CLOTS OF β-FIBRIN

Viscoelastic Properties, Temperature Dependence of Elasticity, and Interaction with Fibrinogen-binding Tetrapeptides

AKIRA SHIMIZU AND JOHN D. FERRY
Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT Clots of human β-fibrin, in which only (or predominantly) the B fibrinopeptide is released, were formed at 14°C by copperhead venom procoagulant enzyme (CVE or venzyme), at pH 8.5, ionic strength 0.45. The shear modulus of elasticity increased slowly and after several days attained a constant value, which was lower than those of α-fibrin or αβ-fibrin under the same conditions. Before studying the temperature dependence of elasticity, the CVE was then inhibited by introducing phenyl methyl sulfonyl chloride (PMSF) by diffusion. With increasing temperature, the modulus decreased progressively from 5°C to nearly zero at 35° and was essentially reversible with temperature change; recovery of elasticity after change from 34.5° to 14° required ~2 d but was considerably faster than the initial buildup of elasticity by CVE at 14°. Creep and creep recovery measurements on unligated clots showed creep rates and irrecoverable deformation that were similar in magnitude to those of α-fibrin clots formed with batroxobin and much larger than those of αβ-fibrin clots formed with thrombin, under the same conditions. During creep and creep recovery, the differential modulus or compliance remained constant, showing that there was no permanent structural damage, and if network strands are severed in slow flow, they must rejoin in new configurations. Introduction (by diffusion) of the tetrapeptides Gly-His-Arg-Pro (GHRP) and Gly-Pro-Arg-Pro (GPRP), which resemble the B and A binding sites on the E domain of fibrin respectively, reduced the shear modulus and increased the creep rate of β-fibrin clots to an extent similar to the effect of GPRP on αβ-fibrin, much more than that of GHRP on αβ-fibrin, but much less than that of GPRP on α-fibrin. A ligated β-fibrin clot formed with Factor XIIIa (in which the activating thrombin had been neutralized by hirudin) showed essentially perfect elastic behavior, with no creep and with complete recovery after removal of stress, and was inert to GHRP.

INTRODUCTION

Thrombin converts fibrinogen to fibrin monomer by cleaving two moles each of the fibrinopeptides A and B (1), uncovering noncovalent binding sites which can interact respectively with sites designated (2) "a" and "b" on other fibrin monomer molecules. The Aa junctions are sufficient for self-assembly of fibrin monomers in the staggered overlapping pattern to form the protofibril, which is the first stage in the construction of a fibrin clot. The B fibrinopeptides are released more slowly than the A. The Bb junctions which are then established have been thought to be involved in the lateral aggregation of protofibrils (3, 4), although such aggregation can take place without them. However, it is evident from recent work that the Bb junctions also stabilize the binding of fibrin monomers together within the protofibrils themselves (5–6a).

The roles of the Aa and Bb junctions can be investigated by studying the action on fibrinogen of certain snake venom enzymes that remove the A or B fibrinopeptides selectively (7). Ancrod (from *Agkistrodon rhodostoma*) and batroxobin (from *Bothrops atrox* or *Bothrops moojeni*) cleave A only; CVE or venzyme (from *Agkistrodon contortrix contortrix*) cleaves B only, or at least highly preferentially, under certain conditions (8). Fibrin monomer lacking both fibrinopeptides is often designated as $(\alpha\beta\gamma)_2$ or αβ-fibrin; lacking A, as $(\alpha B\beta\gamma)_2$ or α-fibrin; lacking B, as $(A\alpha\beta\gamma)_2$ or β-fibrin.

α-Fibrin monomer polymerizes to form oligomers and clots that resemble those from αβ-fibrin (6) except that the clots have very different mechanical properties (6a, 9). Also, β-fibrin polymerizes to form oligomers and clots but only at lower temperatures; both dissociate into smaller oligomers at temperatures approaching 37°C and into monomers when diluted to 0.2 mg/mL at 37°C (10, 11). Electron microscopy of the three types of clots shows striking similarities (6, 12) and it is evident that all three types of protofibrils are based on the staggered overlapping pattern in which A and B sites on the central fibrin nodule interact respectively with "a" and "b" sites on the terminal nodules of two other fibrin monomers.

The association and gelation of β-fibrin, their reversibility with temperature change, and ligation (cross-linking) by Factor XIIIa have been studied in several investigations

by Shainoff, Furlan, Mosesson, and others (5, 8, 10–14). However, there have been no quantitative measurements of mechanical properties of β-fibrin clots. The present paper reports measurements of the shear modulus of elasticity and its dependence on temperature, viscoelastic creep, and creep recovery, and the effects on mechanical properties of the introduction, by diffusion into a clot, of the tetrapeptide Gly-His-Arg-Pro (GHRP) which resembles the N-terminal sequence of the B site and binds to the "b" site (15, 16), as well as Gly-Pro-Arg-Pro (GPRP), which resembles the "a" site and binds to it but also binds to the "b" site (16).

Previous studies (6a, 9, 17–20) of the viscoelastic properties of $\alpha\beta$-fibrin and α-fibrin clots may be briefly summarized as follows. These statements refer to "fine" clots formed at pH 8.5, ionic strength 0.45, where there is minimal lateral aggregation of protofibrils ("fine clot" conditions), and to small shear deformations. Unligated $\alpha\beta$-fibrin clots subjected to constant stress show an initial elastic deformation ("initial" in our experiments means 25 s after imposition of stress) followed by creep (gradual increasing deformation). After removal of stress, the creep recovery is incomplete and the irrecoverable deformation is proportional to the stress duration. Introduction of the tetrapeptide GPRP, which binds to the "a" site and inhibits (15) polymerization of $\alpha\beta$-fibrin monomers, diminishes the shear modulus (ratio of stress to strain at 25 s), and increases enormously the creep rate and irrecoverable deformation. The creep deformation becomes a linear function of time so the clot containing GPRP appears to flow like a highly viscous viscoelastic liquid. By contrast, clots ligated by Factor XIIIa show no creep, only an initial elastic deformation which is completely recovered after removal of stress, and are unaffected by the introduction of GPRP.

Unligated α-fibrin clots have somewhat lower shear moduli and larger creep rates and irrecoverable deformations than $\alpha\beta$-fibrin clots. They are also far more susceptible to the action of GPRP in lowering the shear modulus and further increasing creep rates and irrecoverable deformations. These observations, as well as studies of the interaction of GPRP with oligomers (6a, 20) support the view (5, 6) that Bb contacts stabilize the bonding of individual $\alpha\beta$-fibrin monomers within protofibrils. It is of particular interest, therefore, to study clots in which there are only Bb contacts, and their interaction with GHRP which binds to the "b" sites, as well as GPRP which also binds to the "b" sites.

MATERIALS AND METHODS

Lyophilized human fibrinogen, Imco Lot F176, was generously given us by Drs. B. Blombäck and R. Procyk, New York Blood Center. It was dissolved in Tris-HCl/NaCl buffer, pH 8.5, ionic strength 0.45, of which 0.40 was contributed by NaCl. After dialysis against this buffer at 4°C for 2 d with several changes, aliquots were frozen with liquid nitrogen and stored at −10°C. After each was thawed, fibrinogen concentration was measured spectrophotometrically at 282 nm, with correction for scattering by subtraction of absorbance at 320 nm.

Bovine thrombin was obtained from Parke-Davis and Co., Detroit, MI. CVE enzyme from *A. contortrix cortortrix,* purified by chromatofocusing, was generously provided by Dr. John R. Shainoff, Cleveland Clinic Foundation. Trasylol (plasmin inhibitor) was obtained from FBA pharmaceuticals, New York; phenyl methyl sulfonyl fluoride (PMSF) and hirudin from Sigma Chemical Co., St. Louis, MO; and tosyllysine chloromethyl ketone (TLCK) from Aldrich Chemical Co., Milwaukee, WI. Materials for gel electrophoresis (acrylamide and cross-linking comonomer, ammonium persulfate, and Coomassie Brilliant Blue dye) were obtained from Biorad Laboratories, Richmond, CA. Factor XIII (fibrin stabilizing factor) was generously furnished by Professor L. Lorand, Northwestern University.

Because the fibrin clots were kept for long periods (up to two weeks) during mechanical measurements, inhibitors were added to the fibrinogen solutions, before clotting, to inactivate possible though unlikely traces of foreign enzymes: Trasylol (2 U/mL) for plasmin, hirudin (5 U/mL) for thrombin, and TLCK (0.1 mM) for a fibrinolytic enzyme originally present in the snake venom (10, 12). Unless ligation was desired, the solutions contained 1.5 mM EDTA. When a ligated clot was required, the EDTA was replaced by 3.2 mM calcium chloride plus Factor XIIIa as noted. The latter was prepared by activating Factor XIII (at 150 mg/L) with thrombin (0.6 U/mL) and calcium chloride (0.016 M) for 90 min. and then inactivating the thrombin by hirudin (45 U/mL). The procedures for disc gel electrophoresis have been previously described (20–22).

The apparatus for measurements of elastic modulus and creep has been previously described (18, 20, 23). The solution to be clotted, containing approximately 8.5 mg/mL fibrinogen, was cooled to 14°C, CVE was added, and it was introduced between the parallel circular plates of the apparatus, which had been precooled to 14°C, to avoid release of A fibrinopeptides at higher temperatures (8–10). Separate aliquots were monitored for clotting time and kept for gel electrophoresis measurements. After clotting, the shear modulus G_{25} was measured intermittently by imposing a torque $\mathcal{S}$ for 25 s, measuring the angular displacement α, and returning the torque immediately to zero. Here, G_{25} is calculated by the relation, (23)

$$G_{25} = 2\mathcal{S}h/\pi a^4\alpha \qquad (1)$$

where h is the distance between the plates (about 0.12 cm) and a is the plate radius (2.5 cm). The shear strains in the disc-shaped clot were always very small (maximum, at the disc periphery, about 1% at the beginning of a creep experiment and 6% at the end). After G_{25} had increased to a constant value, requiring several days, a creep experiment was done at constant torque; the angular displacement $\alpha(t)$ was followed as a function of time. The creep compliance $J(t)$ is

$$J(t) = \pi a^4\alpha(t)/2\mathcal{S}h \qquad (2)$$

The initial value, J_1, is simply the reciprocal of G_{25}. The final value at time t_1 is denoted by J_1'. At t_1, a differential compliance J_Δ was determined from an incremental angular displacement 25 s after application of an incremental torque (about half the original torque). Then the torque was removed, and creep recovery began. After 25 s the compliance had decreased to J_s. It gradually decreased further to a final value J_s', a measure of permanent deformation. Finally, after creep recovery, an incremental torque was applied again and the resultant angular displacement was measured to obtain the differential compliance J_Δ final. These various compliances monitor possible changes in clot structure (9, 19).

When measurements were to be made at different temperatures, the CVE in the clot was inactivated at 14°C by diffusing in from the clot periphery PMSF over a period of 40 h to a nominal concentration of 0.5 mM to prevent release of A fibrinopeptide at higher temperatures (8, 10). The arrangement for this diffusion procedure has been described previously (19, 20).

RESULTS

Dependence of Shear Modulus on Time and Temperature

The buildup of shear modulus with time at 14°C after clotting of a solution containing 0.38 TAME units (8) of CVE per mL is shown in Fig. 1. For comparison with clots of $\alpha\beta$ and α-fibrin under the same conditions, formed with thrombin and batroxobin respectively (6a), $G_{25}(t)/G^f_{25}$ is plotted against t/t_c, where G^f_{25} is the final modulus value and t_c is the clotting time. Values of t_c, G^f_{25}, and fibrinogen concentration [F] are given in the legend.

The clotting time for β-fibrin under "fine clot" conditions is much longer than at pH 7.3, ionic strength 0.15 (8, 10). The buildup of rigidity relative to the clotting time is also strikingly slow compared with the behavior of α-fibrin and $\alpha\beta$-fibrin; half the final elastic modulus is attained at $t = 7t_c$, compared with $\sim 2t_c$ for the others. The inset shows results of gel electrophoresis under reducing conditions on aliquots quenched by SDS at times indicated by a to e on the graph for β fibrin and compared with a fully developed thrombin clot (Th) and fibrinogen (O). The displacement of the β chain indicates that the release of the B peptide is complete at b, ~6 times t_c. Thus the release of B is not the rate-determining step in the slow buildup. (Release of A peptide is the rate-determining step in polymerization of $\alpha\beta$-fibrin under similar conditions (24).) At later times, the α chain appears to have undergone some attack in spite of the enzyme inhibitors present. Similar behavior was observed by Mosesson and collaborators (13).

For a similar experiment with CVE, the modulus G_{25} is plotted directly against real time in Fig. 2. The course of buildup agrees quite well with that in Fig. 1, although the final modulus is somewhat higher (670 dyn/cm^2). Creep and creep recovery measurements were made at 14.2° after ~5 d, and on the sixth day PMSF was diffused in to inactivate the enzyme. Then measurements were made at a series of different temperatures in the order 14.2°, 5.0°, 12.2°, 25.0°, 30.0°, 14.2°, 34.5°, and 14.0°. After each temperature change, G_{25} was followed as a function of time and reached a plateau level within 3 to 5 h except when the temperature was lowered from 30° or higher. During this sequence of temperature changes, the age of the clot increased from 7 to 12 d. Unfortunately, the temperature itself is slow to come to equilibrium in this apparatus and no conclusions can be drawn about the kinetics of changes in the fibrin network for times shorter than ~2 h. However, the buildup of G_{25} after the final change from 34.5° to 14.0° required at least 2 d and is plotted with black circles

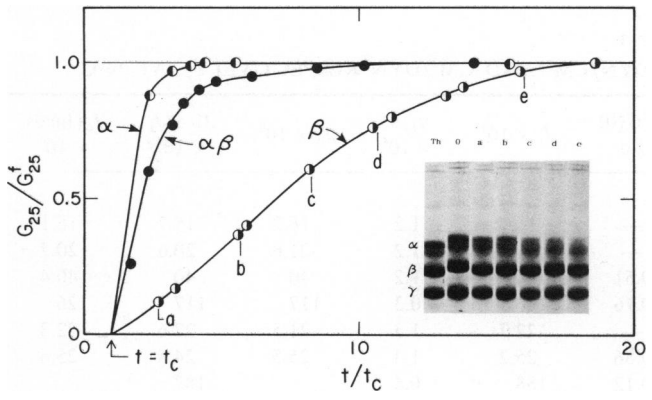

FIGURE 1 Shear modulus relative to its final value plotted against t/t_c. β, CVE 0.38 U/mL, [F] = 8.54 mg/mL, t_c = 9 h, G^f_{25} = 490 dyn/cm^2 (exp. 93); α, batroxobin 0.1 U/mL, [F] = 8.51 mg/mL, t_c = 90 min, G^f_{25} = 1,300 dyn/cm^2 (Exp. 84); $\alpha\beta$, thrombin 0.19 U/mL, [F] = 8.57 mg/mL, t_c = 32 min, G^f_{25} = 1,860 dyn/cm^2 (Exp. 90). *Inset:* reduced gel electrophoresis on aliquots of β-fibrin taken at times indicated by a to e on graph; Th is for fully developed thrombin clot, O is fibrinogen.

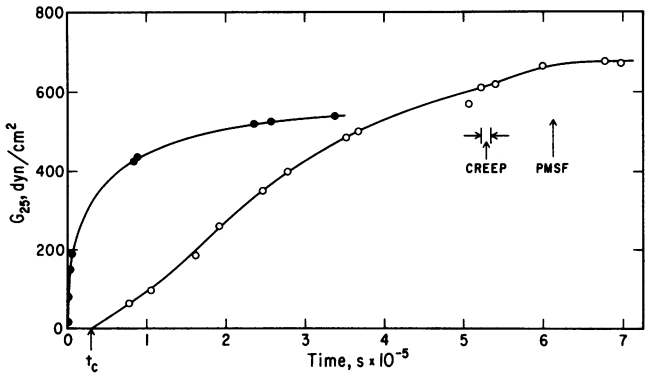

FIGURE 2 Shear modulus plotted directly against time for clot of β fibrin, [F] = 8.60 mg/mL, CVE 0.38 U/mL, t_c = 8.5 h (exp. 87). Times of creep experiment and of introduction of PMSF are shown. Black circles: buildup of G_{25} after temperature change from 34.5° to 14.0°C (cf., Fig. 3). The abscissa unit (10^5 s) is ~1 d.

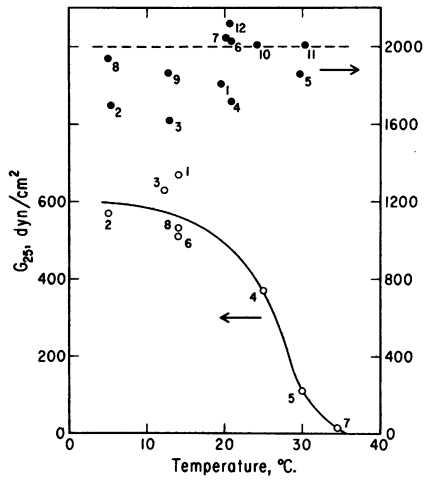

FIGURE 3 Shear modulus plotted against temperature for clot of β-fibrin, Exp. 87 (cf. legend of Fig. 2). Numerals denote sequence of measurements. *Solid circles:* similar measurements for a clot of $\alpha\beta$ fibrin, [F] = 8.57 mg/mL, thrombin 4.2 U/mL, t_c = 27 min. (Exp. 83). Dashed line is average of measurements 7 to 12 inclusive. Note the difference in ordinate scale.

in Fig. 2. The fact that G_{25}, starting from 15 dyn/cm^2 at 34.5°, attains a value at 14.0° (530) nearly as high as the initial value when the clot was first formed at 14.2° (670) gives some reassurance that there has been no deleterious effect of degradation of the protein despite the extreme duration of the experiment. The buildup of rigidity after lowering the temperature, although slow, is much faster than that following the original clotting, despite the conclusion that in the latter the release of B peptide is not the rate-determining step. A similar conclusion was reached by Mosesson and collaborators (12) by following opacity measurements.

The plateau values of G_{25} are plotted against temperature in Fig. 3, with numerals denoting the sequence of measurements. The points below 14° are somewhat erratic but the sharp drop in the range 20°–30° agrees with earlier qualitative observations (8, 10) at lower pH and ionic strength. The sequence 5-6-7-8 shows rather good reversibility between temperatures where the clot is nearly liquefied and where the modulus is near its maximum value.

The fact that, after the CVE had acted for 6d, the clot could be essentially liquefied at 30° is evidence that the results are not affected by any slow release of fibrinopeptide A. (In a qualitative test, a CVE clot was liquefied at 37° and the solution was divided into two aliquots; to one, batroxobin was added to a concentration of 1.25 U/mL; both were incubated at ambient temperature, at which they gelled, for 2 d. Upon warming to 37°, the gel with batroxobin, which releases the A peptides, remained solid, and the other liquefied.) Facilities were not available to determine the A release directly.

Fig. 3 includes, for comparison, data for the temperature dependence of G for a clot of unligated $\alpha\beta$-fibrin formed with thrombin at the same pH, ionic strength, and fibrinogen concentration (but a much shorter clotting time, 27 min). The temperature range here was from 5.2 to 30.3°C. The measurements in sequence 1–6 were made within a period of clot age from 43 to 76 h; those in sequence 7–12 within a period from 115 to 142 h. The scatter within each group appears to be random, and the average for the second group is drawn as a horizontal dashed line. From these measurements, there seems to be no significant dependence of modulus on temperature in a thrombin clot. (In a very early study (25) of bovine fibrin of lower purity at pH 7 by an entirely different experimental method, a moderate reversible decrease in G with increasing temperature was reported. We have no explanation for this difference.)

Interaction with GHRP and GPRP: Shear Modulus and Creep

To determine the effect of GHRP on clot mechanical properties, the tetrapeptide was diffused in from an approximately equal volume of external solution with twice the desired tetrapeptide concentration in the same buffer, as previously described (18, 19), at 14°C. The decrease in G_{25} was followed with time as in earlier experiments where GPRP was introduced into clots of α-fibrin and $\alpha\beta$-fibrin (6a, 19, 20). The concentration is expected to be nearly equalized after 1 d (20), though a small further drop in G_{25} is observed after 2 d. In the first diffusion, the nominal GHRP concentration was 0.5 mM (assuming complete equalization); in the second, after replacing the external solution with a more concentrated charge, it was 2.3 mM (Exp. 93). A similar sequence was performed with the other tetrapeptide, GPRP, in which the nominal concentration after the first diffusion was 0.1 mM and after the second 1.0 mM (Exp. 102). The ratio $G_{25}/G_{25}(0)$, where $G_{25}(0)$ is the modulus before introduction of GHRP, after 1 and 2 d is given with other information in Table I.

In Fig. 4, $G_{25}/G_{25}(0)$ (after 2 d) is plotted against

TABLE I
SHEAR MODULI AND CREEP COMPLIANCES (UNITS DYN/CM^2 AND CM^2/DYN RESPECTIVELY), AT 14°C

Expt.	Fibrin	[F]	Peptide	[P]	$G_{25}(0)$	$G/G(0)$ 1 d	$G/G(0)$ 2 d	$J_1 \times 10^4$	t_1, s $\times 10^4$	$J_\Delta \times 10^4$	$J_1' - J_s$ $\times 10^4$	J_Δ(final) $\times 10^4$
		mg/mL		mM								
87	β	8.6	—	—	670	—	—	16.4	1.2	16.2	15.7	16.1
93	β	8.5	—	—	490	—	—	21.3	1.2	21.6	20.6	20.7
			GHRP	0.5		0.60	0.51	40	1.2	40	40	40.4
			GHRP	2.3		0.21	0.16	126	0.3	117	117	126
102	β	8.5	—	—	460	—	—	22.7	1.3	21.5	22.6	22.3
			GPRP	0.1		0.91	0.86	25.2	1.3	25.5	24.8	25.6
			GPRP	1.0		0.20	0.12	188	0.4	—	187	
98	β*	8.5	—	—	1420	—	—	7.1	1.2	6.9	7.0	7.0
			GHRP	6.0		0.99	0.97	7.25	1.2	7.0	7.3	7.3
84‡	α	8.5	—	—	1300	—	—	7.8	1.2	7.3	7.1	7.9
90‡	$\alpha\beta$	8.6	—	—	1890	—	—	5.2	9.5	5.1	5.4	5.4
96‡	$\alpha\beta$	8.5	—	—	1860	—	—	5.4	1.2	5.1	5.5	5.4

*Ligated by Factor XIIIa and calcium.
‡Measurements at 22 ± 1°C.

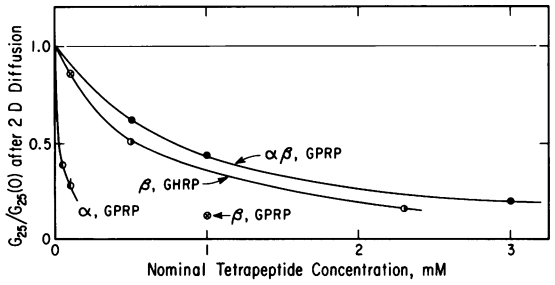

FIGURE 4 Shear modulus ratio after diffusion for 2 d plotted against nominal concentration of tetrapeptide: GHRP and GPRP (*crossed circles*) for β-fibrin, GPRP for α-fibrin and αβ-fibrin (7). Point with pip up is for fibrin concentration 15.0 mg/mL.

tetrapeptide concentration for both tetrapeptides and compared with similar data for introduction of GPRP into α-fibrin and αβ-fibrin clots prepared from Imco fibrinogen. It is evident that β-fibrin is much less susceptible to dissociation by GHRP than α-fibrin is to GPRP. The effect of GHRP on the modulus of β-fibrin is quite similar in magnitude to that of GPRP on αβ-fibrin. However, GPRP lowers the modulus of β-fibrin even more than GHRP does. (A single experiment from Reference 19, not shown, gave the effect of GHRP on αβ-fibrin as $G/G[0] = 0.79$ at 5.6 mM, trivial in comparison with the others; and another showed a synergistic effect of GPRP and GHRP combined.)

Creep and creep recovery data for a β-fibrin clot before introduction of GHRP and at two levels of GHRP concentration are shown in Fig. 5. Numerical data for J_1, t_1, J_Δ, $J_1' - J_s$, and J_Δ (final) are included in Table I, for these as well as two other clots without GHRP, in reasonable agreement, and clots containing GPRP at two different levels, also representative data for α-fibrin and αβ-fibrin clots from Imco fibrinogen from previous measurements (6a).

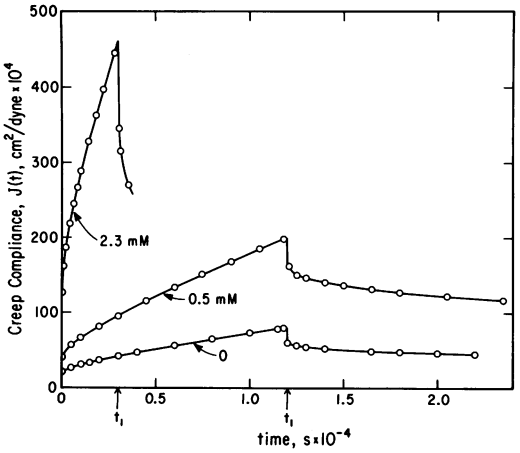

FIGURE 5 Creep and creep recovery of β-fibrin clot before (0) and after introduction of GHRP to nominal concentrations of 0.5 and 2.3 mM (exp. 93). Additional data in Table I.

It is evident from Fig. 5 that GHRP increases both the creep rate and the irrecoverable deformation of β-fibrin, just as GPRP does for α-fibrin and αβ-fibrin (6a, 20). Similar increases were observed for β-fibrin clots containing GPRP, but the latter was somewhat more effective as will be seen from comparison of viscosities. Even without GPRP, the creep and creep recovery of β fibrin, like those of α-fibrin, resemble the behavior of a viscoelastic liquid, and the reciprocal slope of the linear portion of the creep curve represents a steady-flow viscosity. A detailed comparison of the properties of the three types of fibrin will follow. In Table I, the columns J_Δ, $J' - J_s$, and J_Δ (final) are of particular interest. These represent a differential compliance measured 25 s after a step change in stress (the removal of the original torque at time t_1 is equivalent to a negative stress change and $J' - J_s$ is the corresponding change in compliance). All these quantities are essentially equal and in agreement with the initial compliance J_1. This shows, as has been found to be the case for αβ- and α-fibrins both with and without GPRP, that even though substantial flow occurs the network structure has not been damaged, and if network strands have been severed they must form again in new configurations.

In Table II, values of viscosity η and steady-state compliance J_e^0 (a measure of recoverable elastic deformation, given by the intercept of the linear segment of the creep curve on the ordinate axis) are listed, as well as the logarithm of the product ηJ_e^0, which represents a weight-average viscoelastic relaxation time (20). Comparison with α- and αβ-fibrins in Tables I and II shows that both α and β types have somewhat lower moduli and enormously lower viscosities (orders of magnitude) than the normal fibrin formed with thrombin. The moduli and viscosities of α- and β-fibrin are of similar magnitudes, though α-fibrin has a somewhat higher modulus and somewhat lower viscosity. The average relaxation time is somewhat higher for β-fibrin.

The viscosity is plotted logarithmically against concen-

TABLE II
EFFECTIVE VISCOSITIES AND STEADY STATE COMPLIANCES, AT 14°C

Expt.	Fibrin	Peptide	[P]	$\eta \times 10^{-7}$	$J_e^0 \times 10^4$	$\log J_e^0$
				P	cm²/dyne	units s
87	β	—	—	0.37	24	3.95
93	β	—	—	0.25	33	3.91
		GHRP	0.5	0.09	69	3.79
		GHRP	2.3	0.012	216	3.41
102	β	—	—	0.24	35	3.92
		GPRP	0.1	0.17	45	3.89
		GPRP	1.0	0.015	325	3.68
84*	α	—	—	0.19	25	3.68
90*	αβ	—	—	(39)‡	7.4*	5.46*

*Measurements at 22 ± 1°C.
‡A lower limit because it is doubtful whether steady state flow is achieved.

tration for both tetrapeptides in Fig. 6, with comparisons (6a, 20) for α-fibrin and $\alpha\beta$-fibrin with GPRP. Although the reduction of viscosity of β-fibrin by GHRP is substantial, it is relatively less than the effect of GPRP on $\alpha\beta$-fibrin and far less than the spectacular reduction by GPRP in α-fibrin. The viscosity of β-fibrin, like the modulus, is lowered more by GPRP than by GHRP at a comparable concentration. It may be noted that all the viscosities are exceedingly high compared with those of ordinary liquids; for example, that of glycerol is 9 poise at 25.0°C. The product of ηJ_c^0 in Table II will be considered in the Discussion section.

Ligated β-Fibrin Clots

The action of CVE on fibrinogen in the presence of Factor XIIIa and calcium produces a clot with $\gamma-\gamma$ ligation (11). The increase in shear modulus with the progress of time in such a clot is plotted in Fig. 7. The modulus builds up more rapidly than for the corresponding unligated clot, reproduced for comparison from Fig. 2, and reaches a considerably higher limiting value. After 5 d, the $\gamma-\gamma$ ligation was essentially complete. No $\alpha-\alpha$ dimers or polymers were observed, although the α-chains were degraded as in the unligated clots.

Creep and creep recovery data for this clot before and after introduction of GHRP to a very high nominal concentration of 6 mM are shown in Fig. 8, and numerical data are included in Table I. The contrast with the unligated clots (Fig. 5) is striking. There is essentially no creep, just an immediate elastic deformation, and the recovery after removal of stress is almost instantaneous and complete; the behavior is almost that of a perfect elastic solid. Thus introduction of GHRP at a much higher concentration than that which decreases the modulus (Fig. 4) and greatly increases creep rate and irrecoverable deformation (Fig. 5) of unligated clots has practically no effect on the modulus. In the presence of the GHRP, there

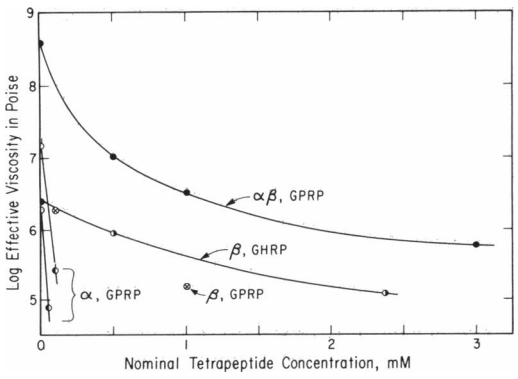

FIGURE 6 Logarithm of viscosity from slope of linear portion of creep curve plotted against nominal concentration of tetrapeptide: GHRP and GPRP (*crossed circles*) for β-fibrin, GPRP for α-fibrin and $\alpha\beta$-fibrin. Fibrin concentrations ~8.5 mg/mL except for points with pips, 15.0 mg/mL.

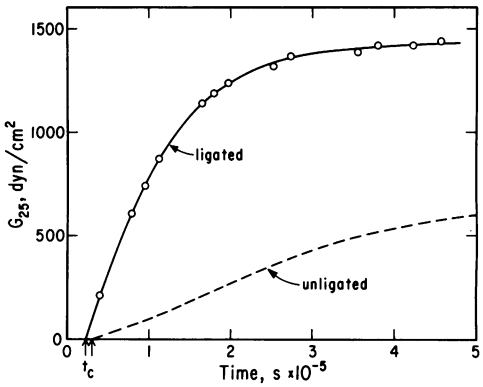

FIGURE 7 Shear modulus plotted against time for ligated clot of β-fibrin, Exp. 98, [F] = 8.45 mg/mL, CVE 0.38 U/mL, Factor XIIIa 4.8 mg/L, $t_c \simeq 6$ h. Dashed curve is for unligated clot, reproduced from Fig. 2. The abscissa unit is ~1 d.

is still almost no creep or flow and nearly perfect elastic recovery. As expected, the various J values in Table I agree since the structure is stable and not rearranging as it does in unligated clots. Similar behavior has been found for ligated fine clots of $\alpha\beta$-fibrin and α-fibrin (6a), which are unaffected by the introduction of GPRP by diffusion.

DISCUSSION

As pointed out in previous communications (6a, 20), the modulus G_{25} is a measure of the ability of the fibrin structure to store elastic energy, while the creep rate and viscosity are measures of the rate (and its reciprocal, respectively) of rearrangements of the structure.

The reversible temperature dependence of G indicates that the structure of unligated β-fibrin is subject to dissociation in an accessible temperature range, and experiments with oligomers (5, 12) indicate that the dissociation is between monomer units within the protofibrils and involves the Bb associations. Two monomers in staggered overlapping configuration are joined by two Bb junctions.

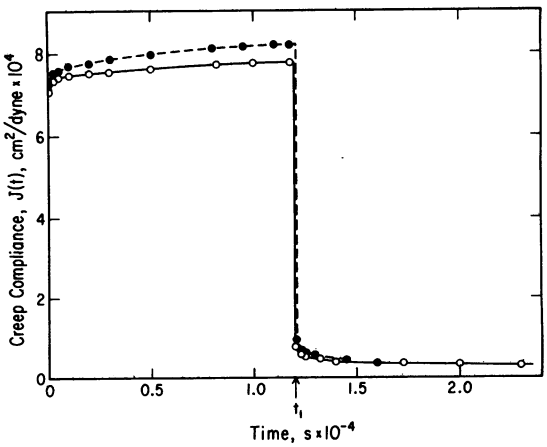

FIGURE 8 Creep and creep recovery of ligated β-fibrin clot before and after introduction of GHRP to a nominal concentration of 6.0 mM (exp. 98). Additional data in Table I.

Various experiments with oligomers of $\alpha\beta$-fibrin (20, 26, 27) or α-fibrin (28) have been interpreted in terms of an equilibrium constant for dissociation of two monomers in which pairs of both Aa and Bb junctions, or pairs of Aa junctions alone, maintain the contact. However, the dissociation equilibrium lies so far on the left (unless disruptive reagents such as GPRP (20) or hexanediol (27) are present) that its dependence on temperature probably cannot be detected. With Bb junctions alone, the binding of two monomers together is evidently much weaker.

Unfortunately the lack of a theory relating the magnitude of G to the degree of dissociation of the β-fibrin structure prevents the use of Fig. 3 to calculate an equilibrium constant and an enthalpy of dissociation. However, it may be noted that a small increase, with increasing temperature, of the proportion of dissociated Bb junction pairs can cause a large change in G, because a single break in a clot network strand can remove the contribution of the entire strand to the elastic modulus (20). Thus G may fall to a very low value at 34.5° when only a modest fraction of Bb junctions are dissociated. This probably explains why the rate of G buildup in Fig. 2 is so much faster after a temperature change from 34.5 to 14° than in the original polymerization at 14°; extensive oligomeric complexes are undoubtedly already present in the former case.

The product ηJ_e^0 represents a weight-average viscoelastic relaxation time related to whatever structural rearrangements are occurring (6a, 20). If the flow process under constant stress is attributed to severance of network strands, ηJ_e^0 can be identified with the average lifetime of an intact strand (20), i.e., the average time between breaks in a single strand, ~1.3 h for α-fibrin and 2.4 h for β-fibrin. One may speculate about the relation of this to the lifetime of an association between two monomer units and hence the rate of dissociation in the formulation of a dissociation equilibrium. If the rate constants for dissociation are similar in magnitude for these two fibrins, and the equilibrium constant for dissociation is much larger for β-fibrin, it follows qualitatively that the rate constant for association is lower for β-fibrin, in qualitative agreement with the comparisons in Fig. 1. Of course, $\alpha\beta$-fibrin has a much longer strand lifetime in this interpretation as well as presumably a much smaller dissociation equilibrium constant. This speculation ignores many aspects, including the number of dissociable monomer contacts in a network strand and the number of protofibrils in a strand bundle. However, one feature is unambiguous, namely, if there is a steady state of dissociation and reassociation of strands in new configurations during flow under constant stress, the actual rates of dissociation and reassociation must be the same because of the equality of J_1, J_Δ, $J' - J_s$, and J_Δ final in Table I.

We now turn to the effects of the tetrapeptides GHRP and GPRP. Binding experiments by Laudano and Doolittle (18, 29) with radioactive peptides, including use of β-fibrin from the action of thrombin on lamprey fibrinogen, show

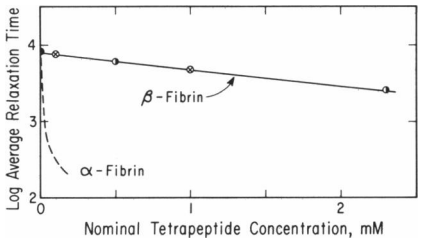

FIGURE 9 Logarithm of weight-average relaxation time plotted against nominal concentration of tetrapeptide. Points half filled, β-fibrin with GHRP; crossed, β-fibrin with GPRP; dashed curve, α-fibrin with GPRP (from Reference 7).

that GPRP is bound noncovalently to the "a" sites and less strongly to the "b" sites; GHRP is bound only to the "b" sites and somewhat less strongly to these sites than GPRP. We postulate as before (6a) that the associations between fibrin monomers are stabilized by both Aa and Bb junctions and that tetrapeptides compete with the A and B sites in accordance with these relative binding strengths.

In α-fibrin, the strong binding of GPRP to the "a" sites displaces A sites and there are no Bb junctions, so G and η are sharply decreased at very low GPRP concentrations. In $\alpha\beta$-fibrin, higher GPRP concentrations are needed for the same effect because the Bb junctions must also be dissociated and the binding of GPRP to the "b" sites is weaker. In β-fibrin, the Bb junctions are dissociated by either tetrapeptide, but somewhat more effectively by GPRP because it binds more strongly than GHRP to the "b" sites. In $\alpha\beta$-fibrin, GHRP has only a small effect (19, 20) and a combination of the two tetrapeptides is more effective than either (19). Thus, qualitatively, all the observed results can be explained in terms of equilibrium displacements.

When the effects of the tetrapeptides on ηJ_e^0 are examined, the difference between α-fibrin and β-fibrin is emphasized, as seen in Fig. 9. With the interpretation of this product as the average lifetime of a network strand, it appears that GPRP greatly increases the rate of dissociation of monomer contacts within α-fibrin protofibrils but both tetrapeptides, in the same concentration range, affect the rate of dissociation in β-fibrin only slightly. Their action on β-fibrin is principally to decrease the equilibrium number of strands contributing to the elasticity, rather than the kinetics of their rearrangement.

Finally, the essentially perfect elastic behavior of ligated clots both with and without GHRP shows that $\gamma-\gamma$ ligation (even without $\alpha-\alpha$ ligation) completely eliminates the structural rearrangements that are responsible for creep and flow in unligated clots, and reinforces the hypothesis that the latter are due to severance of network strands.

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