] Abbreviations: HPLC, high-performance liquid chromatography.

sequenced through to the carboxyl-terminus. For this purpose, 1 mg of each preparation was cleaved with 100 μL of 70% formic acid containing 10 mg of cyanogen bromide; after 2 h the reagents were evaporated with a stream of nitrogen. The resulting fragments were fractionated by gel filtration chromatography on Sephadex G-50SF column (1 × 120 cm) in 0.25% trifluoroacetic acid at a flow rate of 6 mL/h with monitoring at 235 nm. Protein-containing fractions were then subjected to amino-terminal sequence analysis. Relevant pooled fractions were further characterized by reversed-phase HPLC using conditions similar to those described above but with a gradient between 10 and 50% acetonitrile, followed by extended sequence analysis of pure carboxyl-terminal fragments.

Preparations of αC Fragment. To prepare the αC fragment, bovine fibrinogen (20 mg/mL) in 10 mM HEPES, pH 7.2,0.2 M NaCl buffer was digested with plasmin (0.02 caseinolytic unit/mL of mixture) at 25 °C. Digestion was stopped after 6 h by the addition of phenylmethylsulfonyl fluoride to a final concentration of 0.5 mM. At this stage, the primary digestion products were a 40-kDa fragment, with an equal amount of fragment X; although these fragments were only present in small amounts, there were few other digestion products. The 40-kDa fragments were purified by gel filtration on a Sepharose 6BCL column (2.5 × 90 cm) equilibrated with 20 mM HEPES, pH 7.2, 2 M NaCl, 200 mM ε-amino caproic acid and 0.5 mM phenylmethylsulfonyl fluoride. The ionic strength of the digest was adjusted to 2.0 by the addition of NaCl before application to the column; the 40-kDa fragment was eluted in the second, minor peak. The fractions in this peak were pooled, dialyzed against 0.125% acetic acid at 4 °C, and then lyophilized. This fragment comprises the carboxyl-terminal two-thirds of the Aα chain, which includes the αC domain, as reported earlier (Veklich et al., 1993).

Characterization of αC Fragment. The amino-terminal protein sequence was determined by direct analysis of 0.1 mg of material. The carboxyl-terminal end was identified by isolation and sequencing of carboxyl-terminal peptides from a tryptic digest of αC fragment. For this purpose, 0.4 mg of material in 50 μL of water was treated with 2 mg of succinic anhydride added over 2 h, with the pH kept at 8 by addition of 5% trimethylamine in water; after 5 h, 5 μg of trypsin was added and the sample was digested for 16 h. The digest was fractionated by reversed-phase HPLC as described above.

Turbidity Measurements and Transmission and Scanning Electron Microscopy Experiments. The polymerization process was initiated by 10-fold dilution of concentrated monomeric solution with either of two buffers: 50 mM HEPES, pH 6.8, 150 mM NaCl, 0.5 mM $CaCl_2$, or 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$. Turbidity and electron microscopy experiments were carried out similarly, in so far as possible. Turbidity measurements were carried out on Specord M-40 (Carl Zeiss, Jena) or Perkin-Elmer Lambda-4B spectrophotometers at 350 nm. Transmission electron microscopy observations were made on a Hitachi H600 or Philips 400 electron microscope. Pre-formed clots were applied to 300-mesh, glow-discharged carbon-coated Formvar grids and then negatively contrasted with 1% uranyl acetate (Weisel, 1986). Scanning electron microscope experiments were carried out on clots that were fixed, dehydrated, critical point dried, and sputter coated with gold and then observed using an Amray 1400 or JEOL JSM35 microscope (Langer et al., 1988).

Rotary-shadowed samples were prepared by spraying a dilute solution of molecules in a volatile buffer (0.05 M ammonium formate at pH 7.4 or 0.125% acetic acid at pH 3.5) and 30% glycerol onto freshly-cleaved mica and shadowing with tungsten in a vacuum evaporator (Denton Vacuum Co., Cherry Hill, NJ) (Weisel et al., 1985). Samples at acid pH were prepared by dilution of concentrated protein solution (13 mg/mL) with 0.125% acetic acid at pH 3.5 with 30% glycerol to a final concentration of 25 μg/mL. All of the specimens were examined in a Philips 400 electron microscope (Philips Electronic Instruments Co., Mahwah, NJ), usually operating at 80 kV and a magnification of 60000×.

RESULTS

Characterization of Fragment X_1 and X_2 Monomer Preparations. Plasmin digestion of bovine fibrinogen was stopped at an early stage where only small quantities of the desired fragments, designated X_1 and X_2, appear (Figure 1A), but this is necessary to preserve the integrity of the rest of the molecule, which is also susceptible to plasmin cleavage. The separation methods described above allowed the preparation of purified, homogeneous X_1 and X_2 fragments (Figure 1A) that are mostly concentrated in fractions that precipitate at 28–30% and 32–34% saturated $(NH_4)_2SO_4$, respectively. The clottabilities of X_1 and X_2 fragments were 87 and 72%, respectively. However, after conversion into monomeric form and two to three cycles of dissolution/repolymerization as described above, both fragments, like fibrin monomer, became practically 100% clottable, as determined by the amount of protein remaining in the supernatant.

The molecular masses of X_1 and X_2 were ~295 and ~260 kDa, respectively, according to SDS–polyacrylamide gel electrophoresis measurements, using molecular mass standards. Thus, these fragments are ~45 and ~80 kDa smaller than the parent molecule. Taking into account the expected plasmin-sensitive sites, cleavage should remove about two-thirds of the carboxyl-terminal part of the Aα chain, which comprises the αC domain with a molecular mass of 40–45 kDa. Therefore, one would expect that fragment X_1 was missing one αC domain and fragment X_2 was missing both αC domains, a conclusion supported by SDS gel electrophoresis of the preparations under reducing conditions. Fragment X_1 monomer preparations showed a new band with a molecular mass corresponding to about 33 kDa and the α chain in the same position as that of fibrin but reduced in amount (Figure 1B). The β and γ chains appeared to be the same as those of fibrin. The fragment X_2 monomer preparations exhibited a new band with a molecular mass corresponding to about 26 kDa but no band in the position corresponding to the α chain (Figure 1B). Again, the β and γ chains appeared to be the same as those of fibrin.

The polypeptide chain compositions of fibrin monomer and X_1 and X_2 monomers were also compared with those of fibrinogen and X_1 and X_2 fragments. The electrophoretic mobilities of both α (and α derivatives) and β chains in all three monomer preparations were increased, indicating that the A and B fibrinopeptides present in the parent fibrinogen and X_1 and X_2 fragments were removed by thrombin. Quantitative measurements of the release of the fibrinopeptides from similar preparations of these fragments carried out by Suttnar et al. (1991) using HPLC demonstrated that fragments X_1 and X_2 contain, respectively, about 76% and 58% of fibrinopeptide B and both fragments contain 100% of fibrinopeptide A. All these results indicate that the amino-terminal ends of the Bβ chains are intact in most, but not all, of the fragment molecules. However, most of these more degraded fragments have been removed by addition of

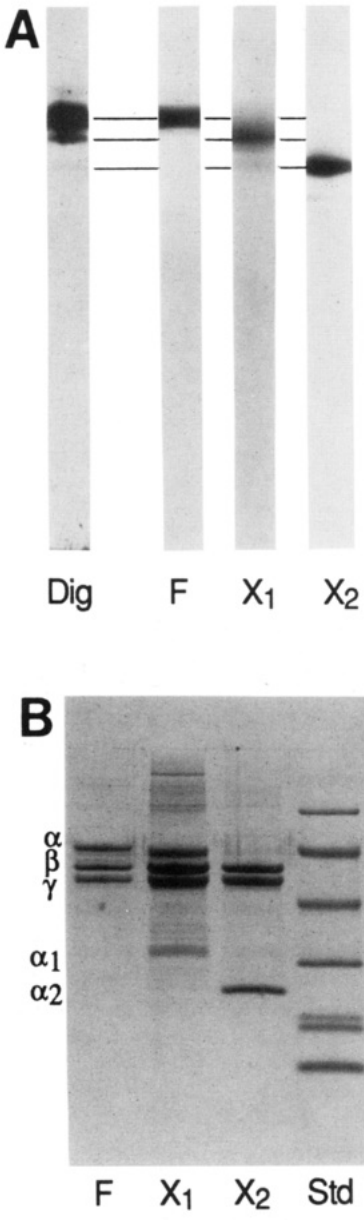


FIGURE 1: Preparations of bovine fragment X_1 and X_2 monomer as characterized by SDS–polyacrylamide gel electrophoresis. (A) Samples prepared and run under nonreducing conditions, left to right: the plasmin digest (Dig), fibrin monomer (F), fragment X_1 monomer (X_1), fragment X_2 monomer (X_2). (B) Samples prepared and run under reducing conditions, left to right: fibrin monomer (F), fragment X_1 monomer (X_1), fragment X_2 monomer (X_2), protein molecular mass standards (Std). The polypeptide chains described in the text are labeled.

thrombin and repeated cycles of monomer polymerization at comparatively high ionic strength with no calcium, as described above. This further purification was possible because of the large differences in clotting properties under these ionic conditions of intact fibrinogen and fibrinogen missing the amino terminus of the Bβ chain (Siebenlist et al., 1990). Hence, the fragment X monomer preparations are considerably more homogeneous. To characterize further the structure and homogeneity of the monomeric fragments, we carried out detailed amino acid sequence analysis of them.

Amino Acid Sequence Analysis of Fragment X_1 and X_2 Monomer Preparations. The polypeptide chains of monomeric fibrin and fragment X_1 and X_2 monomer were separated by reversed-phase HPLC after cleavage of the disulfide bonds (Kehl et al., 1982) (Figure 2), and the components in the peaks and shoulders were identified by amino-terminal

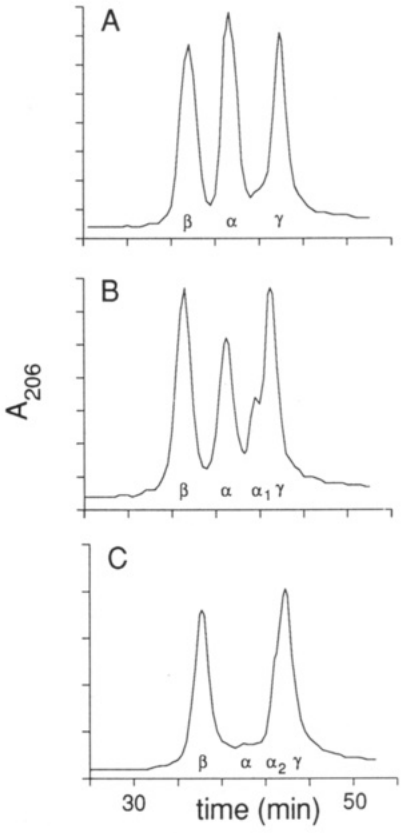


FIGURE 2: Separation of the polypeptide chains of bovine fibrin and fragment X_1 and X_2 monomers by reversed-phase HPLC. (A) Fibrin: the peaks contain β, α, and γ, from left to right. (B) Fragment X_1 monomer: the peaks contain β, α, smaller α chain (shoulder of adjacent peak, denoted α_1), and γ, from left to right. (C) Fragment X_2 monomer: the peaks contain β, trace amount of α chain, smaller α chain (shoulder of adjacent peak, denoted (α_2), and γ, from left to right.

sequencing (Table 1) and SDS gel electrophoresis. So that no components could be missed, the contents of every protein-containing fraction were analyzed. The first peak in all of these preparations contained pure β chain as judged by its amino-terminal sequence. It showed a single band on SDS gel electrophoresis. Approximately 4 and 15% of the β chains of the fragments X_1 and X_2 monomer, respectively, were missing the first 42 amino acids (human numbering), corresponding to cleavage at the earliest plasmic cleavage site in the Bβ chain. The second peak in all preparations contained pure, homogeneous α chain, according to sequence and SDS gel electrophoresis. In fragment X_1 monomer this α chain peak was only half the size of that in the fibrin preparation, and in X_2 monomer only trace amounts of this component were present. In the fibrin sample, the third peak contained pure γ chain; in the X monomer fragments, only the later part of the third main peak corresponded to pure γ chain, while the leading shoulder corresponded to a modified α chain. However, the α and γ chains were sufficiently well resolved to allow the unambiguous conclusion that all amino-termini were intact. SDS polyacrylamide gel electrophoresis analysis of the HPLC components indicated that all β and γ chains and α chains in the second peak were virtually undegraded as judged by their molecular size. However, the α chain components in the shoulder of the third peak were degraded to well-defined, apparently homogeneous components as judged by SDS gel electrophoresis, with their molecular masses being estimated as 33 and 26 kDa for X_1 and X_2, respectively; these cleavage products are termed α_1 and α_2, respectively.

Table 1: Characterization of Bovine Fibrin Monomer, Purified Fragment X_1 and X_2 Monomers, and αC Fragment

peptide	chain	M_r[a]	amino-terminus	carboxyl-terminus	positions[b]	no. of residues
fibrin	α	63.7	GPRLVERQQS	TQKGHTKARP	17–609	560[c]
	β	57.1	GHRPYDKKKE	MKIRPYFPEQ	15–461	447
	γ	52.7	YVATRDNCCI	LGGAKQAGDV	1–411	411
	γ′[e]		YVATRDNCCI	VEHHVEIEYD	1–419	419
X_1	α	63.7	GPRLVERQQS	TQKGHTKARP	17–609	560[c]
	$α_1$	33.1	GPRLVERQQS	GTGLAPGSPR	17–268	252
	β	57.1	GHRPYDKKKE	MKIRPYFPEQ	15–461	447
	β′[d]		ARPATATVGQ	MKIRPYFPEQ	43–461	419
	γ	52.7	YVATRDNCCI	LGGAKQAGDV	1–411	411
	γ′[e]		YVATRDNCCI	VEHHVEIEYD	1–419	419
X_2	$α_2$	25.7	GPRLVERQQS	ITGPVPREFK	17–219	203
	β	57.1	GHRPYDKKKE	MKIRPYFPEQ	15–461	447
	β′[d]		ARPATATVGQ	MIKRPYFPEQ	43–461	419
	γ	52.7	YVATRDNCCI	LGGAKQAGDV	1–411	411
	γ′[e]		YVATRDNCCI	VEHHVEIEYD	1–419	419
αC		38.0	SQLQEAPLEW	GSAIESKHFK	220–581	329[c]
			ALLEMQQTKM	GSAIESKHFK	231–581	318[c]
			MVLETFGGDG	GSAIESKHFK	240–581	309[c]

[a] Determined by SDS–polyacrylamide gel electrophoresis. [b] Although the lengths of the bovine chains are not identical to those of humans, the human numbering of homologous amino acids was used for ease of comparison with previously published data. [c] The number of residues is lower than that of human fibrin because of the shorter bovine α chain. [d] The amount of this form of the β chain was found to be 4 and 15% of the total for the X_1 and X_2 monomers, respectively. [e] Longer variant of the γ chain which is always present at ≈30% of the total in fibrinogen preparations.

The carboxyl-terminal ends of all peptide chain components were identified by isolation and complete sequencing of short carboxyl-terminal fragments. Cyanogen bromide-cleaved samples were fractionated by gel filtration chromatography on Sephadex G-50. All protein-containing fractions were screened for their contents for cyanogen bromide cleavage products by the analysis of the first 10 amino-terminal amino acid residues. The carboxyl-terminal fragment of undegraded α chain, with the starting sequence Glu-Asp-Glu-Ala- (Henschen et al., 1980; Chung et al., 1982) appeared in the pool with the K_{av} 0.48–0.59. It was present both in fibrin and fragment X_1, but absent in fragment X_2. The α chain cyanogen bromide fragments were purified by reversed-phase HPLC and sequenced through to the known carboxyl-terminus of the α chain, proving that fragment X_1 contains one undegraded α chain (Table 1). The largest cyanogen bromide fragment of the undegraded α chain, which starts with the sequence Val-Leu-Glu-Thr- (Henschen et al., 1980; Chung et al., 1982) beginning in position 241 (human numbering), was eluted in the starting peak of the gel filtration column. However, in fragment X_1 the sequence Val-Leu-Glu-Thr– was also present in the pool with the K_{av} 0.48–0.59. HPLC purification followed by sequence analysis showed that the carboxyl-terminus of the degraded α chain corresponded to position 268 (Table 1). Since the last amino acid is an arginine, this carboxyl-terminus would be in agreement with a plasmic cleavage site. No component with the amino-terminal sequence Val-Leu-Glu-Thr– was detected in fragment X_2, indicating a more extensive degradation of the α chain component in agreement with the lower molecular weight. The α chain cyanogen bromide fragment, which starts with the sequence Ser-Thr-Ile-Thr– in position 208, contains two early plasmic cleavage sites in human fibrinogen. This fragment is normally eluted with a K_{av} of 0.59–0.62, but in material from fragment X_2 it was detected in the pool with K_{av} 0.76–0.90, the later elution indicating a smaller size of fragment. Isolation by HPLC and subsequent sequencing showed that this fragment ended with the lysine residue in position 219 at a typical plasmic cleavage site (Table 1). In all three preparations, the carboxyl-terminal cyanogen bromide fragment of the β chain was identified in the pool with K_{av} 0.76–0.90 and that of the γ chain in the pool with the K_{av} 0.48–0.59. All carboxyl-terminal fragments were sequenced through and showed no degradation (Table 1). The two splice-variant forms of the γ chain were present in the same ratios in all preparations.

Thus, while fibrin monomer used in this work had both αC domains intact, X_1 monomer contained only one αC domain and X_2 monomer had none. At the same time both, X_1 and X_2 monomers had mostly intact amino termini of all three chains.

Electron Microscopy of Clots Formed by Fibrin and X Monomers. The clots formed by X_1 and X_2 monomers seemed to be undistinguished visually from those formed by fibrin monomer even at very low concentrations of protein (≈0.05 mg/mL) although they were less resistant to mechanical disturbances. The structural features of clots formed by monomers of X_1, X_2, and fibrin were investigated by transmission and scanning electron microscopy. Transmission electron microscopy of negatively contrasted clots gives a detailed picture of fiber structure (Weisel, 1986), while scanning electron microscopy of gold-coated, critical point dried specimens reveals more of the three-dimensional architecture of the intact clot (Weisel, 1988). Both types of preparations yield information about the extent of lateral aggregation and the nature of branching of fibers. Transmission electron microscope samples show the details of the branch points, while scanning electron microscope preparation methods ensure that any existing linkages between fibers are not damaged. If there is little or no branching, individual fibers will be observed by transmission electron microscopy, but the lack of mechanical integrity of the clot will prevent specimen preparation for scanning electron microscopy.

Negatively contrasted fibrin monomer clots observed by transmission electron microscopy (Figure 3A) were similar in appearance to those made by addition of thrombin to fibrinogen (Weisel, 1986). Low-magnification transmission electron micrographs of X_1 and X_2 monomer clots are shown in Figure 3C,E. Comparison of the clots formed by all three species revealed extensive branching of fibers in all cases, although there were differences in structure. Fibers of X_2 monomer clots appeared to aggregate to yield large fiber bundles, and there were larger pores between fiber bundles. The fiber bundles of clots from X_1 monomer were intermediate in size between those from X_2 and fibrin monomer. The band patterns

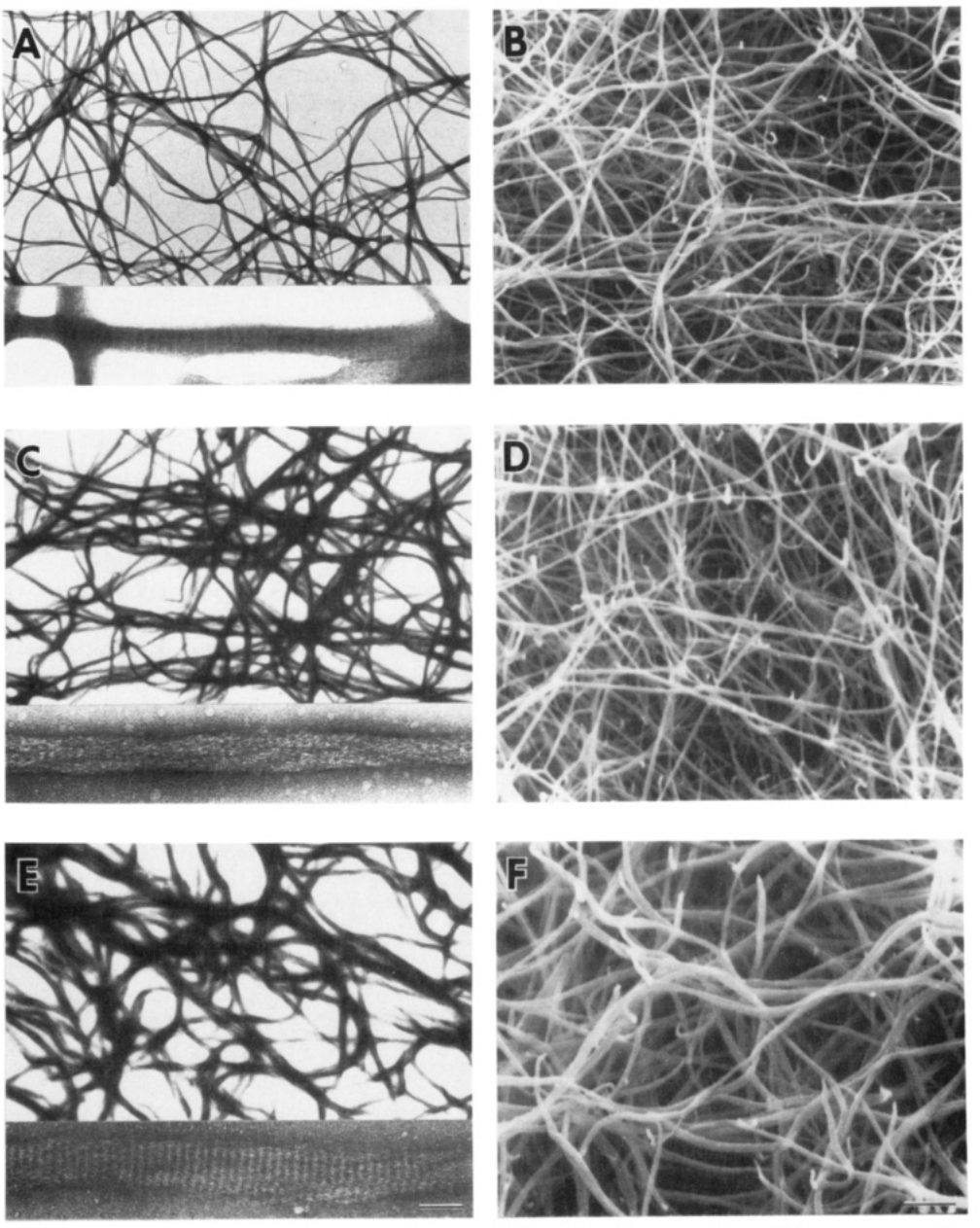


FIGURE 3: Transmission and scanning electron microscopy of clots and fibers from fragment X_1 and X_2 monomers. All preparations were negatively contrasted with uranyl acetate for transmission electron microscopy. The same preparations were critical point dried and sputter-coated with gold prior to examination by scanning electron microscopy: (A) transmission electron micrograph of a clot prepared from fibrin monomer desAB (inset: fibrin band pattern at higher magnification); (B) scanning electron micrograph of a clot prepared from fibrin monomer desAB; (C) transmission electron micrograph of a clot prepared from X_1 monomer (inset: fiber band pattern at higher magnificent); (D) scanning electron micrograph of a clot prepared from X_1 monomer; (E) transmission electron micrograph of a clot prepared from X_2 monomer (inset: fiber band pattern at higher magnification); (F) scanning electron micrograph of a clot prepared from X_2 monomer. Bar for insets, 0.1 μm. Bar for all other micrographs, 1 μm.

of fibers of X_1 and X_2 monomer preparations (insets of Figure 3A,C,E) were different in appearance than those of fibrin monomer. As reported previously for bovine fibrinogen lightly digested by *Pseudomonas* protease Ps1 (Weisel et al., 1981; Weisel et al., 1983), the thin, weak light band in the middle of the dark-staining region appears to be less prominent in fibers from these fragment X monomer preparations than in those from fibrin monomer. In addition, the striations of the X monomer fibers were somewhat wavy in appearance suggesting that the longitudinal order is not quite as good; the striations were also more discontinuous in the lateral direction, indicating that the spacing between protofibrils may be greater. Note also that the fibers from fragment X_2 monomer (Figure 3E inset) are better ordered than those from fragment X_1 monomer (Figure 3C inset).

Since clots are mechanically stable and hence difficult to disperse into a thin layer that the electron beam of a transmission electron microscope can penetrate, we also prepared samples for the scanning electron microscope. With this technique, extensively branched fiber networks were observed for all three samples (Figure 3B,D,F). The degree of lateral aggregation of fibers increased with the cleavage of the αC domain. This increase in the size of fiber bundles and concomitant increase in pore size is apparent in the typical images of Figure 3D,F. In addition, the diameters of several hundred fiber bundles were measured from the micrographs. With this technique, the average diameter for fibrin monomer fiber bundles was 100 ± 19 nm, while the average from X_2 monomer clots was 150 ± 21 nm, with diameters for X_1 monomer fibers intermediate. It should be noted that the

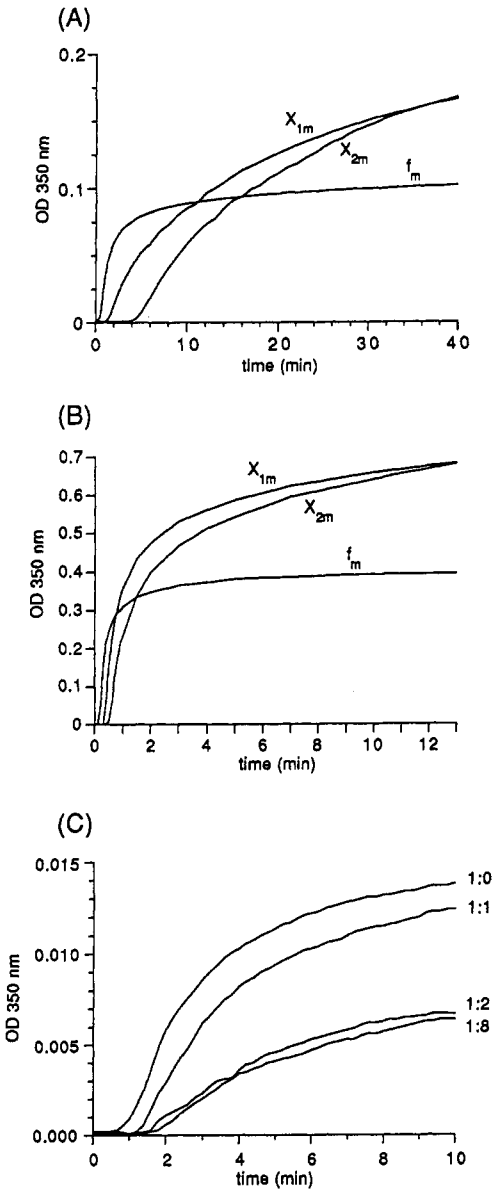


FIGURE 4: Turbidity curves. Optical density (OD) at 350 nm is plotted against time in minutes: f_m = fibrin monomer; X_{1m} = fragment X_1 monomer; X_{2m} = fragment X_2 monomer. (A) Fragment X_1 and X_2 monomer at low protein concentration (0.09 mg/mL). (B) Fragment X_1 and X_2 monomer at high protein concentration (0.50 mg/mL). (C) Turbidity curves for addition of αC fragment to fibrin monomer. The molar ratios of fibrin monomer to αC fragment for each turbidity curve are indicated.

extent of lateral aggregation is reflected in the physical properties of the clots from X monomer, which are more fragile and mechanically unstable than fibrin monomer clots. These results indicate that the αC domains are not necessary for fiber formation and branching but their absence affects the architecture of the clot.

Turbidity Profiles of Clot Formation from Fragment X Monomer. Measurements of turbidity over the time of clot formation allow the monitoring of different stages of polymerization (Hantgan & Hermans, 1979). The results obtained at low concentrations of X monomer (0.09 mg/mL) were consistent with the electron microscope observations, in that the final turbidity was higher than that for fibrin monomer (Figure 4A), indicating thicker fibers. Note that turbidity curves can be compared directly in a quantitative manner because, in all cases, all protein in solution was incorporated into the clots. After the dilution of fibrin monomer to neutral pH to initiate polymerization, there was a short lag period, reflecting the formation of two-stranded protofibrils, and then the turbidity rose rapidly, corresponding to the rate of lateral aggregation of protofibrils to form fibers, and leveled off to a maximum value depending on the protein concentration. For X_2 monomers lacking both αC domains, the clotting time was 12.9× that of fibrin monomer, the lag period was 9× longer, and the maximum rate was 4.8× lower (Table 2). For X_1 monomers having one αC domain, these parameters were intermediates between those of fibrin monomer and X_2 monomer (Table 2). There was a longer lag period, following which the turbidity rise was less rapid than for fibrin monomer but more rapid than for X_2 monomer, indicating its only αC domain participates in fibrin assembly.

Turbidity curves at higher concentrations of X monomer (0.5 mg/mL) were closer to curves observed for fibrin monomer, although significant differences were still present (Figure 4B). For X_2 monomer, the clotting time was 7.7× that of fibrin monomer, the lag period was 3× longer, and the maximum rate was 2.1× lower (Table 2). For X_1 monomer, the corresponding values were again intermediate between those of fibrin monomer and X_2 monomer.

Thus, the above results indicate that the αC domains accelerate all stages of fibrin polymerization and their effects are greater in dilute solution. These findings suggest that αC fragments, which mimic the αC domains, will have inhibitory effects on the polymerization process, especially in dilute solution.

Effects of αC Fragment on Turbidity. The αC fragment employed in the present study was characterized by amino- and carboxyl-terminal sequencing (Table 1). The amino-terminus was heterogeneous, containing three sequences starting at slightly different points. The most amino-terminal sequence corresponds to the same plasmic cleavage site as that defining the carboxyl-terminus of the α_2 polypeptide chain in fragment X_2. At the carboxyl-terminus, the αC fragment was 28 residues shorter than the complete α chain in fibrin, corresponding to an absence of the most carboxyl-terminal plasmic fragment. The structure of similar preparations of αC fragment were characterized by previous electron microscope studies (Veklich et al., 1993), where it was also shown that isolated αC fragment affected the polymerization process by interacting with the αC domains of the fibrin molecule to prevent intermolecular interactions via the αC domains.

To characterize further the influence of the αC domains on assembly of the clot we examined turbidity changes in the mixture of αC fragment and fibrin monomer first incubated at pH 3.5 and then brought to neutral pH to initiate polymerization. At a molar ratio of fibrin:αC fragment of 1:1, there was a striking decrease in the rate of turbidity rise and maximum turbidity and an increase in the lag period (Figure 4C). At a ratio of fibrin:αC fragment of 1:2, these effects were even more pronounced with a maximum turbidity of about half that with no αC fragment. With increasingly greater amounts of αC fragment, there was little or no additional change in the turbidity curves.

These results indicate that αC fragment retarded all stages of polymerization of monomeric fibrin and decreased substantially the turbidity of the final gel in a dose-dependent manner but cannot inhibit the process entirely. Thus, it appears that the αC domains comprise additional sites that accelerate polymerization but are not required for clotting.

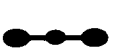
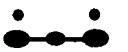
Localization of αC Domains in desA Fibrin Monomer. The results reported above were obtained with monomeric forms

Table 2: Polymerization Parameters for Fragment X Monomer Preparations (X_{1m} and X_{2m}) and Fibrin Monomer (f_m) at Low and High Protein Concentration

	low (0.09 mg/mL)					high (0.50 mg/mL)				
concn of monomer	f_m	X_{1m}	R_1[a]	X_{2m}	R_2[a]	f_m	X_{1m}	R_1[a]	X_{2m}	R_2[a]
lag period (s)	29	76	**2.6**	260	**9.0**	10	19	**1.9**	30	**3.0**
max rate ($\times 10^{-5}$ s^{-1})	95	28	**3.4**	20	**4.8**	2000	1700	**1.2**	950	**2.1**
clotting time (s)	84	720	**8.6**	1080	**12.9**	12	40	**3.3**	92	**7.7**

[a] R_1 and R_2 are ratios of each parameter for X_1 and X_2 monomer, respectively, with that of fibrin monomer. Thus, they indicate the increase of the lag period and clotting time or decrease of the maximum rate of X_1 and X_2 monomer with respect to fibrin monomer.

Table 3: Shapes Observed for Fibrin DesA and Related Molecules under Various Conditions

		% of molecules with the appearance			
sample	no. of molecules examined				
fibrin desA, pH 3.5	365	19	3	**44**	35
fibrin des A, pH 7.4	266	**72**	15	6	7
fibrinogen, pH 7.4[a]	370	**84**	9	1	6
fibrin desAB, pH 3.5[a]	378	13	4	**59**	24
fibrin desAB, pH 7.4[a]	344	22	**71**	1	6

[a] These data were taken from Veklich et al. (1993) for comparison.

of fibrin and X_1 and X_2 fragments. The interpretation of these results is dependent on the extent to which the αC domains of different fibrinogen-derived species may normally interact with each other. Previously, it was demonstrated by electron microscopy that the αC domains of fibrinogen associate with each other and with the central domain, while those of fibrin monomer desAB are freed from the central region so that they can interact intermolecularly (Veklich et al., 1993). Since most of the B fibrinopeptides are usually removed after protofibril formation, it is important also to localize the αC domains in desA fibrin for more accurate assessment of the functional role of the αC domains in the polymerization of fibrinogen with thrombin. Here, we examined desA fibrin monomer prepared by the method of rotary shadowing.

Fibrin monomer desA at acidic pH was rotary shadowed with tungsten and examined by electron microscopy, with particular attention being paid to the extra mass besides the trinodular structure. Only 19% of molecules displayed no additional mass, and 3% had a single large nodule near the central region, while 44% had two small extra nodules and 35% had one small extra nodule (Table 3). Fibrin monomer desA was diluted into neutral pH buffer under conditions such that polymerization was very slow and samples were prepared for microscopy. In this case, in comparison to fibrin monomer desAB (Veklich et al., 1993), only a very small percentage of molecules were involved in oligomer formation because of slower rate of polymerization. At neutral pH, 72% of molecules show no additional mass, while 15% have a single large nodule near the center and 6% and 7% have two or one small nodules, respectively. These results indicate that in fibrin desA most of the αC domains still interact with the central module, which reduces their possibility to participate in protofibril formation.

DISCUSSION

Earlier experiments showed that the carboxyl-terminal portion of fibrinogen's Aα chains play an important role in the fibrin assembly process. Lower molecular weight fibrinogens that normally occur in the circulation have longer clotting times and turbidity curves with longer lag periods and slower turbidity rise (Mosesson et al., 1967; Mosesson, 1983). Polymerization of preparations of high and low molecular weight fractions has been compared (Holm et al., 1985), showing that lag periods are greatly increased with cleavage of the carboxyl-terminal portion of the α chain, termed the αC domain, although these preparations were heterogeneous (Grøn et al., 1988). Another study found that this portion of the molecule accelerates protofibril formation, lateral aggregation, and the production of a three-dimensional network, but does not influence dramatically the structure of intermediates and the final gel (Medved' et al., 1985, 1986). In contrast, another investigation dealing with the structure of the final product revealed that the clot formed from fragment X appears to be markedly different than the normal clot, showing much less branching (Weisel & Papsun, 1987). In still another study (Hasegawa & Sasaki, 1990), a fibrinogen derivative with a molecular mass of about 325 kDa but uncharacterized carboxyl-terminal Aα chain showed much lower turbidity, indicating less lateral aggregation than intact fibrinogen. In these studies, there were differences in the species and concentration of fibrinogen used, the amount of digestion and the preparation methods, and whether polymerization was carried out with fibrin monomer or fibrinogen with thrombin. Remarkably, however, the differences from normal of the turbidity curves for most of these other preparations from many laboratories are similar to those demonstrated here. Most notably, consistently the lag period is increased and the rate of turbidity rise is decreased. The maximum turbidity attained varied, but that might be expected since it is more dependent on the experimental conditions (Weisel & Nagaswami, 1992).

The contradictions between the results obtained by various methods in different laboratories may be accounted for by differences in the structure of the fragment X or low molecular mass preparations used in the studies. Isolated fractions of normally occurring fibrinogen molecules of differing solubility constitute "families" of species with different carboxyl-termini (Nakashima et al., 1992), while fragment X preparations commonly contain some molecules with cleaved amino-terminal Bβ chains as well. It was shown earlier that highly clottable fragment X_1 treated with thrombin forms a "defective" clot in dilute solutions (Gorkun & Medved, 1983). However, X_1 monomer obtained from the fragment by two

to three cycles of dissolution and repolymerization becomes entirely clottable and forms a normal gel (Medved' et al., 1985). It appears that the small amount of nonclottable material that is removed by this repolymerization procedure dramatically influences the structure of the final gel. On the other hand, these preparations were not fully characterized and no experiments were carried out with molecules missing both αC domains, which made the conclusions ambiguous. For example, from the results of the present study it is clear that the X_1 monomers used previously (Medved' et al., 1985) contained one αC domain that was functionally active. So, questions remained about the validity of the conclusions on the function of the αC domain. The present results emphasize the importance of characterization of the X fragments used for such kinds of studies.

In the research presented here, the preparations were well characterized. Some significant features of the preparation procedures used here include the use of bovine rather than human fibrinogen, which seems to give more homogeneous fragments, the sacrifice of yield for minimal damage to the fragments, and the use made of both differences in solubility on removal of the αC domains and the effective purification inherent in fibrin monomer isolation. According to SDS gel electrophoresis and amino acid sequence analysis, intact carboxy-terminal parts of the α chain are absent in X_2 fragment monomer prepared from early plasmin digests. Fragment X_2 definitely lacks both αC domains that are made up of the carboxyl-terminal two-thirds of the Aα chain. In fragment X_1, one αC domain is missing but the other one is present. Amino-terminal parts of the Bβ chains, which are also easily removed at the early stages of fibrinolysis, are present in both monomer fragment preparations. From the known polymerization characteristics of molecules missing Bβ1-42 (Siebenlist et al., 1990), it seems very unlikely that the small percentage of molecules missing this part of the molecule could give rise to the effects observed here. Thus, although both X_1 and X_2 monomers may be regarded as good models for the investigation of the role of the αC domains in the fibrin assembly process, only the use of a new model, namely the highly clottable fragment X_2 monomer missing both αC domains, allows the results obtained to be interpreted unambiguously.

Electron microscopy of the clots prepared from highly purified fibrin and fragment X_1 and X_2 monomers allowed testing of the influence of the αC domains on various aspects of clot formation, including branching, and clarification of existing contradictions (Medved' et al., 1985; Medved' et al., 1986; Weisel & Papsun, 1987). Although it is possible that there are some effects of dilute acetic acid on the molecules other than influences on the αC domains (Veklich et al., 1993), we have now shown that clots formed from fibrin monomer are similar to those produced by addition of thrombin to fibrinogen. There are striated fibers that associate with one another laterally and branch to form a three-dimensional gel. Clots from X_1 and X_2 monomer are similar, but consistent with the turbidity results, the fibers making up the clots aggregate laterally to a greater extent. In particular, X_2 monomer clots contain fiber bundles which are greater in diameter than those observed in fibrin monomer clots, and there are pores of greater size. In summary, it appears that the αC domain is not essential for branching or lateral aggregation but does affect the final clot structure.

Turbidity curves, reflecting the polymerization process of fibrin monomer and X_1 and X_2 monomers (Figure 4), confirmed the results obtained by electron microscopy. The higher final turbidity of the clots formed by X_1 and X_2 monomer in comparison with those formed by fibrin monomer correlates well with the thickness of the fibers in the microscope images. At the same time, the curves are different from one another, indicating dissimilarities in the earlier stages of polymerization. X_1 monomer, missing one of the αC domains, polymerizes more slowly than fibrin monomer but more rapidly than X_2 monomer, lacking both αC domains. Despite these differences in rates of polymerization, all three proteins yield thick fibers and gels that contain substantial numbers of branch points. It is also evident that the single proteolytically modified αC domain in fragment X_1 is functionally active, increasing the polymerization rate of X_1 monomer in comparison with X_2 monomer. Thus, only results obtained with X_2 monomer could be interpreted unambiguously. One of the most striking effects of the removal of the αC domains, seen in the turbidity curves at low protein concentration (0.09 mg/mL), is the increase in the lag period prior to the rise in turbidity. Since the lag period is primarily a consequence of the time required for protofibril formation and growth (Hantgan & Hermans, 1979) (although other assembly steps also occur during this time), it appears that fragment X_2 monomers, lacking both αC domains, are very slow to assemble into protofibrils at low concentrations of protein. On the other hand, at higher concentrations of X_2 monomer (0.5 mg/mL), the lag period is much closer to that of fibrin monomer. A similar dependence was observed for the rate of turbidity increase, which is related to lateral aggregation of protofibrils. It is much higher for fibrin monomer than for X_2 monomer, especially in dilute solution. These results clearly indicate that, in this model system, the αC domains accelerate both protofibril formation and lateral aggregation.

Incubation of fibrin monomer with αC fragment at low molar ratios prolonged the lag period and decreased the rate and amount of lateral aggregation, supporting the above conclusions. These effects increase to a maximum at a molar ratio of two αC fragments for each fibrin, a result consistent with binding of one fragment to each half of the fibrin molecule, which contains two pairs of each polypeptide chain. These experiments, together with previous findings (Veklich et al., 1993) suggest that the αC fragments interact with αC domains of fibrin monomer, preventing intermolecular interactions of this part of the molecule. Nevertheless, it appears that the effects of blocking the αC domain interactions by addition of αC fragment are not identical to the effects of proteolytic removal of the αC domains, although the turbidity curves can only be compared qualitatively since the experimental conditions are somewhat different. In addition, because polymerization via the main complementary binding sites is not affected by the αC domains, even high molar ratios of αC fragment (1:8) are only able to decrease the maximum rate and affect the final clot, but not prevent the process.

Electron microscope images of individual molecules suggest a possible mechanism for the influence of the αC domains on the polymerization process. Only a small fraction of fibrinogen molecules examined by electron microscopy display an additional globular region near the central domain that appears to consist of the two αC domains interacting with each other (Erickson & Fowler, 1983; Weisel et al., 1985). In contrast, in preparations of shadowed desAB fibrin monomers at neutral pH, most molecules have this additional globular domain (Veklich et al., 1993). Additional evidence for interaction between the αC domains comes from microcalorimetry measurements on fibrinogen (Medved' et al., 1983). Furthermore, fibrin monomer preparations at neutral pH also

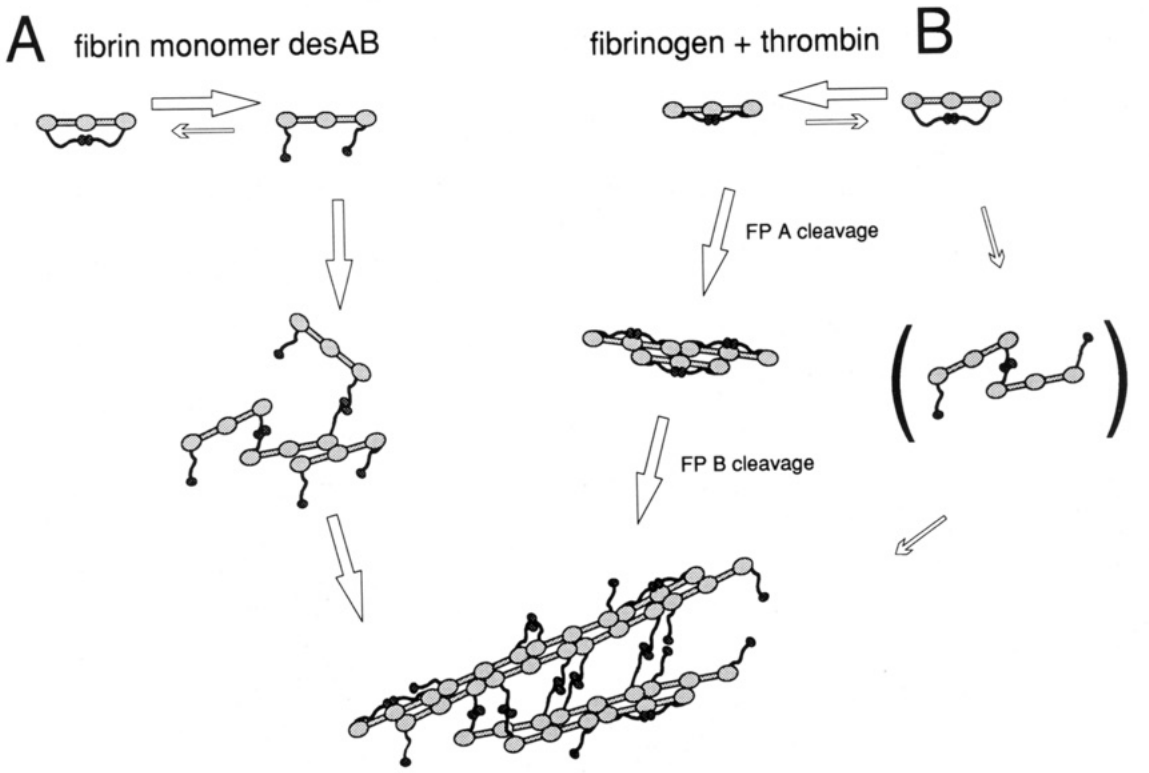


FIGURE 5: Schematic diagram of mechanisms involving the αC domains in fibrin assembly. (A) With fibrin monomer desAB, αC domains are preferentially dissociated from the central domain and from each other. Thus, they are able to promote both protofibril formation and lateral aggregation by their intermolecular interactions. (B) With the sequential cleavage of the fibrinopeptides by thrombin, first desA fibrin, in which the αC domains are still associated with each other and with the central domain, is produced. On protofibril formation and cleavage of the B fibrinopeptides, the αC domains dissociate from the central domain and are free to form intermolecular associations. Thus, *in vivo* they are involved primarily in the lateral aggregation of protofibrils to form fibrils.

shows intermolecular interactions via the αC domains (Veklich et al., 1993). Thus, these results indicate that the αC domains of fibrin desAB are flexible extensions from the main bulk of the molecule that can dissociate and reassociate, changing from intramolecular to intermolecular interactions. This process may be better expressed after conversion of fibrinogen to fibrin since the αC domains of fibrinogen appear to bind to the amino-terminal disulfide knot and may not be accessible for intermolecular interactions. It appears that the intramolecular interactions are different than those responsible for Factor XIIIa-induced ligation, as discussed earlier (Veklich et al., 1993).

At low concentrations of fibrin desAB, where the distances between the molecules are large, the αC domains may function to bring molecules together more efficiently to initiate oligomer formation, which then is carried on mainly by interactions between the main complementary binding sites. The flexible connector anchoring each αC domain to the distal end of the coiled-coil may allow an initial interaction between αC domains of two molecules that are still separated by some distance. This effect is less significant at higher protein concentrations, where the distances between molecules are small enough for the interactions between the main polymerization sites to occur rapidly. After protofibrils are formed, the αC domains again interact intermolecularly to reduce the distance between protofibrils and to promote their lateral aggregation. Aspects of these mechanisms of assembly are illustrated in Figure 5A.

It is clear from all of the results above that the αC domains interact with each other, but these interactions will have very different consequences depending on which stage of clotting they normally occur (Weisel & Nagaswami, 1992). If the αC domains interact during fibrin oligomer formation, they could help to bring molecules together prior to association via the complementary binding sites. On the other hand, if the interactions occur later they could enhance lateral aggregation of protofibrils or fibers. If they occur throughout the assembly process, all steps may be accelerated (Weisel et al., 1993). The model system used here, namely fibrin monomer desAB or fragment X monomer, is necessary for the success of the experiments carried out but does not allow us to distinguish among these possibilities, since the final turbidity depends on the relative effects on different steps. For example, fibrin monomer desAB polymerizes differently than fibrinogen plus thrombin, the system that exists *in vivo*, or fibrin monomer desA with thrombin added during the lag period (Weisel et al., 1993). In other words, the sequence of release of the fibrinopeptides is important.

Additional studies reported here account for these observations and allow us to define the stage at which the αC domain interactions are most significant in situations closer to physiological. The αC domains of most molecules of fibrin monomer desA, like those of fibrinogen, interact primarily with each other and with the central domain, while the αC domains of fibrin monomer desAB are released from the central domain to associate intermolecularly (Veklich et al., 1993). Therefore, for the most part, normally the αC domains are not free to interact intermolecularly during oligomer formation.[2] Once protofibrils are formed and the B fibrinopeptides are cleaved, the αC domains can then interact to enhance lateral aggregation. Thus, when thrombin cleaves the fibrinopeptides in a stepwise manner, the αC domains most probably have their major impact on lateral aggregation (Figure 5B). In fibrinogen and fibrin desA the αC domains are associated with each other and with the central domain. After formation of protofibrils, the B fibrinopeptides are cleaved, releasing the αC domains as well, so that they interact

intermolecularly to enhance lateral aggregation. Results showing that the peptide Gly-His-Arg-Pro (amino terminus of the β chain) binds to the αC domains (Hasegawa & Sasaki, 1990), suggesting that the αC domains may associate with the central region via the amino terminal Bβ chain, are consistent with the release of the αC domains on cleavage of the B fibrinopeptides.

There is now a variety of other results in the literature supporting a role for the αC domains in lateral aggregation. In several previous studies, polymerization of fragment X-like molecules yielded curves with lower final turbidity (Mosesson et al., 1967; Hasegawa & Sasaki, 1990), although these results depend on the details of the experimental procedures (Holm et al., 1985; Medved' et al., 1985). Several dysfibrinogenemias with amino acid substitutions in the αC domains, fibrinogen Caracas II [Aα Ser-434 to N-glycosylated Asn (Maekawa et al., 1991)], fibrinogen Marburg [missing Aα 461–611 (Koopman et al., 1992)], and fibrinogen Dusart [Aα Arg-554 to Cys (Koopman et al., 1993)], all appear to be defective in lateral aggregation. More direct evidence comes from studies of monoclonal antibodies specific for particular regions of the αC domains (Cierniewski & Budzynski, 1992). In addition, results of a very recent study using a 24-kDa fragment isolated from the carboxyl terminal Aα chain (Lau, 1993) are also consistent with the conclusions presented here. It is important to note that the αC domains are not required for lateral aggregation, which can occur when they are completely missing in fragment X_2 monomer, but only promote it. Although the αC domains are important for the formation of fibers of normal thickness under physiological conditions, the extent of lateral aggregation is dependent on the relative rates of the various steps of polymerization (Weisel & Nagaswami, 1992).

The conclusions of these studies may be summarized as follows: (1) Well-characterized, virtually homogeneous fragments X_1 and X_2, missing either one or both of the αC domains, respectively, as well as αC fragment, were used to analyze the function of the carboxyl-terminal α chain of fibrin. (2) The monomeric forms of these fragments formed clots with well-developed branching, but the extent of order and lateral aggregation, as visualized by transmission and scanning electron microscopy were different, indicating that the αC domains affect clot structure. (3) Analysis of turbidity curves demonstrated that both the missing αC domains and the addition of αC fragment to fibrin monomer affect the lag period and rate of turbidity increase, suggesting that the αC domains interact intermolecularly to enhance polymerization. (4) In fibrinogen and desA fibrin monomer, the αC domains are attached to the central domain, while they are free to associate intermolecularly in desAB fibrin, suggesting that the αC domain interactions are most significant for lateral aggregation *in vivo*.

ACKNOWLEDGMENT

We thank Chandrasekaran Nagaswami, Sherin Sabet, and Lourdes de Santiago for technical assistance.

[2] However, the αC domains probably do play some role in oligomer formation. The slightly higher percentage of free large nodules seen in fibrin monomer desA in comparison with fibrinogen (15% versus 9%) suggests that more αC dimers dissociate from the central domain. This increases the probability of dissociation of the αC dimers, which is reflected in the slightly higher percentage of free noninteracting αC domains at neutral pH (6 + 7% = 13% for fibrin desA versus 1 + 6% = 7% for fibrinogen (Table 3)). These results suggesting that the αC domains of some desA fibrin molecules can enhance protofibril formation are consistent with recent results showing that cross-linked α polymers are formed very early in polymerization (Grøn et al., 1992).

REFERENCES

Belitser, V. A., Varetskaja, T. V., & Malneva, G. V. (1968) *Biochim. Biophys. Acta 154*, 367–375.
Blombäck, B., Okada, M., Forslind, B., & Larsson, U. (1984) *Biorheology 21*, 93–104.
Budzynski, A. Z., Marder, V. J., & Shainoff, J. R. (1974) *J. Biol. Chem. 249*, 2294–2302.
Chung, D. W., Rixon, M. W., & Davie, E. W. (1982) in *Proteins in Biology and Medicine* (Bradshaw, R. A., Hill, R. L., Tanget, J., et. al., Eds.) pp 309–328, Academic Press, New York.
Cierniewski, C. S., & Budzynski, A. Z. (1992) *Biochemistry 31*, 4248–4253.
Erickson, H. P., & Fowler, W. E. (1983) *Ann. N. Y. Acad. Sci. 408*, 146–163.
Gorkun, O. V., & Medved, L. V. (1983) *Dokl. Acad. Nauk Ukr. SSR (USSR) Ser. B, N4*, 67–70.
Grøn, B., Bennick, A., Nieuwenhuizen, W., Bjornsen, S., & Brosstad, F. (1988) *Thromb. Res. 52*, 413–424.
Grøn, B., Filion-Myklebust, C., Bennick, A., Nieuwenhuizen, W., Matsueda, G. R., & Brosstad, F. (1992) *Blood Coag. Fibrinolysis 3*, 731–736.
Hantgan, R. R., & Hermans, J. (1979) *J. Biol. Chem. 254*, 11272–11281.
Hasegawa, N., & Sasaki, S. (1990) *Thromb. Res. 57*, 183–195.
Henschen, A., Lottspeich, F., Töpfer-Petersen, E., Kehl, M., & Timpl, R. (1980) in *Protides of the Biological Fluids* (Peeters, H., Ed.) pp 47–50, Pergamon, Oxford.
Henschen, A., & McDonagh, J. (1986) in *Blood Coagulation* (Zwaal, R. F. A., & Hemker, H. C., Eds.) pp 171–234, Elsevier Science, Amsterdam.
Holm, B., Brosstad, F., Kierulf, P., & Godal, H. C. (1985) *Thromb. Res. 39*, 595–606.
Kehl, M., Lottspeich, F., & Henschen, A. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 1501–1505.
Koopman, J., Haverkate, F., Grimbergen, J., Egbring, R., & Lord, S. T. (1992) *Blood 80*, 1972–1979.
Koopman, J., Haverkate, F., Grimbergen, J., Lord, S. T., Mosesson, M., DiOrio, J. P., Siebenlist, K. S., Legrand, C., Soria, J., & Soria, C. (1993) *J. Clin. Invest. 91*, 1637–1643.
Langer, B. G., Weisel, J. W., Dinauer, P. A., Nagaswami, C., & Bell, W. R. (1988) *J. Biol. Chem. 263*, 15056–15063.
Lau, H. K. F. (1993) *Blood 81*, 3277–3284.
Maekawa, H., Yamazumi, K., Muramatsu, S., Kaneko, M., Hirata, H., Takahashi, N., deBosch, N. B., Carvajal, Z., Ojeda, A., Arocha-Pinango, C. L., & Matsuda, M. (1991) *J. Biol. Chem. 266*, 11575–11581.
Marder, V. J., Shulman, N. R., & Carroll, W. R. (1969) *J. Biol. Chem. 244*, 2111–2119.
Marguerie, G., Pouit, L., & Suscillon, M. (1973) *Thromb. Res. 3*, 675–689.
Medved', L. V. (1990) *Blood Coag. Fibrinolysis 1*, 439–442.
Medved', L. V., Gorkun, O. V., & Privalov, P. L. (1983) *FEBS Lett. 160*, 291–295.
Medved', L. V., Gorkun, O. V., Manyakov, V. F., & Belitser, V. A. (1985) *FEBS Lett. 181*, 109–112.
Medved', L. V., Gorkun, O. V., Manyakov, V. F., & Belitser, V. A. (1986) *Mol. Biol. (USSR) 20*, 461–70.
Mosesson, M. W. (1983) *Ann. N. Y. Acad. Sci. 408*, 97–113.
Mosesson, M. W., & Doolittle, R. F., Eds. (1983) *Molecular Biology of Fibrinogen and Fibrin*, New York Academy of Sciences, New York.
Mosesson, M. W., Alkjaersig, N., Sweet, B., & Sherry, S. (1967) *Biochemistry 6*, 3279–3287.
Mosesson, M. W., Siebenlist, K. R., Amrani, D. L., & DiOrio, J. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1113–1117.
Nakashima, A., Sasaki, S., Miyazaki, K., Miyata, T., & Iwanaga, S. (1992) *Blood Coag. Fibrinolysis 3*, 361–370.
Phillips, H. M. (1981) *Can. J. Biochem. 59*, 332–342.
Pizzo, S. V., Schwartz, M. L., Hill, R. L., & McKee, P. A. (1972) *J. Biol. Chem. 247*, 636–645.

Privalov, P. L., & Medved, L. V. (1982) *J. Mol. Biol. 159*, 665–683.
Shen, L. L., McDonagh, R. P., McDonagh, J., & Hermans, J. (1977) *J. Biol. Chem. 252*, 6184–6189.
Siebenlist, K. R., DiOrio, J. P., Budzynski, A. Z., & Mosesson, M. W. (1990) *J. Biol. Chem. 265*, 18650–18655.
Suttnar, J., Medved', L., Gorkun, O. V., & Dyr, J. E. (1991) *Thromb. Haemost. 65*, 897.
Varetskaya, T. V. (1960) *Ukrain. Biokhim. Zh. USSR 32*, 13–24.
Veklich, Y. I., Gorkun, O. V., Medved, L. V., Nieuwenhuizen, W., & Weisel, J. W. (1993) *J. Biol. Chem. 268*, 13577–13585.
Weisel, J. W. (1986) *J. Ultrastruct. Mol. Struct. Res. 96*, 176–188.
Weisel, J. W. (1988) in *Fibrinogen 3. Biochemistry, Biological Functions, Gene Regulation and Expression* (Mosesson, M. W., Amrani, D. L., Siebenlist, K. R., & DiOrio, J. P., Eds.) pp 113–116, Excerpta Medica, Amsterdam.
Weisel, J. W., & Papsun, D. M. (1987) *Thromb. Res. 47*, 155–163.
Weisel, J. W., & Nagaswami, C. (1992) *Biophys. J. 63*, 111–128.
Weisel, J. W., Phillips, G. J., & Cohen, C. (1981) *Nature 289*, 263–267.
Weisel, J. W., Phillips, G. J., & Cohen, C. (1983) *Ann. N. Y. Acad. Sci. 408*, 367–79.
Weisel, J. W., Stauffacher, C. V., Bullitt, E., & Cohen, C. (1985) *Science 230*, 1388–1391.
Weisel, J. W., Veklich, Y. I., & Gorkun, O. V. (1993) *J. Mol. Biol. 232*, 285–297.