The Ultrastructure of Fibrinogen Caracas II Molecules, Fibers, and Clots*

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Fibrinogen Caracas II is an abnormal fibrinogen involving the mutation of αa serine 434 to N-glycosylated asparagine. Some effects of this mutation on the ultrastructure of fibrinogen Caracas II molecules, fibers, and clots were investigated by electron microscopy. Electron microscopy of rotary shadowed individual molecules indicated that most of the αC domains of fibrinogen Caracas II do not interact with each other or with the central domain, in contrast to control fibrinogen. Negatively contrasted Caracas II fibers were thinner and less ordered than control fibers, and many free fiber ends were observed. Scanning electron microscopy of whole clots revealed the presence of large pores bounded by local fiber networks made up of thin fibers. Permeability experiments also indicated that the average pore diameter was larger than that of control clots. The viscoelastic properties of the Caracas II clot, as measured by a torsion pendulum, were similar to those of control clots. Both the normal stiffness and increased permeability of the Caracas II clots are consistent with the observation that subjects with this dysfibrinogenemia are asymptomatic.

Fibrinogen is a plasma glycoprotein with a molecular mass of 340,000 daltons. The molecule consists of three pairs of non-identical polypeptide chains known as αa (64,000 daltons), Bβ (56,000 daltons), and γ (47,000 daltons). These polypeptide chains are linked by 29 disulfide bonds and form two identical halves to the fibrinogen molecule, each half being comprised of one set of αa, Bβ, and γ chains. The chains are folded into several domains to form an elongated molecule 47.5 nm in length. There is a globular central domain containing the amino termini of all six chains linked by disulfide bonds to form a “disulfide knot.” Rod-like regions extend from either side of this central domain and consist of three-stranded α-helical coiled-colls that terminate in two outer nodules. Each αa chain extends from the outer domains to eventually terminate in a globular carboxyl-terminal region known as the αC domain. The two αC domains have been shown to be bound both to each other and to the central domain in normal fibrinogen under physiological conditions (1, 2).

Thrombin, a proteolytic enzyme, cleaves two pairs of peptides from the αa and Bβ chains in the central domain of fibrinogen, called fibrinopeptides A and B, to produce the fibrin monomer. This peptide cleavage exposes binding sites in the central domain that interact with complementary binding sites in the outer domains of other fibrin monomers in a half-staggered arrangement. Fibrin monomers spontaneously polymerize initially to form dimers, followed by longer two-stranded structures termed protofibrils. Lateral aggregation of protofibrils eventually yield fibrin fibers that have a characteristic pattern of striations with a periodicity of 22.5 nm as observed by electron microscopy. These fibers can also undergo lateral aggregation to form thicker fiber bundles. Branching of fibers and fiber bundles result in the formation of a three-dimensional network that is the basic structural framework of the blood clot.

Studies on the mechanism of fibrin polymerization have shown that sequential cleavage of fibrinopeptide A, followed by fibrinopeptide B, is important for the formation of a normal clot structure (3, 4). The release of fibrinopeptide A allows the initial formation of two-stranded protofibrils. Cleavage of fibrinopeptide B is delayed until the fibrin molecules have been incorporated into protofibrils, at which point lateral aggregation to form fibers is enhanced. Fibrinopeptide B cleavage also releases the αC domains from their intramolecular binding sites on the central domain (2). It has been postulated that the αC domains enhance lateral aggregation by intermolecular binding to other αC domains located on adjacent protofibrils, hence pulling the protofibrils together (2). Several studies have provided evidence to support this proposed role for the αC domains in lateral aggregation of protofibrils. Different preparations of fragment X, missing the αC domains, have impaired lateral aggregation (2, 5, 6). Purified fragment Xa monomer, which consists of fibrin monomer lacking both of the αC domains, exhibits a decreased rate of lateral aggregation when compared with fibrin monomer containing both αC domains (2). However, these results also indicated that although the αC domains accelerate the polymerization of fibrin and influence the final structure of the clot, they are not essential for either branching or lateral aggregation. When the αC domains are blocked by αC fragments (1, 7) or monoclonal antibodies specific for the αC domain (8), both the rate and extent of lateral aggregation are decreased. The latter effect, which results in thinner fibers, suggests that modification of the αC domains, as opposed to their removal, may have an additional inhibitory effect on fibrin polymerization.

Several dysfibrinogenemias have been reported that involve mutations in the αC domain, including fibrinogen Caracas II (Aα Ser434 to N-glycosylated Asn) (9), fibrinogen Dusart (Aα Arg554 to albumin-linked Cys) (10), and fibrinogen Marburg (Aα 461-611 missing) (11). All of these mutant fibrinogens appear to be defective in lateral aggregation. Other substi-
tions have also been identified in this region of the Aα chain, but their effects on fibrin polymerization have not been defined (12, 13). The Caracas II and Dusart fibrinogens have additional material on the carbohydrate domains, carbohydrate and albumin, respectively, and both produce fibers that are thinner than normal. Extensive studies have been carried out on the ultrastructure of molecules, fibers, and clots from fibrinogen Dusart, in addition to an investigation of the biomechanical properties of the final clot structure (10, 14–16). These studies have revealed a lack of interactions between the carbohydrate domains of fibrinogen Dusart, the formation of very thin fibers that exhibit less order than normal, and a clot structure that has very short distances between branch points resulting in greatly diminished pore sizes. The viscoelastic and permeation properties of the Dusart clots show a marked increase in stiffness and a much lower permeation rate, reflecting the above ultrastructural observations. Clinical symptoms of subjects who have the Dusart syndrome include a high incidence of thromboembolism and a resistance to thrombolysis, both of which can be related to the defective clot structure. In contrast, fibrinogen Caracas II is a congenital dysfibrinogenemia found in an asymptomatic subject and was originally discovered through a prolonged thrombin time observed in a routine coagulation test (17). The rate of lateral aggregation of protofibrils, as observed by turbidity measurements, was lower than normal, and the final clot was translucent. The fibers formed from this mutant fibrinogen were shown to be thinner and more disordered than normal, although striations were still discernible (18). The defect in the fibrinogen Caracas II molecule was found to be an N-glycosylated asparagine substitution for serine at position 434 of the Aα chain, with the majority of the oligosaccharide consisting of a disialylated biantennary structure (9).

There is considerable evidence that the similar carbohydrate moieties normally present on the Bγ and γ chains of fibrinogen molecules have an inhibitory effect on the polymerization process. Hyperglycosylated fetal fibrinogen aggregates more slowly than normal with reduced turbidity in the final clot (19, 20). Removal of the sialic acid residues from either fetal (21–23) or normal adult (24–26) fibrinogen results in faster polymerization, higher final turbidity, and thicker fibers. Complete de-glycosylation of fibrinogen also promotes lateral aggregation to form thicker fibers, along with a significant reduction in the degree of branching (27). Fibrinogen Caracas II not only has a higher sialic acid content than normal, which would be expected to have an adverse effect on the extent of lateral aggregation, but this additional carbohydrate is also located on the carbohydrate domains, which have been directly implicated in the process of lateral aggregation itself.

The study presented here involves an ultrastructural investigation of fibrinogen Caracas II molecules, fibers, and clots to elucidate the effects this mutation has on the structure and function of fibrinogen at the molecular level. Viscoelastic and permeation properties of the gel network were also measured to relate the ultrastructural observations to the overall physical behavior of the clot. The results provide insight into both the role of the carbohydrate domains in clot formation and the basis for the lack of clinical symptoms exhibited by the fibrinogen Caracas II subjects.

**EXPERIMENTAL PROCEDURES**

Fibrinogen was purified from plasma prepared from the blood of the subject and a normal individual as described previously (9).

Electron Microscopy of Individual Molecules—Fibrinogen samples were prepared for rotary shadowing by spraying a dilute solution in 70% glycerol, 50 mM ammonium formate at pH 7.4 onto freshly cleaved mica. Shadowing was carried out with tungsten in a vacuum evaporator (Denton Vacuum Co., Cherry Hill, NJ) (1, 28, 29), and the specimens were examined in a Philips EM 400 transmission electron microscope (Philips Electronic Instruments Co., Mahwah, NJ) at 80 kV.

Electron Microscopy of Negatively Contrast Fibrin—Thrombin was added to 0.5 mg/ml fibrinogen to a final concentration of 0.35 NIH units/ml in 0.15 M NaCl, 5 mM CaCl2, 50 mM Tris-HCl, pH 7.4, and the mixture was stirred occasionally by gentle agitation with a pasteur pipette to prevent gel formation. An aliquot of the mixture was left undisturbed to determine when clot formation was completed. A drop of the fibrinogen suspension was transferred to a 500 mesh copper grid coated with a thin carbon film, rinsed with several drops of buffer, and negatively stained with 2% uranyl acetate. The specimens were observed in a Philips EM 400 electron microscope operating at 80 kV. Fiber diameters were determined by direct measurement of the negatives projected in the screen of a Datamate 400 Microfiche Reader (Micromage Display, San Diego, CA), with measurements being made only on single fibers that were clearly not aggregated into bundles.

Viscoelastic Measurements—Clots were prepared for scanning electron microscope experiments by fixation, dehydration, critical point drying, and sputter coating with gold as described previously (27, 30). The clots were formed by the addition of thrombin to 0.5 mg/ml fibrinogen to give a final concentration of 2 NIH units/ml, and clotting was allowed to proceed for at least ten times the gelation time in 0.15 M NaCl, 50 mM CaCl2, pH 7.4. Specimens were observed using an Amray 1400 microscope.

**Viscoelastic Measurements—** Clots with a volume of 0.115 ml were formed between 12-mm diameter glass coverslips in a Plazek torsion pendulum (31–33) by adding thrombin to 0.5 mg/ml fibrinogen to a final concentration of 0.35 NIH units/ml in 0.15 M NaCl, 5 mM CaCl2, 50 mM Tris-HCl, pH 7.4. The dynamic storage modulus (G') and loss modulus (G'') were calculated from recordings of the free oscillations induced by the application of a momentary impulse to the pendulum at room temperature (15, 33).

Permeation Measurements—Thrombin (final concentration, 1 NIH unit/ml) was added to fibrinogen Caracas II or control fibrinogen at a concentration of 1 mg/ml in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM CaCl2. Immediately after mixing, 100 µl of solution was transferred to a pre-etched plastic tube with one end sealed with parafilm; the tubes were left in a moist environment for 2 h for clot formation to proceed to completion.

Permeation measurements were made as described previously (34). Tubes containing the gels were placed in a holder and connected via plastic tubing to a reservoir containing the buffer described above. Permeation experiments were performed at different pressure heads, where the pressure was determined by the vertical distance between the buffer reservoir and the tip of the gel; pressure was kept constant during the experiment. A dye, bromphenol blue, was applied to the clot after each experiment to detect leaks between the gel and the walls of the tube or defects in the clot itself, which were grounds for discarding the results from any such gel. Such defects could be detected as a nonuniform progression of dye through the gel. Average rates of flow were determined by multiple measurements of the volume of liquid eluting from the gel in a given period of time. Accurate measurements of the small volumes involved were made by weighing the eluate on an analytical balance. Flow rate (in ml/h) plotted against the pressure head (in dynes/cm²) gave a straight line for any one clot. In these experiments, flow rates at different pressure heads were determined in random order to avoid any potential effects of compression of the gel, although such effects were not generally observed. In most experiments, the results were highly reproducible, with standard deviations of about ±5%. Occasionally, the formation of a defect in a clot (later noticed by the dye) caused a striking increase in flow rate during the course of multiple experiments; the results of these experiments were discarded.

The permeation coefficient or Darcy constant, which represents the pressure drop through a gel, was calculated from the flow measurements, pressure, and geometric parameters of the clot (35, 36).

\[ K_s = \frac{Q \cdot L}{h \cdot A \cdot \Delta P} \]

where Q is the volume of liquid (in ml) having the viscosity \( \eta \) (in poise), flowing through a gel with length L (in cm) and cross-sectional area A (in cm²) in time t (in seconds) under differential pressure \( \Delta P \) (in dyne/cm²). The resulting Darcy constants (\( K_s \)) are in units of cm². In our experiments, L = 1.3 cm, A = 0.061 cm², \( \eta = 10^{-3} \) poise; Q/t was measured for different values of \( \Delta P \).
RESULTS

Ultrastructure of Individual Fibrinogen Molecules—Fibrinogen Caracas II molecules were rotary shadowed with tungsten and examined by electron microscopy (Fig. 1, A–E). The αC domains of most normal fibrinogen molecules interact with the central domain, so they are usually not visible in electron micrographs. The results of observations from several preparations are summarized in Table I. In the control fibrinogen sample about 78% of the molecules were simply trinodular with no discernible extra mass, whereas about 19% showed one or two small nodules near the ends, and 3% showed a larger nodule near the central domain. In contrast, only 46% of the molecules in the Caracas II sample exhibited a simple trinodular structure (Fig. 1B), whereas 48% have one or two additional small nodules adjacent to the molecular backbone (Fig. 1, D and E), and about 6% had a single additional large nodule near the center (Fig. 1C).

Substructure of Negatively Contrasted Fibers—Fibers formed from fibrinogen Caracas II had an average diameter of 48 ± 11 nm (mean ± S.D. of 275 measurements), considerably below the value of 82 ± 19 nm for the control fibers. We measured the diameter of individual fibers only and were careful to omit any fiber bundles from our data. Caracas II fibers exhibited a greatly reduced tendency to form bundles relative to the control fibers (Fig. 2, A and B), which facilitated accurate measurement of their diameters.

The substructure of Caracas II fibers (Fig. 2, D–K) exhibited marked regional heterogeneity as they varied in appearance along their lengths to a much greater extent than control fibers (Fig. 2C). In regions where cross-striations were visible, they were not as prominent as normal, although the spacings within the band pattern were essentially normal, as was the periodicity of 22.5 nm (Fig. 2, D and E). Commonly, longitudinal strands were visible in these regions and probably corresponded to individual protofibrils. A decrease in protofibrillar order apparently contributed to the reduction in the clarity of the cross-striations. Striations were observed over a wide range of diameters and usually extended across the complete width of the fibers. Therefore, ordering did not appear to be correlated with fiber width. Some regions exhibited very pronounced longitudinal strands that retained a fairly high degree of parallel order but lacked discernible striations (Fig. 2F). Other regions showed neither transverse nor parallel order and had relatively wide spaces between the protofibrils, and thus displayed an open “spongy” or “frayed” appearance (Fig. 2G and H). However, it should be noted that in spite of the regional variation along the fibers from a relatively ordered to a very disordered appearance, there was considerable uniformity in substructure across the width of a fiber regardless of the overall nature of the particular region (Fig. 2K).

Another important difference between Caracas II and control fibers was the frequent occurrence of fiber ends within the dysfibrinogenemic sample. Some fibers retained considerable order toward their termini to form a relatively smooth tapered end (Fig. 2I), whereas others became increasingly disordered and terminated with a very frayed appearance (Fig. 2J).

Structure of Whole Clots—Clots formed from fibrinogen Caracas II (Fig. 3, B–D) were also very different in appearance from control clots (Fig. 3A) when examined by scanning electron microscopy. Although there was considerable variation, many fibers were thinner in diameter than normal, and there was less aggregation to form bundles; both of these observations are consistent with the transmission electron microscopy results. In some areas it appeared that the fibers may have aggregated to form bundles that were thicker than in the control clot. However, on closer inspection, these apparent large bundles actually consisted of closely spaced networks of much thinner fibers.

However, the most striking feature of these dots was the presence of large pores or open areas bounded by local fiber networks. The largest of the pores had the appearance of caves or tunnels bounded by long, curved bundles of fibers (Fig. 3B). Along the walls of these channels were a mixture of fine fiber meshworks together with smaller pores (Fig. 3C). Free fiber ends were commonly observed, particularly at the boundary between the fiber meshworks and the pores (Fig. 3D). Owing to the extreme heterogeneity of the Caracas II clot structure, it was not possible to obtain meaningful measurements of average fiber bundle diameters or mean distance between branch points.

Viscoelastic Properties of Whole Clots—A torsion pendulum can be used to measure directly the viscoelastic properties, including the stiffness, of clots. In the method used in this study, viscoelastic parameters are derived from the measurement of a series of damped harmonic oscillations that occur during the recovery of a specimen from the deformation induced by an applied torsional stress.

For a fibrinogen concentration of 0.5 mg/ml, the frequency of free oscillation was ~0.3 radian/sec for both the Caracas II and control clots. The storage modulus (G’) was 20 dyne/cm² for the
Caracas II clot and 28 dyne/cm² for the control clot, whereas the loss modulus (G') was 3 dyne/cm² in both cases. The storage modulus is a measure of the stress stiffness, and the loss modulus is a measure of the energy dissipated by nonelastic, viscous processes. Thus, the viscoelastic properties of the clots formed from fibrinogen Caracas II were similar to those of the control clots, with the storage modulus for Caracas II being about 70% of the control value, and there was no discernible difference between the respective loss moduli.

Permeation Properties of Whole Clots—At a differential pressure of 2650 dyne/cm², the average flow rate in clots made with fibrinogen Caracas II was 7.92 ml/h, compared with control clots made under the same conditions, which had an average flow rate of 0.48 ml/h. The flow rates were a linear function of pressure, although the fibrin Caracas II clots sometimes developed defects, such as channels along the walls or in the middle of the gel, at lower pressures than control clots. The Darcy constant (Kₐ), which is a reflection of the surface area of the clot available for flow, was 17.8 x 10⁻³ cm² for clots from fibrinogen Caracas II and 1.08 x 10⁻³ cm² for control clots, a 16.5-fold difference.

**DISCUSSION**

Localization of αC Domains on Fibrinogen Caracas II Molecules—With control fibrinogen, most molecules appear as simple trinodular structures and only a small proportion exhibit extra nodules (1, 2). However, a significant proportion of the molecules of fibrinogen Caracas II displayed additional mass located away from the trinodular backbone. These observations of individual molecules suggest that the modified αC domains of fibrinogen Caracas II, unlike those of control fibrinogen, do not interact with the central nodule of the molecule or with each other. A similar lack of αC domain interaction has been observed with fibrinogen Dusart molecules, in which an albumin molecule is covalently bound within this region (15). In the case of fibrinogen Caracas II, the prevention of αC domain interactions could be a result of both the additional bulk of the carbohydrate groups and the electrostatic repulsion between the negative charges on the sialic acid.

In a prior study of fibrinogen Caracas II (9), the results were consistent with approximately half of the αC domains being abnormal and half being normal. The evidence (Table I) suggests that the polypeptide chains are assembled such that half of the molecules are completely normal and half contain two abnormal αC domains, because it was observed that about 50% of the molecules of fibrinogen Caracas II displayed normal morphology. A random distribution of chains would result in only 25% of the molecules having completely normal αC domains, whereas 25% would be completely abnormal and 50% would have one abnormal Aα chain.

Substructure of Fibrin Caracas II Fibers—The values we obtained for the average diameters of the Caracas II fibers (48 nm) and control fibers (82 nm) appear to be somewhat at variance with those of Marchi et al. (18), who reported values of 79 and 92 nm for the average diameters of Caracas II and control fibers, respectively, but there are important differences between these two sets of data. Firstly, we measured the diameter of individual fibers and not fiber bundles. Secondly, Marchi et al. (18) measured a sample of only 10 fibers because their data were just part of a broad preliminary screening of several different dysfibrinogenemias. Our sample consisted of 275 fibers, which is a sufficiently high number to reduce any tendency toward weighting of larger, hence more prominent, fibers.

Although the fibers prepared by the addition of thrombin to fibrinogen Caracas II were generally thinner, less ordered, and more variable in appearance than normal, it appears that most molecules in these fibers must still be arranged in a half-staggered manner because the striation spacings and general band pattern are not affected by the mutation. There are often larger spaces between the protofibrils than normal, resulting in fibers that are more open in structure and hence of lower density (Fig. 2, G and H). This may indicate that the extra charge and increased bulk of the carbohydrate on the αC domains prevents the protofibrils from packing close together in the fiber. The modification of the αC domains may also account for the reduction in the average fiber diameter to about half of the thickness of control fibers, because the weaker intermolecular interactions between the αC domains would be expected to inhibit lateral aggregation of the protofibrils.

The appearance of Caracas II fibers varies considerably along their lengths, from relatively ordered to very disordered (Fig. 2K). It is possible that this variability arises from the presence of differing amounts of abnormal molecules in different regions of the fibers, because it is likely that these molecules will assemble more slowly than the normal molecules in these heterozygous individuals. The presence of many fiber ends is also consistent with molecular segregation, because the abnormal molecules may be impeding further growth by “capping” protofibril ends.

Structure and Biophysical Properties of Fibrin Caracas II Clots—Whole clots from fibrinogen Caracas II were dramatically different from control clots when examined by scanning electron microscopy. The most striking characteristic was the presence of large pores or channels in the clots, often bounded by long curving fibers of fiber bundles with associated meshworks of thin fibers. It seems that these pores may be gaps in the gel network, because fiber ends are seen along the pore edges as if the shorter fibers do not allow the formation of a complete fiber network. A greater proportion of Caracas II fibers than normal appeared to be curved, indicating increased flexibility, which may be a result of the thinner fiber diameter and the relatively loose association of the constituent protofibrils. The heterogeneity of the Caracas II networks gives the clots a very open lacy appearance, which could also partially account for the translucent nature of these clots (17). Mathematical treatments of the relationship between clot structure and turbidity assume a homogeneous gel and relate turbidity only to fiber diameter (37). In this case, however, low turbidity may also arise from the decreased fiber density and the large pore sizes observed in the clots.

The viscoelastic properties of the clots formed from fibrinogen Caracas II were similar to those of the control clots, espe-
**Fig. 2.** Electron micrographs of negatively contrasted fibrin fibers. A, fiber network formed from fibrinogen Caracas II. Some examples of free fiber ends are indicated by arrowheads. B, fiber network formed from control fibrinogen. Bar, 1 μm. C, control fibrin fiber that exhibits a
cially with respect to the storage modulus ($G'$), which differed from the control by only about 30%, indicating a similar degree of stiffness between the two types of clot. These results are somewhat surprising when the marked difference between the respective clot structures is taken into consideration. For example, a six-fold difference was observed between both the $G'$ and $G''$ values for clots formed from Dusart plasma and control plasma (15). However, the Caracas II results are consistent with the absence of clinical symptoms observed in the Caracas II subject because there is evidence to suggest that the mechanical properties of clots are related to the propensity of individuals toward thrombotic disease (38, 39).

Although some of the mechanisms responsible for the elasticity of fibrin clots have yet to be elucidated (40), it is possible that the thinner fibers, the relative abundance of free fiber ends, and the spaces left by the large pores in the Caracas II

**Fig. 3.** Scanning electron micrographs of fibrin clots. A, clot formed from control fibrinogen. B, clot formed from fibrinogen Caracas II that shows distinct large pores bounded by smaller secondary networks. C, clot formed from fibrinogen Caracas II that shows thin fibers forming a secondary network. D, clot formed from fibrinogen Caracas II that shows many examples of free fiber ends (indicated by arrowheads). Bar, 5 μm.

distinctive band pattern with a 22.5-nm repeat. E, fibrin Caracas II fiber that shows longitudinal striations that almost obscure the 22.5 nm repeat. F, thin fibrin Caracas II fiber that still shows a 22.5 nm repeat. F, thin fibrin Caracas II fiber that lacks a 22.5 nm repeat but still has distinct edges. G, fibrin Caracas II fiber that lacks a 22.5 nm repeat and has frayed edges and readily discernible gaps between the protofibrils. H, fibrin Caracas II fiber that has a similar appearance to that in G but also exhibits protofibrillar branches (indicated by arrowheads). I, fibrin Caracas II fiber that shows a tapered end. J, fibrin Caracas II fiber that shows a frayed end. Bar, 100 nm. K, fibrin Caracas II fiber that provides an example of regional structural variation. Bar, 200 nm.
The greater degree of branching in the Caracas II clot. The clot structure would contribute to a decrease in clot stiffness and thus lower the value of $G'$. However, this decrease may essentially be offset by an increase in stiffness resulting from the greater degree of branching in the Caracas II clot. The similar values for the loss modulus ($G''$) exhibited by the Caracas II and control clots indicate that energy dissipation by nonelastic, viscous processes is the same in both cases. It has been suggested that the primary contribution to $G'$ arises from slippage between protofibrils (32, 41-43). In the case of Caracas II, there is likely to be more slippage because of weaker interprotofibrillar interactions, but this may be offset by fewer opportunities for slippage as a result of the thinner fibers.

Permeation, a direct measure of the bulk transport of fluid through a gel, is related to the clot structure. We have demonstrated by scanning electron microscopy that clots made from fibrinogen Caracas II have very large pores compared with control clots. Such pores would be expected to yield very high flow rates. From the electron micrographs, the average pore diameter of the larger pores in Caracas II clots is roughly 3.3 ± 2.4 μm, whereas that in control clots is about 0.6 ± 0.5 μm, although it should be noted that it is sometimes difficult to identify what would constitute individual pores. Thus, the average cross-sectional area of the larger pores in Caracas II clots, an approximate measure of the flow rate, is 30-fold higher. On the other hand, electron micrographs also reveal a very fine meshwork of fibers surrounding the pores. As a consequence, the measured flow rate for fibrin Caracas II clots is 16.5 times larger than that of the control clots, somewhat lower than expected from the size of the large pores alone.

These results are considerably different than those of permeation experiments from another dysfibrinogenemia, fibrinogen Dusart, which also has a single amino acid substitution in the αC domains leading to defective lateral aggregation (14). Although the permeation experiments for fibrin Dusart were carried out with plasma rather than purified fibrinogen and the protein concentrations were different, the results may be compared qualitatively. Fibrin Dusart clots, made up of a dense meshwork of thin fibers with small pores, have extremely low rates of permeation ($K_w$ was 175-fold lower than the control). In contrast, even though the fibrin Caracas II clots are also made up of thin fibers, the large pores that are present lead to higher than normal permeation rates. This observation highlights the importance of determining the ultrastructure of clot networks, in addition to permeation values, in order to adequately explain clot porosity (36, 44).

Permeation may be relevant for the clinical consequences of these dysfibrinogenemias. The rate of permeation is an important determinant of how rapidly molecules, such as those involved in fibrinolysis, travel through a clot. Permeability may also affect the neutralization of free thrombin. In addition, cellular interactions with fibrin are likely to be affected by the pore sizes in the clot. Thus, the higher than normal permeation in fibrin Caracas II clots is consistent with the observation that subjects who have this dysfibrinogenemia are asymptomatic, whereas subjects with fibrinogen Dusart, having clots with much lower permeability, have severe thrombotic problems. However, in the case of fibrinogen Dusart, it should be noted that possible anomalous interactions of plasminogen or plasminogen activator with the clot may also provide a significant contribution to the observed clinical symptoms (45, 46).

Molecular Mechanisms of Abnormal Polymerization of Fibrinogen Caracas II—During the initial stages of fibrin network formation under the action of thrombin, the cleavage of fibrinopeptide A allows the resultant fibrin desA monomers to polymerize into oligomers in a half-staggered arrangement via their complementary binding sites. At this early stage, the αC domains appear to remain bound to the central nodule of the molecule and are probably not involved in the initial aggregation process (1, 2). Although the αC domains remain attached to the central domain in desA fibrin, they are free to interact intra- and intermolecularly in desAB fibrin. Studies using fibrin desA fibrin monomer (2), its X-fragments missing one or both αC domains (2, 5, 6), and αC fragments (1, 7) indicate that the αC domains may enhance oligomer formation by bringing individual molecules into closer proximity.

The converse may be true for Caracas II where the αC domains are not only free from the central nodule at all stages of polymerization but were expected to undergo repulsive interactions owing to the negatively charged sialic acid groups, in addition to the steric hindrance induced by the carbohydrate moieties. This interference from the abnormal αC chains is likely to slow down the initial rate of oligomer formation, which is supported by the observation of an extended lag period during turbidity experiments (17). However, half of the molecules present in fibrinogen Caracas II have normal αC domains, and these will probably polymerize at a faster rate than the abnormal molecules, resulting in an initial selective increase in the concentration of oligomers containing predominately normal fibrin molecules. As the oligomers elongate into two-stranded protofibrils, the pool of available normal molecules will eventually become depleted relative to mutant molecules, which may result in capping of the ends of protofibrils with increasing proportions of abnormal fibrin monomers. The end result may be a heterogeneous pool of protofibrils containing varying proportions of normal and abnormal molecules.

Near the completion of the lag period, the protofibrils will begin to undergo lateral aggregation to form fibers; this process is assisted by the normal αC domains but inhibited by the mutant chains. Turbidity experiments have demonstrated a decrease in the rate of lateral aggregation of Caracas II protofibrils compared with controls, as shown by a lowered rate of optical density increase following the lag period, reflecting the probable inhibitory effect of the abnormal αC domains (9, 17). Again, it is likely that the regions of protofibrils containing higher populations of normal αC domains will undergo lateral aggregation at a faster rate than mutant-rich regions, resulting in the fibers being more ordered during earlier stages of formation with a tendency toward decreasing order later on as more of the carbohydrate-rich αC domains become involved. This process may explain the large number of fiber ends observed for Caracas II, because eventually the population of defective αC domains may become so high that their repulsive effects prevent further fiber growth by either protofibril elongation or lateral aggregation. The loose protofibril association observed in Caracas II fibers, combined with the tendency toward fracturing, particularly at fiber ends, is likely to be a result of the repulsion between the abnormal αC domains and suggests another mechanism that may contribute to the formation of this unique clot architecture. Loose, frayed ends could increase the frequency of branching, giving rise to the dense, highly branched meshwork made up of thin fibers that was observed in the areas adjacent to the pores.

The final structure of Caracas II clots, therefore, is probably a result of the effect of the decreased rates of both protofibril elongation and lateral aggregation, combined with the effect that the repulsive forces associated with the additional charge and mass of the carbohydrate bound to the αC domains have on the ultrastructural integrity of the constituent fibers.

Conclusions—The subjects who have the fibrinogen Caracas II mutation have been shown to be clinically asymptomatic, even though this particular modification of the αC domains results in the formation of a unique clot structure (17). The lack
of clinical symptoms may be a consequence of the physical properties of the clots. The viscoelastic properties of Caracas II clots are remarkably similar to those of control clots, despite the very different architecture, resulting in a near normal response to mechanical stress. Also, these clots are even more permeable to fluids than the control, thus allowing relatively unrestricted movement of ions and proteins through the gel while still maintaining an adequate structural framework for the cellular barrier. In contrast, the Dusart mutation, which while still maintaining an adequate structural framework for unrestricted movement of ions and proteins through the gel is much stiffer and also much less permeable than control clots (14, 15). This is likely to be one of the factors contributing to the observation that Dusart subjects are prone to the development of thromboemboli with the attendant complications.

These studies demonstrate that clots made up of fibers with similar diameters can differ dramatically in structure and physical properties. It is also evident that clot structure can be related to some clinical symptoms. Structural studies of these dysfibrinogenemias have revealed important aspects of the molecular mechanisms of assembly of fibrin clots.

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