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Research Article

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Electron Microscopy of Plasmic Fragments of Human Fibrinogen as Related to Trinodular Structure of the Intact Molecule

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ABSTRACT We have examined rotary shadowed, purified plasmic fragments of human fibrinogen with the electron microscope and have determined the relation of these fragments to the intact fibrinogen molecule. Both intact fibrinogen and its earliest cleavage product, fragment X, are trinodular. The next largest product, fragment Y, consists of two linked nodules. The two terminal products, fragments D and E, are single nodules. From measurements of simultaneously shadowed specimens of these different species, we conclude that the outer nodules of the trinodular fibrinogen molecule are the fragment D-containing regions and the central nodule is the fragment E-containing region.

INTRODUCTION

Fibrinogen is a 340,000-mol wt plasma protein with three pairs of disulfide-linked polypeptide chains (Aα, Bβ, and γ), which, after thrombin cleavage of fibrinopeptides A and B, polymerizes to form fibrin. Diverse models have been proposed for the three-dimensional structure of the fibrinogen molecule on the basis of electron microscopic studies. These include the following: a trinodular rod about 47-nm long with two identical outer nodules and a smaller central nodule (1); a sausage-shaped cylinder 45-nm long (2); a disk about 14 nm in Diam and about 5-nm wide (3); a pentagonal dodecahedron or other spherical structure 20–24 nm in Diam (4, 5). The spherical models were proposed in studies of negatively stained fibrinogen. The use of the heavy metal shadowing technique of Hall and Slayter (1), by which the generally accepted trinodular structure of fibrinogen was first proposed, also gave a substantial proportion of monad and dyad structures in their preparations and a variety of different structures in similar preparations from other laboratories (3, 6, 7). These results thus suggested that one or both of these different methods of specimen preparation did not show the true molecular structure of fibrinogen. Fowler and Erickson (8) have recently presented images showing identical trinodular molecules both in negatively stained and in shadowed preparations of fibrinogen. Virtually all the fibrinogen molecules in these preparations are trinodular. That study thus confirmed the trinodular model and reconciled the discrepancy in the appearance of fibrinogen in different electron microscopic preparations.

The nonmicroscopic evidence that is also in accord with the trinodular model includes physical chemical studies of different fibrinogen fragments (9, 10) and demonstration of an asymmetric cleavage of fibrinogen by the proteolytic enzyme, plasmin (11). The earliest product of plasmic digestion of fibrinogen is fragment X (250,000–300,000 mol wt), which is heterogeneous in size, reflecting cleavages at several points in the carboxyl terminal halves of the Aα-chains. Fragment X is asymmetrically cleaved to fragment Y (150,000 mol wt) and fragment D (93,000 mol wt). Fragment Y is further cleaved to fragment E (50,000 mol wt) and another fragment D. Each fibrinogen molecule thus yields one E fragment and two identical D fragments, which are the terminal products under usual digest conditions (12, 13). The single fragment E is dimeric (i.e., two identical halves connected by disulfide bonds) and contains portions of all six polypeptide chains, whereas each fragment D is monomeric and contains portions of one Aα-, one Bβ-, and one γ-chain. The results gave rise to the idea that the E fragment corresponds to the central nodule and the D fragments correspond to the two identical outer nodules of the trinodular model.

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The identification of the fragments in terms of the trinodular model has so far been based on indirect biochemical evidence. In the past, visualization in the electron microscope of trinodular fibrinogen molecules has been too variable to allow similar studies of isolated fragments to be meaningful. Since electron microscopic techniques are now established that reliably show the trinodular structure of the intact molecule, we felt that it was important to look specifically at each fragment using the same techniques (8). We present here images of purified fragments X, Y, D, and E that confirm the asymmetric plastic cleavage scheme and establish the relationships of the fragments to the trinodular structure of fibrinogen.

METHODS

Reagents and enzymes. Unless otherwise specified all reagents were analytical reagent grade and were acquired from commercial sources. Bovine pancreatic trypsin inhibitor (10,000 Kallikrein inactivator U/ml) was obtained from FBA Pharmaceuticals, West Germany. The human fibrinogen used in these studies (grade L, Kabi, Stockholm) contained sufficient plasminogen as a contaminant to permit complete digestion of the fibrinogen by plasmin after activation by urokinase. Plasminogen-free fibrinogen was prepared by affinity chromatography (14) to provide pure samples of the intact molecule for electron microscopy. Human urokinase (Winthrop Laboratories, Sterling Drug Co., New York) was dissolved in Tris-NaCl buffer, pH 7.4, to a concentration of 5000 Ploug U/ml and stored at −20°C.

Polyacrylamide gel electrophoresis. Electrophoresis in the presence of sodium dodecyl sulfate (SDS)[1] was performed as described (15), except that a constant current of 3 mA/gel was used and the gels were stained in Coomassie Brilliant Blue at 65°C for 25 min.

Preparation of fragments X, Y, D, and E. After dialysis at 4°C into 0.15 M NaCl–0.05 M Tris, pH 7.4, fibrinogen at 9.0 mg/ml was incubated at 37°C with urokinase at 90 Ploug U/ml. This mixture was immediately diluted with an equal volume of 0.15 M NaCl–0.05 M Tris–0.01 M $CaCl_2$, pH 7.4 followed by constant stirring at 37°C. For aliquots removed at various times between 20 min and 10 h, the digestion was terminated with bovine pancreatic trypsin inhibitor (50 Kallikrein inactivator U/ml) and stored at 0°C. Approximately 180 mg of the 45-min digest were dialyzed at 4°C into ethylene diamine-acetic acid buffer (ionic strength 0.1, pH 8.0), applied to QAE-Sephadex A-50 (2.6 × 55 cm) at 23°C, and eluted at 40 ml/h as described (16). All pools obtained from this elution were exhaustively dialyzed against 0.15 M ammonium bicarbonate at 4°C, concentrated by ultrafiltration, and rechromatographed on Sephacryl S-200 superfine (1.6 × 86 cm) or in the case of fragment E, on Sephadex G-100 (1.8 × 41 cm). For these gel filtration steps the sample volume applied was kept to 1% of the column volume and the flow rate was 3 ml/h per cm^2.

Electron microscopy. Samples were diluted to 30–110 μg/ml in 50 mM ammonium formate, pH 7. Portions of these solutions were mixed with glycerol and a 50 μg/ml solution of tobacco mosaic virus in a 1:1:1 ratio, sprayed on freshly cleaved mica, dried in vacuo, and rotary shadowed with platinum as described (8). The tobacco mosaic virus was used as an aid in selecting areas of the specimen for further study. Specimens were examined in a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.) at 80 kV accelerating voltage using a 50 μm objective aperture. Micrographs were taken at ×44,000 nominal magnification. Magnification was calibrated as described (8).

RESULTS

SDS gel analyses of fibrinogen digests. Fig. 1 shows the SDS gel electrophoretic analyses of fibrinogen digestion mixtures that were terminated at various times. Four major bands are observed in the 45-min digest: the doublet migrating just below the position of intact fibrinogen corresponds to early and late species of fragment X; the next two bands, which are about equal in intensity, correspond to fragments Y and D. A minor band near the top of the gel appears resistant to limited digestion by plasmin and is attributed to cold insoluble globulin, which is present in trace amounts in Kabi fibrinogen. The minor band in the bottom half of the gel, which becomes more prominent at later digestion times, corresponds to fragment E. The relative molar quantities of fragments X, Y, D, and E in the 45-min digest were 33, 21, 38, and 8%, respectively, as estimated from densitometry of the gel and the molecular weights of the fragments (see below). In the presence of 5 mM calcium fragment D is resistant to prolonged digestion by plasmin (18). Thus, the species of fragment D that is present in the 45-min digest is the largest fragment D and appears at the same position in a gel of the 10-h digest.

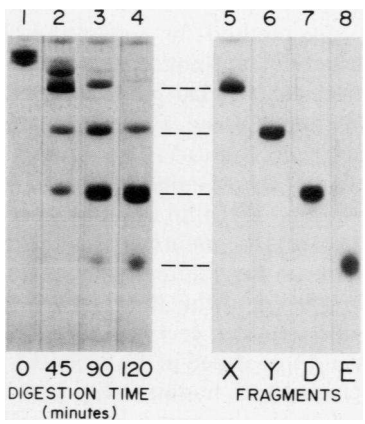

FIGURE 1 SDS-5% polyacrylamide gel electrophoresis of nonreduced samples of fibrinogen (gel 1), whole plasmic digests of fibrinogen (gels 2–4), and the final preparations of the purified fibrinogen fragments (gels 5–8) that were examined by electron microscopy. The digestion was performed in the presence of 5 mM calcium. The slight size heterogeneity of fibrinogen observed in gel 1 results from in vivo cleavage of the Aα-chain (17). The amount of protein applied to each gel was 9 μg for gel 1, 32 μg for gels 2–4, and 15 μg for gels 5–8.

[1] *Abbreviation used in this paper*: SDS, sodium dodecyl sulfate.

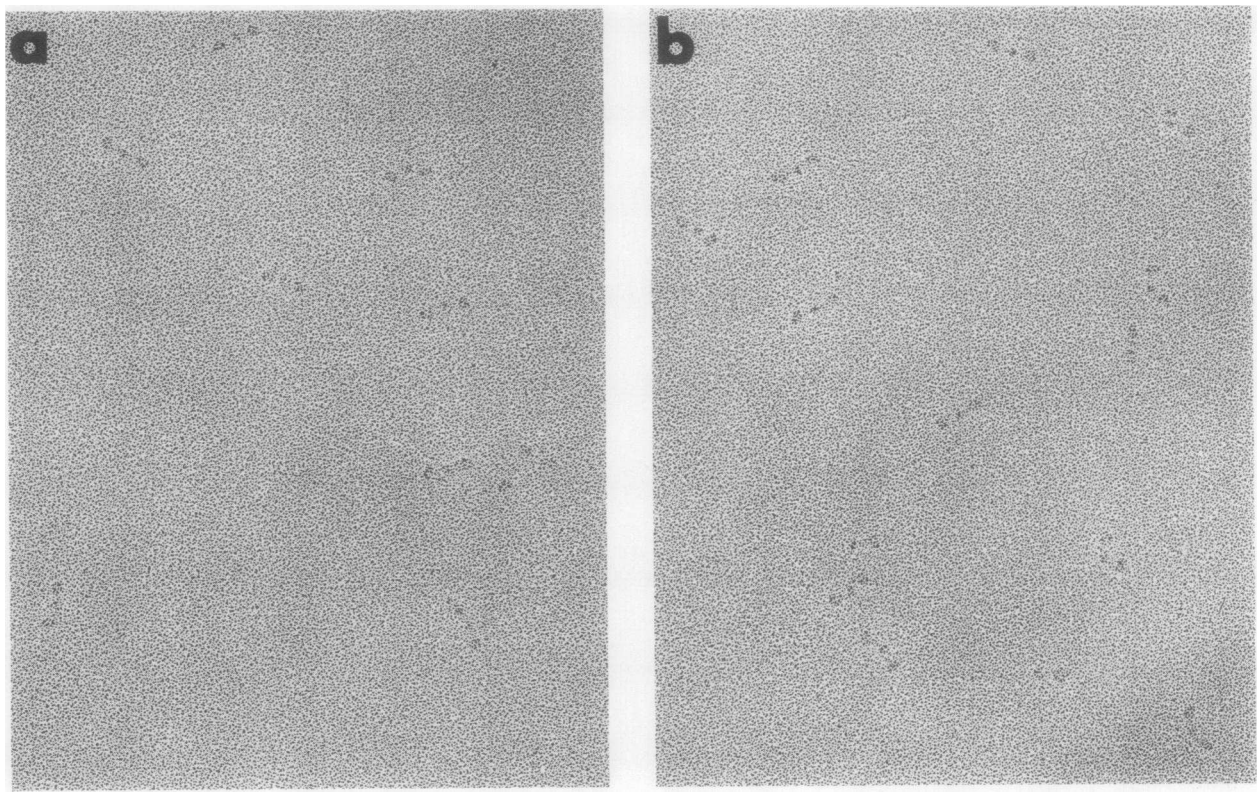

FIGURE 2 Electron micrographs of rotary shadowed preparations of (a) plasminogen-free human fibrinogen sprayed onto mica at a final concentration of 33 μg/ml and (b) purified fragment X sprayed at a final concentration of 36 μg/ml. Each trinodular structure is 1 molecule. Preparations shown in Figs. 2–4 were all rotary shadowed simultaneously. ×150,000.

Isolation of fibrinogen fragments. An ion-exchange chromatography method for separating the terminal plasmic products of fibrinogen (16) was used to fractionate limited digests that contained, in addition to fragments D and E, the intermediate degradation products, fragments X and Y. The elution profile from QAE-Sephadex of the 45-min digest differed from that of the terminal digest (16) in three respects: only trace amounts of protein (fragments D, X and Y) were eluted at pH 8.0 with the first buffer; most of fragment D in the digest appeared in the first of two well-resolved peaks generated by the second buffer (pH 5.0); and fragments X and Y coeluted in the second of these peaks and also appeared with fragment E in a small peak that was obtained with the third buffer (pH 4.4, ionic strength 0.2).

The first and third peaks from the QAE-Sephadex step were each gel filtered once to obtain fragment D and fragment E, respectively, with purity > 96%. Fragment X and fragment Y were only partially resolved when the major peak from the ion-exchange column was chromatographed on Sephacryl S-200. However, when fractions from the leading and trailing halves of this peak were rechromatographed separately on the same gel filtration column, these fragments were each obtained ~96% pure.

Fig. 1 shows SDS gel electrophoretic analyses of the purified fragments, which were subsequently examined by electron microscopy. Each appears as a single band by this method of analysis and clearly represents the major species of each fragment present in the whole digest. The smaller of the two forms of fragment X observed in the 45-min digest was isolated with an overall yield of 35%. The fragment Y was isolated at about 20% yield and was the only form of fragment Y observed at any time of digestion. The fragment D and E species were also unique and were obtained with 50 and 40% yields, respectively. SDS gel electrophoretic analyses of these isolated fragments after reduction with β-mercaptoethanol provided accurate assessment of their subunit molecular weights (13), which were then summed to give the following total molecular weights: fragment X = 250,000; fragment Y = 150,000; fragment D = 93,000; fragment E = 50,000.

Electron microscopy of fibrinogen fragments. The principal plasmic degradation products of fibrinogen, fragments X, Y, D and E, are all clearly distinguish-

able in our rotary shadowed preparations. Although the resolution of rotary shadowed specimens is limited by the size of the platinum grains, the major structural features such as the number, size, and shape of the globular regions present in each of the fragments are sufficiently distinct to identify them with respect to the trinodular model. Comparison of intact fibrinogen and fragment X in Fig. 2 and Table I demonstrates that they are both trinodular and are indistinguishable at this level of resolution. As in fibrinogen, the outer nodules of fragment X appear to be equal in size and larger than the central nodule. In general, the corrected average length and nodule dimension of fragment X are somewhat less than those of fibrinogen, but these differences are not significant given the standard deviations of these measurements and our overall estimate of the possible error of the correction factor (Discussion).

Fragment Y, the next largest intermediate, consists of two linked nodules as shown in Fig. 3. As with fibrinogen, the actual linkage between nodules cannot be resolved in the rotary shadowed specimen. Most of the molecules have one nodule clearly larger than the other. The sizes of these nodules and the distance between them are very similar to those obtained by measuring one outer nodule and the central nodule in the intact fibrinogen molecule (Table I).

As shown in Fig. 4, both terminal plasmic digestion products, fragments D and E, consist of single nodules. Fragment D is almost exactly the same size as the outer nodules of intact fibrinogen (Table I) and is elongated, a characteristic that has also been reported for the outer nodules of the fibrinogen molecule (8, 19). Fragment E is clearly smaller than fragment D and, as shown in Table I, is comparable in size to the central nodule of intact fibrinogen. The orientation of fragment E molecules could not be determined; therefore, the only dimension given in Table I for fragment E is its diameter, which is midway between the values measured for the length and width of the central nodule of intact fibrinogen.

TABLE I
*Comparison of the Nodule Dimensions of the Plasmic Fragments with Those of Intact Fibrinogen**

	Overall length	Outer nodule		Central nodule	
		Length	Width	Length	Width
		nm		*nm*	
Fbg	43.8±1.7	11.3±1.3	6.8±1.3	6.1±0.6	4.0±0.6
X	41.9±2.6	10.3±1.3	7.0±1.3	5.1±1.0	4.0±0.8
Y	22.7±2.5	9.3±1.3	6.4±0.8	4.6±1.0	3.5±0.8
D		11.3±1.1	6.1±0.8		
E				diameter 5.3±0.8	

* Values are ±SD and were obtained by subtracting 4 nm from the actual measured value. As discussed in the text, this correction reflects the contribution of two thicknesses of a 2-nm thick platinum shell surrounding each molecule. At least 25 molecules from preparations that were rotary shadowed simultaneously were measured in the determination of each value.

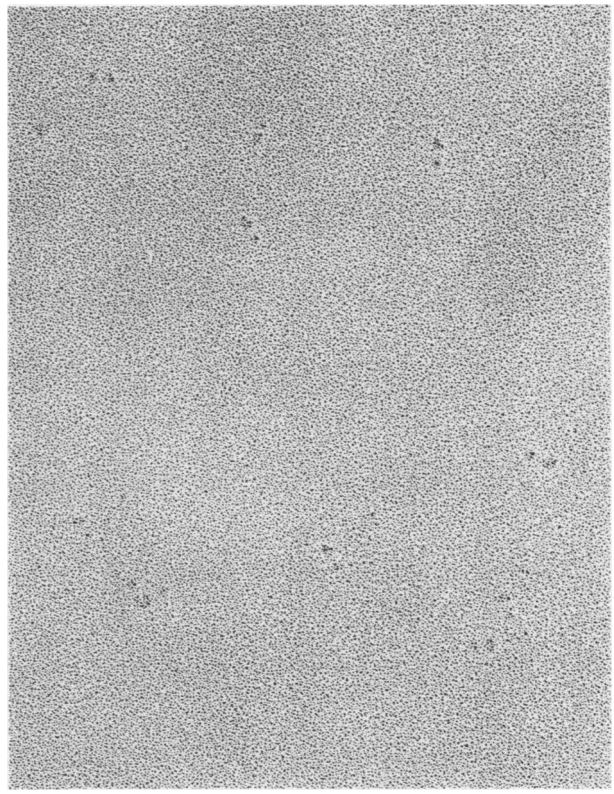

FIGURE 3 Electron micrograph of a rotary shadowed preparation of purified fragment Y sprayed onto mica at a final concentration of 36 μg/ml. ×150,000

DISCUSSION

The action of plasmin on fibrinogen results in the sequential appearance of a series of high and low molecular weight fragments (20). The low molecular weight fragments have been reported to possess several different physiologic activities (21, 22) and the larger fragments have been shown to have inhibitory effects on fibrin formation (23, 24). It has been suggested that during proteolysis the globular regions of the intact molecule are retained in the large terminal fragments (9, 10).

Two electron microscopic studies of fibrinogen degradation products have been reported to date. Niewiarowski et al. (25) examined whole digests and did not determine the structural characteristics of the various fragment species. Tranqui-Pouit et al. (26) interpreted their micrographs in terms of a globular

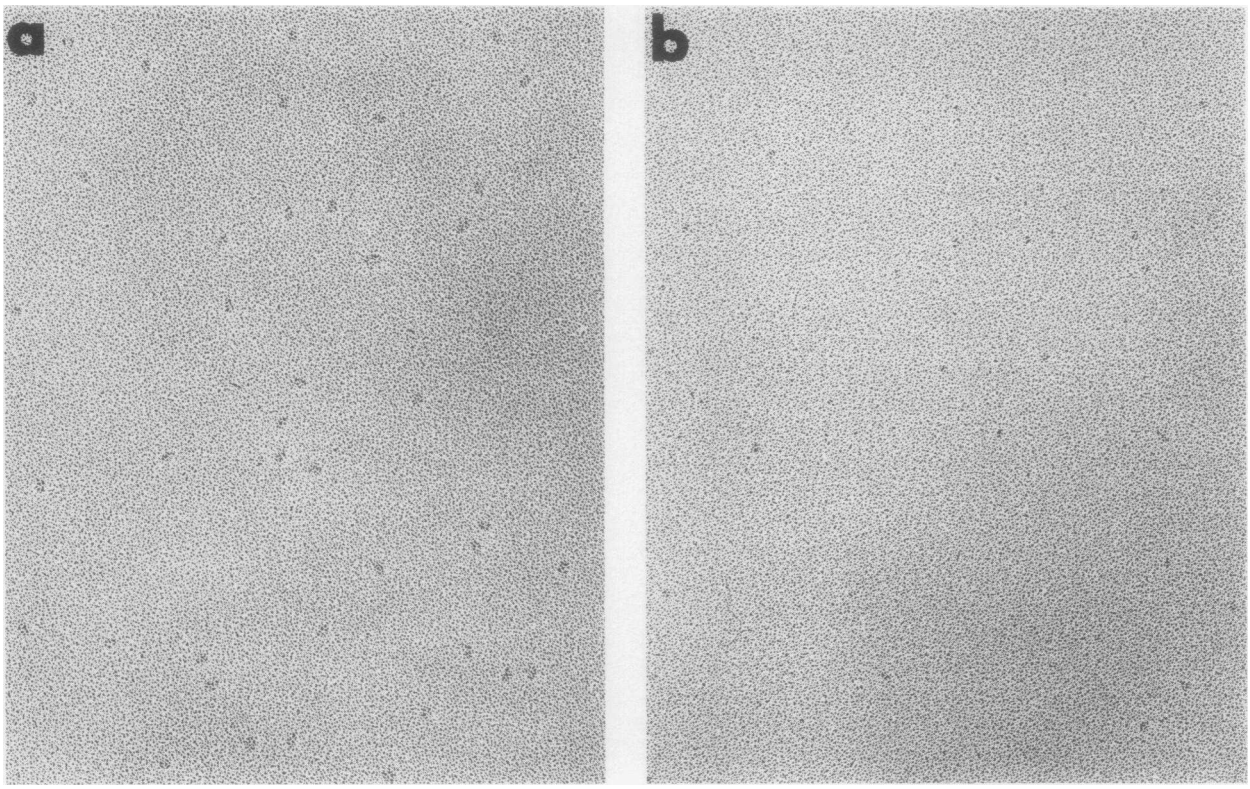

FIGURE 4 Electron micrographs of rotary shadowed preparations of (a) purified fragment D sprayed onto mica at a final concentration of 20 μg/ml and (b) purified fragment E sprayed at a final concentration of 10 μg/ml. Note the apparent elongation of the fragment D molecules. ×150,000.

model 24 nm in Diam for the intact fibrinogen molecule and for an early fragment X. A later fragment X was reported to consist of a nodular filament 60-nm long and all subsequent plasmic cleavage products were reported to be shorter filaments in various configurations. These investigators suggested that the fibrinogen molecule underwent an overall conformational change from a sphere to an extended nodular rod both during plasmin digestion and during cleavage of the fibrinopeptides. These results were obtained with a negative staining technique that we now believe is invalid because it involved the use of a very high concentration of fibrinogen. This caused aggregation and superposition of molecules in their specimens, as discussed (8).

Although fragment X, like fibrinogen, has a readily identifiable trinodular structure in our negatively stained preparations, smaller globular particles that lack a distinctive shape are more difficult to distinguish with certainty from the background granularity in such preparations. Since small globular particles such as fragments D and E can be distinguished much more reliably in our shadowed specimens, we have used this technique for the present study. Our shadowing technique differs from that of Hall and Slayter (1) by the inclusion of glycerol in the protein solution during drying, which seems to produce a much clearer distribution of separate, undistorted molecules. The rotary shadowed specimens in Figs. 2–4 were all prepared and shadowed simultaneously, thereby obviating the need to account for a variable thickness of platinum when comparing the relative sizes of the different fragments. However, the absolute molecular dimensions are obviously affected by the platinum thickness and the values in Table I have therefore been corrected for a 2-nm platinum shell, which completely surrounds each molecule. This correction factor was determined by comparison of the dimensions of intact fibrinogen as measured by three different electron microscopic techniques including rotary shadowing (8). Our estimate of the possible error in this correction factor is 0.6 nm per 2 nm thickness. The plausibility of the corrected dimensions can be tested by calculating the volume of an ellipsoid with axes equal to the dimensions of fragment D given in Table I (i.e., 2a = 2b = 6.1 nm, 2c = 11.1 nm). If a specific volume of 1.3 cm^3/g

is assumed for this protein, an ellipsoid of this volume can contain up to 102,000 g/mol. A compact ellipsoidal protein with these dimensions is thus consistent with the molecular weight of 93,000 for the fragment D in our preparations.

The finding that rotary shadowed fibrinogen and fragment X are indistinguishable (Fig. 2) indicates that the carboxyl terminal halves of the two Aα-chains of fibrinogen, which are absent from fragment X, are not identifiable with any specific part of the trinodular structure seen in preparations of intact fibrinogen. This is not unreasonable, since each of these carboxyl terminal regions, being only 20,000–40,000 daltons, would be visible only if it were a discrete projection. Furthermore it has been suggested that these regions are "free-swimming appendages" not associated with the trinodular core of the molecule (27–29), in which case they would be difficult to visualize in the electron microscope because of their random orientation. The presence in fragment Y of two nodules, one larger than the other (Fig. 3), and the uninodular structures of fragments D and E, with D being larger and elongated (Fig. 4, Table I), are entirely consistent with the asymmetric cleavage scheme outlined earlier.

It has been proposed on the basis of biochemical data that fragments E and D correspond to the middle and outer nodules of the trinodular fibrinogen molecule, respectively. The present study confirms this proposed identification of the plasmic fragments with the trinodular structure of fibrinogen as well as the asymmetric plasmic cleavage of fibrinogen. This will permit the functional characteristics of the different fragments, such as their effects on fibrin polymerization, to be interpreted in structural terms.

ACKNOWLEDGMENTS

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