Human Fibrinogen Heterogeneities

I. STRUCTURAL AND RELATED STUDIES OF PLASMA FIBRINOGENS WHICH ARE HIGH SOLUBILITY CATABOLIC INTERMEDIATES*

(Received for publication, January 29, 1972)

M. W. Mosesson;† J. S. Finlayson, R. A. Umfleet, and D. Galanakis

From the Department of Internal Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York 11203, and the Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland

SUMMARY

The heterogeneity of human plasma fibrinogen manifested as differences in solubility reflects the presence of early catabolic intermediates, which are more soluble, have a longer thrombin-clotting time, and are of a lower molecular weight than the parent material from which they are formed. In this study the S-sulfo subunits from fibrinogen fractions of low (I-4) and high (I-8 and I-9) solubility were compared. Separation of Aα, Bβ, and γ chains was achieved by gradient elution chromatography on CM-cellulose. The tryptic peptide maps were characteristic for each type of chain. The only observable differences were in the maps of Aα chains; in those of I-8 and I-9 a considerable number of spots were absent or reduced relative to I-4. NH₂-terminal analyses of isolated chains before and after thrombin treatment showed the characteristic residues of the Aα, Bβ, and γ chains, respectively. Thus the NH₂-terminal portions of all chains of I-4, I-8, and I-9 were intact.

Molecular weight estimation of subunit chains was made by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate under conditions in which differences in mobility due to the presence or absence of A or B peptides were distinguishable. Electrophoresis in this medium before and after reptilase or thrombin treatment served for identification of the chains. The γ chains moved as a single band in this system (molecular weight, 50,700) and were indistinguishable from one another; the same was true of Bβ chains (molecular weight, 60,400). However, electrophoresis of Aα chains revealed the presence of 13 more-or-less distinct bands (numbered in order of increasing anodal mobility), of which all but two (Band 3, a Bβ contaminant and Band 5, a γ chain contaminant) were shown to be intact Aα chain or COOH-terminally degraded Aα remnants. The molecular weights ranged from that of intact Aα chain (70,900) to 15,400 (Band 13). The highest proportion of intact Aα chains was in I-4; the lowest was in I-9, which had virtually no detectable intact Aα chain. Conversely, I-9 contained the highest proportion of Aα remnant chains; I-4, the lowest. These data suggested that cleavage at any or all of at least 10 sites along the Aα chain resulted in the formation of early catabolic intermediates.

Comparisons with highly clottable derivative fractions I-8D and I-9D produced by plasmin treatment of I-4 in vitro showed that they were quite similar to their respective plasma counterparts (I-8 and I-9) in that the γ and Bβ chains appeared to be intact, but few or no intact Aα chains were present. These observations thus strengthen the notion that it is plasmin which catalyzes the formation of early intermediates in vivo. Examination of Fragment 'X,' another fibrinogen derivative produced by plasmin digestion, revealed considerable depletion of intact Bβ as well as of Aα chains, indicating that Fragment 'X' was more extensively degraded than I-8D or I-9D.

Human plasma fibrinogen, as well as that of bovine and rabbit origin, is heterogeneous from the standpoint of solubility (3-7). This heterogeneity is a reflection of the relatively lower molecular weight of material with higher solubility (7-9). Two of the most highly soluble plasma subfractions have been named I-8 and I-9 (3). Because Fraction I-8 was obtained in a more highly clottable form, many of the biophysical and biochemical studies (8, 10) were carried out on it, although I-9 exhibited the higher solubility. Comparisons of human plasma Fraction I-4 with I-8 (3, 8) and a remarkably similar fraction (I-8D) obtained after limited plasmin degradation of Fraction I-4 (11), led to the hypothesis that the higher solubility material was a catabolic derivative produced by plasmin digestion, revealed considerable depletion of intact Bβ as well as of Aα chains, indicating that Fragment 'X' was more extensively degraded than I-8D or I-9D.

* This research was supported by National Institutes of Health Grant HE-11409. The results of these investigations were presented in part at the 2nd Annual Meeting of the International Society on Thrombosis and Haemostasis, Oslo, Norway, July 1971 (1), and at the 44th Annual Meeting of the American Heart Association, Anaheim, Calif., November 1971 (2).
† National Institutes of Health Career Development Awardee No. 32,569.

1 Sherman et al. (11) termed the plasmin-degraded material "I-8DI" to indicate that it was a derivative (D) and that there were five subfractionation (DI) steps involved in its isolation and repurification to high clottability. In the present paper, that fraction is referred to simply as I-8D; by the same token, therefore, plasmin-degraded material having solubility equivalent to that of plasma fraction I-9 is termed I-9D.
intermediate derived from higher molecular weight species. Conclusive support for the catabolite hypothesis was provided by turnover studies of radioactively labeled rabbit fibrinogen; a classical precursor-product relationship (12) was shown between the low and higher solubility forms of clottable material (7). The fact that in both I-8 and I-SD the transformation from low to higher solubility material occurred by release of peptide material from the COOH-terminal portion of the parent species (8, 11) suggested that the formation of the in vivo derivatives was catalyzed by plasmin. More recent studies of the sequence of plasmin degradation of fibrinogen (13, 14) have shown that the α chain is the first to be degraded; related studies of fibrin subunits formed from human fibrinogen of high and low solubility indicated that the high solubility material lacked intact α chains (13), an observation which further supported the notion that formation of these metabolites in vivo is catalyzed by plasmin.

The studies to be reported here were concerned with the detailed subunit structure of fibrinogen fractions of low (I-4) and high solubility (I-8 and I-9). These were compared to ascertain the exact features which account for previously shown differences in solubility and thrombin-clotting times (3). Also included were comparisons with certain plasmin-mediated in vitro derivatives, viz. Fractions I-SD and I-9D and Fragment X' (15), to determine their structural relationships to one another and to the intermediates formed in vivo.

MATERIALS AND METHODS

Fibrinogen Subfractions and Derivatives—Fractions I-4, I-8, and I-9 were prepared from outdated human ACD plasma as described by Moesson and Sherry (3). The clottability of I-9, usually about 90% when prepared by the original method, was increased to more than 96% (four preparations) by the following modifications. After the usual procedure of precipitating Fraction I-8 at 2.1 M NaCl-0.01 M sodium phosphate buffer, pH 6.4, to a protein concentration of 1 to 2%; reprecipitation by adding solid β-alanine to a final concentration of 2 M (250 g of β-alanine per liter of solution) was carried out at room temperature, followed by cooling to 1–2°C for 30 min or more and centrifugation at 10,000 to 12,000 × g. The resulting Fraction I-9 was more than 96% coagulable with thrombin (range for four preparations, 96 to 99%).

Limited plasmin degradation of Fraction I-4 (original clottability >98%) was carried out as previously described (11). Clottability of the digest prior to fractionation was 88%. Fractions I-SD and I-9D isolated from this digest by ethanol and centrifugation at 10,000 to 12,000 × g were used in the following study.

NH2-terminal Analyses—NH2-terminal analysis was performed by an adaptation of the DNS method (17), previously described in detail (18). The relative amounts of NH2-terminal residues were estimated visually. Thrombin-treated chains for NH2-terminal analysis were prepared as follows. Freeze-dried subunit chains (about 1 to 1.5 mg samples) were suspended in 0.2 ml of 0.2 M N-ethylmorpholine, pH 8.4 ± 0.1, and treated with two successive additions of 10 μl of thrombin. The suspension was incubated overnight at room temperature, then dissolved by addition of 0.4 ml of freshly deionized 10 M urea, and carried through the DNS-labeling procedure. Human thrombin (lot H-1) was provided by Dr. D. L. Aronson, Division of Biologics Standards, National Institutes of Health; it was reconstituted with water to a concentration of approximately 100 U.S. units per ml and stored at -20°C.

Tryptic Peptide Mapping—Mapping of the S-sulfo chains was done by a modification (19) of the method of Katz et al. (20). Digestion was carried out with trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone (Warthington Biochemicals Corp.); maps were stained with either a collidine-ninhydrin or a cadmium acetate-ninhydrin stain.

Electrophoretic and Related Procedures—Gel electrophoresis was carried out at 20° (5% polyacrylamide gel slabs, 3 mm thick) in Tris-EDTA-borate buffer (21) containing freshly deionized urea (8 M; buffer pH 8.6) or no urea (buffer pH 8.4). The former conditions were very similar to those described by Takagi and Iwanaga (22). A vertical electrophoresis assembly (E-C apparatus Corp., Philadelphia, Pa.) and a pulsed power supply (Ortec, Oak Ridge, Tenn.) were employed. Samples were applied in 40 μl of 20% (w/v) sucrose.

Polyacrylamide gel electrophoresis in acetic acid-urea solutions (23) was carried out in a disc gel apparatus with a regulated power supply (Buchler Instruments) in tubes, 5 × 75 mm, at a gel concentration of 10% (pH 2.7, 2 M urea).

SDS-polyacrylamide gel electrophoresis was performed essentially as described by Weber and Osborn (24) in 9% gels. For disulfide bond reduction, when desired, DTT (14 mM final concentration) was employed. Samples for application were made by mixing with an equal volume of 50% (v/v) glycerol-0.1 M sodium phosphate, pH 7.0.

Protein markers used were phosphorylase A (rabbit muscle), molecular weight 94,000 (24), and aldolase (rabbit muscle), 40,000 (24), from Worthington Biochemicals Corp., albumin (bovine), 68,000 (24), from Sigma, chymotrypsinogen A (bovine pancreas), 25,700 (24), and cytochrome c (horse heart), 11,700 (24), from Mann Research Laboratories, the γ chain, 47,000 (25), and β chain, 56,000 (25), of human fibrinogen. The precision in estimating the molecular weight of unknown bands from these markers was ±1.5 to 3.5%.

Isolated S-sulfofibrinogen chains were reacted with reptilase (prepared from the venom of Bothrops atrox; a gift from Penta-pharm Ltd. Basel, Switzerland) or thrombin in the following way. Freeze-dried samples were solubilized in deionized 10 M urea to a concentration of about 10 mg per ml and then dialyzed overnight against 300 to 500 volumes of 0.2 M sodium phosphate, pH 7.0. After dialysis samples contained a uniform fine granular suspension. To 50 μl of each sample (estimated final protein was provided by Dr. Victor Marder and was about 40% clottable.

S-Sulfofibrinogen was prepared according to the method of Pechère et al. (16).

2 The nomenclature used for the subunit chains of fibrinogen, that is, Aα, Bβ, and γ, conforms to the recent recommendations of the Committee on Nomenclature of the International Society on Thrombosis and Haemostasis (Oslo, Norway, 1971). Formerly, the chains had been designated α(A), β(B), and γ. 3 The abbreviations used are: PEG, polyethylene glycol; DTT, dithiothreitol; DNS, 1-dimethylaminonaphthalene-5-sulfonyl; SDS, sodium dodecyl sulfate.
concentration, 4.5 mg per ml) was added two 5-ml portions of reptilase at an interval of 30 to 60 min, or 2.5 ml of thrombin. The suspension was allowed to incubate with stirring for 3 hours at room temperature and overnight at 5°C. The amount of enzyme added caused unmodified fibrinogen (Fraction I-4, 2 mg per ml, which had been carried through the same dialysis procedure) to clot 30 s after the first reptilase addition and in less than 10 s after treatment with thrombin. A portion of the enzyme-treated S-sulfo sample was mixed with an equal volume of 9 M urea, 2% SDS solution prior to application as described above. Standard markers in the same solvent were run in separate gels.

Gels were stained with Amido schwarz 10B or Coomassie brilliant blue. Amido schwarz-stained gels could readily be counterstained with Coomassie blue (26) which in turn could be displaced by staining with Amido schwarz; this provided a useful system for examining the same bands stained with a more or less sensitive stain.

Densitometric scans of the gels were carried out with an Aminco filter fluorometer equipped with a horizontal thin layer scanning attachment; a Wratten 23A filter was employed. To eliminate variable light refraction encountered during scanning of cylindrical gels laid directly on glass plates, gels were submerged in water in a clear plastic trough specially constructed for that purpose. Gel slabs could be placed directly on glass plates over the light source.

Agarose electrophoresis (proteins stained with light green) and immunoelectrophoresis (27) were performed at room temperature in 1% agarose in barbital buffer (pH 8.6) on flexible plastic film (Cronar, 70-mm movie film, Du Pont), 90 to 100 mm in length, for 1 to 2 hours at 60 to 80 volts. Immunodiffusion was carried out at room temperature for 24 to 48 hours with rabbit anti-human fibrinogen serum which had been made specific by absorption with human serum.

Chromatographic Procedures—DEAE-cellulose (Whatman DE 23) analytical column chromatography of unmodified fibrinogen was carried out at 20°C on columns, 0.9 x 30 cm, employing a combined pH and phosphate gradient (18). Fractions corresponding to 1% of the total gradient were collected.

CM-cellulose gradient chromatography (Whatman CM 23) for the separation of S-sulfofibrinogen chains was carried out on columns, 0.9 x 30 or 1.5 x 30 cm, at room temperature in 8 M urea. A sodium acetate gradient from 0.005 to 0.15 M with respect to Na+ was formed with a nine-chamber gradient device (Buchler Instruments) containing 50 or 75 ml per chamber. The Na+ molarity in each chamber was, consecutively: 0.005, 0.02 x 4, 0.04 x 3, 0.15. Dilution of the stock buffer solution (0.2 M sodium acetate, 8 M urea, pH 5.3 ± 0.1) with 8 M urea to prepare the individual buffers resulted in a fall in pH: e.g. the pH in Chamber 1, 0.005 Na+, was 4.7. Urea solutions were prepared from reagent grade material and deionized on a mixed bed resin (Biorex AG 501-X8) immediately prior to use. Fractions corresponding to 1% of the total gradient were collected. The void volume of the system, was assumed from previous estimates (18) to be 6% of the total gradient. Columns were regenerated by flushing with stock 0.2 M sodium acetate buffer followed by starting buffer. Pooled fractions were extensively dialyzed against water and freeze-dried.

RESULTS

Chromatographic, Electrophoretic, and Immunochemical Characterization of Unmodified I-4, I-8, and I-9—These experiments were carried out to extend the results of previous studies of I-4 and I-8 (8) by comparing them with I-9, which has an even higher solubility and longer thrombin clotting time than I 8 (3).

Electrophoresis of these unmodified fractions in 5% polyacrylamide gels at pH 8.4 showed that they migrated as single bands; Fractions I-8 and I-9 were indistinguishable from one another although both had a slightly greater anodal mobility than I-4. Agarose electrophoretic studies indicated a marginally greater anodal mobility of I-8 and I-9 compared with I-4, but this difference could not be shown by the positions of the fibrinogen precipitin arcs in concomitant immunoelectrophoretic experiments. Immunodiffusion experiments comparing I-4, I-8, and I-9 resulted in fibrinogen precipitin lines of complete identity. Thus, by these several criteria, I-9 could not be distinguished from I-8.

The chromatographic elution pattern of I-8 on DEAE-cellulose was very similar to that of I-4 (Fig. 1) except for the previously observed (8) tendency for slightly earlier elution of the first major peak of I-8. I-9 displayed a shoulder on the ascending limb of Peak 1 which was not present in either I-4 or I-8 (8) by comparing them with I-9, which has an even higher solubility and longer thrombin clotting time than I 8 (3). Fragments 'X' also migrated as a single band with the same mobility as I-8 and I-9; Fragment 'X' also migrated as a single band but had a significantly greater mobility than any of these fractions.

Fragment 'X' also migrated as a single band with the same mobility as I-8 and I-9; Fragment 'X' also migrated as a single band but had a significantly greater mobility than any of these fractions.

In all such chromatographic experiments, the frontal fraction varied from 3 to 25% of the total eluted protein depending upon the particular preparation and upon the amount of material loaded on the column. The nature of the frontal fraction is not relevant to these studies but is considered in Paper III of this series (28) which relates primarily to γ chain heterogeneity.
Chains were consistent with their elution behavior during CM-Sepharose chromatography. In addition to the major band corresponding to each S-sulfato chain, small amounts of other bands were detected in I-4 and I-8 Aα chains but not in those from I-9. The presence of a γ chain contaminant, shown by other techniques to be present in all Aα preparations (see below), was suggested in a single I-8 Aα preparation by the presence of detectable terminal tyrosine in thrombin-treated or untreated samples. Because the NH₂-terminal pyroglutamylation of the Bβ chain is unreactive with DNS chloride, Bβ contamination of Aα chains could not be evaluated by this analysis, but other data have indicated the consistent presence of small amounts of Bβ chains in Aα preparations (see below).

Owing to the unreactive NH₂-terminal residue, Bβ chains (two preparations of each fraction) revealed only trace amounts of terminal amino acids. The most evident of these was tyrosine after thrombin treatment. Prior to thrombin treatment, small amounts of NH₂-terminal glycine were detected in I-4 and I-8 Aα chains but not in those from I-9. The presence of a γ chain contaminant, shown by other techniques to be present in all Aα preparations (see below), was suggested in a single I-8 Aα preparation by the presence of detectable terminal tyrosine in thrombin-treated or untreated samples. Because the NH₂-terminal pyroglutamylation of the Bβ chain is unreactive with DNS chloride, Bβ contamination of Aα chains could not be evaluated by this analysis, but other data have indicated the consistent presence of small amounts of Bβ chains in Aα preparations (see below).

The γ chains (two preparations of each fraction) revealed only tyrosine detectable before or after thrombin treatment.

Electrophoretic Analysis (Figs. 4 to 9)—Urea-polyacrylamide gel electrophoresis at pH 8.6 resulted in clear separation of I-4 S-sulfo subunit chains (Fig. 4). The relative mobilities of the chains were consistent with their elution behavior during CM-Sepharose chromatography. In addition to the major band corresponding to each S-sulfo chain, small amounts of other bands were usually seen.

The γ chains of I-4, I-8, and I-9 were indistinguishable from one another in this analytical system, as were the respective Aα chains. In contrast to that of I-4, the Aα bands of I-8 and I-9 were more diffuse, somewhat more anodal than that of I-4 and tended to overlap the Bβ position. Additionally, in one of two preparations of I-8 (Fig. 4) there was a second major band which migrated more slowly than any Aα band.

Fig. 2. Elution patterns of S-sulfo chains of I-4 (39 mg), I-8 (75 mg), and I-9 (84 mg) subjected to gradient elution chromatography in 8 M urea on CM-sepharose (columns, 1.5 X 30 cm). The theoretical Na⁺ gradient is at the top. The point at which the gradient was begun is indicated by the vertical arrow. Each chromatogram was obtained separately under identical conditions. Each tube contained 4.5 ± 0.1 ml; pooling was as indicated by the shaded areas.

6 The tryptic peptide maps of the Bβ and γ chains from I-4, I-8, and I-9 (in addition to the Aα chain shown here) are available as JBC Document Number 72M-81, in the form of 1 microfiche. Orders for supplementary material should specify the title, author(s), and reference to this paper and the JBC Document number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014 and must be accompanied by remittance to the order of the Journal in the amount of $2.50 per microfiche.

7 Reaction of any S-sulfo chain with DTT usually results in bands with slower anodal mobility (pH 8.6) because of the lower anionic charge resulting from reductive displacement of SO₂⁻ groups. After such DTT treatment, the two main bands in the I-8 Aα sample (Fig. 4) migrated as a single band with a mobility intermediate between those shown in the figure. This experiment suggested that the slower band was a disulfide-bonded polymorphic form of the Aα chain separated by molecular sieving, and it effectively excluded the possibility that this might have represented α polymer (30, 31).
The foregoing results suggested that the differences between Fractions I-4, I-8, and I-9 were related to differences in the Aα chain population. This notion was investigated, and ultimately substantiated, by the following series of experiments in which SDS-polyacrylamide gel electrophoresis was the principal technique. It had already been shown (see above) that the NH₂-terminal portions of the Aα and Bβ chains were intact and could be cleaved by thrombin. It was also established at the outset, through analyses of isolated Aα, Bβ, and γ chains, that the individual chains, regardless of their molecular size, could be differentiated by comparing their gel patterns before and after reaction with reptilase or thrombin. This was possible because the electrophoretic technique was capable of distinguishing molecular weight differences in chains resulting from the loss of an A or B peptide (molecular weight 1500 to 1600); actual computed reductions were between 700 and 2800, a result consistent with the precision of the method.

The Bβ chains of I-4, I-8, and I-9 moved as single bands and were indistinguishable from one another; the same was true of γ chains (Fig. 5). Upon treatment with thrombin, the position of Bβ chains shifted toward the anode whereas that of the γ chains did not. After reaction with reptilase, most of the Bβ band underwent no change in mobility; however, two additional minor bands (arrows, Fig. 5) were detected. The band of higher molecular weight was barely appreciated as a faint band continuous with the anodal portion of the Bβ band in I-4 and I-9 or as a faint but discrete band in I-8. Its appearance was consistent with cleavage of Peptide B from a small percentage of Bβ chains. The lower band, molecular weight 56,000, differed from that of the intact Bβ chain (60,400) by 4,400, an amount suggesting that it had been derived from a Bβ chain by cleavage at a site more internal than the thrombin-susceptible site at position 14 (perhaps at position 42). Thus, in spite of the fact that reptilase does not act exclusively on the Aα chain (35), the reaction was sufficiently specific to enable Aα and Bβ chains (and by inference, γ chains as well) to be distinguished from one another.

In contrast to Bβ and γ chains, a complex band pattern was obtained with chromatographically isolated Aα chains. There were 11 to 13 more-or-less distinct bands, which were numbered consecutively in order of increasing anodal mobility (Figs. 6 and 7). For clarity, bands were further classified as Group I, II, or III if their relative migration was close to that of intact...
FIG. 5 (upper left). SDS-polyacrylamide gel electrophoresis (9% gel) of chromatographically isolated S-sulfo Bβ and γ chains before and after treatment with reptilase (R) or thrombin (T). The Bβ and γ chains were recombined for this experiment prior to enzyme treatment. The arrows point to minor bands which became visible after reptilase treatment. The gel was stained with Coomassie blue.

FIG. 6 (upper right). SDS-polyacrylamide gel electrophoresis (9% gel) of chromatographically isolated Aα chains of I-4 and I-9 (15 μg each). The gel was stained with Coomassie blue. On the right the isolated, recombined Bβ and γ chains from I-4 are shown for reference. Eleven distinct bands were visible in this experiment and are numbered consecutively in order of increasing anodal mobility.

FIG. 7 (lower). SDS-polyacrylamide gel electrophoresis (9% gel) of chromatographically isolated S-sulfo Aα chains of I-4, I-8, and I-9 before and after treatment with reptilase (R) or thrombin (T). The gel was stained with Coomassie blue. Thirteen more-or-less distinct bands were visible and are numbered consecutively according to increasing anodal mobility. Bands 1 to 11 correspond to those in Fig. 6 and Table I in which Bβ (Band 3) and γ chain (Band 8) contaminants are indicated. The estimated molecular weights (before enzyme treatment) are indicated on the left.
TABLE I
Summary of the data relating to SDS-polyacrylamide gel electrophoretic estimation of the molecular weight of S-sulfo chains of I-4, I-8, and I-9. The numbered bands are further classified as Group I, II, or III if their relative migration is close to that of intact Aα, Bβ, or γ chains, respectively, and as Group IV if their migration is considerably faster than that of the intact chains. Inasmuch as Group IV bands were difficult to resolve by densitometric scanning, their relative staining intensities have been estimated visually and graded Tr (trace) to ++++. Failure to detect a given band is indicated by -.

<table>
<thead>
<tr>
<th>Group</th>
<th>Band No.</th>
<th>n</th>
<th>Mean mol wt X 10^2</th>
<th>Relative distribution of Group IV bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>7</td>
<td>70.9</td>
<td>I-4 I-8 I-9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>67.3</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>3 (Bβ)</td>
<td>8</td>
<td>60.4</td>
<td>I-4 I-8 I-9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
<td>57.7</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>5 (γ)</td>
<td>8</td>
<td>50.7</td>
<td>I-4 I-8 I-9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7</td>
<td>46.5</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>7 (γ)</td>
<td>8</td>
<td>37.6</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7</td>
<td>33.9</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>7</td>
<td>31.8</td>
<td>++++++++</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7</td>
<td>29.2</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6</td>
<td>25.0</td>
<td>Tr Tr ++</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>6</td>
<td>22.6</td>
<td>-- -- Tr</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>3</td>
<td>15.4</td>
<td>-- -- Tr</td>
</tr>
</tbody>
</table>

| a | Number of gels in which this band was measured. |
| b | This band has the same molecular weight and susceptibility to reptilase and thrombin as the Bβ chain. |
| c | This band has the same molecular weight and susceptibility to reptilase and thrombin as the γ chain. |
| d | This band was frequently difficult to appreciate as a discrete band when there was smearing of the stain in that region (cf. Gel 1, Fig. 6). |

Aα, Bβ, or γ chains, respectively; Group IV bands were those which migrated considerably faster than any of the intact chains.

There were usually two Group I bands (occasionally this group was resolved into a triplet) both of which were enzymically identifiable as Aα chains. Of these bands, Number 1 was assumed to correspond to intact Aα chain whereas Number 2 was regarded as an Aα remnant of slightly reduced molecular size (Table I). Of the Group II bands one proved to be a Bβ chain (Band 3) because it reacted only with thrombin, while the other (Band 4) met the enzymic criteria for an Aα chain. Band 5 (Group III), on the basis of its characteristic molecular weight and its nonreactivity with reptilase or thrombin, is most likely a γ chain contaminant. Band 6 is an Aα remnant which can also be observed in unchromatographed fibrinogen chain preparations as a discrete band or as a minor band continuous with the γ chain at its anodal border (Fig. 9); it becomes clearly evident after treatment with reptilase or thrombin. Of the Group IV chains, Bands 12 and 13 were the faintest which could be enzymically identified as Aα remnants, whereas Bands 8 and 9 were the predominant species (e.g., Fig. 7). The relative proportion of the total Aα chain population represented by Group IV varied directly (Table I; Fig. 8) with the solubility of the preparation (i.e., I-9 > I-8 > I-4).

From the foregoing data it was evident that the differences between I-4, I-8, and I-9 were in the degree of intactness of their Aα chains. Densitometric scans (Fig. 8) indicated that 66%

Owing to limitations on the narrowness of the illuminating band width obtainable with the densitometric scanning equipment employed, the absorption characteristics of Coomassie blue cannot be expected to adhere to Beer's law except at low concentrations (36, 37). For this reason only less intensely stained gels were selected for densitometric study (maximum absorbance of any band scanned was 0.88). From the experience of others (36),

**Fig. 8.** Relative distribution of Aα chains in I-4, I-8, and I-9 based upon densitometric analysis of SDS gels stained with Coomassie blue. The Aα chains are classified as Group I, II, or III if their relative migration is close to that of intact Aα, Bβ, or γ chains, respectively, and as Group IV if their migration is considerably faster than that of any intact chain (see Table I). The I-9 and I-4 gels were those shown in Fig. 6; the I-8 gel scanned was obtained by running a 45-μg sample in the same experiment as the 112-μg sample of I-8 shown in Fig. 7.

**Fig. 9.** SDS-polyacrylamide gel electrophoresis (9% gel) of DTT-reduced preparations of I-4, I-8, I-9, I-8D, I-9D, and Fragment 'X'. Intact Aα, Bβ, and γ chains are shown, as well as certain identifiable Aα remnant chains which are indicated by appropriately numbered arrows. The Aα remnant ("11") seen in I-8D, I-9D, and 'X' corresponds to Band 11.
of all I-4 Aα chains was in Group I but only 19% of I-6 and 11% of I-9 chains were of this size order. Furthermore, it could be estimated visually that Fraction I-9 had virtually no intact (i.e. Band 1) Aα chains whereas about 50% of the Group I bands in I-8 and most of those in I-4 were of this variety. Understandably, I-9 had the highest proportion of the smaller Aα remnants.

**Comparisons with I-8D, I-9D, and Fragment 'X'**—Comparisons of whole I-8 and I-9 with the plasmin-mediated Fractions I-8D and I-9D and Fragment 'X' were carried out by SDS-gel electrophoresis to assess and compare the type and degree of degradation of their component chains (Fig. 9). I-8D and I-9D were quite similar to their respective counterparts, I-8 and I-9, in that the Bβ and γ chains appeared to be intact and few Group 1 Aα chains were detectable. The I-8D and I-9D bands which were visible and identifiable as Aα remnants by enzymic analysis corresponded in size to the Aα remnants 2, 8, 9, 11, and 12 which had been identified in whole preparations of I-8 or I-9 or their isolated chains. In contrast to I-8 and I-9 there was little indication of the presence of Bands 6 and 10 in I-8D or I-9D. Furthermore, in I-8D and I-9D there were considerable quantities of the Aα remnant corresponding in size to Band 11, whose concentration was too low to be detected in equivalent amounts of whole I-8 and I-9 (Fig. 9).

Fragment 'X' (two preparations) displayed considerable depletion of intact Bβ chains as well as of Aα chains. In some gels (not shown), the γ zone was resolved into two bands, probably representing degraded Bβ chains as well as γ chains. It seems reasonable to conclude from these patterns, albeit tentatively, that I-8D and I-9D resemble their plasma counterparts in lacking intact Aα chains and that Fragment 'X' differs in that substantial amounts of intact Bβ chains are lacking as well.

**Discussion**

In the method previously described (3) for the isolation and purification of high solubility plasma fibrinogen fractions, Fraction I-7 was treated with 2.1 M glycine to precipitate Fraction I-8; the subsequent precipitate from the resulting supernatant solution was designated I-9. The proportion of fibrinogen precipitating in the I-8 fraction was largely dependent upon the protein concentration prior to glycine addition. Thus, Fractions I-8 and I-9 were both regarded as representative of fibrinogen of the highest solubility (3). The present studies permit the further conclusions that the measurable differences between I-8 and I-9 are of a relatively minor quantitative nature involving the degree of intactness of Aα chains. Although both are representative of the same over-all process, in view of the facts that I-9 may now be prepared in highly clottable form, is more completely catabolized than I-8 (in the sense of virtually complete lack of intact Aα chain), has a higher solubility and exhibits a longer thrombin-clotting time (3), it would appear that I-9 is a more suitable fraction than I-8 for future comparative studies (e.g. with Fraction I-4 or Fragment 'X'). Based upon the molecular weights of the component Aα chains (Table 1) and their distribution in each preparation (Fig. 8), the mean molecular weight of I-9 can be calculated to be about 10,000 less than that of I-8. Thus, based upon a molecular weight for Fraction I-8 of 271,000, obtained by averaging the previously reported values 269,000 and 273,000 (8, 11), Fraction I-9 may tentatively be assigned a mean molecular weight of 261,000.

There were no differences in the tryptic peptide maps of Bβ chains isolated from I-4, I-8, and I-9 or in those of the γ chains isolated from these fractions. These findings have supported the NH-terminal, electrophoretic, and chromatographic data in indicating that the Bβ and γ chains are intact. In contrast, the peptide map of Aα chains from I-4 contained several spots which were clearly missing or faint in the Aα chains isolated from I-8 and I-9. The fact that there were no new peptide spots in I-8 or I-9 suggests that the primary sequence of the smaller Aα chains is present in (and thus derived from) larger Aα chains. These observations, combined with NH-terminal and electrophoretic data, add to an already convincing body of evidence (3, 7, 8, 11, 13) that lower molecular weight plasma fibrinogens are clottable catalytic intermediates.

There is as yet no conclusive evidence that plasmin mediates the catabolism of fibrinogen in vivo, although the similarities between early products of plasmin digestion in vitro (namely I-8D, I-9D) and their plasma counterparts make such a surmise most attractive (11, 39). The present studies have shown that the structural features of the chains of I-8 and I-9 are essentially the same as those of I-8D and I-9D. Differences between the naturally occurring and plasmin-mediated derivatives (e.g. the presence of larger amounts of Band 11 Aα remnant in the latter) appear to be quantitative rather than qualitative, and could reflect the modifying influences of the plasma environment on plasmin action. In a recent brief report, Mills and Karpatkin (40) concluded that plasmin was not the enzyme responsible for early degradation of fibrinogen in vivo and that thrombin or a contaminant nonplasmin fibrinolytic activity present in their thrombin preparation might be responsible. The idea that thrombin itself could mediate such a reaction in vivo is entirely inconsistent with the widely accepted specificity of thrombin and the available data on the early plasma intermediates (3, 8, this study). The conclusion that the fibrinolytic activity discernible in their experiments might not be plasmin will require more rigorous documentation.

Aα chain heterogeneity manifested as a doublet (namely Bands 1 and 2) in the Group I Aα position in SDS-electrophoretic experiments of Fraction I-4 or its equivalent has been reported from other laboratories (13, 30). Heterogeneity has also been shown by gel electrophoresis of S-carboxymethylated (31, 41) and S-sulfo chains (25) under acid conditions. Since an ob-
from these data. It is therefore convenient to regard cleavages and degradation sequence (or sequences) cannot be deduced recognized by the demonstration of at least ten Aα remnants presented studies at least ten cleavage sites on

I-8D and I-9D (11) occurs in this manner. Following this there occurs by COOH-terminal attack without loss of clottability the BP chain loses a fragment containing the Acβ chain which is first degraded (Step 1, Fig. 10) and that this as well as those of others (13, 14) clearly indicate that it is the clottable derivatives (11, 15, 43) to be made. The present data, as occurring in three relatively distinct stages (15), in the first and third stages are characterized in part by the absence of clotting

necessarily in the same relative positions.

13 Unpublished results.

servable transition from Band 1 to Band 2 has been reported to occur during plasmin hydrolysis (13) and since the ratio of Band 2 to Band 1 is greater in I-8 than in I-4 and almost infinite in I-9 (Fig. 7), only Band 1 can be regarded as an intact chain. All other identifiable Aα-bands contain clottable catabolites which are COOH-terminally degraded. In recent EDE-gel electrophoretic experiments13 with Blomback Fractions I-1 and I-3 (4) from fresh-frozen plasma, a doublet has been noted in the Aα position. Thus, all plasma fibrinogen fractions appear to contain at least some degraded Aα chains. As a result of the present studies at least ten cleavage sites on the Aα chain (presumably lysyl-X or arginyl-X bonds; see Fig. 10) have been recognized by the demonstration of at least ten Aα remnants of discrete molecular weight. Although conversion to Bands 2 and 9, for example, occurs with relatively high frequency, the exact degradation sequence (or sequences) cannot be deduced from these data. It is therefore convenient to regard cleavage of any or all of these bands as “Step 1” in the formation of the early catabolic intermediates (Fig. 10).

Plasmin degradation of fibrinogen in vitro is currently viewed as occurring in three relatively distinct stages (15), in the first of which (Stage I) clottable derivatives are present; the second and third stages are characterized in part by the absence of clottable derivatives. Presently available information permits a more complete picture of the composition of Stage I components to be drawn and a comparison of the well characterized early clottable derivatives (11, 15, 43) to be made. The present data, as well as those of others (13, 14) clearly indicate that it is the Aα chain which is first degraded (Step 1, Fig. 10) and that this occurs by COOH-terminal attack without loss of clottability (3, 11). The formation of the plasmin-mediated Fractions I-SD and I-9D (11) occurs in this manner. Following this there is hydrolysis of the Bβ chain (13, 14) which is not associated (at least initially) with the loss of clottability (13, 44). Shainoff and co-workers (44, 45) have reported that prior to appreciable loss of clottability the Bβ chain loses a fragment containing the B peptide indicating that the early fragmentation of the Bβ chains occurs in the NH2-terminal region (Step 2, Fig. 10); however, evidence relating to the presence or complete absence of the B peptide in the remaining core molecule has not as yet been reported.14

Fletcher et al. (43) have defined “first derivative” as that product retaining clottability. This definition therefore includes derivatives in which degradation of Aα chain or of both Aα and Bβ chains has occurred. The Fragment X’ (13) preparations (two) which we studied had extensively degraded Bβ and Aα chains (Fig. 9). Since it is not highly clottable, other degradative steps must have occurred. This material is therefore a more advanced Stage I derivative than those characterized by Sherman et al. (11) or defined by Fletcher et al. (43).

Recent studies (6, 9) of bovine plasma fibrinogen of high solubility have suggested that the catabolic pathway outlined above (cf. Fig. 10) may not obtain in this species. Previous investigations of early intermediate human fibrinogen (3, 8, 11) had shown that the NH2-terminal region of the molecule containing the A and B peptides remained intact and that the rate of release of these peptides by thrombin was the same as for “native” Fraction I-4 (8). The lengthening of the thrombin time was therefore attributable solely to delayed aggregation as was shown by direct experimentation (8). Although as in the corresponding human fractions, the molecular weight of bovine I-9 is lower than that of I-4 (9) and intact Aα chain is lacking in the former, the NH2-terminal residues (approximately 5 moles of tyrosine per 350,000 g) are considerably different from those found for Fraction I-4 (6). More importantly, there is no change in NH2-terminal residues after reaction of bovine I-9 with thrombin, although the fraction itself is highly clottable. If this material proves to be an intermediate metabolite analogous to human material, a different catabolic pathway than that for human fibrinogen must exist, and site (or sites) of thrombin action different from those generally recognized must be postulated for this species of fibrinogen. The additional possibility that bovine I-9 could be a “fetal fibrinogen” has been raised (9) and must also be considered.

Acknowledgments—We are grateful to Dr. Michael Potter, National Cancer Institute, for the use of his laboratory facilities, and to Miss B. Lynne Armstrong for skilled technical assistance.

Addendum—Since this manuscript was submitted, Pizzo et al. (49) have also confirmed that degradation of the Aα chain precedes that of the Bβ chain (13, 14) and have presented their deductions of the sequence of fibrinogen hydrolysis by plasmin in vitro. Since these investigators assumed that their fraction lacking intact Bβ chains was the same as fraction I-SD (see 14 The known sequence of the NH2-terminal portion of the Bβ chain (40, 47) indicates that among the plasmin-susceptible sites are three (Arg-Ala, position 42, and Lys-Ala, positions 47 and 58) whose disruption would result in release of a fragment containing Peptide B and the formation of new NH2-terminal alamine in the core. In recent studies we have observed that new NH2-terminal alamine is formed in the core molecule of the early plasmin-catalyzed derivatives in which varying degrees of Bβ chain fragmentation have occurred. The presence of new alamine might not have been suspected unless specific steps (such as NH2-terminal analysis before and after thrombin treatment) were taken to differentiate it from the alamine at the NH2 terminus of the Aα chains. Thus, the fact that the NH2-terminal residues of Fragment X’ were reported to be the same as those of fibrinogen (48), does not contradict the proposed degradative sequence.
Footnote 1), they concluded that the initial Bβ attack occurred in the COOH-terminal region of the molecule. Our demonstration of the intactness of the Bβ chains in L-D and I-D, as well as our subsequent studies in which the Bβ remnant corresponding to their B chain has been shown to be unreactive with thrombin, strongly indicates that initial cleavage of the Bβ chain takes place in the NH2-terminal region, as originally reported by Shainoff and co-workers (44, 45).

REFERENCES

Human Fibrinogen Heterogeneities: I. STRUCTURAL AND RELATED STUDIES OF PLASMA FIBRINOGENS WHICH ARE HIGH SOLUBILITY CATABOLIC INTERMEDIATES

M. W. Mosesson, J. S. Finlayson, R. A. Umfleet and D. Galanakis


Access the most updated version of this article at http://www.jbc.org/content/247/16/5210

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/16/5210.full.html#ref-list-1