Early Events in the Plasmin Digestion of Fibrinogen and Fibrin

EFFECTS OF PLASMIN ON FIBRIN POLYMERIZATION*

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LINUS L. SHEN,‡ RICHARD P. MCDONAGH, JAN MCDONAGH, AND JAN HERMANS

From the Departments of Biochemistry and Pathology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

We have studied the effects of limited plasmic digestion of fibrinogen and fibrin on the physical properties of fibrin gels. After limited digestion the rigidity (elasticity) of the clot formed from degraded fibrinogen is 1% of the rigidity obtained from undegraded fibrinogen, whereas the decrease in clottability as a result of this limited digestion is only about 10%. When plasmin digests fibrin in gel form, the degradation process is significantly prolonged; the rigidity of the attacked gel increases and remains high (hyper-rigidity) for 2 to 4 h and then disappears relatively abruptly. Plasmin cleaves the α (or Aα) chain and releases the hydrophilic, COOH-terminal portion (M, = 44,000). Plasmin apparently has a high affinity for a site about one-third of the way from the NH₂ terminus of the α chain of fibrin; plasmin also prefers fibrin to fibrinogen as a substrate in a system where both species are present in equal amounts. The high affinity for the first site of lysis causes the digestion of fibrin to be orderly, in the sense that in the first phase of digestion only α chains are split, and the rigidity of the gel does not fall. The low rigidity of a fibrin gel formed from fibrinogen digested briefly with plasmin has been reproduced in a fibrin gel prepared by first briefly digesting fibrin with plasmin, then dissolving this material in NaBr, and finally allowing the fibrin to gel again. Fibrin molecules lacking the COOH-terminal two-thirds of the α chain form an abnormal network of fibrin fibers of low rigidity, whereas the normal fibrin network does not lose its rigidity when these same peptides are removed.

It is suggested that the hydrophilic COOH-terminal two-thirds of the α chain serves to maintain a fibrin polymerization site in the adjacent portion of the NH₂-terminal part of this chain exposed on the surface of the fibrin molecule.

The mechanism of plasmic digestion of fibrinogen has been studied extensively in recent years (for reviews, see Refs. 1 and 2). Most investigations have emphasized changes in the subunit structure of fibrinogen during the course of plasmic digestion, which ultimately produced the terminal products, D and E. Some studies have dealt with the advanced stage of degradation of these end products, and others have dealt with the early stage of digestion in which fibrinogen is degraded to species that still possess about 87% of the original clottability (3–7). The plasmic degradation of bovine fibrinogen and non-cross-linked fibrin in solution and in gel form has been studied by Inoue et al. (8). They found that fibrinogen and fibrin monomer in solution are degraded in a similar manner; the degradation of fibrin gel follows a different pattern, in which the β chains of fibrin in the gel remain intact longer in the early stage of digestion.

We have investigated the plasmic degradation process by measuring changes in the clot rigidity with a sensitive elastometer. This study focused on the structural changes that occur in fibrinogen and fibrin gels in the early stage of plasmic digestion, during which the loss of clottability is about 10%. The observed differences in the physical changes in fibrinogen and fibrin have been correlated with changes in the subunit structure caused by fibrinolysis.

EXPERIMENTAL PROCEDURES

Materials—Human fibrinogen (grade L, 94% clottable) was purchased from A. B. Kabi (Stockholm, Sweden). This preparation contained trace amounts of Factor XIII. Bovine thrombin was obtained from Parke-Davis. Plasmin in 50% glycerol (Michigan Department of Public Health Lot ERD 166) contained 55 casein units/ml Lyophilized plasmin (A. B. Kabi Lot 30232) contained approximately 18 casein units/mg of protein and was dissolved in 50% glycerol and stored at –25°. Trypsin inhibitor (Tryptol, aprotinin; RBI Pharmacueticals) contained 10,000 KIU/ml. Ancrod (Venacil, Lot 09-005-DH) was a gift from Abbott Laboratories. Fibrinogen and thrombin stock solutions were prepared as previously described (9).

The intactness of the fibrinogen was monitored by DEAE-polyacrylamide gel electrophoresis. Fibrin monomer was prepared as described by Donnelly et al. (10) and modified by Lorand and Ong (11). The catability of the fibrin monomer was 90%. Fibrinogen, Fraction I-II (12), was kindly given to us by Dr. M. W. Mosesson (SUNY Downstate Medical Center).

Measurements—The method used for the rigidity measurements has been described (13). The degradation of fibrin, as followed by rigidity measurements, can be performed by simultaneously adding a relatively large amount of thrombin and a varying amount of plasmin to the fibrinogen solution to make the clotting time of the fibrinogen solution approximately 20 s and to vary the dissolution

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‡ Present address, Physical Biochemistry Laboratory, Experimental Biology Division, Abbott Laboratories, North Chicago, Ill. 60064.

1 The abbreviations used are: KIU, kallikrein inhibitor unit; SDS, sodium dodecyl sulfate.

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The mechanical strength of the fibrin gel reaches 90% of its control gel throughout the entire clotting period, indicating that the observed hyper-rigidity is not due to a change in the thickness of the fibrin fibers making up the network.

**Digestion of Fibrin by Small Amounts of Plasmin: Rigidity**

The clotting of intact fibrinogen (Mₙ = 344,000) by thrombin, as followed by rigidity measurement, is shown in Fig. 1. The mechanical strength of the fibrin gel reaches 90% of its maximum in about 1 h. In corollary experiments it was found that the rigidity of fibrin gels prepared from Fraction I-8 (Mₙ = 275,000), which has somewhat shorter A₂ chains (15) than the principal fibrinogen fraction (I-4), is, within experimental error, the same as that of an intact fibrin clot. It is also seen in Fig. 1 that varying amounts of plasmin added simultaneously with the thrombin cause subsequent clot dissolution. As expected, the addition of larger amounts of plasmin results in more rapid dissolution of the clot, even before the rigidity is fully developed. Under certain conditions a hyper-rigidity phenomenon is observed in the clotting-dissolution process (Fig. 2). At a low fibrinogen concentration, a transient stage is observed during which the clot develops a higher rigidity than normal under the action of a small amount of plasmin. With increasing concentrations of plasmin, the hyper rigidity effect diminishes and eventually is lost. The absolute concentration of fibrin is also important, as well as the plasmin/fibrin ratio. This specific effect was observed at a fibrin concentration of about 0.6 mg/ml but not at a higher fibrin concentration nor at a higher pH and ionic strength. However, hyper-rigidity was observed under conditions that did not lead to covalent cross-linking, that is, at a low calcium concentration or when ancrord was used instead of thrombin but again only at a low fibrinogen concentration. This indicates that the development of hyper-rigidity is not an effect of the covalent cross-linking of the fibrinogen produced strikingly different effects on the elasticity of the fibrin gel that was subsequently formed, as compared to the effect of limited digestion of the preformed gel (Fig. 3). Fibrin gels formed from slightly digested fibrinogen do not show the characteristic hyper-rigidity phenomenon nor the apparent delay in digestion observed with fibrin. The loss of rigidity increases steadily with the incubation time. The action of plasmin on fibrinogen molecules appears to be straightforward: plasmin damages the molecules, and the damaged molecules form weaker gels. The measured elastic modulus is a sensitive indicator of the damage done by plasmin to fibrinogen.

**Digestion of Fibrin by Small Amounts of Plasmin: Rigidity**

For comparison of the effect of plasmin digestion on fibrinogen in solution to that of fibrin gel, experiments were performed under the same conditions as described above, except that plasmin (0.005 casein unit/mg of fibrinogen) was added to the fibrinogen solutions first, and the solutions were incubated for 1, 3, 15, and 30 min prior to the addition of thrombin together with trypsin inhibitor to inhibit further action of plasmin. The inhibition of plasmin with trypsin inhibitor appears to occur instantaneously at this concentration (10 KIU/ml), since the addition of plasmin to a fibrinogen solution containing the same concentration of trypsin inhibitor did not alter the mechanical strength of the resulting gel after the addition of thrombin. Limited plasmin digestion of fibrinogen produces strikingly different effects on the elasticity of fibrin gel that was subsequently formed, as compared to the effect of limited digestion of the preformed gel (Fig. 3). Fibrin gels formed from slightly digested fibrinogen do not show the characteristic hyper-rigidity phenomenon nor the apparent delay in digestion observed with fibrin. The loss of rigidity increases steadily with the incubation time. The action of plasmin on fibrinogen molecules appears to be straightforward: plasmin damages the molecules, and the damaged molecules form weaker gels. The measured elastic modulus is a sensitive indicator of the damage done by plasmin to fibrinogen.
Limited Plasmin Digestion: Gel Electrophoresis—Plasmin digestion of fibrinogen and gelled fibrin was further characterized by analysis of the time dependence of the changes in subunit structure with SDS-polyacrylamide gel electrophoresis. The conditions were chosen so that hyper-rigidity could best be observed. Figs. 4 and 5 show the results of gel electrophoretic analyses designed to follow changes in the subunit structure of fibrinogen and fibrin gel at different stages of plasmin digestion. The scans correspond to approximately 82% clottability. The gels for fibrinogen show the disappearance of Aα chain in about 65 min with the concomitant appearance of a band with a molecular weight of 29,000. The set for fibrin of Fig. 4 shows considerable γγ dimer formation, which results from the action of contaminating Factor XIIIa in the presence of calcium ion. The band corresponding to α chain disappears almost completely after 35 min of reaction, while bands with molecular weights of 44,000 and 26,000 appear and increase.

Similar results were obtained when ancrd was used instead of thrombin. The gels of ancrd fibrin still show a small degree of γγ dimerization, which is probably due to activation of Factor XIII by an impurity in the ancrd preparation.

In agreement with others (16, 17), we conclude that the bands with molecular weights of 30,000 and 26,000 contain the NH₂-terminal portions of the Aα and α chain, respectively. These NH₂-terminal portions of the α chains are attached to other parts of the fibrinogen molecule via disulfide cross-links. The other, COOH-terminal portion of the α chain is not covalently attached to other chains, at least until α chain cross-links are introduced by Factor XIIIa. Although the identity of the band with a molecular weight of 44,000 cannot be deduced with certainty, other data, reported in the next section, support the interpretation that it contains the COOH-terminal part of the α chain. There is no evidence for β chain degradation in these early digests, and therefore there is no reason to assume that this band contains a β chain fragment. This band was also observed by Inoue et al. (8) and by Pizzo et al. (17, 18) and was reported by them to appear more strongly in the fibrin digests than in the fibrinogen digests, as we also find.

We shall call the COOH-terminal part of the α chain, of molecular weight 44,000, the α₁ peptide for convenience. The question of whether this peptide is indeed observed intact in early fibrin digests, as our experiments suggest, must be settled by further experimentation.

Release of Fragments into the Clot Supernatant—Clot supernatant fragments are released more rapidly from fibrinogen than from fibrin (Figs. 6 and 7). The digestion of both fibrinogen and fibrin shows a rapid release of from 10 to 15% of the ultraviolet absorbing material as nonclottable material. After this, the release of fragments from fibrin levels off for some time. In contrast, with fibrinogen the initial rapid release is followed by an apparently steady digestion of all the clottable material to nonclottable fragments.

The breakdown of the α chains of fibrin coincides with the release of the ultraviolet-absorbing material into the clot supernatant. SDS-polyacrylamide gel electrophoresis of the supernatant material has revealed the presence of a band with the mobility expected of the α₁ peptide. The increase in absorbance of the supernatant is between 10 and 15% of that obtained after complete digestion of fibrin, while the mass of the two α₁ peptides is 29% of that of the fibrinogen molecule. Mihalyi has recently shown that tryptic digestion of fibrinogen releases low molecular weight peptides derived from the COOH-terminal part of the Aα chain, which constitute 23% of the total nitrogen but only 13% of the ultraviolet absorption. We conclude that all available α₁ peptide has been released by the time the amount of ultraviolet-absorbing material released into the supernatant of fibrin gel temporarily levels off. The release of α₁ peptide correlates with the appearance of hyper-rigidity or the period in which no loss of rigidity is noticed, depending on the fibrinogen concentration, of fibrin gel and with the period in which the rigidity of the fibrin formed from predigested fibrinogen drops to a small fraction of the native value.

The results shown in Fig. 7b also indicate that the release of supernatant peptides of a 1:1 mixture of fibrin and fibrinogen proceeds at first as the digestion of fibrin alone. This indicates that plasmin has a considerably greater affinity for certain bonds in fibrin than for any bonds in fibrinogen.

Importance of the Order of Gelation and Lysis for the Rigidity of Fibrin—The results of the analysis by SDS-gel electrophoreses of the subunit structure of the early plasmin split product of fibrin and fibrinogen do not provide a basis for explaining the large difference in rigidity resulting from the split. For further study of this, fibrin monomers were prepared from intact fibrin gels and from fibrin gels that exhibited maximal hyper-rigidity following a small degree of plasmin digestion. These fibrin monomer solutions were then allowed to regel by a 10-fold dilution of the NaBr solution in the elastometer, and the development of clot elasticity was measured. The reclothed control gel, to which no plasmin had previously been added, showed greater than 90% of the rigidity of a fibrin gel obtained by clotting fibrinogen with thrombin in the same solvent. In contrast, the reclothed fibrin monomer that had been the constituent of a partly lysed gel showing hyper-rigidity exhibited only about 10% of the rigidity of the control clot. This indicates that the fiber conformation in the gel remains essentially intact when the COOH-terminal portions of the α chains are removed but that this fiber conformation is not regained when the partially digested fibrin is dissolved and the solution is subsequently regelled.

DISCUSSION

The hydrolysis of fibrin by plasmin is generally regarded as the primary in vivo catabolic pathway for the destruction of fibrin gels. It has also been observed that activation of plasminogen is initiated at the same time as activation of the early phase of the clotting system and that plasmin generation and fibrin formation are linked by complex feedback control mech-
Fig. 4 (left). a, time dependence of the rigidity of fibrin gel in the absence and presence of plasmin (0.0085 casein unit/mg of fibrinogen); arrows, times at which trypsin inhibitor was added to different samples, which were then used for gel electrophoresis. Conditions were as for Fig. 2. b, Coomassie blue-stained SDS-polyacrylamide electrophoresis gels of these samples (reduced) and of fibrinogen solutions digested under the same conditions. S, mixture of molecular weight standards. c, densitometer scan of the two gels indicated with arrows (—, fibrin; —, fibrinogen). Molecular weight estimates are shown for each peak. Peaks which correspond to native constituent chains and dimers of chains are identified.

Fig. 5 (right). Same as Fig. 4, except that ancrod was used for clotting rather than thrombin.

The most salient finding is that the first plasmic split appears to have no deleterious effect on the rigidity of the gel and presumably does not interfere directly with hemostasis. The first event in the plasmin degradation of both fibrin and fibrinogen is the cleavage of a peptide (designated $\alpha$, molecul-
Limited Plasmin Digestion of Fibrin: Role in Polymerization

Fig. 6. Plasminic digestion of fibrin (○) and fibrinogen (△). Release of ultraviolet-absorbing material into the clot supernatant in the case of fibrin or release of nonclottable material in the case of fibrinogen is shown on the ordinate. These results were obtained in parallel with those reported in Figs. 2 and 3, under the same experimental conditions.

Fig. 7. Same as Fig. 6. Fibrin was formed from fibrin monomer solution (△). Fibrinogen was in the same solvent (○). Results for a sample containing equal amounts of fibrinogen and fibrin are also given (□). Concentration of fibrin plus fibrinogen, 0.6 mg/ml; 0.1 M NaBr; 0.05 M Tris (pH 7.6); plasmin, 0.0085 casein unit/mg of fibrinogen; thrombin, 4 NIH units/ml. Trypsin inhibitor (40 KIU/ml) was added to stop the reaction. Results obtained when heparin (0.5 mg/ml) was added to the samples are indicated with closed symbols.

It must be kept in mind that the damaging effect of fibrinolysis is delayed only when plasmin is attacking fibrin in the gel state. There is no delay in the effect on gel rigidity produced by exposing fibrinogen to limited plasmin digestion. The simplest explanation for this is that the conformation of fibrin formed from predigested fibrinogen differs from that of native fibrin, whereas fibrin conformation remains essentially intact during the attack by plasmin if the fibrin has already been converted to a gel. Earlier studies also lend support to this interpretation. Fletcher, et al. (25) studied an early fibrinogen degradation product with a molecular weight of 286,000, which yielded a more slowly clotting fibrin. On the basis of sedimentation and diffusion constants, they concluded that the conformation of this derivative differed from that of native fibrinogen. More recently, Tranqui-Poit et al. (26) have used electron microscopy to show that during plasmin degradation the shape of the molecule changes from spherical to linear. These investigators observed shape changes even with their earliest degradation product (molecular weight 286,000).

The part of the α chain where the primary plasmin split occurs also contains one of the α cross-linking acceptor sites, and the acceptor glutamine residue is located between two plasmin-sensitive bonds that are only 10 residues apart (27). At present it is not clear which of the four plasmin-sensitive points in this region is the one that is primarily cleaved when the αi peptide is released from fibrin. It appears that any of the four sensitive bonds within this 34-residue segment may be the first cleaved in a somewhat random fashion. This means that release of the cross-linking site would also be variable and would occur at least part of the time.

The presence of α cross-links introduced by Factor XIIa slows down the digestion by plasmin (28), and hence the amount of Factor XIIa in the clot may regulate the lysis of the clot. Conversely, plasmin interferes with the action of Factor XIIa. Since α cross-links are formed rather slowly, a small amount of plasmin, present at the time of gelation, may regulate the extent of α cross-linking achieved by removing the major COOH-terminal portions of many α chains. Such a finely tuned control mechanism has many obvious advantages.

We conclude with a description of a possible structural basis for the differences in rigidity of fibrin gels observed when the COOH-terminal portions of the α chains are removed. A premise of this hypothesis is that the rigidity of a fibrin gel is determined by the number and quality of the fibrin polymerization sites and that an increase of the intermolecular contacts

3 A high affinity does not imply a high rate of cleavage. In fact, the initial cleavage of fibrin is slower than that of fibrinogen. It would be tempting to propose that the lower rate is due to shielding of plasmin-sensitive bonds from the enzyme by intermolecular contacts in the fibrin fiber. We find no support for this hypothesis in the results of our experiments.

polar weight 44,000) from the COOH-terminal end of the α (or Aα) chain. There are no interchain disulfide bonds in this fragment, and it is released from the parent molecule. During plasminic digestion of fibrinogen, αi is rapidly degraded to small peptides. This observation is in agreement with the description of fibrinogen degradation that has been developed by various groups over the past several years (1, 2, 4, 7). Our observations suggest that, during the degradation of fibrin, αi is perhaps not degraded quite as rapidly but instead accumulates and that nearly all available αi peptide is released before other bonds are cleaved.

Our results suggest that plasmin has a high affinity for the bond in the α chain that is to be split. The high affinity of plasmin for fibrin has also been described by Amburs and Markus (22), who proposed that this may serve to concentrate and retain active plasmin on the fibrin in the clot. It has also been proposed, in vivo, plasmin has a higher affinity for fibrin than for fibrinogen (23). The present results demonstrate that in the presence of fibrin there is no free pool of plasmin available for the degradation of fibrinogen. Because fibrin is cleaved preferentially over fibrinogen, the affinity for the site of the first split in fibrin is higher than for cleavage sites in fibrinogen. Since no other peptides appear until the α chains have been cleaved, the affinity is higher than that for splits in other regions of fibrin.

The initial cleavage of the α chains of fibrin is a step with this important function: it delays both the weakening and the lysis of the clot for a considerable time (but not indefinitely), and it prevents plasmin from diffusing out of the clot during this time. Once the α chains have been cleaved, the cleavage of β and γ chains appears to proceed at a similar rate (24). The observed lowering of the rigidity begins some time after the α chains have been split, and the further release of peptides into the clot supernatant occurs somewhat later. This indicates that several splits, presumably of adjacent chains, are needed to cause a lowering of the rigidity and that even more are required before the core fragments, D and E (or dimers of fragment D held together by cross-linked γ chains), are released. Since the rigidity or modulus of elasticity is representative of the various mechanical properties of fibrin that are of physiological importance, it is apparent that the destructive effects of plasmin digestion are delayed by the high preference of plasmin for cleavage of one section of the α chain of fibrin.
also gives an increase of the rigidity. This premise is a natural one; a theory designed to relate the elasticity of fibrin to structure of fibers and network would have it as a postulate. With this premise, the observed changes in the rigidity are interpreted as follows: (a) removal of the COOH-terminal portions of the alpha chains of fibrinogen causes a loss of certain polymerization sites; (b) in fibrin polymerized to a network of fibers, these same sites participate in intermolecular contacts, and when the COOH-terminal portions of the alpha chains are removed the contacts not only remain but increase slightly (in number or quality).

The second of the above conclusions excludes the possibility that the polymerization site is formed by the COOH-terminal portion of the alpha chain. This chain segment has an unusually high hydropathic amino acid composition (29). We suggest that it is one of the functions of this chain to maintain a polymerization site on the surface of the fibrin molecule. The enzymatic removal of the soluble chain section would allow the polymerization site to move to a less accessible position in the fibrin molecule. On the other hand, once the contacts have been formed as a part of the fiber structure, they stabilize the exposed conformation of the sites, obviating the solubilizing role of the COOH-terminal parts of the alpha chains. As judged from the increase of the rigidity that may accompany the removal of these soluble chains from inside the fibrin fiber, this removal appears to lead to an improvement of the intermolecular contacts. Since the split causes removal of predominantly hydrophilic chains totaling some 30% of the remaining mass, this is not an unreasonable consequence. It appears that only a short segment of the hydrophilic alpha peptide is crucial for exposure of the polymerization site, since fibrinogen Fraction I-8, which has alpha chains with molecular weights of 31,000 to 34,000 (30), forms fibrin with a rigidity indistinguishable from that of intact fibrin (Fraction I-4). This means that a hydrophilic segment with a molecular weight of only 6000 to 9000 is all that is essential for exposure of the polymerization site.

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