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IDENTIFICATION OF FIBRIN OLIGOMERS IN SONICATED FIBRIN CLOTS

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Clots of bovine fibrin, with both coarse and fine structure, and ligated to different extents by fibrinoligase, have been broken up by ultrasonic agitation and the sonicates have been examined by ultracentrifugal sedimentation. Sonication is followed by gross aggregation of the fragments unless guanidine hydrochloride is introduced (order of 1 M). In that case, sonicates of γ-ligated fine clots contain two species whose sedimentation coefficients correspond to fibrin monomer and an oligomer with twice the monomer cross-section area and at least 20 monomer units, presumably with the structure of lateral dimerization with staggered overlapping. If the γ ligation is incomplete, shorter oligomers are identified. The monomer and oligomer with degree of polymerization > 20 appear also in sonicates of coarse clots, but in smaller amounts, the principal product consisting of larger aggregates. The implications of these results with respect to metastability of the fine clot and the pattern of polymerization are discussed.

1. Introduction

Intermediate soluble polymers of fibrin can be observed during the polymerization process following activation by thrombin; they can be stabilized by 1,6-hexanediol [1] or other reagents [2] and identified by sedimentation in the ultracentrifuge. From certain old observations on 1,6-hexanediol-stabilized oligomers, it may be inferred that these can be dissociated by dilution if they have not been ligated by fibrinoligase in the course of their formation, but if ligated are rather stable [3,4]. Ligated oligomers can be identified by sedimentation in fibrin which has been solubilized in urea at an early stage of polymerization [5], and, by agarose gel electrophoresis, when fibrin polymerization is interrupted at an early stage [6].

In the present study, fibrin oligomers have been obtained from the opposite direction by breaking up fibrin clots with sonication. Oligomers are identified by sedimentation after sonication of clots with fine, transparent structure (in which the fibrous structure is believed to be the protofibrils formed by lateral dimerization of fibrin monomers with staggered overlapping, with minimal lateral aggregation [7]), and also clots with coarse, opaque structure (in which the fibrous structure is much thicker owing to extensive lateral aggregation of the protofibrils). The character of the breakdown products depends on the coarseness and also on the degree of α and γ ligation.

2. Materials

Bovine fibrinogen, about 60% clottable, was obtained from Miles Laboratories and purified either by precipitation with β-alanine in a modification of the method described by Straughn and Wagner [8] or by ethanol precipitation following Blombäck and Blombäck [9]. The clottability was 93–96%. Aliquots of solution were frozen for storage at −20°C but with one exception were not kept for more than about two months. Thrombin (bovine, topical) was obtained from Parke–Davis and Company; it was dialyzed against Tris buffer, pH 7.5, ionic strength (μ) 0.15 of which 0.10 was sodium chloride. Fibrin stabilizing factor (FSF) was generously furnished us by Professor L. Lorand of Northwestern University. It was kept at 4°C in a Tris buffer solution of pH 7.5, ionic strength 0.15, containing 0.001 M ethylene diamine tetraacetate (EDTA). When used for clot ligation, it was preactivated to fibrinoligase by thrombin and calcium as described previously [10]. Trasylol was obtained from Worthington

Biochemicals; guanidine hydrochloride from Aldrich Chemical Company.

Clots were prepared by rapid mixing of fibrinogen solution with a suitable amount of thrombin to give a clotting time of 10 to 15 min. The fibrinogen concentration was 10 g/ℓ. All contained 0.5 unit/ml of Trasylol to inactivate any plasmin which might be present. For fine, transparent clots, the fibrinogen was in Tris buffer pH 8.5, μ0.45, of which 0.40 was sodium chloride. For coarse, opaque clots, the buffer was imidazole pH 6.5, μ0.15, of which 0.10 was sodium chloride. Two different degrees of ligation were obtained: one by adding 2.5 mM calcium chloride, relying on residual FSF in the fibrinogen for partial ligation; the other with 2.5 mM calcium chloride plus activated FSF (10 or 25 mg/ℓ). When ligation was to be avoided, the solution contained 1 mM EDTA.

3. Methods

Each clot (3 ml volume) was formed in a narrow-bottom centrifuge tube. After a lapse of 3 hr to allow the clot structure to become essentially completely established, it was sonicated by introducing the titanium microtip of a Sonifer Cell Disrupter (Model W185, Heat Systems Ultrasonics Inc.). The drive level was 4 to 5 watts and the period usually 1 min when guanidine hydrochloride was added as described below; otherwise the time was about 30 min and an ice bath was used to avoid heating. Aliquots were then taken for ultracentrifugation and gel electrophoresis.

Sedimentation velocity measurements were made in a Spinco Model E ultracentrifuge at a speed of 42 040 RPM and temperature of 25°C. The data were reduced to water at 20°C by the usual density and viscosity corrections, based on measurements of densities and viscosities of the corresponding solvents, made up with guanidine hydrochloride when appropriate. The partial specific volume of the protein was taken as 0.71. Polyacrylamide electrophoresis was performed after reduction with dithiothreitol and solubilization by sodium dodecyl sulfate [11]. The degree of ligation was estimated roughly by visual observation of the stained gels and comparison with fibrinogen controls.

4. Results

4.1. Fine clots sonicated without use of guanidine hydrochloride

Fine clots, either unligated, partially γ-chain ligated by residual FSF in the fibrinogen, or completely γ-chain ligated with additional activated FSF, were dispersed by sonication but gave milky suspensions indicating the presence of aggregates which were large on a molecular scale; some large clumps were visible. Ultracentrifugation showed a trace of material with a sedimentation coefficient characteristic of monomeric fibrinogen or fibrin and no specific polymers.

4.2. Fine clots sonicated with guanidine hydrocloride

In an effort to eliminate the aggregation described above, guanidine hydrochloride [12] was introduced just before sonication; the clot was crushed and to it was added 0.5 cm^3 buffer containing a weighed amount of guanidine hydrochloride. This procedure gave sonicates from partially or fully γ-ligated clots which remained clear for at least several hours although for lower guanidine hydrochloride concentrations they eventually became somewhat turbid. The fibrin concentration was reduced by the additional volume to 8.5 g/ℓ. In these sonicates, ultracentrifigation identified two or three peaks, provided the final guanidine hydrochloride concentration was at least 0.95 M. Their sedimentation coefficients, reduced to viscosity and density of water at 20°C, are listed in table 1. From unligated clots, only monomer fragments are to be expected since the guanidine dissolves the unligated fibrin as well as dissociating the intermediate polymers [12].

The sedimentation coefficients at finite concentrations are smaller than the infinite-dilution values which characterize monomeric fibrin and intermediate polymers [1], and it would be difficult to appraise the concentration dependence in these mixtures or extrapolate to zero concentration. However, those near 6 of 7 S can clearly be identified with the monomer ($n = 1$). The others are characterized by the ratio s_n/s_1 of the sedimentation coefficients of oligomer and monomer in each experiment. Values of this ratio were calculated on the basis of hydrodynamic models for several values of n and matched to the nearest experimental value.

Table 1
Sedimentation coefficients of sonicated clots

Type [a]	Expt. no.	Fibrin conc. (g/ℓ)	GCl [b] (M)	γ lig. [c] (%)	α lig. [d] (%)	$s_{20,w}$	s_n/s_1 obs.	n [e]	s_n/s_1 calc.
F	15	8.5	0	100	0	6.3	1	(1)	(1)
F	9	8.5	0.49	10	0	7.4	1	(1)	(1)
F	10	8.5	0.95	10	0	6.4	1	(1)	(1)
						11.7	1.85	4	1.89
F	11	8.5	1.38	10	0	6.5 [h]	1	(1)	(1)
						9.8	1.51	2	1.43
						12.3	1.89	4	1.89
F	13	8.5	1.38	10	0	6.8	1	(1)	(1)
						10.9 [h]	1.60	3	1.70
						14.8	2.17	6	2.16
						25.4	3.17	>20	–
F	12	8.5	1.78	10	0	7.1 [h]	1	(1)	(1)
						9.6	1.35	2	1.43
						11.6	1.62	3	1.69
F	14	8.5	1.78	100 [g]	9	6.3	1	(1)	(1)
						18.4 [h]	2.78	16	2.80
F	16	8.5	1.78	100 [g]	0	6.4	1	(1)	(1)
						21.9 [h]	3.42	>20	–
C	19	10	0	100 [g]	60	6.6	1	(1)	(1)
C	20	10	0	0	0	6.4	1	(1)	(1)
						23.4	3.64	>20	–
C	18	8.5	1.75	100 [g]	80	6.4	1	(1)	(1)
						20.1	3.13	>20	–
C [f]	17	8.5	1.75	100 [g]	80	5.6	1	(1)	(1)
						20.3	3.63	>20	–

a) F = fine, C = coarse.
b) Molar concentration of guanidine hydrochloride in final sonicate.
c) Percent γ ligation estimated from polyacrylamide gel electrophoresis.
d) Percent α ligation estimated from polyacrylamide gel electrophoresis.
e) Degree of polymerization of oligomer estimated by matching s_n/s_1 with model hydrodynamic calculation.
f) Intermediate between coarse and fine; ionic strength 0.20.
g) Activated FSF added.
h) Component in larger amount.

The calculation was performed as follows. The theoretical sedimentation coefficient was calculated for the trinodular rod model [13] assuming the frictional coefficient to be the sum of those for the nodules, taking the molecular weights of the center and terminal spheres to be 55 000 and 142 500 respectively; the result was $s_1 = 9.3$ S. This is not far from the value of 8.4 obtained experimentally for the monomer unit in inhibited clotting mixtures [1]. The friction coefficient relative to that of a sphere of equal volume is 2.044, so if one changes now to an ellipsoid of revolution model the corresponding axial ratio a/b is 21 [14]. For each oligomer with degree of polymerization n, the half length a_n was assumed to be $\frac{1}{2}(n+1)a_1$ corresponding to lateral dimerization with staggered overlapping [13,15], and the ellipsoid

Table 2
Calculated sedimentation coefficient ratios

n	2	3	4	5	10	15	20
s_n/s_1	1.43	1.70	1.89	2.04	2.50	2.76	2.95

minor axis b_n was calculated from the requirement that the volume is n times that of the monomer. The ratio s_n/s_1 is then given by

$$s_n/s_1 = n\left(\frac{a_1^2 - b_1^2}{a_n^2 - b_n^2}\right)^{1/2} \frac{\ln[a_n/b_n + (a_n^2/b_n^2 - 1)^{1/2}]}{\ln[a_1/b_1 + (a_1^2/b_1^2 - 1)^{1/2}]} \quad (1)$$

Values of s_n/s_1 are listed in table 2. The ratio becomes insensitive to n for $n > 20$.

From table 2 it is evident that the sonication fragments from fine clots with complete γ ligation consist of monomer plus rather large oligomers of the size commonly found in inhibited clotting mixtures ($n = 20$ or more). When ligation is only about 10%, smaller oligomers with $n = 2$ to 6 are seen, and the larger oligomers are usually absent. There was no α ligation in any of these fine clots. Since the sedimentation coefficient is primarily determined by the width of a rod-like molecule, and a side-by-side combination of more than two would result in higher s than observed here, it may be concluded that there is little further lateral aggregation of the lateral dimer pattern with staggered overlapping.

No attempt was made to estimate the amounts of the different species, but the polymers usually exceeded the monomer as judged qualitatively from peak areas.

4.3. Coarse clots

Coarse clots sonicated without addition of guanidine hydrochloride also gave milky suspensions with gross aggregates. Ultracentrifugation showed a small amount of monomer, somewhat more than obtained in the corresponding experiments with fine clots. In addition, when ligation was prevented by use of EDTA, a trace of polymer with $n > 20$ was identified.

When the sonication was performed with added guanidine hydrochloride, both monomer and polymer with $n > 20$ were identified, but unlike the corresponding experiments with fine clots the monomer was in excess, and both species were relatively sparse in comparison with large aggregates with an apparent broad distribution of sizes. The sedimentation coefficients and s_n/s_1 ratios for these experiments are also given in table 1.

5. Discussion

5.1. Metastability of fine clots

The fact that the fine clot aggregates as soon as its structure is broken up, if guanidine hydrochloride is absent, suggests that the structure is metastable with respect to lateral aggregation. In fact, a system of long fibrillar units with random orientations is expected to be thermodynamically unstable unless exceedingly dilute; calculations of Flory [16] show that it tends to undergo phase separation into a dilute isotropic and a concentrated ordered phase. We suggest that the polymerization process forms an interpenetrating array of long fibrillar units. Under the conditions of high pH and ionic strength corresponding to fine clots, lateral aggregation is suppressed in the early stages and the tendency to aggregate later when long polymers are achieved is prevented simply by their steric blocking in random orientations, which makes rearrangement to seek parallel configurations impossible. When sonication breaks up the fibrils into somewhat shorter lengths, aggregation is spontaneous.

5.2. Fragments from fine clots

The identification of fragments from γ-ligated fine clots which correspond to twice the cross-section area of fibrinogen or fibrin monomer gives further support to the view that the lateral dimer pattern with staggered overlapping is the primary protofibril structure. The stability of these fragments in guanidine hydrochloride indicates that the γ ligation joins successive overlapping units and is not involved in any further lateral aggregation.

When the γ ligation is complete, the fragments appear to contain at least 20 monomers, though their length (or distribution of lengths) remains uncertain. It is probably determined by the rupture of junctions by the hydrodynamic forces of the sonication, which become more severe with increasing length [17]. Monomer fragments also exist, but in relatively small amounts. When the γ ligation is incomplete, shorter

fragments are identified. On the basis of probability, one can expect incomplete sequences of γ-linked pairs which would break up in this manner. In this case, the monomer fragments generally exceed the polymers in amount.

5.3. Fragments from coarse clots

In the ligated coarse clots, there is both γ and α ligation, and the latter presumably binds protofibrils together in lateral aggregates [18,19]. The identification, in sonicates of coarse clots, of polymeric fragments (though in relatively small amounts) similar to those derived from fine clots suggests that the α–α linkages are mechanically weaker and more susceptible to hydrodynamic disruption than the γ–γ linkages. Somewhat larger amounts of monomer fragments are produced, though in the coarse clots the majority of the sonicated protein remains in the form of grosser aggregates.

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References

[1] S. Shulman and J.D. Ferry, J. Phys. Coll. Chem. 55 (1951) 135.
[2] J.D. Ferry, Physiol. Rev. 34 (1954) 753.
[3] J.D. Ferry, S. Shulman, K. Gutfreund and S. Katz, J. Amer. Chem. Soc. 74 (1952) 5709.
[4] S. Katz, S. Shulman, I. Tinoco, Jr., I.H. Billick, K. Gutfreund and J.D. Ferry, Arch. Biochem. Biophys. 47 (1953) 165.
[5] L. Lorand, D. Chenoweth and A. Gray, Ann. N.Y. Acad. Sci. 202 (1972) 155.
[6] M. Moroi, N. Inoue and M. Yamasaki, Biochem. Biophys. Acta 379 (1975) 217.
[7] M.E. Carr, Jr., L.L. Shen and J. Hermans, Biopolymers, in press.
[8] W. Straughn III and R.H. Wagner, Thromb. Diath. Haemorrh. 16 (1966) 198.
[9] B. Blombäck and M. Blombäck, Ark. Kemi. 10 (1956) 415.
[10] W.W. Roberts, O. Kramer, R.W. Rosser, F.H.M. Nestler and J.D. Ferry, Biophys. Chem. 1 (1974) 152.
[11] P. McKee, P. Matlock and R.L. Hill, Proc. Nat. Acad. Sci. U.S. 66 (1970) 738.
[12] S. Shulman, S. Katz and J.D. Ferry, J. Gen. Physiol. 36 (1953) 759.
[13] R.F. Doolittle, Adv. Protein Chem. 27 (1973) 1.
[14] C. Tanford, Physical Chemistry of Macromolecules (Wiley, New York, 1961) p. 327.
[15] J.D. Ferry, S. Katz and I. Tinoco, J. Polymer Sci. 12 (1954) 509.
[16] P.J. Flory, Proc. Roy. Soc. A234 (1956) 73.
[17] R.E. Harrington and B.H. Zimm, J. Phys. Chem. 69 (1965) 161.
[18] C. Gerth, W.W. Roberts and J.D. Ferry, Biophys. Chem. 2 (1974) 208.
[19] L.L. Shen, R.P. McDonagh, J. McDonagh and J. Hermans, unpublished.