Partial Chemical Characterization of Rat Fibrinogen*

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HESSEL BOUMA, III, AND GERALD M. FULLER
From the Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77550

Rat fibrinogen has been purified and compared with bovine and human fibrinogen with respect to a number of chemical characteristics, including molecular size, charge distribution, N-terminus-terminal amino acids, total amino acid composition, and interspecies immunological cross-reactivity. Although human and bovine fibrinogen demonstrated three nonidentical polypeptide chains by sodium dodecyl sulfate gel separations and by CM-cellulose separations, rat fibrinogen Αα, Ββ, and γ chains exhibited identical molecular weight sizes as well as identical charges. The presence of two nonidentical chains in these preparations was shown by qualitative N-terminus-terminal sequence analyses. The γ chain of rat fibrinogen was also shown to be quite distinct from the γ chains of human and bovine fibrinogen in its elevated content of cysteinyl and methionyl residues. Rat fibrinogen possesses the first reported blocked γ chain N-terminus-terminal amino acid of any species. It is concluded that, although many chemical properties of rat fibrinogen are unique, the basic molecular structure has remained consistent when compared with that of fibrinogen from the vertebrates studied thus far. Moreover, the inducibility of this system, together with the partial chemical characterization of the fibrinogen molecule, provides important information for the use of rat fibrinogen as a model system in studying the biosynthesis and assembly of this complex molecule.

Many of the chemical features of fibrinogen from a number of vertebrate species have been characterized (1-3). It is known, for example, that fibrinogen from vertebrates is composed of three pairs of nonidentical polypeptide chains (Αα, Ββ, γ) which are bound together through a complex set of disulfide bridges. Fibrinogen has a mass of approximately 340,000 ± 20,000 daltons. The physical properties of the molecule remain fairly consistent throughout those species that have been studied. It is not surprising that the bulk of the information concerning the physiochemical properties of fibrinogen has been established with the use of either the human or bovine molecules, since the plasma from these sources is readily available in large quantities. More recently, interests of several laboratories have been directed toward gaining an understanding of the processes of assembly of this complex molecule as well as learning more about the control of its biosynthesis. The clottable molecule, fibrinogen, purified from the laboratory white rat, was characterized with respect to several of its chemical properties in an effort to establish a convenient laboratory model for the study of its biosynthesis and assembly. Several unique chemical features of this fibrinogen are described in this report.

Materials and Methods

Rat fibrinogen was purified from plasma obtained from adult rats which had been induced into a hyperfibrinogenemic state with a subdermal injection of 1.0 ml of commercial turpentine (4) 48 hours prior to exsanguination. Whole blood was withdrawn from the femoral artery into heparinized syringes and immediately chilled to 4°C. Human fibrinogen was purified from the pooled outdated plasma obtained from the University of Texas Medical Branch blood bank. Bovine fibrinogen was purified beginning with commercially prepared Cohn Fraction 1 (Pentex; 90% clottable).

The concentration of plasminogen in the plasma was reduced by passing rat and human plasma over a lysine-coupled Sepharose 4B affinity column, according to the method of Deutsch and Mertz (5). Fibrinogen was precipitated from plasma with a 25% (w/v) ammonium sulfate fractionation. This crude fibrinogen preparation was dissolved into 0.10 M sodium phosphate (pH 7.2)-0.15 M NaCl buffer, dialyzed against one change of the same buffer, and then precipitated with a 20% (w/v) ammonium sulfate fractionation. The resulting fibrinogen precipitate was redissolved in a minimum amount of the phosphate buffer, dialyzed against the same buffer, and then subjected to chromatography on Sephadex G-200 (50 x 80 cm). In a control experiment, 1 ml of 10 mM L-cysteine was added to all buffers during the purification procedure. When L-cysteine was added, the step using the lysine-Sepharose chromatography was omitted. The commercial bovine fibrinogen was purified by a 20% (w/v) ammonium sulfate precipitation followed by (G-200 Sephadex) chromatography.

Isolation of Fibrinogen Chains—Rat and human fibrinogen samples were reduced and alkylated to the S-carbamylmethyl derivatives using iodoacetamide, essentially according to the procedure of McDonagh et al. (6), except that 1.64 mmol of 3-mercaptoethanol/g of fibrinogen were used for reduction. Alkylation was accomplished by using a molar excess of iodoacetamide 5 times that of the reducing agent, added

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†The International Committee on Nomenclature of Blood Clotting Factors designates the fibrinogen chains with the fibrinopeptides still attached as Αα and Ββ. The third polypeptide chain is designated γ. Fibrin polypeptide chains are called α, β, and γ respectively.
directly to the fibrinogen solution. The alkylated protein was dialyzed against five changes of 0.10 M ammonium bicarbonate at 4°C, after which the protein was lyophilized and stored at -20°C. Complete reduction and alkylation were verified by amino acid analyses which revealed an absence of half-cystine.

The reduced and blocked polypeptide chains were then separated by CM-cellulose chromatography in 8.0 M urea, as described by McDonagh et al. (6), except that a simple two-chamber gradient was used consisting of 500 ml of starting buffer (50 mM sodium acetate, pH 4.8) and 500 ml of the final buffer (170 mM sodium acetate, pH 5.3). Each protein region was pooled, dialyzed against several changes of 0.10 M ammonium bicarbonate, lyophilized, and stored at -20°C.

Polyacrylamide Gel Electrophoresis-Polyacrylamide gel electrophoresis carried out in sodium dodecyl sulfate was routinely used for the analytical determination of reduced rat Cm-fibrinogen. Molecular weight determinations were performed on 10% acrylamide gels containing 0.1% of the anionic detergent Na dodecyl-SO4 according to the method of Weber and Osborn (7), using the reported molecular weights for pancreatic ribonuclease (13,700), immunoglobulin light chain (25,000) and heavy chain (50,000), and human fibrinogen Aa chain (73,000), Bβ chain (56,000) and γ chain (47,000) as reference standards (8). The gels were scanned on a Gilford linear scanner and the molecular weight was calculated from the gel scans, using pancreatic ribonuclease (13,700) as a migratory front. Five percent polyacrylamide gels were also used for determining the purity of the proteins and their constituents. The buffer system for these gel analyses as well as those used for molecular weight determinations was 0.06 M sodium phosphate buffer, pH 7.2, containing 0.1% Na dodecyl-SO4. Acrylamide gels were run with additional buffer systems: acid urea gels, pH 5.8; 8 M urea, phosphate gels, pH 7.2; 8 M urea, 0.1% Na dodecyl-SO4, Tris-glycine gels, pH 8.3; and 8 M urea, 0.1% Na dodecyl-SO4, phosphate gels, pH 7.2. The acrylamide gel concentration for 5% were for these experiments.

Amino Acid Analyses—The total amino acid composition of rat and human Cm-fibrinogen as well as the isolated Cm-fibrinogen polypeptide chains were determined from 1-kg samples hydrolyzed in 1.0 ml of 5 N HCl at 110°C for 24 hours in a sealed dinitrogen atmosphere, treated with 1.0 M NaOH. The protein was then washed three times using 6 ml of 0.05 M sodium phosphate buffer, pH 7.2, and 500 ml of the final buffer (170 mM sodium acetate, pH 5.3). Each protein region was pooled, dialyzed against several changes of 0.10 M ammonium bicarbonate, lyophilized, and stored at -20°C.

_polymerization_ and the formation of a peptide bond between the epsilon amino group of lysine and the carboxamido group of glutamine on adjacent γ chains, it is possible to identify the γ chain on Na dodecyl-SO4 gel electrophoresis by its conversion to the heavier γ-γ dimer. The faster migrating band of rat and bovine non-cross-linked fibrin is completely converted to the γ-γ dimer in cross-linked fibrin. In comparing bovine Aa chain with rat Aa chain (contained in the rat Aa-Bβ band), it is shown that the latter migrates faster, reflecting a mass difference of approximately 10,000 to 15,000 daltons. Quantitative comparison from gel scans of reduced rat fibrinogen re-

**RESULTS**

The purification of bovine, human, and rat fibrinogen by the procedures previously outlined resulted in essentially pure protein when analyzed on polyacrylamide gels. Purified human, bovine, and rat fibrinogen was at least 93 to 95% clottable. In a control experiment a known plasmin inhibitor (13) was added to the syringe during blood collection. In addition, the inhibitor was maintained throughout the purification process in order to prevent any plasminolytic cleavage. Fibrinogen from hyperfibrinogenemic and normal controls as well as from plasmin-inhibited animals all showed identical chemical and physical properties. Induction to a hyperfibrinogenemic state was performed only in rats, the yield of rat fibrinogen and is accomplished through an incompletely understood acute phase reaction to various trauma states (14, 15).

An electrophoretic comparison of reduced bovine and rat fibrinogen and fibrin reveals a unique characteristic of the latter species (Fig. 1). All three component polypeptide chains were clearly separated in reduced bovine fibrinogen and fibrin, as well as that from other mammalian species observed so far. Reduced rat fibrinogen and fibrin, on the other hand, exhibited only two bands which could not be further resolved on a number of different gel systems. Since fibrin stabilization is mediated through the formation of a peptide bond between the epsilon amino group of lysine and the carboxamido group of glutamine on adjacent γ chains, it is possible to identify the γ chain on Na dodecyl-SO4 gel electrophoresis by its conversion to the heavier γ-γ dimer. The faster migrating band of rat and bovine non-cross-linked fibrin is completely converted to the γ-γ dimer in cross-linked fibrin. In comparing bovine Aa chain with rat Aa chain (contained in the rat Aa-Bβ band), it is shown that the latter migrates faster, reflecting a mass difference of approximately 10,000 to 15,000 daltons. Quantitative comparison from gel scans of reduced rat fibrinogen re-
FIG. 1. Comparative Na dodecyl-SO₄ polyacrylamide (5%) gel electrophoresis of bovine and rat fibrinogen and fibrin. A, bovine fibrinogen; B, rat fibrinogen; C, reduced bovine fibrinogen; D, reduced rat fibrinogen. On gel scans, the Aα-Bβ-γ ratio is 1.85:1. E, reduced cross-linked bovine fibrin; F, reduced cross-linked rat fibrin. Reduction was accomplished by the addition of 2-mercaptoethanol to a 1% concentration. Cross-linked fibrin was prepared by clotting fresh plasma with topical thrombin (Parke, Davis), then washing and reducing the fibrin clot with 2-mercaptoethanol.

veals that there is 1.85 times as much stained material in the Aα-Bβ band than in the γ-band, suggesting an equimolar ratio of the three component chains of fibrinogen.

The molecular weights of rat fibrinogen chains were estimated by 10% acrylamide Na dodecyl-SO₄ gel electrophoresis (Fig. 2), using established molecular weight markers. Rat fibrinogen Aα-Bβ chains had an estimated molecular weight of 51,700, and the γ chain had an estimated molecular weight of 47,500. The summation of the component molecular weights of the peptide chain yields a total molecular weight of 302,000 for the whole rat fibrinogen molecule. No attempt was made to determine the molecular weight of whole fibrinogen on Na dodecyl-SO₄ gel electrophoresis directly, since molecular weights in this size range are generally inaccurate and quite variable according to carbohydrate content (16).

The preparative separation of reduced and alkylated human and rat fibrinogen on CM-cellulose is shown in Fig. 3. Gel electrophoresis of human fibrinogen resolves three peaks; however, rat fibrinogen is resolved into only two symmetrical peaks. Thus, both Aα and Bβ chains appear to be very similar in size as well as in charge, as shown by Na dodecyl-SO₄ gel electrophoresis and in CM-cellulose chromatography, respectively. Fig. 4 shows a comparative electrophoretic analysis of human and rat fibrinogen before and after separation into individual chains.

Quantitative NH₂-terminal analyses of rat fibrinogen, fibrin, and the isolated fibrinogen chains were performed using two different coupling reagents and procedures. The consistent finding in all experiments was the presence of 4 mol of alanine/mol of fibrinogen; 1 mol of alanine/mol (M, 52,000) of Aα-Bβ chains; and 4 mol of glycine/mol of fibrin. Neither isolated γ chains nor fibrinogen or fibrin revealed the presence of any detectable NH₂-terminal group for the γ chain polypeptide. To our knowledge, this constitutes the first report of such a blocked NH₂ terminus of the γ chain of the fibrinogen molecule of any species.

To verify that two different polypeptides were present in the rat Aα-Bβ band observed on Na dodecyl-SO₄ gel electrophoresis and CM-cellulose chromatography, purified Aα-Bβ chains were subjected to NH₂-terminal amino acid sequence analyses. Seven cycles were performed; each cycle yielded two amino acids the identity of which was in agreement with the reported sequences (17) for the A and B fibrinopeptides of rat fibrinogen. These results confirm the presence of two nonidentical chains in the preparations.

The separated polypeptide chains of rat Cm-fibrinogen were generally quite similar in amino acid composition to the
reported compositions of human (see Table I) and bovine fibrinogens (18–20). The values for serine and threonine were derived after extrapolation of the time course of hydrolyses back to zero times, while those values for valine, leucine, and isoleucine were averaged from the 72- to 96-hour hydrolysates only. All other amino acid values are averages from all hydrolysates. Two features of the rat fibrinogen molecule are noticeably different from human fibrinogen. First, published data for human fibrinogen suggest the presence of only 36 disulfide bridges representing both inter- and intrachain disulfide bonds. The amino acid compositions reported here, however, suggest that there may be as many as 42 disulfide bridges in both human and rat fibrinogen. Second, the comparative amino acid composition of the chains of fibrinogen indicates the presence of twice as many cysteine and methionine residues in the rat γ chain as reported in the human γ chain. The difference in methionine content is supported by comparative electrophoretic analysis of rat and human cyanogen bromide cleavage products of rat and human γ chains (Fig. 5). Although the bands are insufficiently distinct to quantitate, it is apparent that extensive differences between the rat and human γ chains exist, with the rat protein exhibiting significantly more bands in agreement with its elevated methionine content. Particular care had been taken during reduction and alkylation of rat and human fibrinogen to avoid the oxidation of methionine residues. Hence the number of cyanogen bromide-cleaved polypeptide chains significantly reflects the number of methionine residues present within the γ chain.

Immunologically, bovine, human, and rat fibrinogen all behaved as classical antigens. When monospecific antibodies directed against bovine, human, and rat fibrinogen were examined by double immunodiffusion gels using plasma from these species, there were varying degrees of immunological cross-reactivity (Fig. 6). All interspecies cross-reactivity tests demonstrated incomplete immunological identity except for the rat fibrinogen antibodies, which did not cross-react with bovine fibrinogen.

**DISCUSSION**

As a result of shortened Aα chains, the molecular weight of rat fibrinogen is less than that reported for other fibrinogens. The 10% decrease in molecular weight is not particularly striking if one considers that the molecular weight of fibrinogen is generally accepted to be 340,000 ± 20,000. It does, however, represent one of the smallest molecular weight sizes reported, although Mosesson et al. (20) have demonstrated certain size and solubility variations in human fibrinogen. Others have demonstrated that the Aα chain of fibrinogen is most susceptible to proteolytic cleavage by plasmin (22). The difference in molecular size of the Aα chain in the rat could be the result of plasmin cleavage. This possibility has been offered to explain the co-electrophoresis of Aα and Bβ chains observed in avian systems (23). However, in the experiments presented here, considerable care was taken to prevent such cleavage. Although limited plasmin cleavage cannot be conclusively ruled...
TABLE I
Comparative amino acid composition of human and rat fibrinogen and their polypeptide chains

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Human³</th>
<th>Rat⁵</th>
<th>Human³</th>
<th>Human⁵</th>
<th>Rat⁵</th>
<th>Human³</th>
<th>Human⁵</th>
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<tr>
<td>Lysine</td>
<td>77</td>
<td>76±3</td>
<td>33</td>
<td>31</td>
<td>31±3</td>
<td>214</td>
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<tr>
<td>Histidine</td>
<td>22</td>
<td>19±3</td>
<td>9</td>
<td>8</td>
<td>10±3</td>
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<tr>
<td>Arginine</td>
<td>59</td>
<td>50±3</td>
<td>18</td>
<td>18</td>
<td>13±2</td>
<td>126</td>
<td>136±4</td>
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<tr>
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<td>--</td>
<td>--</td>
<td>--</td>
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<td>134</td>
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<td>56±3</td>
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<td>Threonine</td>
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<td>56±2</td>
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<td>21</td>
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<td>174</td>
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<tr>
<td>Serine</td>
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<td>22</td>
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<td>234</td>
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<td>Glutamic Acid</td>
<td>120</td>
<td>109±5</td>
<td>44</td>
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<td>11</td>
<td>25±4</td>
<td>160</td>
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<tr>
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<td>94±2</td>
<td>35</td>
<td>33</td>
<td>43±1</td>
<td>274</td>
<td>258±14</td>
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<tr>
<td>Alanine</td>
<td>54</td>
<td>39±6</td>
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<td>24</td>
<td>24±2</td>
<td>126</td>
<td>123±6</td>
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<tr>
<td>Half Cystine</td>
<td>26</td>
<td>-----</td>
<td>8</td>
<td>6</td>
<td>-----</td>
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<tr>
<td>Valine</td>
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<td>16</td>
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<td>105±7</td>
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<tr>
<td>Methionine</td>
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<td>14</td>
<td>16</td>
<td>19±1</td>
<td>80</td>
<td>96±4</td>
</tr>
</tbody>
</table>

³Taken from McKee et al. (18), sulfonated fibrinogen.
⁵Taken from Mills and Liener (19), sulfonated fibrinogen.
Carboxymethylated fibrinogen. Molecular weights are: rat fibrinogen, 302,000; Aα-Bβ chains, 103,000; γ chains, 47,500; human fibrinogen, 330,000.
Proline residues for whole rat fibrinogen molecule are based on the sum of the residues from the individual chains.

Fig. 6. Immunological cross-reactivities of monospecific bovine, human, and rat fibrinogen antibodies with interspecies plasma. A, anti-human fibrinogen (A) versus human fibrinogen (1, 4), bovine plasma (2), rat plasma (3), bovine plasma (5), and ovalbumin (6). B, anti-bovine fibrinogen (A) versus bovine fibrinogen (1, 4), human plasma (2), rat plasma (3), bovine plasma (5), and ovalbumin (6). C, anti-rat fibrinogen (A) versus rat fibrinogen (1, 4), bovine plasma (2), human plasma (3), rat plasma (5), and ovalbumin (6).

out, we consider it unlikely, since all fibrinogen samples purified from many unstimulated as well as stimulated rats demonstrate the same physical and chemical properties. In addition, those experiments in which L-aminocaproic acid was present throughout purification showed the same size and charge properties as the controls. Moreover, antibodies to purified rat fibrinogen behaved with complete immunological identity to the fibrinogen in fresh rat plasma.

A number of investigations have been directed toward NH₂-terminal analyses of fibrinogen (24). One of the most consistent findings has been variations in the NH₂-terminal residues of Aα and Bβ chains from species to species as well as considerable heterogeneity of the A and B fibrinopeptides. Tyrosine is the NH₂-terminal residue of the γ chain in all vertebrates thus far examined with the exception of the two perissodactyls, tapir and rhinoceros, which have leucine and arginine end groups, respectively, and the lamprey, which has a nonacetylated serine NH₂-terminal (25). By inference, one would predict the γ chain NH₂-terminal of the rat to be either an acetylated serine or threonine, or a pyrroolidone carboxylic acid. Although serine could represent a single base mutational change from tyrosine, both arginine and leucine in the two

*P. O'Neill and R. F. Doolittle, personal communication.
perissodactyls represent at least double base changes or a base deletion or addition. This suggests that the NH2-terminal region of the γ chain is not as highly conserved as the thrombin cleavage site of Arg-Gly and theoretically makes the other end groups equally likely. Such a suggestion also has implications on possible modes of fibrinogen biosynthesis and assembly, since one theory (1) proposes the existence of a single polypeptide chain, profibrinogen molecule, in which the conserved NH2-terminal of the γ chain appeared to be indicative of a proteolytic cleavage site resulting in the free γ chain polypeptide.

In addition to the charge and size homogeneity observed in Ace-Bβ chains of rat fibrinogen, in contrast to bovine and human Ace-Bβ chains, the comparative amino acid compositions of the γ chains demonstrate that the rat fibrinogen γ chain is involved in significantly more disulfide bonding than is its human and bovine counterpart. The methionine contents of the γ chains also differ, as verified by cyanogen bromide cleavages, and clearly indicate that the rat fibrinogen molecule is distinctly different from those of other vertebrate species studied so far.

Antibodies directed against rat fibrinogen derived from three different rabbits all showed the same unique immunological patterns. The cross-reactivity results may be explained in part from the realization that the lagomorph and rodent are more closely related phylogenetically than is the lagomorph to either the primates or artiodactyls. Thus, to respond immunologically to rat fibrinogen, the rabbit capitalizes on minor but unique differences which evidently either are not present or are concealed in the bovine molecule, but are present in the human molecule.

The information provided in this report characterizes the rat fibrinogen molecule in regard to a number of chemical features which appear to be unique. More recently, several laboratories have been directing their attention toward understanding how fibrinogen is assembled and how its biosynthesis is controlled. The laboratory rat represents a useful model for studying how fibrinogen synthesis and assembly are regulated. The identification of these unique molecular features as well as the ability to induce fibrinogen synthesis should provide important information for those interested in fibrinogen assembly and biosynthesis using this animal model.

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H Bouma, 3rd and F M Fuller


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