MOLECULAR SUBSTRUCTURE OF FIBRINOGEN

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Recent studies have provided much new information on the structure of the plasma protein fibrinogen. N-terminal analyses of fibrinogen and fibrin (Blombäck and Yamashina, 1958) have suggested that the native molecule is a dimer consisting of three pairs of polypeptide chains. This idea is supported by studies on the conversion of fibrinogen to fibrin by thrombin which have shown that two A peptides and two B peptides are released per fibrinogen molecule (Blombäck, 1958; Gladner et al., 1959).

This report describes two methods for the dissociation of fibrinogen and a partial characterization of the products by physical means. The results support the model given above and suggest that the polypeptide chains of fibrinogen are linked by disulfide bridges.

Experimental

Purified bovine fibrinogen of 94% clottability was obtained from Pentex Corporation. Conversion to the S-sulfo derivative was carried out by the method of Swan (1957) as modified by Pechère et al. (1958). The final product was lyophilized from distilled water.

Sedimentation velocity experiments were performed with a Spinco Model E Ultracentrifuge using Schlieren optics and a double sector cell. Sedimentation coefficients are reported as $S_{20,w}$ in Svedbergs (S); the

values have not been extrapolated to infinite dilution or corrected for possible binding of a third component.

In the Archibald molecular weight determinations, the interference and Schlieren optical systems were used to measure concentration (C_m) and concentration gradient, respectively, at the meniscus. The values were obtained from the photographic plates with a Nikon microcomparator by linear extrapolation into the region corresponding to the meniscus.

The original concentration, C_o, and the amount of bound sodium dodecyl sulfate (SDS) were obtained as follows: The protein was dialyzed against buffer overnight. Equal aliquots of a concentrated SDS solution and of water were then added to equal volumes of protein solution and buffer, respectively. A synthetic boundary cell determination of the refractive index across this boundary (in fringes) was used for the C_o determination and includes the contributions of protein, bound SDS and free SDS. The number of fringes corresponding to protein and SDS bound to protein was then determined from an interference sedimentation velocity run at 52,050 rpm. By the time the fringe pattern in the boundary region of the protein-SDS complex could be resolved, the fringes in the region between the meniscus and the boundary were horizontal (constant refractive index difference between solvent and solution), but those on the centrifugal side of the boundary had a slight continuous upward curvature indicating non-specific aggregation or an association-dissociation phenomenon (accounting for about 10% of the total fringes for C_o). Correction for radial dilution and loss of material due to sedimentation of aggregates was obtained by calculating the vertical fringe shift about halfway between the meniscus and the bottom of the cell from photographs taken at 2 minute intervals. This value was added to the number of fringes

across the boundary to give the number of fringes due to protein and bound SDS. The amount of free SDS is the difference in fringes between the velocity run and the C_o run. The number of fringes of SDS added (usually 13.5 corresponding to a 0.5% solution of SDS) was determined by a separate synthetic boundary cell experiment on SDS alone. The concentration of the S-sulfo derivative was determined from the C_o run after subtracting the number of fringes due to the added SDS; aggregation of the S-sulfo derivative in high salt prevented a C_o determination of protein alone.

Starch gel and cellulose acetate strip electrophoresis were carried out with a commercial apparatus (E. C. Company) using partially hydrolyzed starch from Connaught Laboratories and cellulose acetate strips from Gelman Instrument Company.

Dissociation in Alkaline Solution

Although fibrinogen readily forms a complex with sodium dodecyl sulfate at neutral pH (with about 12 ± 1 out of 13 fringes of SDS bound to 6 mg/ml of protein), sedimentation velocity patterns showed a single hypersharp boundary at 6.6S, indicating that no apparent dissociation had occurred. However, dissociation into discrete subunits sedimenting at 3.5S was readily effected by heating the protein at pH 10 for about 10 min. at 80° in the presence of SDS. When the detergent was added just before cooling, identical results were obtained; however, when the solutions were cooled in the absence of SDS, aggregation occurred which could not be reversed by addition of SDS to a concentration of 1% or urea up to 6 molar. Thus, the detergent apparently serves only to prevent re-aggregation of the subunits.

The dissociation reaction appears to be a two-step process. In a typical experiment in which the protein was heated for 3 min. at 75°

(pH 10, SDS/protein = 1), centrifugation showed three components: undissociated fibrinogen (6.6S) amounting to about 47% of the total protein; a shoulder on this peak at 5.6S with about 27% of the total area; and the remaining 26% sedimenting at 3.5S. After 6 min. at 75° the 3.5S component accounted for 67% of the total protein; the remainder was about equally distributed between 5.6S and 6.9S components. After prolonged heating at 75° or at 90° all material sedimented at 3.5S; no species with an S rate lower than this was ever observed. Although dissociation experiments were carried out under a variety of conditions (varying ionic strength, pH, temperature and time of incubation at the high pH), no significant enrichment of the intermediate component at 5.6 S was achieved. Further experiments are required in order to determine whether this component results from a swelling of fibrinogen or actually represents a partial degradation product.

The results suggested that the product of the dissociation might represent the separated chains of fibrinogen; however, further resolution on the basis of molecular weight differences did not seem feasible, and the possibility of separation on the basis of charge properties was considered. The chemistry of the dissociation needs further study; however, we believe it involves an alkali-catalyzed dismutation of disulfide bridges. Because of the variety of products which can be formed by this reaction at the cysteinyl residues, any given chain might carry a number of different charges. This difficulty could be eliminated if in place of alkaline rupture the disulfide bridges were cleaved to form a single product quantitatively.

Dissociation Accompanied by Sulfitolysis

S-sulfo-fibrinogen was found to be soluble but highly aggregated in aqueous solutions. Lyophilized samples were dissolved in solutions

containing SDS. In this solvent the sedimentation behavior of the S-sulfo derivative was essentially identical to that of the end-product obtained in the high pH-high temperature dissociation. When the ratio of SDS to protein was lowered from 1 to 0.5 the sedimentation rate increased from 3.5 S to 4.5 S and the concentration gradient centrifugal to the boundary was raised above the reference gradient. This behavior suggests the existence of association-dissociation equilibria.

Values of the molecular weight (using one component formulation) varied from 97,000 at $C_m = 0.9\,C_o$ to 73,000 at $C_m = 0.5\,C_o$. Correction for SDS binding ($\bar{V}_{SDS}$ taken as 0.89), according to the equation given by Hersh and Schachman (1958), reduces the molecular weight range to 65,000 to 50,000. This is consistent with a mixture of the six polypeptide chains of fibrinogen (e.g., the number average value would be 57,000 based on a molecular weight of 340,000 for fibrinogen).

Further efforts have been made to characterize and identify the material obtained by S-sulfonation of fibrinogen. Because of the strong tendency of this material to aggregate, electrophoresis studies have been limited thus far to solvents containing 6 M urea. Sedimentation studies in 6 M urea showed that the S-sulfo-protein sediments at about 3 S, compared with a value of 6 S for fibrinogen in the same solvent. The shape of the boundary was identical to that observed for this material in SDS solution. It would therefore appear that the chains remain separated in 6 M urea solutions. More exact analysis of the data will require correction for preferential binding. However, the results present the distinct possibility that the chains of fibrinogen are held together by inter-chain disulfide bridges. An alternative is that sulfitolysis merely alters the protein conformation (for example, by breaking intra-chain disulfide bonds) to facilitate denaturation by urea or SDS.

Starch gel electrophoresis of S-sulfo-fibrinogen in 6 M urea solutions was carried out in three buffers: 0.02 M phosphate, pH 7.5; 0.03 M borate, pH 8.7; and 0.02 M carbonate, pH 9.2. Five hundred to 1500 volts were applied to a 16 x 20 cm. gel of 4 mm. thickness for periods of 16 to 50 hours. In all cases only two bands were visible when developed with nigrosin. The slower moving component had the greater protein concentration as indicated by the degree of staining. Identical results were obtained using cellulose acetate strip electrophoresis; in this case resolution into two distinct components was achieved in 1 to 5 hours at the same applied voltage. The optical density as a function of distance from the origin was obtained by scanning the nigrosin stained strips with a Beckman Model RB Analytrol. The area under the curve representing the more slowly moving component was about twice that of the other component.

Our electrophoretic results agree well with those of Henschen (1962) and Clegg and Bailey (1962) on S-sulfo-fibrinogen prepared in a similar manner. From examination of S-sulfo-fibrin prepared with Reptilase, Clegg and Bailey were able to conclude that chain separation had been achieved by sulfitolysis. Their evidence indicated that two chains of S-sulfo-fibrinogen travel in starch gel as a single band.

Our investigations are being continued in the areas of the mechanism of dissociation of fibrinogen, properties of the individual chains, and the arrangement of the chains in the native molecule.

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