Cassels' thesis and other unpublished information on the NMR and X-ray crystallographic studies.

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Sites of D-Domain Interaction in Fibrin-Derived D Dimer†

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ABSTRACT: We have examined the plasmic digestion products of fibrin formed in the presence of dansylcadaverine, the fluorescent D dimer, to determine whether they are held together not only by the cross-link region on the γ chain but also by other interactions on the D domain. Antibodies to the D dimer reacted 8× more strongly with sites on the D dimer (purified or in the presence of E) than with sites on fibrinogen or plasmin-digested fibrinogen. The reactivity of this surface site was lost when the γ chain was cleaved by plasmin after the molecule had been destabilized by the removal of calcium ions, thus breaking the covalent linkage of the homodimer. The noncovalent D dimer retained its dimeric structure by the criteria of molar volume, measured by fluorescence polarization, and molecular sieving. The noncovalently attached, cross-link-containing peptide bound tightly to the parent molecules at higher temperatures but rotated more freely below 15 °C, and could be lost from the parent molecules without destroying the dimeric structure. We therefore propose that the forces maintaining the dimeric structure of the noncovalently joined molecule are not solely located at the γ-chain cross-link region. These other sites on the D domain are therefore candidates for the initial fibrinogen polymerization site and may also have a role in fibrinogen half-molecule assembly.

The early stages of the fibrinogen to fibrin conversion can be divided into three separate steps (Doolittle, 1973). The first, catalyzed by the proteolytic enzyme thrombin, involves cleavage of the A and B fibrinopeptides from the α and β chains of the fibrinogen molecule to yield a fibrin monomer. Cleavage of only the A fibrinopeptide is required for the second step, polymerization of fibrin monomers to form a urea-dissociable gel, to proceed (Blomback et al., 1978). The third step, stabilization of this gel, is carried out by calcium-requiring transglutaminase (fibrin-stabilizing factor XIIIa), which covalently links the γ chains on adjacent molecules by an isopeptide bond (Lorand, 1972). The later stages of fibrin formation involve lateral association of the initially formed linear polymers and covalent cross-linking of the α chains, through an acceptor site in the highly mobile COOH-terminal end of

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0006-2960/80/0419-4051$01.00/0

the chain and site(s) elsewhere in the α chain (Doolittle et al., 1979). Since the α chain cross-link sites are readily inhibited, form slowly relative to γ chain cross-linking, and are susceptible to proteolytic digestion even in the presence of calcium ions, it is unlikely that they are the site of initial polymerization.

Whereas the mechanism of steps 1 and 3 has been elucidated, that of step 2 is poorly understood. It has been proposed that release of the fibrinopeptides A results in exposure of the initial polymerization sites that are situated on the D domains on opposite sides of the symmetrical fibrinogen molecule (Doolittle, 1973). Evidence has been adduced for this from the fact that fragment D and fragment Y (covalent D:E) inhibit fibrin polymerization. It has been suggested that these molecules participate in the formation of the linear polymer, but since they only have one binding site they thereby effect chain termination (Doolittle, 1973). Fibrinogen and D-monomer, D-dimer, and Y fragments can also bind to insolubilized fibrin monomer (or to fibrinogen fragments containing the E domain such as NDSK, the N-terminal disulfide knot), and it has been proposed that this interaction is also a manifestation of the initial polymerization event (Blomback et al., 1978; Kudryk et al., 1974). Most other evidence has pointed to an interaction between the E and D domains, at one or more sites (Heene & Matthias, 1973; Olexa & Budzynski, 1979), as being the initial polymerization event. Therefore, there must be sites on both D and E, with the E site probably being divalent, if this theory is correct. The theory is reinforced by the apparent lack of interaction between members of the D species, and this is interpreted by other authors as excluding a major interaction between the D domains. To account for the inhibition of clotting by fragment D dimer using the aforementioned theories, it must be postulated that the initial polymerization site is either superficial or has become reexposed as a result of a conformational change either after polymerization, after cross-linking, or after proteolytic digestion.

We have previously shown that fragment D dimer contains two calcium-specific binding sites (Lindsey et al., 1978a) and that calcium ions protect the molecule against further plasminolytic cleavage even when the progression of destabilization-induced cleavages has commenced (Purves et al., 1978a,b). The extended cleavage is restricted to the γ chain, and excision of the cross-link was found to be an early postdestabilization event. The excised cross-linked peptide was found to bind extremely tightly to the parent molecule, and this suggested to us that the cleaved D dimer might retain its dimeric structure despite destabilization and proteolytic digestion, and thereby provide evidence for another interaction between the D domains.

This paper reports studies on the surface topology and the molecular size of fragment D dimer during plasmin digestion that follow destabilization of the molecule by the removal of calcium ions.

Materials and Methods

Preparation of Fibrinogen. Blood was collected into 25 mM sodium citrate (1 part per 4 parts of blood), and the plasma was immediately separated by centrifugation. The fibrinogen was precipitated by the slow dropwise addition of saturated ammonium sulfate (pH 7.4) to a final concentration of 25% (w/v). The precipitate was washed with 25% ammonium sulfate, and the spun precipitate was redissolved in a roller with a solution containing 5 mM sodium citrate, 150 mM NaCl, and 50 mM Tris at pH 7.4. The solution was clarified by centrifugation, and the process was repeated five times but using 20% ammonium sulfate. The final solution was into a 50 mM Tris buffer, pH 7.4, containing 150 mM NaCl. The whole process was performed at room temperature in 6 h and yielded a product that was at least 95% clottable and contained less than 0.1% fibronectin measured by radioimmunoassay (unpublished method). Ammonium sulfate was removed by dialysis or by rapid passage through a large-volume, short Sephadex G25 column equilibrated with the appropriate buffer.

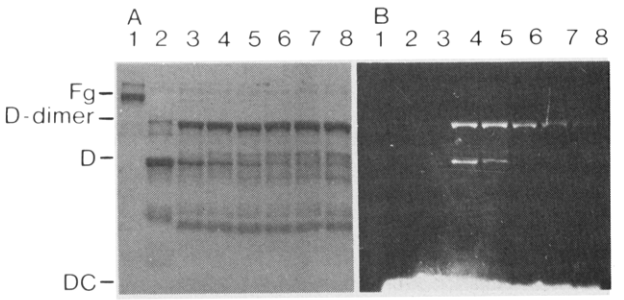

FIGURE 1: The inhibiton of polymerization by dansylcadaverine. To 0.8 mL of fibrinogen solution (2 mg/mL) was added 0.1 mL of thrombin (50 U/mL), calcium (50 mM), and dansylcadaverine (50 mM) to give final dansylcadaverine concentrations of (lane 2) 5 mM; (3) 2.5 mM; (4) 1 mM; (5) 0.5 mM; (6) 0.25 mM; (7) 0.1 mM; (8) 0.05 mM. After 8 h at 37 °C, 50 μg of plasmin was added and left for 24 h. Aliquots were applied directly to the polyacrylamide gel with the starting material in lane 1. The gels were photographed for fluorescence under UV light in the wet state (B) and stained with coomassie blue (A): Fg, fibrinogen; D, D monomer; DC, free dansylcadaverine.

Preparation of Fluorescent Fragment D Dimer. Fluorescent fragment D dimer was prepared as described previously (Purves et al., 1978a,b). A fibrinogen preparation was clotted in the presence of 2.45 mM dansylcadaverine and subsequently digested with plasmin. Calcium was added to all buffers to prevent destabilization (Purves et al., 1978a,b) and further digestion. The reaction mixture consisted of 200 mg of fibrinogen, 50 units of bovine thrombin (Parke-Davis), 4 mg of factor XIII concentrate (Behring), 5 mM $CaCl_2$, 0.15 M NaCl, and 0.1 M Tris, pH 7.4, in a volume of 20 mL. The clot, formed at 37 °C for 18 h, was homogenized, washed, and digested with plasmin for 18 h at 37 °C. The digestion mixture consisted of 1.2 mg of plasminogen (Sigma), 500 Ploug units of urokinase (Leo), 5 mM $CaCl_2$, 0.15 M NaCl, and 0.1 M Tris, pH 7.4, in a volume of 20 mL. The digested mixture was dialyzed against 0.01 M Tris, pH 8.6, containing 1 mM $CaCl_2$ and chromatographed on a DEAE-52 column to remove fragment E (Nussenzweig et al., 1961). The eluate was analyzed by electrophoresis on $NaDodSO_4$–polyacrylamide gels.[1] Those fractions containing no fragment E were pooled, concentrated by dialysis against poly(ethylene glycol), mol wt 20 000, and chromatographed on a Sephadex G200 column (100 × 2.5 cm) in 0.1 M Tris buffer, pH 7.4, containing 0.5 mM $CaCl_2$. The peak of fragment D dimer was pooled, concentrated by dialysis against poly(ethylene glycol), and rechromatographed.

Nonfluorescent fragment D dimer was prepared by omission of the dansylcadaverine during clotting.

Preparation of Fluorescent D Monomer. Fluorescent fragment D monomer was a byproduct of D-dimer purification. The yield of D monomer was determined by the amount of dansylcadaverine present (Figure 1). If the concentration of dansylcadaverine is over 1 mM, fluorescent D monomer appears, and at 5 mM clotting as well as cross-linking are com-

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

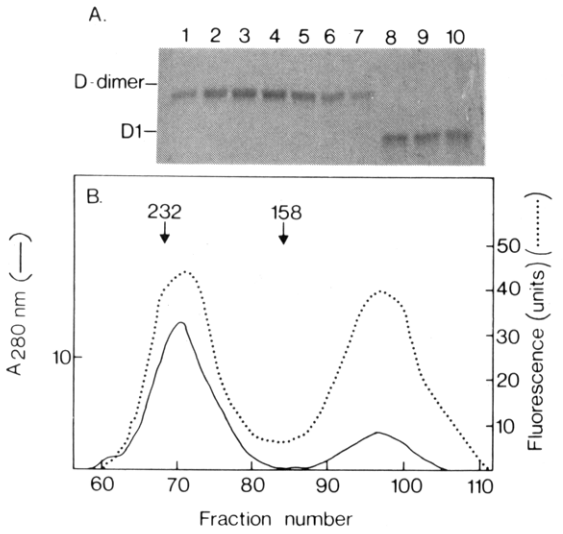

FIGURE 2: The separation of fluorescent D monomer and D dimer on molecular sieving. The fluorescent proteins obtained from a calcium-stabilized digest of fibrinogen clotted in the presence of 1.5 mM dansylcadaverine were eluted from DEAE-52 Sephadex, concentrated, and applied to an Ultrogel AcA 34 column (100 × 2.5 cm) in a 1-mL volume and eluted with a Tris buffer (see text). The position of elution of two other proteins is shown: catalase, mol wt 232 000, and aldolase mol wt 158 000. The fractions at positions 63, 67, 79, 71, 73, 75, 77 and 97, 99 and 101 (lanes 1–10) were subjected to polyacrylamide gel electrophoresis and stained with coomassie blue (A). In B the relative fluorescent intensities of the monomer and dimer can be seen. The absorbance at 280 nm and fluorescence were measured on each sample.

pletely inhibited. The D monomer is probably formed directly and not as a result of degradation of D dimer, since the fluorescent γ chains, under reducing conditions in NaDodSO_4–polyacrylamide electrophoresis, are exactly the same size as those of fibrinogen-derived D monomer. The molecule elutes in the monomer position if the plasmin-digested dansylcadaverine-inhibited fibrin is applied directly to the molecular sieving column (Figure 2). The mechanism of inhibition has not been investigated further yet. The fluorescent group in the D monomer can be shown (Purves et al., 1978b) to be in the expected position at the C-terminal end of the γ chain. We have previously shown that the only fluorescent D species derived from D dimer is one of the earliest products of destabilization-induced cleavage, where the γ chain is larger than usual due to unilateral cross-link cleavage (Purves et al., 1978b). The D monomer was eluted from the Sephadex G200 column after fragment D dimer, pooled, concentrated, and rechromatographed as for fluorescent fragment D dimer. The nature of the fluorescent D monomer is not a primary concern of this paper, but since this protein was conveniently available it seemed more appropriate to compare it with D dimer than with a chemically dansylated protein.

Plasmin Digestion of Destabilized D Species. Plasminogen (Sigma) was activated for 1 h at room temperature with urokinase (Leo) (Castellino & Sodetz, 1976). The reaction mixture consisted of 5 mg of plasminogen, 200 Ploug units of urokinase, 0.1 M Tris, and 0.15 M NaCl, pH 7.4, in a volume of 1 mL. Plasmin activity was determined before any digestion by measuring the rate of increase of absorbance at 247 nm of a 1 mM solution of *p*-tosyl-L-arginine methyl ester hydrochloride (Sigma), in 0.1 M Tris and 0.15 M NaCl buffer, pH 7.4, as described by Walsh (1970) for trypsin. Fragment D dimer was digested with preactivated plasmin at 37 °C with a molar ratio of 100:1. The reaction mixture consisted of 6 mg of D species, 3 mM EGTA, plasmin (activity equivalent to 60 μg of fully activated plasminogen), and 0.15 M NaCl, pH 7.4, in a volume of 2 mL. The reaction was stopped by the addition of 5000 Kallikrein units of Trasylol (Bayer) (Kunitz plasmin inhibitor; Vogel et al., 1968).

Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of 0.1% NaDodSO_4 was carried out on slab (200 × 140 × 2 mm), 5–20% density gradient gels according to the method of Laemlli (1970). The gels were photographed for dansyl fluorescence under ultraviolet illumination using an orange and UV filter before coomassie blue staining.

Molecular Sieving. Gel filtration of fluorescent D dimer and digests was carried out on a column (100 × 2.5 cm) loaded with either Sephadex G200 or Ultrogel AcA 34, using a Tris buffer, at pH 7.4, containing 0.15 M NaCl, 5 mM CaCl, and 50 units/mL Trasylol. The separation of these D species has been described in previous papers (Purves et al., 1978a,b). Calibration of the column was carried out with protein standards of known molecular size (B.D.H. and Pharmacia), viz., ferritin, fibrinogen, catalase, albumin, carbonic anhydrase, and myoglobin, dissolved in the column buffer and applied in the same volume as for the D species. The interpolated Stokes radii yielded molecular weights that were within 10% of the values in the literature. The separation could be improved using Ultrogel AcA 34 (Figure 2). The flow rate was 13 mL/h and 3.5-mL fractions were collected, diluted with radioimmunoassay buffer, and assayed using the D-dimer radioimmunoassay.

Fluorescent Measurements. Fluorescence was determined with a Hitachi MFP 44A fluorescence spectrophotometer using 1-cm quartz cells (Helma). Dansyl fluorescence was determined by exciting the sample at 335 nm and measuring the light emitted at 518 nm. Fluorescence polarization was determined in three separate readings on each sample at each temperature with the Hitachi instrument using a Polacoat filter for the exciting light and a Polaroid filter for the emitted light. The polarizing filters were rotated manually, and the sample chamber was flushed with dry nitrogen in order to remove condensation. This was the limiting factor in operating at low temperatures. Polarization was calculated using the equation $P = (V - gH)/(V + gH)$, where V represents vertical excitation and emission polarizer, H represents vertically polarized excitation and horizontally polarized emission, and g represents a correction factor consisting of the ratio of vertically to horizontally polarized emission with excitation in the horizontal plane (Brand & Witholt, 1967). Samples were diluted 1:1 with buffer containing 40% w/v sucrose. The sample temperature was maintained by means of an external water bath with the temperature controlled by a Haak E52 pump/heater and a flow-through cooler. The sample temperature was measured with a precalibrated digitherm digital thermometer (Taeuber and Corssen, Johannesburg) with the probe inserted into the spare cuvette that was filled with 20% sucrose. The sample temperature could be measured to ±0.05 °C and was maintained by the water bath at ±0.1 °C.

Properties of the Antisera. A number of antisera prepared by immunizing rabbits with human fibrinogen, D monomer, D dimer, and fragment E were tested. The purpose of the project was to find antisera capable of detecting the cross-linked site on the D dimer—the only obvious feature distinguishing fibrin—from fibrinogen degradation products. This was not successful. The antiserum used in this study had the highest titer in the detection of the D domain but did not differ qualitatively from the other D domain antisera and could be completely absorbed with D monomer and almost completely with fibrinogen. The residual activity in the latter case was at a very low titer and is the anti-D neospecificity described

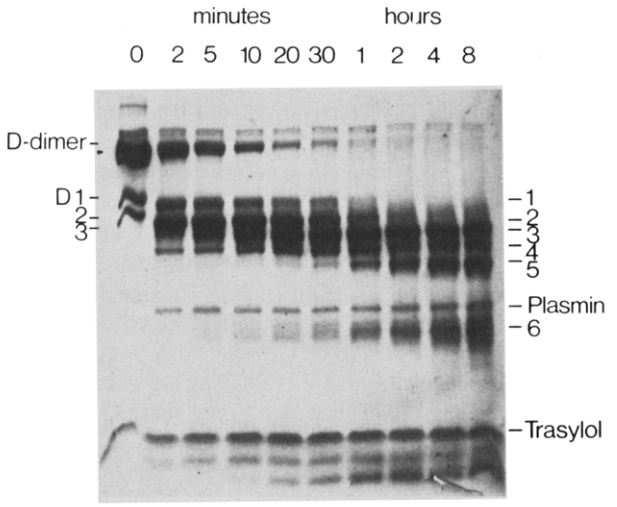

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of D dimer after destabilization. Fluorescent D dimer was destabilized by the chelation of calcium ions with buffered EGTA and digested with preactivated plasmin. Samples were taken at timed intervals and the reaction was stopped with Trasylol. A slab gradient gel 5–20% (200 × 140 × 2 mm) was used, and the proteins were stained with Coomassie blue. D1–6 refer to the D species of decreasing size due to progressive cleavage of the γ chain. Not shown is the larger fluorescent D species due to unilateral cleavage of the γ chain that is only detectable at an early stage with heavy applications of protein (Purves et al., 1978b). Plasmin and Trasylol components and the D-monomer contaminant of the D-dimer preparation can also be seen.

by Plow & Edgington (1975), but it could be quantitatively ignored in this study due to the high dilution of the antiserum used. Fragment E was unreactive to the antiserum. The relative affinities and potencies of the fibrinogen derivatives are described under Results. Various isolated preparations of mixtures of D2, 3, 4, and 5 were tested and found to have virtually no activity, but this appears to be a function of denaturation, since the unresolved, freshly degraded D dimer derived D species are quite reactive, although not in a predictable manner, in different preparations.

Radioimmunoassay Procedure. The antiserum used had been prepared by injecting fragment D dimer into rabbits using standard techniques. The D dimer was labeled with ^{125}I by the Chloramine T procedure (Greenwood et al., 1963), and labeled D dimer was separated from free ^{125}I by Sephadex G25 filtration using the radioimmunoassay buffer. The labeled D dimer was shown to have the same molecular sieving properties as unlabeled D dimer. All samples were assayed in duplicate, and dilutions were made using the radioimmunoassay buffer 0.05 M Tris, 0.15 M NaCl, 10 mM ε-aminocaproic acid, 5 mM $CaCl_2$ or 5 mM EGTA (where appropriate), 0.1% NaN_3, 10 g of bovine albumin, 100 000 units of Trasylol, and 2000 units of heparin, pH 7.5 (volume 2 L). Bound and free ligands were separated by precipitation of the bound ligand with a second antibody consisting of a donkey antiserum against rabbit γ-globulin (Burroughs-Wellcome). The precipitates were counted for a minimum of 10 000 counts per tube. Binding data were calculated using a Univac computer and the analytical procedure of Rodbard (1974).

Results

Plasmin Digestion. $NaDodSO_4$–polyacrylamide gel electrophoresis showed that plasmin digestion of fluorescent fragment D dimer destabilized by calcium ion chelation with EGTA resulted in degradation to smaller molecular weight species, D2–6 (Figure 3). The fluorescent cross-link-containing peptide migrated as a species with a molecular weight of less than 10 000 (Purves et al., 1978b). After digestion for 20 min less than 2% of the D dimer had not been degraded, and the digestion mixture consisted mainly of D2 and D3. After digestion for 1 h, the chief digestion product was D3. All subsequent experiments on partially digested D dimer were carried out with the samples digested to the same extent as the 20-min and 1-h digests.

Molecular Sieving of Degraded D Dimer. Identical volume aliquots of the starting material and 20-min and 1-h plasmin digests of D dimer were subjected to gel filtration on a Sephadex G200 column previously calibrated with D-monomer and D-dimer fragments and standards of known molecular weight. Trasylol was included in the buffer to prevent digestion during chromatography. Fractions of the eluate were assayed by a D-dimer radioimmunoassay and for fluorescence. The absorbance traces were the same in all three cases, but since Trasylol had been included in the buffers and applied samples, the recovery of the protein was checked by subjecting peak samples to polyacrylamide gel electrophoresis, where it could be confirmed that the proteins maintained the same relative amounts as the applied aliquots. Therefore, despite plasmin digestion, all the samples were eluted by the same volume of buffer (Figure 4), confirming that the digested D species were of the same combined molecular size as the parent molecule. Despite this, polyacrylamide gel electrophoresis confirmed that the D species in the 20-min and 1-h sample peaks were monomeric and that the fluorescence was of low molecular weight. The fluorescence, however, eluted in the same peak as the radioimmunoassay positive material. About 50% of the fluorescence was lost in the 1-h sample, presumably by gradual dissociation during molecular sieving and subsequent binding to the Sephadex. Attempts to dissociate the fluorescent peptide prior to application to the column, by repeated freezing and thawing, did not yield a low molecular weight fluorescent peak. The technique of molecular sieving on Sephadex G200 is not sufficiently sensitive to indicate the loss of the 10 000-dalton peptide from the dimer of molecular weight 180 000. More of the fluorescent material (up to 90% of that applied) is detectable in the dimer position if the sample is freshly prepared and not stored at low temperatures.

The antigen recognized in the radioimmunoassay decreased with time, however, to approximately 14 and 10% at 20 min and 1 h, respectively, but this was not matched by the appearance on molecular sieving of a monomer species in addition to the already present D-monomer contaminant.

These results suggest that the dimeric structure of the D dimer is maintained even if the molecule has been cleaved or the cross-linking peptide has been lost by absorption to the column material.

Fluorescence Polarization. The fluorescence polarization of D-monomer and D-dimer fragments was determined at temperatures between 5 and 30 °C with either 0.5 mM calcium ions or 1 mM EGTA present in the medium. The Perrin plot of $1/P$ vs. T/η, where P is the observed polarization, T is the absolute temperature (Kelvin), and η is the viscosity (poise), is shown in Figure 5. The ordinal intercepts of the linear regression of the D monomer and D dimer were similar ($p < 0.05$, $n = 6$). The Perrin equation predicts that the ordinal intercept (a function of P_0) is characteristic of the fluorophore and independent of the molar volume associated with the fluorescence. The Perrin equation is

$$\left[\frac{1}{P} - \frac{1}{3}\right] = \left[\frac{1}{P_0} - \frac{1}{3}\right]\left[1 + \frac{RT}{\eta V}\tau_0\right]$$

where P_0 is polarization P extrapolated to $T/\eta = 0$, R is the gas constant, T is the temperature, τ_0 is the lifetime of the

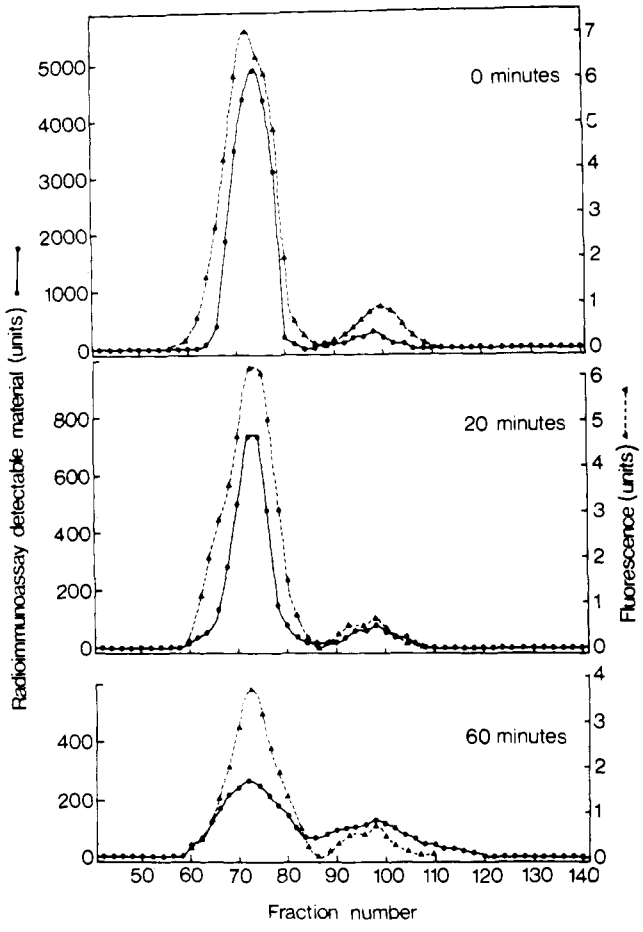

FIGURE 4: Gel filtration of fluorescent D dimer and destabilized, digested D species. Samples were eluted from the same column (100 × 2.5 cm) containing Sephadex G200, in 3.5-mL fractions at a flow rate of 13 mL/h. The samples were assayed by the ^{125}I-labeled D-dimer radioimmunoassay (—) and by dansyl fluorescence (- - -). The absorbance traces were indistinguishable in each case (not shown). The decrease in fluorescence was from 100 to 86 to 50% and the decrease in the D-dimer assay-positive material was from 100 to 14 to 10% in the samples taken at 0 time, 20 min, and 1 h. The fluorescence, antibody-reactive, or protein content of the material in the monomer contaminating the applied sample did not change. Polyacrylamide gel electrophoresis of the peak material confirmed the presence of the protein in the same ratios as the applied sample, and no protein was detectable in later fractions or in the column wash. The fluorescent peptide bound to the column was very difficult to remove.

excited species, V is the molar volume (milliliters per mole), and η is the viscosity of the solvent.

No difference could be detected by excluding calcium ions from the medium, demonstrating that resulting conformational changes were small. Marguerie (1977) using circular dichroism could detect few if any conformational changes brought about by the binding of calcium to fibrinogen. The slopes of the Perrin plot are expected to be proportional to the molar volumes only if spherical geometry is assumed (Brand & Witholt, 1967), but were in the ratio 1.74:1 (mean of three determinations).

Fluorescent D monomer was used as a conveniently available reference protein, but a chemically dansylated one would also have been suitable.

Since the monomeric and dimeric species can be easily distinguished using fluorescence polarization, this technique was applied to partial plasmic digests of destabilized fragment D dimer (Figure 6). The digestion was stopped by the addition of Trasylol. Plasmin and Trasylol were also simultaneously added to fragment D dimer as a zero time control.

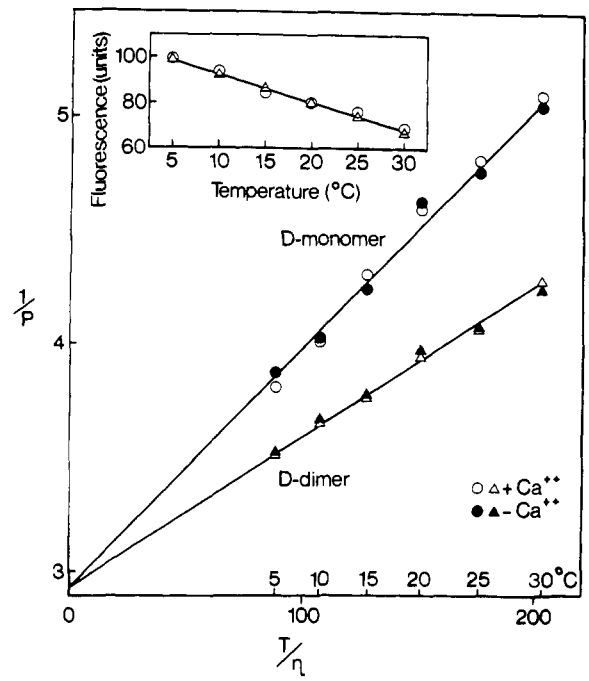

FIGURE 5: Perrin plots of the fluorescence polarization of fluorescent D monomer and D dimer. The upper graph refers to D monomer and the lower to D dimer (see Materials and Methods) in the presence (○, △) and absence (●, ▲) of calcium ions but in the presence of plasmin inhibitors. The horizontal axis is T/η, where T is the temperature (Kelvin) and η is the viscosity (poise) of 20% sucrose at that temperature. The temperature in degrees centrigrade is also shown. The vertical scale is the reciprocal of polarization (see text). The ordinal intercepts are not significantly different at the 95% confidence level: emission, 518 nm; excitation, 335 nm. Inset: The change in absolute fluorescence with temperature normalized to 100% at 5 °C is shown for both species.

$NaDodSO_4$–polyacrylamide gel electrophoresis of samples taken immediately after the digestion had been stopped and samples taken after the fluorescence polarization had been determined showed that no further digestion occurred once Trasylol had been added. The Perrin plots for the undigested and digested material were unaffected by the presence or absence of calcium ions. As the temperature was decreased to below 15 °C the digested samples showed less polarization, and the Perrin plot became nonlinear (Figure 6). This tendency was greater for the more digested species. The degree of dissociation of the peptide from the D domains was studied on a 1-h digest of D dimer (Figure 7) at temperatures down to −1.3 °C. The polarization was similar to that of the D dimer at higher temperatures, but below 3 °C it matched that of the D monomer. Because this could represent dissociation to monomers, the degree of dissociation was calculated using the extrapolated values of the D monomer and dimer Perrin plots (Figure 7, inset). On raising the temperature there was recovery of polarization equivalent to that of the dimer. This result was reproducible with different preparations of D dimer and after repeating the experiment with the same sample in the opposite direction of temperature change.

These results suggest that the totally cleaved fluorescent peptide can rotate independently of at least one of the monomers at low temperatures but that reassociation of the monomers is complete at higher temperatures. It should be noted that independent rotation does not imply complete loss of contact and that the molar volume appropriate for the fluorescence polarization is apparent rather than actual, since degrees of independent rotation are possible. The free peptide itself would have negligible fluorescent polarization. The fluorescent peptide could be rotating independently of the

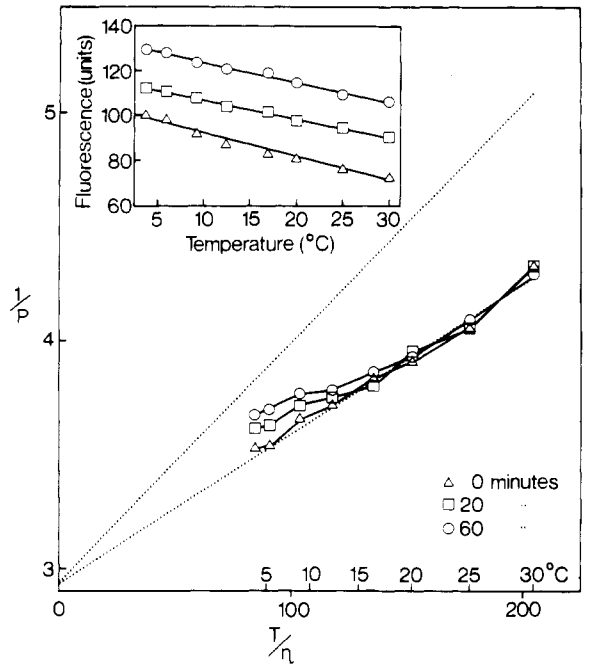

FIGURE 6: Change of fluorescent polarization of digested D dimer at low temperatures. The scales are the same as in Figure 5, and the Perrin plots of D dimer and D monomer have been repeated for comparison. Identical quantities of D dimer were destabilized by the chelation of calcium ions with EGTA and digested with plasmin (see text): 0 time (△); 20 min (□); 60 min (○). Inset: The total fluorescence of the D species is shown at increasing temperatures. The highest fluorescence of the undigested, 0-time D dimer has been used to normalize the results: emission, 518 nm; excitation, 335 nm.

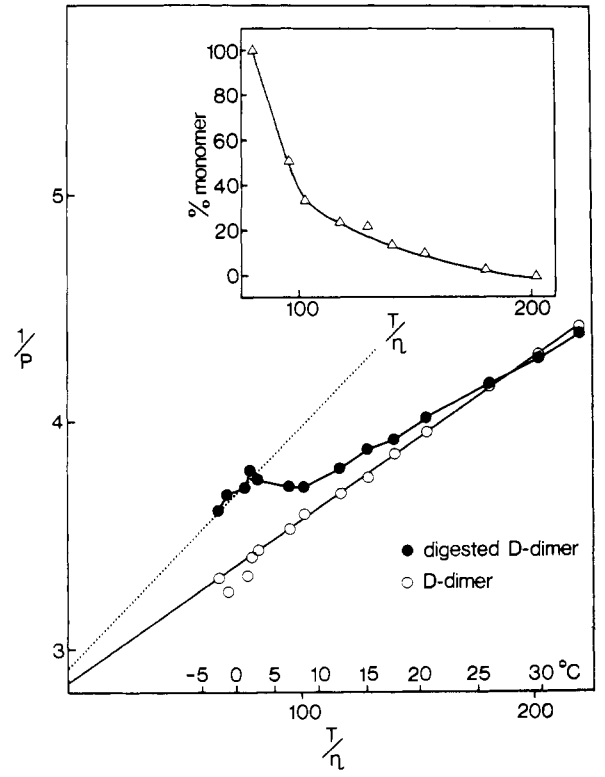

FIGURE 7: The dissociation of digested D dimer at low temperatures. The Perrin plots of D dimer and monomer from Figure 5 have been repeated for comparison. A 60-min digest of D dimer was used. The experiment was performed by dropping the temperature to the lowest level possible and then allowing the temperature to increase. Inset: The amount of dissociation to monomer was calculated assuming that this was the reason for the change in fluorescence polarization using the extrapolated linear regressions of the D dimer and D monomer: emission, 518 nm; excitation, 335 nm.

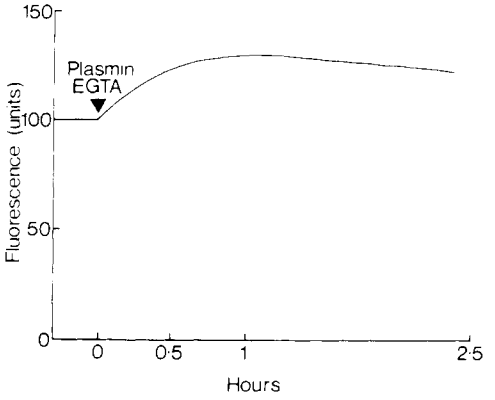

FIGURE 8: The enhancement of fluorescence of fluorescent D dimer during plasmin digestion after destabilization. The sample was continuously monitored for dansyl fluorescence after EGTA and plasmin had been added to the carefully buffered system. There was no change on adding EGTA or plasmin alone. At the peak the sample consisted mainly of D3 monomer in polyacrylamide electrophoresis (see text): emission, 518 nm; excitation, 335 nm.

Table I: Comparison of Logit Transform Slopes and Potencies

sample	slope	potency	range upper	range lower
Fg	−0.7638	1	0.881	1.131
pFg	−0.7564	1.058	0.968	1.156
pFb	−0.9901	8.806	7.642	10.172
pure D dimer	−0.9847	8.8190	7.337	9.529

still-associated dimer or the peptide could be bound to and rotating with an independently rotating monomer.

Fluorescence. It was noticed during fluorescence polarization measurements (Figure 6, inset) that the digested samples had increased fluorescence relative to that of the starting material. The fluorescence of a D-dimer sample was therefore continuously monitored during plasmin digestion (Figure 8). No change in fluorescence was detected as a result of chelation of calcium ions with EGTA, but as plasmin digestion proceeded an increase in the fluorescence occurred reaching a maximum of 135% enhancement after 1 h. At this point the digestion mixture consisted mainly of D3 (cf. Figure 3). Continued digestion led to a slight decrease in fluorescence. After 2.5-h digestion, the enhancement of fluorescence had decreased to 128%. No differences among the individual samples in the slight temperature changes in the emission or excitation spectra were observed during these experiments. On the basis of the increased fluorescence, it can be proposed that the fluorescent cross-link peptide retreats to a more hydrophobic interior environment from a surface site.

Analysis of Radioimmunoassay Results. The antiserum to D dimer was unabsorbed with other fibrinogen antigens and could not detect purified fragment E. The assay gave the same results in the presence of calcium ions or EGTA as long as plasmin was inhibited. In the assay using radiolabeled D dimer (Figure 9), purified D dimer and fibrin digested by plasmin (pFB) showed identical affinity and extent of reaction and were 8× more reactive than fibrinogen (Fg) and plasmin-digested fibrinogen (pFG), which were both equivalent. Complete inhibition of radioligand binding was observed at high enough concentrations of all the proteins used, and the logit plots were linear over a wide range (Figure 9, inset). The assay curves were sufficiently precise to demonstrate the identity of the D dimer and digested fibrin (pFB) curves and the similarity of the fibrinogen (Fg) and digested fibrinogen (pFG) curves. In an assay using radiolabeled fibrinogen instead of D dimer (not shown), the results were similar, but there was only a 4×

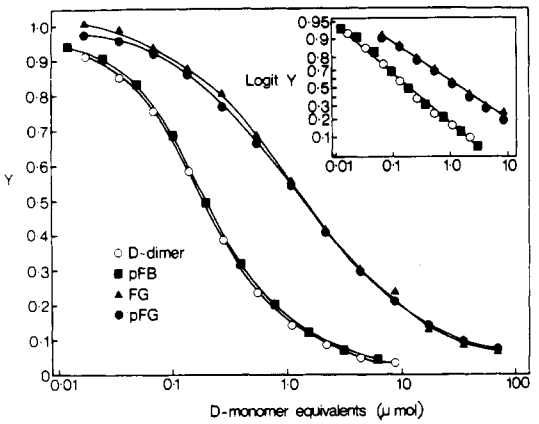

FIGURE 9: The radioimmunoassay of the fibrinogen species. The quantities of material assayed were identical aliquots: Fg (fibrinogen), pFG (fibrinogen digested with plasmin with the late addition of calcium ions), and pFB (fibrinogen clotted and digested with plasmin in the presence of calcium ions). The results were expressed in terms of the content of D domain using molecular weights: fibrinogen, 340000; D, 90000. ^{125}I-labeled D dimer was used as the radioligand and anti-D dimer in all cases. The samples were doubly diluted. The horizontal scale is given in equivalents of D-monomer content (micromoles) and the vertical scale is given in counts precipitated, expressed as a fraction of uninhibited precipitation with nonspecific binding substracted. The graphs of the logit transforms of the radioimmunoassay data are shown in the inset. A comparison of logit transform slopes and potencies is shown in Table I. The slope of the logit transform was used to compare affinities. The fibrinogen and fibrin derivatives were distinctly different ($p < 0.01$) using an F test and Student's t test for homogeneity of variance. The potencies have been calculated by the method of Finney (1964) and normalized; the upper and lower 95% confidence limits are given. The logit transformation is obtained by plotting the logarithm of the dose against ln $(y/(1 - y))$, where y is the response (counts precipitated) expressed as a fraction of the uninhibited response with nonspecific binding subtracted from all results.

difference in potencies between fibrin and fibrinogen derivatives.

These results suggest that the same antigen is operative on the surface of native fibrinogen, digested fibrinogen (without the exposure of more sites), digested fibrin, and on pure D dimer in the absence of fragment E. The differences in affinities of the cross-linked and unlinked D domains could be due to different conformations and not necessarily due to the cross-link itself that does not appear to contribute a new antigen. The reactivity of other D species has been commented on under Properties of the Antiserum. The presence of the E domain in any form does not appear to affect reactivity.

Discussion

Our previous studies (Lindsey et al., 1978a) showed that a single calcium-specific binding site is present on the D domain and that calcium ions stabilize the γ chain against further plasmin proteolysis (Purves et al., 1978a,b). The cross-link-containing peptide can be cleaved by plasmin if the γ chain is destabilized by the removal of calcium ions. This cross-link peptide binds tightly to the parent molecule(s) and, when separated by detergent treatment (Lindsey et al., 1978b), can be shown to contain the glutamine–lysine cross-link site as well as the fluorescent lysine analogue, viz., dansylcadaverine, that is presumably attached at the spare cross-link site (Glu-14) on the γ chain.

Our evidence for an inter-D-domain interaction in addition to the covalent cross-link on the γ chain (between Glu-14 and Lys-6, from the C-terminal end) consists of the demonstration that (1) D dimer with a number of cleavages of the cross-linking γ chain remains associated as a hydrodynamic dimer, (2) the actual loss of the cross-link-containing peptide does not produce dissociation to monomers, (3) the (fluorescent) cross-link-containing peptide can rotate independently of at least one of the D domains at low temperatures, (4) the cross-link region is superficial because of susceptibility to plasmin (possibly at Arg-21 and -37 or Lys-27, -31, -32, -39, etc., from the C-terminal end) and because the fluorescent group increases fluorescence when the cross-link is cleaved, and the peptide therefore probably moves to a more hydrophobic interior environment, and (5) since the antibody-reactive site seems to be close to the cross-link site, they must both be fairly superficial.

The first three points suggest that the cross-link site is not essential for the rest of the molecule to remain associated. Since the cross-link site is probably superficial, it should not be able to contribute a great deal to the forces maintaining the dimer, especially if a large surface area needs to be involved.

The contiguity or identity of the cross-link site and the antibody-reactive site (point 5) has been deduced from the concomitant loss of the antibody reactivity at a time when the cross-link is cleaved. This is more obvious if the cleaved dimer is stored at low temperatures for a time prior to radioimmunoassay. In addition, there is evidence that at an early stage (Purves et al., 1978a) destabilization-induced γ-chain cleavage also destroys the ability of the D dimer to act as an anticoagulant (Dray-Attali & Larrieu, 1977) or to bind to insolubilized E domain (in the form of fibrin monomer or the N-terminal disulfide knot (NDSK)). It seems likely, therefore, that the cross-link region is also an important part of the site for interactions between D and E domains and must therefore be superficially situated.

Our conclusions are that the cross-link site itself and a region on the γ-chain close by cannot be the sole contributors to inter-D-domain interactions.

Thermodynamic studies have shown that fibrinogen polymerization is a highly exothermic reaction, in contrast to an antibody–antigen reaction, and must, therefore, involve many residues in a large surface area (discussed by Doolittle, 1973).

We have only been able to produce dissociation and reassociation of the noncovalently linked D dimer under controlled conditions at low temperatures that are known to cause the dissociation of hydrophobic bonds (reviewed by Bock & Frieden, 1978). No reassociation of the D domain in any other form has otherwise been observed by us. It has been claimed (Ferguson et al., 1975) that cross-linked D dimer can be produced from D monomer, i.e., the fibrinogen-derived species; however, we have been unable to confirm this even under conditions that permit the cross-linking of fibronectin to fibrinogen by the fibrin-stabilizing factor. It would appear that the ability of the D domains to interact (in the putative initial polymerization site) is determined by an unstable or transient configuration in the monomeric condition and that progressive conformational changes may occur preventing reversible polymerization.

The sites that would contribute to the noncovalent forces maintaining the dimer would include the large β-chain fragment, the small α-chain fragment, and the rest of the γ chain with the unlikely participation of the rigid "coiled coil" region at the N-terminal end of the molecule (Doolittle et al., 1978).

Uncertainty still surrounds the actual shape of the fibrinogen molecule (especially since the role of calcium has been largely ignored in this context), and the mode of assembly of this complex molecule is not known, so that it is possible to speculate on the role that an inter-D-domain interaction might play in contributing to the intramolecular fibrinogen structure.

Further studies to locate the calcium-binding site and the initial polymerization site will be greatly facilitated by the recently published completion of the fibrinogen primary sequence (Doolittle et al., 1979).

Acknowledgments

We were ably assisted by N. Linton.

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Reaction of Brain Hexokinase with a Substrate-like Reagent. Alkylation of a Single Thiol at the Active Site†

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ABSTRACT: An analogue of the substrate glucose, *N*-(bromoacetyl)-D-glucosamine (GlcNBrAc) inactivates bovine brain mitochondrial hexokinase completely and irreversibly in a pseudo-first-order fashion at pH 8.5 and 22 °C. The rate of inactivation of hexokinase by this reagent does not increase linearly with increasing reagent concentration but exhibits an apparent saturation effect, suggesting the formation of a reversible complex between the enzyme and the reagent prior to the inactivation step. The pH dependence of the rate of inactivation suggests that a group on the enzyme with pK_a = 9.1 is being modified by this reagent. At pH 8.0 the rate of inactivation by this reagent is very slow, and it can be shown to be a competitive inhibitor of the hexokinase reaction with respect to the substrate glucose. The substrates glucose and ATP strongly protected the enzyme against the inactivation reaction. The inactivation of the enzyme was found to be accompanied by the alkylation of two sulfhydryl residues as shown by the formation of ~2 mol of *S*-(carboxymethyl)-cysteine/mol of inactivated enzyme. Treatment of the enzyme with ^{14}C-labeled reagent results in the incorporation of ~2 mol of reagent/mol of inactivated enzyme. However, the enzyme protected by glucose still shows the incorporation of ~1 mol of the labeled reagent/mol of the enzyme. From a tryptic digest of the enzyme inactivated by this reagent, two labeled peptides were obtained, one of which was absent if the labeling reaction was carried out in presence of glucose. These results indicate that the affinity reagent reacts with two thiols, only one of which is crucial for the activity of the enzyme and is located in the region of its active site.

Previous reports from this laboratory provided evidence for the presence of thiol groups at or near the active site of bovine brain mitochondrial hexokinase, type I (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1). This evidence was mostly based on an investigation of the inactivation kinetics of the enzyme using 5,5′-dithiobis(2-nitrobenzoic acid) and tetranitromethane as protein modification reagents (Redkar & Kenkare, 1972, 1975; Subbarao et al., 1973; Subbarao & Kenkare, 1977a). Further documentation for the presence of an essential thiol at the active site of this enzyme came from experiments in which the enzyme was inactivated by an affinity reagent, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate, which is an analogue of ATP (Subbarao & Kenkare, 1977b).

Our search to identify glucose analogues as affinity reagents for brain hexokinase led us to explore the use of haloacetyl

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0006-2960/80/0419-4058$01.00/0