Studies on the Structural Abnormality of Fibrinogen Paris I

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ABSTRACT The structural properties of an inherited fibrinogen abnormality designated fibrinogen Paris I were investigated. Dodecyl sulfate gel electrophoresis of unmodified fibrinogen samples revealed no discernible differences in molecular weight from normal; this implied that in fibrinogen Paris I, the normal fibrinogen architecture of six covalently linked chains per molecule is preserved. Examination of dithiothreitol reduced samples before and after treatment with Reptilase or thrombin revealed that the Aα- and Bβ-chains could release the A and B peptides, respectively. A mutant chain (mol wt 52,500, termed γParis I) which replaces a large proportion of γ-chains (mol wt 49,400) was shown, like normal γ-chains, to lack thrombin- and Reptilase-sensitive sites. The γ-chains and α-chains of Paris I fibrin underwent Factor XIIIa-catalyzed cross-linking slowly; this behavior was not attributable to an intrinsic abnormality of these chains themselves but rather to the inhibitory effect of the mutant γParis I chains on this process. Results of DEAE-cellulose gradient elution chromatography of Paris I fibrinogen preparations revealed the presence of small amounts of normal fibrinogen molecules and also indicated that the γParis I chains possessed structural overlap with γ-chains. Unlike γ-chains however, the γParis I chains did not incorporate dansylcadaverine in the presence of Factor XIIIa, nor, as previously reported, did they undergo cross-linking. The observations indicate that the amine acceptor site found in the COOH-terminal region of the γ-chain is either not present on the γParis I chain or is unavailable for cross-linking. Further support for localization of the abnormality in the COOH-terminal region of the molecule was obtained from the observation that during plasmin hydrolysis of Paris I fibrinogen, at least one unique form of core Fragment D (DParis I) was evolved, whereas Fragment E did not differ from normal.

INTRODUCTION

In 1963 Ménaché (1) described an inherited abnormality of plasma fibrinogen which has come to be known as fibrinogen Paris I (for recent comprehensive reviews on this general subject see references 2 and 3). The functional defect is featured by a marked prolongation of the thrombin time (1) which is evidently due to delay of the aggregation phase of fibrin derived from such plasma (4). An additional important characteristic is its profound ability to delay the aggregation phase of normal fibrin as well (4). In this regard, in terms of its ultimate ability to form a clot, at least two subpopulations of Paris I fibrinogen have been recognized (4).

Biochemical analyses suggest that the NH2-terminal amino acids and thrombic peptides A and B of fibrinogen Paris I are qualitatively the same as those of normal fibrinogen (2, 5). Furthermore, no abnormalities of that portion of the Aα-chain of fibrinogen Paris I isolated with the NH2-terminal disulfide knot have been found (5, 6). Preliminary results of gradient elution ion-exchange chromatography on DEAE-cellulose (7) indicate that fibrinogen Paris I has a greater anionic-binding capacity than does normal fibrinogen. Most recently, results of dodecyl sulfate gel-electrophoretic analyses of reduced fibrin clots of Paris I fibrinogen have revealed the presence of an abnormal chain that is somewhat larger than the γ-chain (8). Since densitometric scans indicate that it replaces the normal γ-chain, it has been termed γParis I, although it does not participate in fibrin cross-linking as do normal γ-chains (8).

In the studies to be reported, we present experiments which extend those cited above. They permit additional conclusions to be drawn with respect to structural and functional properties of the fibrinogen Paris I molecule, and provide evidence that the defect in the γParis I chain is localized in its COOH-terminal region.

METHODS

Chromatographic analysis. DEAE-cellulose (Whatman DE-23) column chromatography of unmodified samples was
carried out at 2°C on 0.9 × 30-cm columns employing a combined pH and phosphate gradient from 0.005 M phosphate, pH 8.6 to 0.50 M phosphate, pH 4.1 to 4.3 (9, 10). Fractions corresponding to 1% of the total gradient were collected.

**Electrophoretic and related procedures.** Dodecyl sulfate-polyacrylamide gel-electrophoresis was performed essentially as described by Weber and Osborn (11) in gels whose final acrylamide concentration was 5 or 9%. In all figures showing results of such experiments, the gels are aligned with the anode at the bottom of the figure. For disulfide bond reduction, when desired, dithiothreitol (14 mM, final concentration) was employed. Gels were stained with amido-Schwartz 10B and then counterstained with Coomassie Brilliant Blue (12). Densitometric scans of stained gels were made in a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a Linear Transport apparatus. Determination of the radioactivity of the stained bands of radiiodinated gel samples (5% acrylamide) was accomplished by slicing out the bands (approximately 2-3-mm slices) with a razor blade and then counting their radioactivity in a Picker Autowell II gamma counter (Picker Corp., Cleveland, Ohio).

Protein markers used were the various fibrinogen subunit chains and their derivatives whose molecular weights had been determined (12, 13) by calibration against standard marker proteins. Identification of chains retaining peptide A or B was made by comparison of the mobility of reduced samples in dodecyl sulfate electrophoresis before and after treatment with Reptilase (Pentapharm, Ltd., Basel, Switzerland) or thrombin (lot H-I, provided by Dr. D. L. Aronson, Bureau of Biologies, Food and Drug Administration) as previously described (12).

Immunoelectrophoresis (14) and agarose (Behring Diagnostics, Somerville, N. J.) electrophoresis (1% agarose) were carried out in a standard manner (12, 13) with rabbit antihuman fibrinogen serum and rabbit antihuman cold-insoluble globulin serum (9). Immunoelectrophoresis (15) for determination of the plasma fibrinogen level was performed as previously described (9).

**Radioactive labeling procedures.** Normal fibrinogen fraction I-4 and fibrinogen Paris I (chromatographic peak 1) were radiiodinated with 125I (carrier free, New England Nuclear, Boston, Mass.) by the iodine monochloride method of McFarlane (16). Labeling efficiency was 30-50%. The iodine content of the labeled fibrinogen was less than 0.5 atom/molecule. After the labeling procedure, as suggested by the studies of Krohn et al. (17), the radiiodinated fibrinogen sample was diluted by adding “cold” fibrinogen in an amount equal to that in the labeling mixture. Extensive dialysis against 0.3 M NaCl was then carried out. The labeled protein was stored at −20°C.

**Preparation of fibrinogen for analysis.** Since Paris I fibrinogen clots poorly, assays based upon recovery of a clot from plasma (18) grossly underestimate its actual level (i.e. levels determined in this study were approximately 0.3 mg/ml, normal range 1.8-4.0 mg/ml). A more accurate measurement of the fibrinogen level of Paris I plasma samples and the amount of fibrinogen precipitated in Cohn ethanol fraction I was made possible by electroimmunoassay (15) before and after removal of Cohn fraction I (19). After fractionation of one such plasma (fibrinogen level 3.88 mg/ml) 81% of immunoassayable material had been precipitated with Cohn fraction I; this value is within the range found upon fractionation of normal plasma fibrinogen (viz., 70-88%). However, it does not appear that the subtle differences in solubility which have been reported (20) could be discerned by this type of analysis.

Fraction I, obtained on three occasions from two samples of Paris I plasma, was extracted twice with the glycine-citrate-ethanol buffer described by Blombäck and Blombäck (21) to prepare fraction I-0. This material was then subfractionated into fractions I-1 and I-2 by the procedure of Laki (22). The mean yield of protein in fraction I-2 (0.7, 0.9, 1.3 mg/ml starting plasma, respectively) was in the same range found for two control samples of normal plasma (0.8 and 1.1 mg/ml plasma, respectively). The coagulability (23) of Paris I fraction I-2 was considerably lower (mean = 55%, range = 49-60%) than that found for normal fibrinogen fraction I-2 (92-95%, respectively). The Paris I "clot" which did form appeared as a fine to medium granular precipitate. Nevertheless, agarose electrophoresis and immunoelectrophoresis indicated that purification of Paris I fraction I-2, comparable to that for normal fraction I-2, had been achieved. That is, for both samples, agarose electrophoresis demonstrated a major fibrinogen band (confirmed by immunoelectrophoresis) and a small amount of a faster migrating band. This latter band was identified by immunoelectrophoresis as “cold-insoluble globulin” (9), a nonfibrinogen component of the fraction. DEAE-cellulose gradient elution chromatography (Fig. 1) was employed.

**Figure 1** DEAE-cellulose gradient elution chromatogram of 54 mg fibrinogen Paris I fraction I-2 (upper panel) and 19 mg normal fibrinogen fraction I-2. Chromatograms were developed separately under identical conditions. Fractions were pooled as shown by the hatched areas. The electrophoretic band pattern of reduced samples in 5% acrylamide gels containing dodecyl sulfate is shown for the peak 1 and 2 pools, respectively.
to characterize the fibrinogen itself as well as to provide a means for additional purification. Eluate fractions were pooled in two batches corresponding to the major fibrinogen-containing chromatographic peaks (24) (peaks 1 and 2, respectively), concentrated by precipitation of protein at 35% saturated (NH₄)₂SO₄, redissolved in 0.3 M NaCl, dialyzed against the same solution, and stored at −80°C until further use. Analytical procedures were carried out with the peak 1 pool unless otherwise indicated.

Normal human fibrinogen fraction I-4 was prepared by standard procedures as previously described (23).

Cross-linking of fibrin. Dilution of fibrinogen samples and other components of the clotting mixture were made with 0.1 M Tris-HCl buffer, pH 7.5. The final concentration of the components of the cross-linking mixture were: thrombin 0.4 U. S. U/ml, Factor XIII, 36 Loewy (25) U/ml; Ca²⁺, 12 mM; cysteine HCl (adjusted to pH 7 with Tris), 12 mM; fibrinogen, 0.6 or 1.2 mg/ml (in mixtures of Paris I and normal fibrinogen). Human plasma Factor XIII (26) was a generous gift from Dr. S. I. Chung, National Institute of Dental Research, Bethesda, Md. The clotting mixture was incubated for 18 h at room temperature before terminating the cross-linking reaction and solubilizing the clot by adding an equal volume of a dithiothreitol-urea-sodium dodecyl sulfate (2%/10 M%/2%) solution. Portions of this solution were then subjected to dodecyl sulfate gel electrophoresis.

Fluorescent amine incorporation into fibrin. Factor XIIIa-catalyzed incorporation of the fluorescent amine, dansylcadaverine (dansylcadaverine sulfate, a gift from Dr. Roll Huseby), into acceptor sites on fibrin (27) was carried out under the general conditions for cross-linking of fibrin described above. The final concentration of Factor XIII was 4 Loewy (25) U/ml; monodansylcadaverine, 9 mM. Inasmuch as peak 1 Paris I fibrinogen was no longer available at the time this particular experiment was carried out, chromatographic peak 2 of Paris I fibrinogen was used at a final concentration of 1 mg/ml. The Paris I clots which formed were centrifuged, dialyzed against 0.1 M Tris-HCl buffer, pH 7.4 containing 0.01 M EDTA (Tris-EDTA-Gl), and then dissolved with an equal volume of dithiothreitol-urea-sodium dodecyl sulfate solution for electrophoretic analysis. After electrophoresis, gel samples were fixed for 2–3 h in 10% trichloroacetic acid (wt/vol), equilibrated against 0.1 M Tris-HCl buffer, pH 7.4, and examined under UV light for fluorescence. Selected gels were also stained with Coomassie Brilliant Blue to be assured of the identity of each fluorescent band and of the presence of substantial amounts of the γParis I band in the case of fibrinogen Paris I samples.

Plasmin hydrolysis of fibrinogent. This procedure was carried out in 0.05 M Tris-HCl buffer, pH 8.6 at 37°C.

RESULTS

Molecular size and subunit structure of Paris I fibrinogen. Comparison of unreduced samples of Paris I fibrinogen with normal fibrinogen revealed no discernible differences in their anodal migration rates in sodium dodecyl sulfate-containing gels (Fig. 2). This indicated that Paris I fibrinogen had a molecular size which was the same (or nearly so) as that of normal fibrinogen and implied that the essential fibrinogen architecture (six covalently linked chains per molecule) had been preserved. The Paris I sample was resolved into two major bands corresponding in anodal migration rate to bands I and II of normal fibrinogen. Recent studies with normal human fibrinogen have characterized band II as containing intermediate catabolic species characterized by loss of COOH-terminal portions of Aα-chains (31). This therefore suggests that Paris I fibrinogen undergoes similar catabolic processes to those which have been identified for normal fibrinogen (12, 23, 31).

Treatment of fibrinogen samples with Reptilase or thrombin followed by reduction and dodecyl sulfate gel-electrophoretic analysis, permits a functional identification of the presence of dodecyl sulfate, are indicated by numerical subscripts; the higher the number, the smaller the fragment (e.g., D₀ through D₄). A Fragment D band unique to Paris I fibrinogen digests is termed DParris I. Core species larger than Fragment D, identifiable by their migration as bands in sodium dodecyl sulfate-containing gels, have been designated with roman numerals corresponding to decreasing size (i.e., I, largest; II, next largest; and so forth), as suggested by Mills and Karpatkin (30). Digest bands VI and VII (31) appear to correspond to the band identified as Fragment X by Marder et al. (32). Band VIII corresponds to Fragment Y.

The intact subunit chains of fibrinogen are designated Aα, Bβ, and γ; the mutant chain replacing the normal γ-chain in Paris I fibrinogen is termed γParis I. Cleavage at sites other than those attacked by thrombin is indicated by a diagonal line on the COOH- or NH₂-terminal side. Thus Aα→Aα/+α; Bβ→Bβ/+/β, and so forth. Individual chain fragments are further designated with a numerical subscript (12, 13, 31).

Increased in the size of core species resulting from the substitution of γParis I mutant chains for γ-chains are probably too small to be distinguished in unreduced samples. For example, the difference between unmodified fibrinogen (mol wt ~340,000) and fibrin (mol wt ~334,000) under such electrophoretic conditions, is very difficult to appreciate.

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tion of Aα- and Bβ-chains (12). That is, Aα-chains or their catabolic remnants containing peptide A (Aα/), after treatment with Reptilase or thrombin, display a measurable molecular weight reduction (i.e., increase in anodal migration) of approximately 2,000 reflecting release of peptide A from the parent chain. Bβ-chains exhibit an increased anodal migration reflecting the release of peptide B, but only after thrombin treatment; γ-chains which lack a thrombin- or Reptilase-sensitive site, display this by their failure to change their migration rate after treatment with either enzyme.

Paris I and normal fibrinogen were examined after treatment with Reptilase and thrombin (Fig. 3). By the criteria outlined above, Paris I fibrinogen possessed Aα-, Bβ-, γ-chains, and Aα/catabolic remnants (e.g., Aα/), plus other more faintly stained remnants such as Aα/), which were indistinguishable from those of normal fibrinogen. However as reported by Budzynski et al. (8), the γ-chains were markedly reduced in amount relative to Aα- and Bβ-chains. This observation plus the presence of an additional band (γParis I; mol wt 52,500 ±700) indicated that γ-chains (mol wt 49,400, reference 13) had been replaced by a mutant chain. Like a γ-chain, the γParis I band did not change its migration rate after Reptilase or thrombin treatment. Assessment of band distribution of stained gels by densitometric scanning also indicated that mutant γParis I chains had replaced γ-chains, since the γ + γParis I area approximated that found for the γ-chain position of normal fibrinogen.

The relatively close spacing of the γParis I and γ-bands resulted in a complex densitometric peak, the components of which were not well resolved by this technique; nevertheless, it was possible to calculate approximately the proportion of the γParis I mutant chains relative to the γ-chain population of normal fibrinogen by visual correction for peak overlap. For material from chromatographic peak 1 (Fig. 1) the value obtained was 65-75%; for chromatographic peak 2 fibrinogen, 67-82%; for plasma subfraction I-0, 60-70%; for plasma subfraction I-1, 73-78%.

Cross-linking of fibrin catalyzed by Factor XIIIa. Cross-linking of fibrin in the presence of Factor XIIIa results in the introduction of ε-(γ-glutamyl)lysine bridges (34, 35) between γ-chains (γ-dimer) and a-chains (α-polymer) of neighboring molecules (36-39). The capacity of Paris I fibrin to form such bridges was tested by supplementing a Paris I sample with an excess of Factor XIII before addition of Ca" and thrombin. Comparable supplements were added to normal fibrinogen fraction I-4 (Fig. 4), which was then subjected to cross-linking under identical conditions. At a time when virtually complete cross-linking of normal fibrin had occurred, only γ-chains of Paris I fibrinogen had undergone cross-linking. Small amounts of monomeric γ-chain were still evident, and there was no evidence of a-polymer formation. In other experiments (not shown) complete γ-dimerization of the γ-chains in Paris I fibrin was obtained as well as some degree of α-polymer formation. Under no circumstance was there ever any indication that the γParis I mutant chain had participated in the cross-linking process.

Mixtures of normal and Paris I fibrin (Fig. 4, gel 3) showed incomplete γ-dimer formation, and minimal α-polymer formation. Determination of the radioactivity of gel slices from a mixture of 131I-labeled normal fibrin and unlabeled Paris I fibrin indicated that at a time when normal fibrin should have undergone complete γ-dimerization (e.g., gel 1), only 55% of the 131I-labeled γ-chain...
population had formed γ-dimers. Thus, when cross-linked in the presence of Paris I fibrin, the cross-linking of normal fibrin is grossly impaired.

Cross-linking in the presence of dansylcadaverine. Since the γParis I chain does not participate in cross-linking, this mutant may also lack the glutamine acceptor site normally available on γ-chains (36). This possibility was explored by examining the reduced gels of normal and Paris I fibrin after they had been subjected to the action of Factor XIIIa in the presence of monodansylcadaverine (Fig. 5). Under the conditions of this experiment, extensive labeling of acceptor sites on the monomeric γ-chain of normal fibrin had occurred; a small proportion of γ-chains had also formed fluorescent γ-dimers (gel 1). Some incorporation of fluorescent label into α-chains was also evident and there were some faint, higher molecular weight fluorescent bands, presumably due to incorporation of fluorescent label into α-polymers. The Paris I fibrin sample exhibited a fluorescent band in the position of the γ-chain and the α-chain. The intensity of fluorescence in the α-chain position relative to that in the γ-chain position was greater than in normal fibrin, presumably because so many fewer γ-chains were present in the Paris I fibrin preparation. There was no indication that dansylcadaverine had been incorporated into the γParis I position.

Plasmic hydrolysis of normal and Paris I fibrinogen. Hydrolysis with plasmin has been used in many investigations to probe the structure of normal fibrinogen (13, 28-32, 40-43 inter alia) as well as that of congenitally abnormal fibrinogen molecules such as fibrinogen Giessen (44, 45). Such an analysis was carried out to compare normal and Paris I fibrinogen with respect to the sequence of attack on the molecule and the types of core derivatives formed. Limited amounts of purified Paris I fibrino-
than one unique form of Fragment D may be evolved in Paris I digests. Additional observations include the absence from Paris I digests of material corresponding in position to band D₃. At a more advanced digestive phase, the DParis I band as well as the heterogeneity observed in the D₄ region were no longer features of the gel samples (gel 8) which, except for a relatively increased staining intensity of the D₂ band, became indistinguishable from their normal counterparts.

During the digestive phases described above, Fragment E also is evolved. As digestion proceeds its size is reduced. At no phase of hydrolysis was there any indication of a difference in the electrophoretic characteristics of Fragment E species derived from Paris I digests and those generated from normal fibrinogen.

**Gradient elution chromatography of normal and Paris I fibrinogen.** DEAE-cellulose gradient elution chromatography (four chromatograms of two preparations) of Paris I fraction 1-2 was carried out (Fig. 1) to characterize the preparations and as a purification procedure (see Methods). Highly purified normal fibrinogen (fraction 1-4) ordinarily exhibits two major fibrinogen peaks; less purified preparations (such as fraction 1-2) also exhibit a third minor fibrinogen peak (Fig. 1, tube 60-61) and another later eluting peak (tube 71-78) containing nonfibrinogen proteins such as cold-insoluble globulin (9). Analyses of normal fibrinogen chains (46) have indicated that the differences in elution characteristics of peaks 1 and 2 fibrinogen can be accounted for by the absence (peak 1) or the presence (peak 2) of a γ-chain variant termed γ'. Paris I fibrinogen, like normal, exhibited two major fibrinogen-containing peaks. However, the Paris I fibrinogen peaks emerged later than their presumed normal counterparts. After correction for peak overlap, it was estimated that 80% of the fibrinogen-related material in the normal fraction 1-2 chromatogram was eluted as peak 1, the remainder eluting in peaks 2 and 3. The Paris I fibrinogen chromatogram exhibited a similar distribution, since its peak 1 fibrinogen amounted to 81% of the total.

In three of five chromatograms, as illustrated by Fig. 1, there was a slight “shoulder” on the ascending limb of peak 1, suggesting the possible presence of a population of normal fibrinogen molecules (i.e. having two γ-chains). Its presence was confirmed by demonstrating (data not shown) that material obtained from the ascending limb of peak 1 has a considerably higher proportion of γ-chains than of γParis I chains.

**DISCUSSION**

Based upon previous experiments with samples of fibrinogen Baltimore (47), which also manifested delayed elution of chromatographic peaks 1 and 2, it appears that the amount of normal fibrinogen emerging in the ascending portion of Paris I fibrinogen peak 1 does not amount to more than 10% of the total fibrinogen population in the preparations we have analyzed. This value seems reasonable in view of the preponderance of γParis I chains in all plasma subfractions examined. However, since we have not examined fibrinogen of relatively high solubility (i.e. not precipitated in Cohn fraction I), we cannot yet make a final assessment of the proportion of the total fibrinogen which is normal.
The γParis I chains comprise a high proportion of the γ-chain population in both the peak 1 and 2 fibrinogen pools (65-75% of peak 1; 67-82% of peak 2 fibrinogen). Although the presence of the mutant chain almost certainly accounts for the increased anionic-binding behavior of the preparation itself, the heterogeneity manifested as peaks 1 and 2 is not attributable to the presence of the γParis I chain per se. The results are consistent with the view that the region of the γ-chain accounting for the chromatographic behavior of peak 2 fibrinogen (viz., γ'variant chain, reference 46) is present in both the γ- and γParis I chain populations. Thus, this property of γParis I chains suggests that it has structural overlap with γ-chains.

Apart from its apparent lack of a thrombin- or Reptilase-sensitive site, other properties of γParis I differ sharply from those of γ-chains. First, γParis I is a larger chain than γ, and this by itself permits it to be distinguishable in dodecyl sulfate gel-electrophoretic experiments (reference 8, this study). Second, unlike a γ-chain, the γParis I chain does not participate in γ-dimer formation (reference 8, this study); it also fails to incorporate monodansylcadaverine in the presence of Factor XIIIa (Fig. 5). These latter two observations indicate that the amine acceptor site in the COOH-terminal region of the γ-chain (36) is either not present on this mutant chain or is unavailable* for cross-linking. Further support for the localization of the abnormality in the COOH-terminal region of the molecule is provided from examination of the products of plasmin digestion. That is, plasmin Fragment E derived from Paris I fibrinogen does not differ from normal, whereas a unique form of Fragment D (DParis I) has been identified in Paris I digests (Fig. 6). Fragment E consists only of structures derived from its NH2-terminal region, whereas Fragment D consists of chain fragments whose structure overlaps that of the COOH-terminal region of the molecule (13, 32, 40-43 inter alia).

In contrast to the abnormality demonstrated in the γParis I chain, when scrutinized from the standpoint of their electrophoretic migration rates, and functional qualities upon treatment with Reptilase or thrombin, the Aα-, Bβ-, and γ-chain populations of fibrinogen Paris I are indistinguishable from those in normal fibrinogen. The γ-chains however, are reduced in amount. The demonstration of delayed γ-dimer and α-polymer formation of Paris I fibrin is not attributable to an intrinsic abnor-

* Steric availability of such a site is an important consideration. Only one glutaminy1 residue on the γ-chain participates in cross-linking (36), although several are present in the COOH-terminal region. Despite the fact that β-chains of fibrin do not participate in cross-linking (37, 38, 48) and fail to incorporate monodansylcadaverine in the presence of Factor XIIIa (27), S-sulfo-β-chains can undergo considerable cross-linking (49).

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