Molecular Characterization of an Abnormal Fibrinogen by Two-Dimensional Electrophoresis

B. Polack, O. Vailron, E. Concord, J. M. Freyssinet, and G. Hudry-Clergeon

We examined normal and abnormal fibrinogen (fibrinogen "Grenoble") by two-dimensional gel electrophoresis to obtain data on possible defects at the molecular level. Fibrinogen Grenoble is characterized by an abnormal rate of fibrin monomer aggregation. The electrophoretic analysis revealed the presence of abnormal \( \gamma \) chains. Two kinds of \( \gamma \) chains can be detected in fibrinogen Grenoble: (a) normal \( \gamma \) chains and (b) \( \gamma \) chains Grenoble (\( \gamma_\text{G} \)) with a greater molecular mass but no modification in isoelectric point. The latter chain can be detected in whole plasma by two-dimensional gel electrophoresis. Metrological analysis was performed in an attempt to quantify observed differences between normal fibrinogen and fibrinogen Grenoble. On use of gels stained either with Coomassie Brilliant Blue or with silver, the partly qualified evaluation gives about 60% normal \( \gamma \) chain and 40% \( \gamma \) chain Grenoble.

Additional Keyphrases: dysfibrinogenemia • heritable disorders • fibrinogen "Grenoble" • clotting • quantification of observed differences

Fibrinogen, a 340 000-Da glycoprotein, is involved in the last step of the coagulation pathway. Its polymerization, after proteolytic activation by thrombin, forms the fibrin network of the blood clot. All fibrinogens from vertebrates are composed of three paired polypeptidic chains, designated A\( \alpha \), B\( \beta \), and \( \gamma \), linked by disulfide bridges (1).

Fibrinogen heterogeneities are of two kinds.

First, there is a heterogeneity in molecular mass ascribable to partial proteolysis of the C-terminal region of the A\( \alpha \) chain. The susceptibility to proteolysis of this C-terminal part of the A\( \alpha \) chain is such that many proteases can catalyze its cleavage, even in circulating blood. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) of purified fibrinogen usually shows two A\( \alpha \) bands differing by 2000 Da (2).

The second kind of heterogeneities involves both post-transcriptional and post-translational modifications. The post-transcriptional modification results in an alteration in the primary sequence of the \( \gamma \) chain: the \( \gamma \) variant, also called "\( \gamma \) chain." This \( \gamma \) chain is characterized by a C-terminal elongation that results in a more acidic isoelectric point and an increase in molecular mass of 1500 Da (3). The \( \gamma \) and \( \gamma \) chains are produced by differences of splicing of the m-RNA. About 8 to 10% of total \( \gamma \) chain is \( \gamma \) chain. The post-translational modifications are responsible for heterogeneities in isoelectric points of the three chains. They involve either different degrees of sulfation and phosphorylation or variations in neuraminic acid content (4, 5).

Dysfibrinogenemia is a generic designation for inherited abnormal molecular forms of fibrinogen, which are distinguished by addition of the name of the city in which they were first discovered. The molecular abnormality may alter the proteolytic activation by thrombin, the polymerization stage, or the stabilization by factor XIIIa (6).

Fibrinogen Grenoble is one such abnormal fibrinogen. We have characterized it by investigations of the biochemical properties of fibrinogen and by two-dimensional gel electrophoresis (2-D GE). The latter revealed the molecular abnormality of fibrinogen Grenoble. Then, in an attempt to evaluate quantitatively the differences visually observed in spots from normal and fibrinogen Grenoble, we performed a metrological study: digitalized examination of 2-D gels.

Propositus, Materials, and Methods

Case summary. A 61-year-old white woman was referred to a hematologist because of abnormal results for routine clotting tests revealing hypofibrinogenemia. No other disorders were evident. Hepatic function was normal. Results of clinical examination were normal. There was no history of abnormal bleeding or thromboembolism (three pregnancies, with one miscarriage).

Shortly after these tests she had pelvic surgery for prolapse with urinary incontinence, with no complications: no bleeding, no thrombosis, and normal wound healing.

Sample collection. Venous blood was collected in one-twentieth volume of a 38 g/L solution of trisodium citrate. Plasma was separated by centrifugation (3000 \( \times \) g, 20 min). Coagulation studies were either performed without delay or samples were stored at \( -70 \) C, with thawing at \( 37 \) C just before assay. For fibrinogen purification, plasma was obtained by plasmapheresis.

Blood clotting. Most of the coagulation tests were routine ones (7). Fibrinogen was determined in plasma by different methods: by heat precipitation (8), kinetically (9), and immunologically (10).

Biochemical characterization. Fibrinogen was purified according to Blömback and Blömback (11). The final buffer was Tris HCl (50 mmol/L, pH 7.5) containing 100 mmol of NaCl per liter. Fibrinogen concentration was determined spectrophotometrically (\( e_{280}^{1\%} = 1.55 \)). "Clottability" was determined by measuring the difference in \( A_{380} \) nm before and after coagulation induced by thrombin. Fibrin polymerization was studied by recording the transmittance at 350 nm (12). Either plasma or purified fibrinogen was used. Fibrin monomers were obtained as described by Gralnick et al. (13) and their aggregation was monitored spectrophotometrically. Kinetics of fibrinopeptide A release was studied according to Jandrot-Perrus et al. (14), but it was quantified immunoenzymatically according to Soria et al. (15) with use of a kit from Diagnostic Stago, Asnières, France. Fibrin cross-linking experiments were performed according to Finlayson and Morton (16) and analyzed by sds-PAGE according to Webber and Osborne (17). Digestion of fibrinogen by

---

1 Laboratoire d'Hématologie, Centre Hospitalier Régional et Universitaire, 217 X 38043 Grenoble Cedex, France.
2 Laboratoire d'Hématologie, Département de Recherche Fondamentale, U 217 INSERM, Centre d'Etudes Nucléaires, 85 X 38041 Grenoble Cedex, France.
3 LETI/MCTE, Centre d'Etudes Nucléaires, 85 X 38041 Grenoble Cedex, France.

Received June 21, 1984; accepted August 13, 1984.
plasmin was studied as described by Denninger et al. (18) and the digest was analyzed by SDS-PAGE.

Electrophoresis. For 2-D GE, samples were prepared by the method of Anderson and Anderson (19, 20). Plasma or purified fibrinogen, 10 μL, was added to 30 μL of the pH 9.5 dissociation buffer containing, per liter, 20 g of sodium dodecyl sulfate, 20 g of dithiothreitol, 100 g of glyceral, and 50 mmol of 2(1-N-cyclohexylamino)ethane sulfonic acid (Sigma Chemical Co., St. Louis, MO). The mixture was heated at 95 °C for 1 min. After cooling, 30 μL of the sample was applied to the isoelectric focusing gel.

2-D GE was according to Anderson and Anderson (19, 20). All reagents were from Bio-Rad Laboratories, Richmond, CA, and were of "electrophoresis" grade. Distilled de-ionized water was used throughout. The ISO-DALT equipment was purchased from Nuclear-Sciences, Oak Ridge, TN.

For the iso dimension the pH gradient was established in 42.4 g/L acrylamide gels containing, per liter, 9 mol of urea, 20 g of NP-40 surfactant, and 50 g of 3-10 ampholytes (Pharmalytes; Pharmacia Fine Chemicals, Uppsala, Sweden). These gels were prefocused at 200 V for 1 h. Then their tops were gently washed and the samples were layered under the catholyte, which was a 20 mmol/L solution of sodium hydroxide. The anolyte was 10 mmol/L phosphoric acid. Focusing was for 10 000 V h.

For the DALT dimension, we used homogeneous 100 g/L acrylamide gels. ISO gels were equilibrated in 4 mL of the SDS mixture (per liter, 100 g of glyceral, 20 g of sodium dodecyl sulfate, 1.3 g of dithioerythritol, 125 mmol of Tris) for 30 min. The gels were stored at −70 °C after equilibration and thawed at 37 °C immediately before being applied to the top of the DALT gels.

For staining we used either Coomassie Brilliant Blue or the silver staining method of Oakley et al. (21). After staining, the gels were soaked in 20 g/L aqueous glycerol and dried under reduced pressure.

Digitalization. For our metrological studies of dried 2-D gels we used an Optronics film scanner, 100 × 100 μm grid, for digitalization. Each pixel yielded a byte (256 gray levels are possible).

Results

Biochemical characterization. The only coagulation test for which results were abnormal was that for fibrin formation. The concentration of fibrinogen in the plasma was significantly abnormal when measured by heat precipitation or kinetically (Table 1). For other tests the values were normal.

Clottability at an ionic strength of 150 mmol/L was estimated to be 61% for purified propositus fibrinogen vs 94% for normal fibrinogen. Decreasing the ionic strength increased the clottability of fibrinogen Grenoble to 89% at 37.5 mmol/L.

Curves for normal plasma and plasma from the propositus, for fibrin polymerization induced by thrombin, were quite different. Indeed, the polymerization reaction of the control plasma was over before that of the propositus' plasma has started. Curves for polymerization of purified fibrinogen Grenoble showed only a subnormal rate and a lower final absorbance, but at an ionic strength of 75 mmol/L the kinetic profile was almost within normal limits. Fibrin monomers also aggregated at a decreased rate and the final absorbance was lower than for the control. Measurement of release of fibrinopeptide A showed no difference between normal fibrinogen and fibrinogen Grenoble. SDS-PAGE analysis did not reveal any significant differences for fibrin cross-linking by factor XIIIa. Fibrinogen degradation products obtained by plasmin proteolysis did not exhibit detectable changes.

2-D GE analysis of fibrinogen was the only technique by which we could reveal directly the nature of the fibrinogen Grenoble abnormality (Figure 1), showing important differences between normal fibrinogen and fibrinogen Grenoble. The AA and BB chains were almost identical with respect to molecular mass and isoelectric point, but the γ chains were split into two, showing normal γ and γ' chains as well as abnormal γ0 and γγ chains. The abnormal γ0 and γγ' chains showed no change in isoelectric point, but their molecular mass was greater than normal. The difference in molecular mass between γ and γ0 chains and between γ and γγ' chains seemed to be identical (1200 to 1300 Da).

Between γ0 and γγ' chains we found the same increase in molecular mass as between γ and γ' chains, about 2200 Da. The splitting of γ chains was also visible in 2-D GE of whole plasma, even when the AA and BB chains were not well resolved.

Metrological study of fibrinogen Grenoble. Figure 2 shows the diagram of a gel and an identification for each spot.

Gels stained with Coomassie Brilliant Blue. Using purified fibrinogen, we measured absorbance in the 0 to 2 A range. Gray level 0 corresponds to 0 A for the background; gray level 100 corresponds to 0.78 A for the darkest spots. Possible gray levels range from 0 to 255, so the entire dynamics of measure was not used.

Weakly stained parts of the gels were not detected and therefore do not appear on digitalized images. This was sometimes the case for Aα2 chains and spot 1 from Bβ chain, and always so for the γ' chain variant. It was also true for streaks between spots.

For each visible spot, the sum of the gray levels was computed for its whole surface. Theoretically, this value is proportional to the quantity of protein if the amount of protein-bound dye is proportional to the quantity of protein.

Table 1 gives the results we obtained for chains Aα, Bβ, and γ on three gels P-NORM 1, 2, and 3 (from normal fibrinogen) and on three gels P-GRE 1, 2, and 3 (from fibrinogen Grenoble). These results were normalized, assuming the sum of gray levels for each chain to be equal to 100. In Table 2, the results for the Aα, Bβ, and γ chains show fluctuations from one gel to another. Evaluation of scanner reproducibility showed that these variations are indeed ascribable to the gels themselves.

We estimate that the γ0 chain makes up about 40% of the total γ chain of fibrinogen.

For P-NORM 3 and P-GRE 2, where all the spots were available, the sum of gray levels was computed. Table 3 shows these values normalized, based on the total sum of gray levels (Aα + Bβ + γ) being equal to 100.

Silver-stained gels. Using purified fibrinogen and plasma, we measured these gels in the 0 to 3 A range. The values cover the whole measurement dynamics. Anything visible to the eye on the gels would appear also on the digitalized images, including the weakest spots, streaks, and other artefacts. Images were not filtered with a view to subtracting background or artefacts, so results are given with an error of about 5%.

---

Table 1. Fibrinogen as Measured by Three Techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Propositus</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen, g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat precipitation</td>
<td>1.2</td>
<td>2–4</td>
</tr>
<tr>
<td>Kinetics</td>
<td>0.5</td>
<td>2–4</td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td>2.1</td>
<td>2–4</td>
</tr>
</tbody>
</table>
Fig. 1. Two-dimensional gel-electrophoretogram of normal fibrinogen (A), fibrinogen Grenoble (B), normal plasma (C), and propositus plasma (D)

Fig. 2. Two-dimensional gel electrophoretogram of purified fibrinogen Grenoble
Table 2. Dye-Stained Gels: Sum of Gray Levels for Each Spot of the Aα, Bβ, and γ Chains of Normal Fibrinogen (F-norm) and Fibrinogen "Grenoble" (F-gre) (Three Gels of Each)

<table>
<thead>
<tr>
<th>Spots</th>
<th>Aα</th>
<th>Bβ</th>
<th>γ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>F-NORM 4</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>34</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>16</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>10</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>F-GRE 4</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>19</td>
<td>25</td>
<td>13</td>
<td>17</td>
</tr>
</tbody>
</table>

No entry for non-exploitable spots (presence of background, streaks, artefacts or overlapping spots).

Table 3. Dye-Stained Gels: Sum of Gray Levels for Each Type of Chain

<table>
<thead>
<tr>
<th>Chains</th>
<th>Aα</th>
<th>Bβ</th>
<th>γ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel F-NORM 3</td>
<td>31</td>
<td>46</td>
<td>23</td>
<td>100</td>
</tr>
<tr>
<td>Gel F-GRE 2</td>
<td>37</td>
<td>39</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Mean value</td>
<td>34</td>
<td>42.5</td>
<td>23.5</td>
<td></td>
</tr>
</tbody>
</table>

Fibrinogen

For each spot, the sum of gray levels was computerized on its whole surface, when outlines were well defined. It was possible to delineate a "window" including all spots of a chain, even those for which outlines were not clear.

Table 4 summarizes our results for Aα, Bβ, and γ chains for two gels F-NORM 4,5 (normal fibrinogen) and two gels F-GRE 4,5 (fibrinogen Grenoble). These results were normalized on the basis of the sum of gray levels of the same window being equal to 100.

Table 5 shows the ratios between chains (we normalized values assuming the total sum of gray levels for Aα + Bβ + γ to be equal to 100).

For Aα chain spots, a maximum intensity had probably been reached for the silver stain.

There are inter-gel variations that cannot be explained easily, because no relationship is evident between the amount of fixed silver and the quantity of protein. Thus weak spots can be overestimated while strong ones can be underestimated.

As can be deduced from Table 4, γb chain represents about 45% of total γ chain.

Table 4. Silver-Stained Gels: Sum of Gray Levels (Normalized) for Each Spot of the Aα, Bβ, and γ Chains of Normal Fibrinogen (F-norm) and Fibrinogen “Grenoble” (F-gre)

<table>
<thead>
<tr>
<th>Chain</th>
<th>Shope</th>
<th>Gels</th>
<th>Aα</th>
<th>Bβ</th>
<th>γ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>F-NORM 4</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>*</td>
<td>13</td>
<td>11</td>
<td>14</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>*</td>
<td>7</td>
<td>13</td>
<td>10</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>12</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>F-GRE 4</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>*</td>
<td>11</td>
<td>8</td>
<td>10</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

Plasma

In GE of plasma, γ chains are located in a region where only few proteins are revealed. Aα and Bβ chains are much more difficult to identify. Thus the abnormality in fibrinogen Grenoble can be directly detected.

Sums of gray levels (normalized) are shown in Table 6.

Discussion

Dysfibrinogen Grenoble is asymptomatic. The associated functional defect appears to be an alteration of the aggregation of fibrin monomers. Liberation of fibrinopeptide A by thrombin, stabilization by factor XIIIa, and degradation by plasmin are all nearly normal.

2-D GE allowed detection of abnormal γ chains. In normal fibrinogen, two forms of γ chains have been found, γ and γ' chains (3). In fibrinogen Grenoble, two additional chain variants are also present: γ3 and γ5' chains. The molecular

Table 5. Silver-Stained Gels (Purified Fibrinogen): Sum of Gray Levels of the Three Chains (Total Window)

<table>
<thead>
<tr>
<th>Chains</th>
<th>Aα</th>
<th>Bβ</th>
<th>γ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>F-NORM 4</td>
<td>43</td>
<td>29</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>F-NORM 5</td>
<td>48</td>
<td>33</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>F-GRE 4</td>
<td>49</td>
<td>30</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>F-GRE 5</td>
<td>46</td>
<td>30</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Mean value</td>
<td>46.5</td>
<td>30.5</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Total window: Sum of gray levels in a window, including the spots of the whole chain. *: non-exploitable spots.
mass of the γ₀ and γ₁ chains is about 1250 Da greater than that of normal γ and γ₁ chains, probably because of an elongated polypeptic sequence in both γ₀ and γ₁ chains. Because there is both an abnormal γ chain and a polymerization defect, the elongation of the γ chains might be confined to the C-terminal part, which is involved in fibrin monomer aggregation. The C-terminal part of normal γ chains is also involved in the stabilization by factor XIIIia. Because fibrinogen Grenoble shows normal stabilization, the portion of abnormal elongation responsible for it should then differ from the portion responsible for the stabilization.

In our metrological analysis, we attempted to quantify the three kinds of chains—Aα, Bβ, and γ—in purified normal fibrinogen and fibrinogen Grenoble after use of two staining techniques, dye and silver staining.

For technical reasons, not all spots were so exploitable: artefacts on images (background, streaks) or variations on individual gels. Some data are thus incomplete (Aα₂ chains or γ chain variant), and this study concerns the main spots.

Results differ according to staining procedure. Table 7 shows, for each chain, the ratios of staining with Coomassie Brilliant Blue or silver. Theoretically, these ratios should be identical for each chain.

For fibrinogen Grenoble the partly qualified evaluation gives about 40% of γ₀ chain and 60% of normal γ chain and reveals an heterozygous status.

This analysis was also performed with normal plasma and plasma Grenoble. We have tried to quantify spots of fibrinogen γ chains. The ratios differ somewhat from those obtained with purified fibrinogens, but they remain within the limit of error of the measurement.

We conclude that our study describing the characterization of a molecular variant of a plasma protein by 2-D GE provides another example of the potential of this high-resolution technique (22, 23). The detection is even possible with whole plasma as the sample. Once the parameters of reproducibility are well controlled in 2-D GE, reliable results can be obtained relatively quickly.

This work was supported by the Institut National de la Santé et de la Recherche Médicale under contract no. GR-771.183.

### Table 6. Silver-Stained Gels (Plasma): Sum of Gray Levels of the Spots of γ-Chain

<table>
<thead>
<tr>
<th>Chains</th>
<th>γ₀</th>
<th>γ₁</th>
<th>γ₀ + γ₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma norm</td>
<td>42</td>
<td>58</td>
<td>—</td>
</tr>
<tr>
<td>Plasma GRE</td>
<td>29</td>
<td>36</td>
<td>17</td>
</tr>
</tbody>
</table>

### References


