Cunningham, L., Catlin, B. W., & Privat de Garilhe, M. (1956) *J. Am. Chem. Soc. 78*, 4642-4645.
Englander, S. W., & Wand, A. J. (1987) *Biochemistry 26*, 5953-5958.
Hinck, A. P., Loh, S. N., Wang, J., & Markley, J. L. (1990) *J. Am. Chem. Soc. 112*, 9031-9034.
Hynes, T. R., & Fox, R. O. (1991) *Proteins: Struct., Funct., Genet. 10*, 92-105.
Loll, P. J., & Lattman, E. E. (1989) *Proteins: Struct., Funct., Genet. 5*, 183-201.
Markley, J. L., & Jardetzky, O. (1970) *J. Mol. Biol. 50*, 223-233.
Rance, M., Sørensen, W. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479-485.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167-339.
Shon, K., & Opella, S. J. (1989) *J. Magn. Reson. 82*, 193-197.
Sklenar, V., & Bax. (1987) *J. Magn. Reson. 71*, 379-383.
Torchia, D. A., Sparks, S. W., & Bax, A. (1989) *Biochemistry 28*, 5509-5524.
Tucker, P. W., Hazen, E. E., Jr., & Cotton, F. A. (1979a) *Mol. Cell. Biochem. 23*, 3-16.
Tucker, P. W., Hazen, E. E., Jr., & Cotton, F. A. (1979b) *Mol. Cell. Biochem. 23*, 67-86.
Ulrich, E. L., Markley, J. L., & Kyogoku, Y. (1989) *Protein Sequences Data Anal. 2*, 23-37.
Wagner, G. (1983) *J. Magn. Reson. 55*, 151-156.
Wagner, G., Neuhaus, D., Wörgötter, E., Vasak, M., Kagi, J. M. R., & Wüthrich, K. (1986) *J. Mol. Biol. 187*, 131-135.
Wang, J., LeMaster, D. M., & Markley, J. L. (1990a) *Biochemistry 29*, 88-101.
Wang, J., Hinck, A. P., Loh, S. N., & Markley, J. L. (1990b) *Biochemistry 29*, 102-113.
Wang, J., Hinck, A. P., Loh, S. N., & Markley, J. L. (1990c) *Biochemistry 29*, 4242-4253.
Wang, J., Mooberry, E. S., Walkenhorst, W. F., & Markley, J. L. (1992) *Biochemistry* (preceding paper in this issue).
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Wüthrich, K., Billeter, M., & Braun, W. (1984) *J. Mol. Biol. 180*, 715-740.

Factors Affecting γ-Chain Multimer Formation in Cross-Linked Fibrin†

Kevin R. Siebenlist* and Michael W. Mosesson
University of Wisconsin Medical School, Milwaukee Clinical Campus, Sinai Samaritan Medical Center, Milwaukee, Wisconsin 53233

Received July 1, 1991; Revised Manuscript Received September 12, 1991

ABSTRACT: The major covalently linked multimolecular D fragments found in plasmic digests of factor XIIIa cross-linked fibrin formed under physiological pH and ionic strength conditions consist of D dimers, D trimers, and D tetramers. These fragments are linked by ε-amino-γ-glutamyllysine bonds in the carboxy-terminal regions of their γ chains, which had originated in the cross-linked fibrin as γ dimers, γ trimers, and γ tetramers, respectively. In this study, factors affecting the degree and rate of formation of these three classes of cross-linked γ chains were determined by analyzing the D-fragment content of plasmic digests of cross-linked fibrin that had been sampled after all γ-chain monomers had been consumed in the cross-linking process. D trimers and D tetramers, expressed as a proportion of the total D-fragment content, both increased at the expense of the D-dimer population as a function of increasing factor XIII concentration, the time of cross-linking, or the $CaCl_2$ concentration. Their levels decreased as the ionic strength was raised by NaCl addition. However, the ionic strength effect could be reversed by concomitantly raising the $CaCl_2$ concentration. Digests of clots prepared from recalcified fresh citrated plasma also contained each type of cross-linked D fragment, and the proportion of D trimers and D tetramers in the digest increased with increasing clot incubation time. These results indicate that γ-trimer and γ-tetramer formation is a dynamic physiological process. Such cross-linked trimeric and tetrameric γ-chain structures may function in vivo to stabilize the fibrin matrix at interfiber contacts and/or at branch points, thereby enhancing mechanical strength, elasticity, and/or clot resistance to fibrinolysis.

Following thrombin-catalyzed conversion of fibrinogen to fibrin, polymer assembly commences with formation of double-stranded fibrils in which fibrin molecules associate by noncovalent intermolecular interactions between the outer D domains and the central E domains (Blombäck et al., 1978; Laudano & Doolittle, 1978, 1980; Shainoff & Dardik, 1979, 1983; Olexa & Budzynski, 1980) forming a staggered overlapping fibrillar array (Ferry, 1952; Stryer et al., 1963; Krakow et al., 1972; Hantgan & Hermans, 1979; Fowler et al., 1981; Williams, 1981, 1983). The resulting two-stranded fibrils subsequently associate laterally, forming thick fibers that constitute the major structural elements of the branched three-dimensional fibrin matrix (Carr et al., 1977; Hantgan & Hermans, 1979; Hantgan et al., 1980, 1983; Hermans & McDonagh, 1982; Erickson & Fowler, 1983; Hewat et al., 1983). Multiple factors affect the ultimate structure of the fibrin matrix. Rapid assembly and thick fibrin fibers result when clotting occurs at or below physiological ionic strength (Ferry & Morrison, 1947; Hangtan et al., 1980, 1983; Müller et al., 1984; Carr et al., 1986) and when Ca^{2+} is included in the reaction medium (Boyer et al., 1972; Okada & Blombäck, 1983; Carr et al., 1986). Slowing the conversion of fibrinogen

† This investigation was supported by NHLBI Program Project Grant HL-28444.
* Address correspondence to this author at the Sinai Samaritan Medical Center, Winter Research Building, 836 North 12th St., Milwaukee, WI 53233.

0006-2960/92/0431-936$03.00/0

to fibrin at low thrombin concentrations increases thick fiber formation (Rosser et al., 1977; Wolfe & Waugh, 1981; Shah et al., 1985), but since the fibrin assembly rate is slow under these conditions, an extensive fine fibril matrix develops in conjunction with thick fibers (Mosesson et al., 1987).

In the presence of plasma transglutaminase (factor XIIIa) and Ca^{2+}, fibrin molecules undergo covalent cross-linking within the clot matrix by formation of ε-amino-γ-glutamyllysine isopeptide bonds (Matačič & Loewy, 1968; Pisano et al., 1968). Intermolecular cross-linking at the carboxy-terminal regions of γ chains in the D domain occurs rapidly and results primarily in dimers (Chen & Doolittle, 1969) formed by bridging between a lysine at position 406 of one γ chain and a glutamine at position 398 or 399 of the other (Chen & Doolittle, 1971; Doolittle et al., 1971; Doolittle, 1973; Purves et al., 1987). Reciprocal bridging between properly aligned γ chains occurs by using both of these donor–acceptor positions, although formation of only one such bond is necessary to form covalent γ-chain dimers. In addition, isopeptide bond formation between α and γ chains results in hybrid cross-linked chains (Mosesson et al., 1989; Shainoff et al., 1991), whereas cross-linking among α chains creates oligomers and larger α-chain polymers (McKee et al., 1970; McDonagh et al., 1971; Schwartz et al., 1971).

Plasmin digestion of cross-linked fibrin in the presence of Ca^{2+} initially gives rise to a series of cross-linked high molecular weight E-containing intermediate fragments (e.g., DY, YY, and DXD) that are larger than γ-chain-cross-linked D dimers (Mosesson & Finlayson, 1976; Alkjaersig et al., 1977; Regañon et al., 1978; Gaffney et al., 1980; Francis et al., 1980a,b; Gaffney, 1983). At advanced stages of proteolysis, E-containing fragments are consumed, and D dimers persist and predominate, since Ca^{2+} confers resistance to plasmin cleavage within the γ-chain segment containing the intermolecular covalent cross-link between D domains (Haverkate & Timan, 1977; Purves et al., 1978; Nieuwenhuizen et al., 1981, 1982).

In a recent study of plasmin digests of fibrin that had been cross-linked under physiological buffer conditions (Mosesson et al., 1989), two previously unrecognized plasmin-resistant cross-linked D fragments larger than D dimer were identified. They corresponded to D trimers and D tetramers held together by cross-linked remnants of γ trimers and γ tetramers, respectively. Recently, Shainoff et al. (1991) demonstrated γ trimers and γ tetramers in cross-linked fibrin by immunolabeling. In these present studies, we systematically explored the conditions that affect fibrin matrix structure in order to correlate fiber size and matrix branching behavior with γ-trimer and γ-tetramer formation in cross-linked fibrin, as measured by the D-trimer and D-tetramer content in plasmic digests of fibrin.

Materials and Methods

Tris(hydroxymethyl)aminomethane (Tris),[1] glycine, urea, and DTT were purchased from Aldrich Chemical Co., Milwaukee, WI. Phenylmethanesulfonyl fluoride and Coomassie Brilliant Blue R250 were obtained from Sigma Chemical Co., St. Louis, MO. Trasylol (aprotinin) was obtained from Mobay Chemical Corp., New York, NY, and DE-52 cellulose was from Whatman Inc., Clifton, NJ. Human α-thrombin was a generous gift from Dr. J. Fenton II (Division of Laboratories and Research, New York State Department of Health, New York). All other chemicals were the highest purity available from commercial sources.

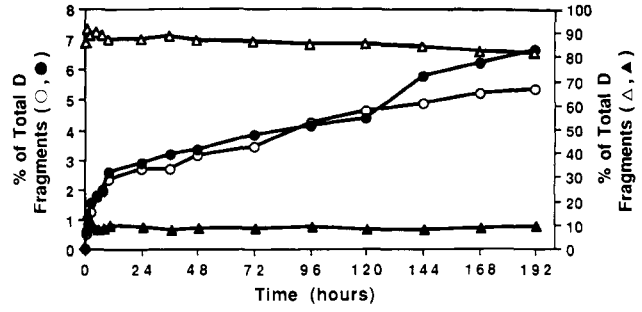

FIGURE 1: D-trimer and D-tetramer formation as a function of the duration of cross-linking was carried out at room temperature under standard conditions (μ = 0.17). At the various times indicated on the abscissa, clot samples were digested with plasmin for 18–24 h, subjected to SDS–PAGE, and analyzed by densitometry. D tetramer (○); D trimer (●); D dimer (△); D monomer (▲). The results shown are the average of five separate experiments.

Human fibrinogen fraction I-2 was isolated from pooled citrated plasma by glycine precipitation (Kazal et al., 1963) and further purified as described by Mosesson and Sherry (1966). Fraction I-2 fibrinogen was subfractionated into peak 1 and peak 2 fibrinogen by chromatography on DE-52 (Finlayson & Mosesson, 1963; Mosesson et al., 1972). Fibrinogen clottability was greater than 98%. Soluble fibrin monomer (15–20 mg/mL in 20 mM acetic acid) was prepared by the method of Belitser et al. (1968). Fibrinogen or soluble fibrin concentrations were determined spectrophotometrically at 280 nm using an absorbance coefficient ($A^{1\%}_{1cm}$) of 15.1 (Mosesson & Finlayson, 1963). Plasminogen was purified from pooled citrated human plasma by affinity chromatography on lysine–Sepharose (Robbins & Summaria, 1976), activated to plasmin by incubation with streptokinase (1:800 molar ratio) for 20 h at room temperature (Robbins & Summaria, 1970), and assayed colorometrically using Kabi S-2251 (Helena Laboratories, Beaumont, TX) as substrate. Factor XIII was purified from human plasma (Lorand & Gotoh, 1970), and assayed as described by Loewy et al. (1961). Its specific activity ranged from 2500 to 3000 units/mg. Factor XIIIa was prepared by addition of thrombin, 5 units/mL, followed by thrombin inhibition with 25 units/mL hirudin (Kanaide & Shainoff, 1975), 25 units/mL.

SDS–PAGE was performed by the method of Weber and Osborn (1969) for tube gels (5-mm diameter) or in 1.5-mm slab gels by the method of Laemmli (1970). Five percent polyacrylamide gels were used for nonreduced samples and 7.5 or 9% gels for completely reduced samples. The gels were stained with 0.5% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (5:5:2) for 1 h at room temperature and destained in methanol/water/acetic acid (5:5:2) with continuous shaking.

Standard conditions for preparing cross-linked fibrin clots consisted of fraction I-2 fibrinogen (3 mg/mL) in 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, 4 mM DTT, and 10 units/mL Trasylol, pH 7.4, buffer (μ = 0.17) supplemented with factor XIII (50 units/mL). The factor XIII present in the I-2 preparations (~3 units/mg) along with the added enzyme brought the final activity into the range observed in plasma (~60 units/mL). Clotting and cross-linking were initiated by adding thrombin (0.25 unit/mL). The mixtures were incubated at room temperature for up to 8 days and sampled periodically for evaluation of the degree of γ- and α-chain cross-linking. Cross-linked clots formed from fibrin monomer were prepared by adding the solubilized monomer under

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

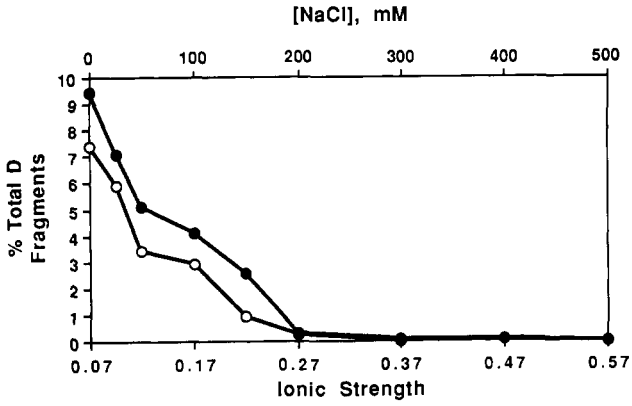

FIGURE 2: Effect of ionic strength on the formation of D trimer and D tetramer. Clotting and cross-linking of fibrinogen at 3 mg/mL were carried out for 24 h at room temperature in 50 mM Tris, 10 mM $CaCl_2$, 4 mM DTT, and 10 units/mL Trasylol, pH 7.4, buffer (μ = 0.07) to which factor XIII (50 units/mL) had been added. The ionic strength was varied by adding NaCl (up to 500 mM). D tetramer (○); D trimer (●). The results represent the mean of five separate experiments.

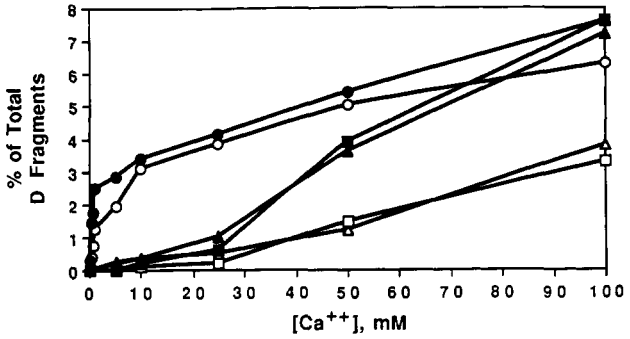

FIGURE 3: Effect of calcium ions on the formation of D trimer and D tetramer. The clotting and cross-linking reaction was carried out at room temperature at a fibrinogen concentration of 3 mg/mL and a factor XIII concentration of 50 units/mL in 50 mM Tris, 4 mM DTT, and 10 units/mL Trasylol, pH 7.4, buffer, with 100 mM NaCl (○, ●), 200 mM NaCl (△, ▲), or 500 mM NaCl (□, ■). The $CaCl_2$ content was varied from 0 to 100 mM. D tetramer (open symbols); D trimer (closed symbols). The results represent the average of three separate experiments.

standard buffer conditions (supplemented with factor XIIIa, 50 units/mL). Under these conditions, monomeric γ chains were consumed in the cross-linking process within 30 min and monomeric α chains within 10 h. For other cross-linking experiments, thrombin (0.001–10 units/mL), factor XIII (up to 100 units/mL), NaCl (up to 500 mM), or $CaCl_2$ (up to 100 mM) concentrations were varied.

Plasmin digestion of cross-linked clots was carried out on clots that had been wound on glass rods and incubated for 1 h in 50 mM Tris/10 mM $CaCl_2$, pH 8.6, buffer containing 5 mM NEM to inactivate factor XIIIa (complete loss of factor XIIIa activity occurred within 30 min). The NEM-treated clot was then washed 3 times with the pH 8.6 buffer and digested in the same buffer with plasmin (10 caseinolytic units/mL) for 18–24 h. Under these digestion conditions, no E-containing intermediates remained at the termination of digestion, and no significant changes in the distribution of digest components were observed over more extended digestion periods.

Total D fragments (D tetramers, D trimers, D dimers, and D monomers) present in the plasmic digests were quantified by densitometric gel scanning using a Gilford response spectrophotometer equipped with a Gilford autoradiogram scanning kit. Tube gels were scanned by photographing the gels, printing the negative on a sheet of Kodak type 4489 film, and scanning the positive film print. This method for scanning tube gels yielded linear plots for peak area versus protein load in the range of 0–500 μg/lane.

Table I: Content of D Fragments in Digests of Cross-Linked Clots Formed at Various Thrombin Concentrations or from Repolymerized Fibrin Monomer[a]

cross-linked fibrin formed with	fragment content (%)			
	D monomer	D dimer	D trimer	D tetramer
IIa, 0.001 unit/mL	10.0	70.2	10.4	9.4
IIa, 0.01 unit/mL	6.9	75.4	9.9	7.7
IIa, 0.10 unit/mL	6.9	77.8	9.1	6.2
IIa, 1.0 unit/mL	5.5	80.7	8.1	5.6
IIa, 10 units/mL	7.2	81.2	6.9	4.7
fibrin monomer	12.6	77.2	6.0	4.2

[a] Clots were prepared from fibrinogen solutions supplemented with factor XIII (50 units/mL) under standard buffer conditions (μ = 0.17) by the addition of thrombin (IIa). In the case of polymerizing fibrin monomer solutions, the buffer was supplemented with factor XIIIa (50 units/mL). All cross-linking mixtures were incubated for 24 h at room temperature prior to plasmin digestion. The results represent the average of three separate experiments.

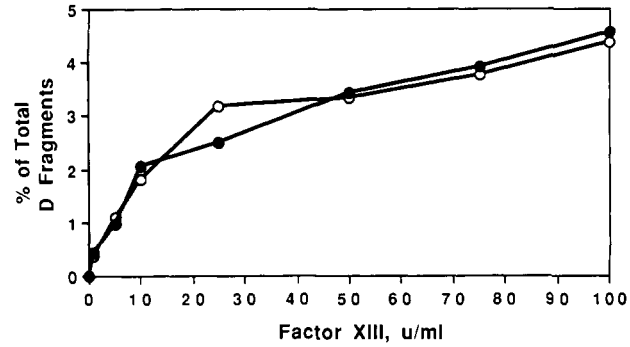

FIGURE 4: Effect of factor XIII concentration on formation of D trimer and D tetramer. The cross-linking reaction was carried out at room temperature under standard buffer conditions (μ = 0.17). Factor XIII additions were varied from 0 to 100 units/mL, final concentration. D tetramer (○); D trimer (●). The results represent of the mean of three separate experiments.

Cross-linked plasma fibrin clots were formed in citrated fresh single human donor plasma either by recalcifying the plasma (10 mM $CaCl_2$ excess over the citrate concentration) or by adding $CaCl_2$ (10 mM $CaCl_2$ excess over citrate) plus thrombin (0.25 unit/mL). The mixtures were incubated at room temperature for up to 5 days, and the resulting clots were washed, digested, and analyzed as described above.

Results

Determination of the evolution of D trimer and D tetramer in the plasmic digest as a function of clot incubation time (Figure 1) was carried out under cross-linking conditions in which complete consumption of γ monomers had occurred within 30 min of incubation. The D-trimer and D-tetramer content of the cross-linking mixture increased rapidly over the first 10 h of incubation and then continued to increase more gradually during the entire sampling period. The increase in D-trimer and D-tetramer content was accompanied by a corresponding decrease in the amount of D dimer, whereas D-monomer levels remained constant at about 10% of the total D-containing fragments. The D monomer in the digest probably originated by plasmic cleavage of polymeric D structures, as previously noted (Mosesson et al., 1989).

Changes in ionic strength and Ca^{2+} concentration are known to produce significant changes in fibrin matrix structure (Ferry & Morrison, 1947; Boyer et al., 1972; Hangtan et al., 1980, 1983; Okada & Blombäck, 1983; Müller et al., 1984; Carr

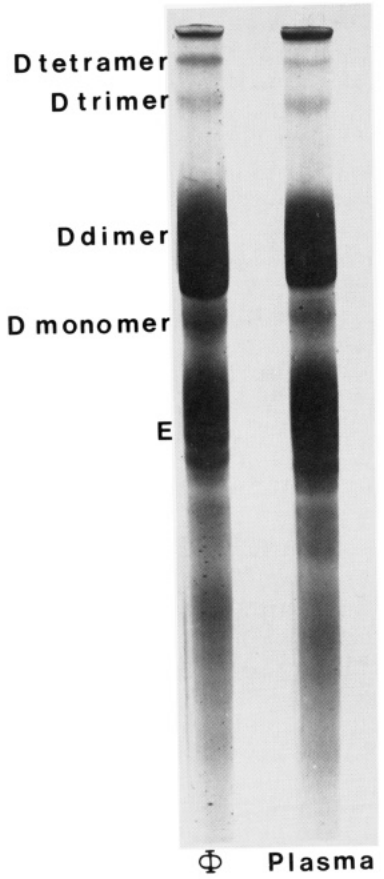

FIGURE 5: Analysis of plasmin digests of cross-linked fibrin clots. Gel 1, plasmin digest of a cross-linked fibrin clot formed from fraction I-2 fibrinogen; gel 2, plasmin digest of a recalcified plasma clot. The material at the top of the gels has not yet been characterized.

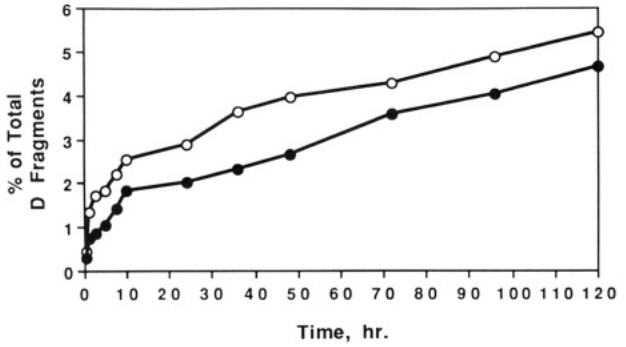

FIGURE 6: Formation of D trimer and D tetramer in digests of plasma clots formed from recalcified citrated fresh single donor human plasma to which thrombin had also been added. The plasma factor XIII concentration was ~60 units/mL. The clots were incubated at room temperature for up to 120 h prior to digestion with plasmin. D tetramer (○); D trimer (●). The results presented are the average of three separate experiments.

et al., 1986). As the ionic strength was raised with NaCl, increasingly translucent "fine" clots were formed. The D-trimer and D-tetramer population formed from these clots decreased progressively, with a concomitant rise in the D-dimer content (Figure 2). At NaCl concentrations at or above 200 mM ($\mu \geq 0.27$), negligible amounts (<0.5%) of D trimers and D tetramers were found. Raising the concentration of $CaCl_2$, which is known to favor "coarse" clot formation (Boyer et al., 1972; Okada & Blombäck, 1983; Carr et al., 1986), resulted in a progressive increase in the content of D trimer and D tetramer (Figure 3), even though the ionic strength at the higher $CaCl_2$ concentrations (i.e., $\mu = 0.44$ at 100 mM NaCl and 100 mM $CaCl_2$) was well above the ionic strength at which NaCl addition nearly completely inhibited D-trimer and D-tetramer production. Moreover, the addition of increasing amounts of $CaCl_2$ to high ionic strength NaCl-containing buffers resulted in increased clot turbidity and concomitantly increased amounts of D trimer and D tetramer in plasmin digests (Figure 3). Thus, Ca^{2+} appears to act on fibrin in a manner that is independent of the effects of ionic strength alone.

The D-trimer and D-tetramer contents in digests of clots formed at varying thrombin concentrations were inversely related to the thrombin concentration (Table I), with the highest amount of D trimers and D tetramers occurring at the lowest thrombin concentration. Soluble fibrin monomer preparations repolymerized at a rate that was even faster than that of fibrinogen at 10 units/mL thrombin, and the D-trimer and D-tetramer content of cross-linked clots prepared from this material was slightly less than that formed at 10 units/mL thrombin.

The level of factor XIII also affected the D-trimer and D-tetramer content in plasmic digests of cross-linked clots (Figure 4). At factor XIII concentrations up to 10 units/mL, the D-trimer and D-tetramer content increased nearly linearly with the level of enzyme. Above this level, there was a progressive but more gradual increment in the amount of D trimer and D tetramer in the digest.

D-Trimer and D-tetramer formation in recalcified cross-linked clots formed from fresh plasma was also examined. The types of D fragments formed in plasma clot digests were the same as those observed in digests of the fibrin clot system (Figure 5). Moreover, the time course of γ-trimer and γ-tetramer formation in plasma clots (Figure 6), as reflected by the amount of D trimer and D tetramer in plasmin digests, was similar to the time-dependent rise observed in the fibrin clot system (Figure 1).

Discussion

The appearance of D dimers, D trimers, and D tetramers in plasmic digests of cross-linked fibrin can be taken as a direct indication of γ-dimer, γ-trimer, and γ-tetramer formation, respectively, in the polymerized fibrin matrix (Mosesson et al., 1989). Immunolabeling experiments directly demonstrating their existence in cross-linked fibrin have recently been reported (Shainoff et al., 1991). Our findings on the evolution of D trimers and D tetramers in plasmic digests of cross-linked fibrin clots indicate that the factor XIIIa catalyzed formation of γ trimers and γ tetramers is a dynamic and continuous physiological process occurring well after all γ monomers have been incorporated into γ dimers in the assembling fibrin matrix. Dimerization is very rapid relative to subsequent γ-chain trimerization and tetramerization and occurs by formation of ε-amino-γ-glutamyllysine isopeptide bonds between γ406 lysine in one chain and γ398 or 399 glutamate in another (Chen & Doolittle, 1971; Doolittle et al., 1971; Doolittle, 1973; Purves et al., 1987). Covalent bridging at these positions between properly aligned γ-chain pairs could result in two such isopeptide bonds, although formation of only one bond per chain pair is sufficient for isopeptide-linked γ dimers to form. The data of Pisano et al. (1968, 1969) support the possibility that γ-trimer and γ-tetramer formation can occur through utilization of unpaired, γ-chain dimer donor–acceptor units, since they found fewer than two isopeptide bonds per mole of fibrin at a time when complete γ-dimer formation had occurred. The observation that γ-trimer and γ-tetramer formation is augmented by increasing the factor XIII concentration (Figure 4) is also consistent with this possibility. However, factor XIIIa catalyzed rearrangement

of formed isopeptide bonds, such as occurs in the case of α_2-plasmin inhibitor cross-linked to fibrin(ogen) (Ichinose & Aoki, 1982), remains an additional potential γ-chain cross-linking mechanism. This mechanism may apply, for example, in the case of γ trimers, which continue to form after all available γ monomers have been consumed. Experiments to evaluate these alternative but not mutually exclusive mechanisms of cross-linking are currently under way.

Our present results provide more information relating to the conditions under which γ trimerization and γ tetramerization occur. Low ionic strength, increasing $CaCl_2$ concentration, or slow fibrin polymerization resulted in substantial augmentation of γ-trimer and γ-tetramer formation. This event appears to be correlated with increases in fiber thickness and/or tight fibril packing, either of which could bring about increased interfibrillar contacts or branched structures having the appropriate γ-chain configurations for γ trimerization or γ tetramerization. The effect of $CaCl_2$ on the formation of γ trimers and γ tetramers is not dependent upon the ionic strength, per se. In fact, increasing the concentration of $CaCl_2$, which increases thick fiber formation despite a concomitant rise in ionic strength, reverses the inhibitory effects of high NaCl concentration on γ-trimer and γ-tetramer formation (Figure 3). The basis for this effect reversal is probably due to binding of Ca^{2+} to sialic acid residues (Dang et al., 1989), whose presence in fibrin tends to impair lateral fibril associations.

Several investigators have noted that the rate of plasmin lysis of cross-linked clots is decreased when compared to that of non-cross-linked clots (Henderson & Nussbaum, 1969; Rampling, 1978; Gaffney & Whitaker, 1979; Gladner & Nossal, 1983; Sakata et al., 1984; Francis & Marder, 1987, 1988; Jansen et al., 1987) and this has been correlated with the formation of high molecular weight α polymers (Francis & Marder, 1988; Gaffney & Whitaker, 1979). Gormsen et al. (1967) demonstrated progressive increases in resistance to lysis of cross-linked clots that had been incubated in vitro for periods of time exceeding 24 h. Furthermore, it is known that in vivo resistance to fibrinolysis increases over a period of days rather than hours (Marder et al., 1977). These last observations suggest that the time course of development of resistance to lysis of cross-linked clots may be more closely related to the rate of formation of γ trimer and γ tetramers that it is to α-polymers. This important point will require careful validation.

In summary, our results indicate that factor XIIIa catalyzed formation of γ trimers and γ tetramers is a dynamic physiological process occurring after all γ monomers have been incorporated into γ dimers. The process of γ-trimer and γ-tetramer formation can be augmented under conditions resulting in increased fiber thickness or tight fibril packing, and diminished under conditions favoring fine fibril matrix formation. These findings provide new insights into the mechanism of fibrin polymerization and cross-linking, and they have uncovered still another new aspect of its dynamic nature.

ACKNOWLEDGMENTS

We are most grateful to Diane Bartley for technical assistance, to Angela Banks-Mallett for assistance in preparation of the manuscript, to Karen Mickey Higgins for graphic arts, and to William Semrad for photographic services.

Registry No. Ca^{2+}, 7440-70-2; factor XIII, 9013-56-3.

REFERENCES

Alkjaersig, N., Davies, A., & Fletcher, A. P. (1977) *Thromb. Haemostasis 38*, 524–535.

Belitser, V. A., Varetskaja, T. V., & Malneva, G. V. (1968) *Biochim. Biophys. Acta 154*, 367–375.

Blombäck, B., Hessel, B., Hogg, D., & Therkildsen, L. (1978) *Nature 275*, 501–505.

Boyer, M. H., Shainoff, J. R., & Ratnoff, O. D. (1972) *Blood 39*, 382–387.

Carr, M. E., Shen, L. L., & Hermans, J. (1977) *Biopolymers 16*, 1–15.

Carr, M. E., Gabriel, D. A., & McDonagh, J. (1986) *Biochem. J. 239*, 513–516.

Chen, R., & Doolittle, R. F. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 420–427.

Chen, R., & Doolittle, R. F. (1971) *Biochemistry 10*, 4486–4491.

Dang, C. V., Shin, C. K., Bell, W. R., Nagaswami, C. & Weisel, J. W. (1989) *J. Biol. Chem. 264*, 15104–15108.

Doolittle, R. F. (1973) *Thromb. Diath. Haemorrh., Suppl. No. 54*, 155–165.

Doolittle, R. F., Chen, R., & Lau, F. (1971) *Biochem. Biophys. Res. Commun. 44*, 94–100.

Erickson, H. P., & Fowler, W. E. (1983) *Ann. N.Y. Acad. Sci. 408*, 146–163.

Ferry, J. D. (1952) *Proc. Natl. Acad. Sci. U.S.A. 38*, 566–569.

Ferry, J. D., & Morrison, P. R. (1947) *J. Am. Chem. Soc. 69*, 388–397.

Finlayson, J. S., & Mosesson, M. W. (1963) *Biochemistry 2*, 42–46.

Fowler, W. E., Hantgan, R. R., Hermans, J., & Erickson, H. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4872–4876.

Francis, C. W., & Marder, V. J. (1987) *J. Clin. Invest. 80*, 1459–1465.

Francis, C. W., & Marder, V. J. (1988) *Blood 71*, 1361–1365.

Francis, C. W., Marder, V. J., & Martin, S. E. (1980a) *Blood 56*, 456–464.

Francis, C. W., Marder, V. J., & Barlow, G. H. (1980b) *J. Clin. Invest. 66*, 1033–1043.

Gaffney, P. J. (1983) *Ann. N.Y. Acad. Sci. 408*, 407–422.

Gaffney, P. J., & Whitaker, A. N. (1979) *Thromb. Res. 14*, 85–94.

Gaffney, P. J., Joe, F., & Mahmoud, M. (1980) *Thromb. Res. 20*, 647–662.

Gladner, J. A., & Nossal, R. (1983) *Thromb. Res. 30*, 273–288.

Gormsen, J., Fletcher, A. P., Alkjaersig, N., & Sherry, S. (1967) *Arch. Biochem. Biophys. 120*, 654–665.

Hantgan, R. R., & Hermans, J. (1979) *J. Biol. Chem. 254*, 11272–11281.

Hantgan, R., Fowler, W., Erickson, H., & Hermans, J. (1980) *Thromb. Haemostasis 44*, 119–124.

Hantgan, R., McDonagh, J., & Hermans, J. (1983) *Ann. N.Y. Acad. Sci. 408*, 344–365.

Haverkate, F., & Timan, G. (1977) *Thromb. Res. 10*, 803–812.

Henderson, K. W., & Nussbaum, M. (1969) *Br. J. Haematol. 17*, 445–453.

Hermans, J., & McDonagh, J. (1982) *Semin. Thromb. Hemostasis 8*, 11–24.

Hewat, E. A., Tranqui, L., & Wade, R. H. (1983) *J. Mol. Biol. 170*, 203–222.

Ichinose, A., & Aoki, N. (1982) *Biochim. Biophys. Acta 706*, 158–164.

Jansen, J. W. C. M., Haverkate, F., Koopman, J., Nieuwenhuis, H. K., Kluft, C., & Boschman, Th. A. C. (1987) *Thromb. Haemostasis 57*, 171–175.

Kanaide, H., & Shainoff, J. R. (1975) *J. Lab. Clin. Med. 85* 574–597.

Kazal, L. A., Amsel, S., Miller, O. P., & Tocantins, L. M. (1963) *Proc. Soc. Exp. Biol. Med. 113*, 989–994.
Krakow, W., Endres, G. F., Siegel, B. M., & Scheraga, H. A. (1972) *J. Mol. Biol. 71*, 95–103.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Laudano, A. P., & Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3085–3089.
Laudano, A. P., & Doolittle, R. F. (1980) *Biochemistry 19*, 1013–1019.
Loewy, A. G., Dunathan, K., Kriel, R., & Wolfinger, H. L. (1961) *J. Biol. Chem. 236*, 2625–2633.
Lorand, L., & Gotoh, T. (1970) *Methods Enzymol. 19*, 770–782.
Marder, V. J., Soulen, R. L., Atichartakarn, V., Budzynski, A. Z., Parulekar, S., Kim, J. R., Edward, N., Zahavi, J., & Algazy, K. M. (1977) *J. Lab. Clin. Med. 89*, 1018–1029.
Matačič, S., & Loewy, A. G. (1968) *Biochem. Biophys. Res. Commun. 30*, 356–362.
McDonagh, R. P., McDonagh, J., Blombäck, M., & Blombäck, B. (1971) *FEBS Lett. 14*, 33–36.
McKee, P. A., Mattock, P., & Hill, R. L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 738–744.
Mosesson, M. W., & Finlayson, J. S. (1963) *J. Lab. Clin. Med. 62*, 663–674.
Mosesson, M. W., & Sherry, S. (1966) *Biochemistry 5*, 2829–2835.
Mosesson, M. W., & Finlayson, J. S. (1976) *Prog. Hemostasis Thromb. 3*, 61–107.
Mosesson, M. W., Finlayson, J. S., & Umfleet, R. A. (1972) *J. Biol. Chem. 247*, 5223–5227.
Mosesson, M. W., DiOrio, J. P., Müller, M. F., Shainoff, J. R., Siebenlist, K. R., Amrani, D. L., Homandberg, G. A., Soria, J., Soria, C., & Samama, M. (1987) *Blood 69*, 1073–1081.
Mosesson, M. W., Siebenlist, K. R., Amrani, D. L., & DiOrio, J. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1113–1117.
Müller, M. F., Ris, H., & Ferry, J. D. (1984) *J. Mol. Biol. 174*, 369–384.
Nieuwenhuizen, W., Vermond, A., & Haverkate, F. (1981) *Biochim. Biophys. Acta 667*, 321–327.
Nieuwenhuizen, W., Voskuilen, M., Vermond, A., Haverkate, F., & Hermans, J. (1982) *Biochim. Biophys. Acta 707*, 190–192.
Okada, M., & Blombäck, B. (1983) *Thromb. Res. 29*, 269–280.
Olexa, S. A., & Budzynski, A. Z. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1374–1378.
Pisano, J. J., Finlayson, J. S., & Peyton, M. P. (1968) *Science 160*, 892–893.
Pisano, J. J., Finlayson, J. S., & Peyton, M. P. (1969) *Biochemistry 8*, 871–876.
Purves, L. R., Lindsey, G. G., Brown, G., & Franks, J. (1978) *Thromb. Res. 12*, 473–484.
Purves, L. R., Purves, M., & Brandt, W. (1987) *Biochemistry 26*, 4640–4646.
Rampling, M. W. (1978) *Thromb. Res. 12*, 287–295.
Regañon, E., Vila, V., & Aznar, J. (1978) *Thromb. Haemostasis 40*, 368–376.
Robbins, K. C., & Summaria, L. (1970) *Methods Enzymol. 19*, 184–199.
Robbins, K. C., & Summaria, L. (1976) *Methods Enzymol. 45*, 257–273.
Rosser, R. W., Roberts, W. W., & Ferry, J. D. (1977) *Biophys. Chem. 7*, 153–157.
Sakata, Y., Mimuro, J., & Aoki, N. (1984) *Blood 63*, 1393–1401.
Schwartz, M., Pizzo, S. V., Hill, R. L., & McKee, P. A. (1971) *J. Clin. Invest. 50*, 1506–1513.
Shah, G. A., Nair, C. H., & Dhall, D. P. (1985) *Thromb. Res. 40*, 181–188.
Shainoff, J. R., & Dardik, B. N. (1979) *Science 204*, 200–202.
Shainoff, J. R., & Dardik, B. N. (1983) *Ann. N.Y. Acad. Sci. 408*, 254–267.
Shainoff, J. R., Urbanic, D. A., & DiBello, P. M. (1991) *J. Biol. Chem. 266*, 6429–6437.
Stryer, L., Cohen, C., & Langridge, R. (1963) *Nature 197*, 793–794.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Williams, R. C. (1981) *J. Mol. Biol. 150*, 399–408.
Williams, R. C. (1983) *Ann. N.Y. Acad. Sci. 408*, 180–193.
Wolfe, J. K., & Waugh, D. F. (1981) *Arch. Biochem. Biophys. 211*, 125–142.