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Location of Plasminogen-Binding Sites in Human Fibrin(ogen)†

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ABSTRACT: Affinity chromatography of various fibrinogen and fibrin fragments on Lys-plasminogen–Sepharose was used to localize the plasminogen-binding sites in human fibrin(ogen). The fragments studied in the present investigation were derived from the central (E) and the terminal (D) globular domains of fibrinogen and fibrin. Our results showed that these two different, sequentially nonidentical domains of fibrin(ogen) both carry plasminogen-binding sites. Competitive affinity chromatography of fragment D_1 and fragments derived from it by proteolytic modification of its D γ-chain revealed that this modification causes an 11-fold increase of the association constant of the interaction with Lys-plasminogen–Sepharose. This suggests that the carboxy-terminal region of the D γ-chain is involved in controlling the plasminogen-binding site of the D domain. In contrast with its fragments, intact fibrinogen is not retained by Lys-plasminogen–Sepharose, indicating that the plasminogen-binding sites present in the constituent E and D domains are not fully functional in the parent molecule. It seems possible that the plasminogen-binding sites are present but hidden in fibrinogen and proteolytic dissection of the molecule uncovers these sites in E and D fragments by removing peptides masking the plasminogen-binding regions.

It is generally accepted that the main intravascular function of plasmin is the dissolution of fibrin clots and removal of fibrin deposits from vessel walls. Plasmin eliminates fibrin deposits by cleaving the fibrin molecule at a large number of different peptide bonds with concomitant conversion of the insoluble fibrin polymer into soluble fragments. Despite its trypsin-like sequence specificity, in vivo plasmin normally does not attack other plasma proteins, not even fibrinogen, the soluble precursor of fibrin. A unified molecular model was proposed to explain why plasmin activity is restricted to fibrin (Wiman & Collen, 1978). According to this proposal, plasminogen and plasminogen activator are bound to the fibrin polymer via fibrin-specific binding sites, and plasminogen activation occurs only on the fibrin surface. Plasmin remains bound to fibrin through the same fibrin-binding sites, as well as its active site region, until dissolution of the clot.

The specific interaction of fibrin and plasminogen is thus crucial to the above regulatory mechanism. The subject of the present investigation is the localization of the plasminogen-binding sites in the fibrin(ogen) molecule.

Fibrinogen is an M_r 340 000 protein composed of two sets of three nonidentical chains (Aα, Bβ, γ)$_2$, connected by a network of disulfide bridges [for a review, see Doolittle et al. (1978)]. By electron microscopy, the molecule appears as an elongated trinodular structure (Price et al., 1981; Telford et al., 1980; Fowler et al., 1980); the central nodule contains the amino-terminal globular domain where the two symmetrical halves of the molecule are linked. Thrombin-catalyzed removal of fibrinopeptides A and B unmasks two pairs of polymerization sites in the central nodule (Laudano & Doolittle, 1978; Olexa & Budzynski, 1980). Through these sites, the bivalent central domain binds terminal globular domains of two other

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0006-2960/83/0422-2440$01.50/0

fibrin monomers by strong noncovalent bonds. The half-molecule staggered overlap between reciprocally paired fibrin units (Williams, 1981) ensures that polymerization can proceed in both directions to form an insoluble polymer.

In vitro, the susceptibility of fibrinogen and fibrin to plasmin permits the dissection of these proteins into defined structures (Nussenzweig et al., 1961; Marder et al., 1969; Takagi & Doolittle, 1975a). The initial plasmic cleavages of fibrinogen remove random-coil peptide extensions but do not affect the trinodular structure (fragment X). Further plasminolysis attacks the three-stranded rope connecting the central and terminal nodules but does not damage the globular domains (Fowler et al., 1980; Marder et al., 1969; Takagi & Doolittle, 1975a), leading to the release of 2 mol of the terminal globule (fragment D) and a fragment corresponding to the central nodule (fragment E).

The ease with which individual domains of fibrin(ogen) can be obtained by plasmic digestion facilitated the assignment of the plasminogen-binding sites to various domains of fibrin(ogen). Affinity of fibrin(ogen) fragments for plasminogen was determined by affinity chromatography. Fibrin has much greater affinity for Lys-Pg[1] than for Glu-Pg (Thorsen, 1975; Lijnen et al., 1981), and it is assumed that the binding of Lys-Pg to fibrin has greater physiological significance in fibrinolysis. Therefore, in the present study we used immobilized Lys-Pg as the affinant.

Experimental Procedures

Preparation of Plasminogen, Plasmin, and Lys-Pg–Sepharose 4B. Human native plasminogen, Glu-Pg, was prepared from fresh citrated plasma by affinity chromatography on lysine–Sepharose 4B (Deutsch & Mertz, 1970). All purification steps were performed in the presence of pancreatic trypsin inhibitor (Trasylol, Bayer) to prevent proteolysis of plasminogen. Proteolytically modified plasminogen, Lys-Pg, was obtained by treatment of Glu-Pg with Sepharose-bound plasmin. The conversion of Glu-Pg to Lys-Pg was checked by polyacrylamide slab gel electrophoresis in urea/acetic acid, pH 3.2 (Walther et al., 1975).

Glu-Pg was activated to plasmin with urokinase–Sepharose 4B (Wiman & Wallén, 1973). The active enzyme was stored in 50% glycerol solution at −20 °C. Plasmin activity was assayed with tripeptide substrate, D-Val-Leu-Lys-pNa (S-2251, Kabi, Stockholm), according to the manufacturer's instructions.

Lys-Pg was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer and was stored at 4 °C in 0.1 M ammonium bicarbonate containing 100 KIU/mL Trasylol and 0.1% sodium azide. The degree of substitution was 5.5 mg of Lys-plasminogen bound per mL of settled gel. Aliquots (0.5 mL) of Lys-Pg–Sepharose were activated with urokinase, and the active site titration of the urokinase-treated Lys-Pg–Sepharose was performed with NPGB (Chase & Shaw, 1967). The NPGB titration indicated that 39% of the immobilized Lys-Pg was converted to plasmin.

Preparation of Plasmic Fragments of Fibrinogen. Human fibrinogen (grade L, Kabi, Stockholm) was dissolved in 0.05 M Tris-HCl/0.15 M NaCl, pH 7.4 buffer at a final concentration of 20 mg/mL, and the solution was exhaustively dialyzed against the same buffer. The fibrinogen concentration of the dialyzed solution was adjusted to 10 mg/mL by dilution with buffer, $CaCl_2$ was added to a final concentration of 5 mM, and the mixture was incubated at 37 °C. Since this fibrinogen preparation contains plasminogen sufficient to permit plasmic digestion after activation, the digestion was started by addition of urokinase (Leo, Ballerup) to a final concentration of 100 Ploug units/mL, and the mixture was incubated at 37 °C. After 30 (early digest) or 240 min (terminal digest), proteolysis was arrested by the addition of Trasylol, 100 KIU/mL, ϵ-ACA, 20 mM, and EDTA, 20 mM (all final concentrations). Fragment E_{fge} was prepared from the early digest, whereas fragment D and fragment E_{fgl} were obtained from the terminal digest by ion-exchange chromatography on QAE-Sephadex A50 (Pharmacia), followed by gel-filtration on Sephacryl S-200 (Pharmacia), according to the method of Fowler et al. (1980). The proteins were lyophilized from 0.3 M ammonium bicarbonate solution and were stored at −20 °C. The isolated fragments were subjected to $NaDodSO_4$ gel electrophoresis under both reducing and nonreducing conditions to check their purity.

Preparation of Plasmic Fragments of Cross-Linked Fibrin. Fibrinogen (98% clottable) was prepared from plasminogen-depleted plasma by the plasma-fractionation procedure of Chen & Mosesson (1977). This preparation of fibrinogen contained factor XIII sufficient to form cross-linked fibrin when treated with thrombin. Fibrinogen was dissolved in 0.05 M Tris-HCl, 0.15 M NaCl, and 10 mM $CaCl_2$, pH 7.4 buffer at a final concentration of 8 mg/mL, and human plasminogen (0.025 mg/mL) and urokinase (50 Ploug units/mL) were added to this solution immediately before addition of thrombin, 4 NIH units/mL (Topostasin, Roche). The reaction mixture was incubated at 37 °C, and the digestion was terminated after 60 (early digest) or 300 min (late digest) by addition of Trasylol, 100 KIU/mL, ϵ-ACA, 20 mM, and EDTA, 20 mM (all final concentrations). DD–E complex was purified by gel filtration from the early digest (Olexa & Budzynski, 1979). The complex was dissociated by incubating the complex in 0.05 M sodium citrate/3 M urea, pH 5.5, at 37 °C for 180 min, and fragments DD and E_2 were separated by gel filtration on a Sephacryl S-300 (Pharmacia) column equilibrated with the dissociating buffer. The pool containing E_2 was desalted on a Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate and was lyophilized. Fragment E_3 was purified from the late digest by ion-exchange chromatography on QAE-Sephadex as described for the purification of plasmic fragments of fibrinogen (Fowler et al., 1980); fragment E_3 was present in fractions eluted at pH 5.4–4.8 and was further purified by gel filtration on Sephacryl S-200 equilibrated with 0.3 M ammonium bicarbonate and lyophilized. The isolated fragments were subjected to $NaDodSO_4$ electrophoresis under both reducing and nonreducing conditions to check their purity.

Plasmic Digestion of Fragment D_1 in the Presence of EDTA. Fragment D_1 was converted to fragments D_2 and D_3 essentially as described by Olexa & Budzynski (1981). Fragment D_1 (10 mg/mL) was dissolved in 0.05 M Tris-HCl, 0.15 M NaCl, and 20 mM EDTA, pH 7.4 buffer and was digested with plasmin (0.06 CU/mL) at 37 °C for 180 min. During this time, fragment D_1 disappeared completely and was

[1] Abbreviations: Glu-Pg, native plasminogen (Glu_1–Asn_{790}); Lys-Pg, proteolytically modified plasminogen (Lys_{77}–Asn_{790}); fragment D_1, plasmic fragment corresponding to the carboxy-terminal globular domain of fibrin(ogen); fragment D_{EDTA}, carboxy-terminal globular fragments of fibrin(ogen) obtained from fragment D_1 by plasmic digestion in the presence of EDTA; fragment E_{fge} and fragment E_{fgl}, amino-terminal fragments obtained from early (E_{fge}) and late (E_{fgl}) plasmic digests of fibrinogen; E_2 and E_3, amino-terminal fragments obtained by plasmic digestion of cross-linked fibrin; NDSK, NH_2-terminal disulfide knot, obtained by cyanogen bromide cleavage of fibrinogen; ϵ-ACA, ϵ-aminocaproic acid; $NaDodSO_4$, sodium dodecyl sulfate; D-Val-Leu-Lys-pNA, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

converted to a mixture of fragments D_2 and D_3 as evidenced by $NaDodSO_4$ gel electrophoresis (this mixture of fragments D_2 and D_3 is hereafter referred to as fragment D_{EDTA}). The digestion was arrested by the addition of 100 KIU/mL Trasylol, and the sample was gel filtered on a Sephacryl S-200 column equilibrated with 0.3 M ammonium bicarbonate and lyophilized.

Preparation of Amino-Terminal Disulfide Knot of Fibrinogen. NDSK was prepared by cyanogen bromide cleavage of human fibrinogen according to the method of Blombäck et al. (1968) as modified by Olexa & Budzynski (1979). Thrombin treatment of NDSK was performed by incubating the fragment (0.5 mg/mL) with thrombin (Calbiochem), 5 NIH units/mL, in 0.1 M ammonium bicarbonate buffer at 25 °C for 180 min. Release of fibrinopeptides A and B was verified by the high-pressure liquid chromatographic assay. For plasminolysis of NDSK, the fragment (0.5 mg/mL) was digested with plasmin (0.12 CU/mL) in 0.1 M ammonium bicarbonate buffer at 25 °C for 60 min; then, the digestion was arrested by addition of Trasylol, 100 KIU/mL. The effect of plasmin on NDSK was followed by the decrease of its molecular weight as detected by $NaDodSO_4$ gel electrophoresis.

Preparation of Plasminogen-Free Fibrinogen. In the assay of the affinity of fibrinogen for Lys-Pg–Sepharose, fibrinogen not contaminated with plasminogen was used. Plasminogen-free fibrinogen was prepared by passing the fibrinogen solution (Kabi, grade L) through a lysine–Sepharose column before use.

Assay of Fibrinopeptide Release. Fibrinopeptides A and B liberated from fibrinogen and fibrinogen fragments by thrombin treatment were quantitated by high-pressure liquid chromatography (Martinelli & Scheraga, 1979). The liquid chromatograph used was built up of an Altex pump (Model 110), a Rheodyne sample-injector valve with a 20-mL sample loop (Model 70-10), and a variable-wavelength detector, Spectrometer II (Laboratory Data Control). A reverse-phase LiChrosorb RP 18, 10-μm, 250 × 4.6 mm column (Merck) was used.

Affinity Chromatography. Affinity chromatography of fibrinogen and fibrinogen and fibrin fragments on a Lys-Pg–Sepharose 4B column was performed in 0.1 M ammonium bicarbonate buffer, pH 8.0 at 25 °C. In the experiments aimed at determining the capacity of Lys-Pg–Sepharose for fibrin(ogen) derivatives, the column (1 mL) was overloaded with the protein in question, and after the column was washed with 5 bed volumes of buffer, the bound protein was eluted with buffer containing ϵ-ACA (10 mM) and quantitated.

Competitive Affinity Chromatography. The relative plasminogen affinity of different fibrinogen fragments was determined by competitive affinity chromatography on Lys-Pg–Sepharose 4B. Competitive affinity chromatography is based on the principle that if different proteins compete for the same sites of the affinant and the availability of these binding sites limits binding (i.e., the column is overloaded), then the ratio of the proteins in the bound fraction will be shifted in favor of the component with higher affinity according to the equation

$$[P_iL]/[P_1L] = K_i[P_i]/(K_1[P_1]) \quad (1)$$

where $[P_1]...[P_i]$ are the free concentrations of different proteins in equilibrium with the affinant, $K_1...K_i$ are the association constants of the protein–affinant interactions, and $[P_1L]...[P_iL]$ are the concentrations of the protein–affinant complexes. If we overload the column with an equimolar mixture of the proteins to be compared to the extent that the free concentrations of the proteins (determined from the effluent) practically equal the concentrations of these proteins in the sample, then, eq 1 simplifies to

$$[P_iL]/[P_1L] = K_i/K_1 \quad (2)$$

Under these conditions the ratio of the proteins in the bound fraction reflects the relative association constants of their interaction with the affinant.

The sample containing an equimolar mixture of the proteins to be compared (4-mL sample; protein concentration was 9 μM for both proteins) was continuously loaded onto the Lys-Pg–Sepharose column (0.4 mL), and fractions of 0.2 mL were collected. After passage of the whole sample through the column, the column was washed with 5 bed volumes of buffer, and the bound proteins were eluted with buffer containing 10 mM ϵ-ACA. The compositions of the sample, the fractions of the effluent, and the bound fraction were analyzed by densitometric evaluation of their $NaDodSO_4$ gel electrophoretic pattern.

Gel Electrophoresis and Densitometry. Aliquots containing ~5 μg of protein material were analyzed by 6–16% linear polyacrylamide gradient, $NaDodSO_4$ slab gel electrophoresis, and the gels were stained with Coomassie brilliant blue G-250. Densitometry of the gels was performed by a Varian 634 double-beam recording spectrophotometer equipped with a Varian gel scanner. Gels were scanned at least 3 times, and calculations were made from the average values. The peak areas were integrated manually and were converted to amount of protein by means of calibration curves prepared by running various known amounts of the given protein on the same slab gel. The plots of density vs. amount of protein were found to be linear up to 5 μg of protein/band. For an equimolar mixture of fragments D_{EDTA} and D_1, the ratio of the densities of the stained bands was 0.75:1, while in the case of an equimolar mixture of fragments E_{fgl} and D_1 it was 0.36:1. The molar ratios of these proteins were calculated from the densitograms with these staining factors.

Results

Specificity of Interaction between Immobilized Lys-plasminogen and Different Fibrin(ogen) Fragments. Fragments E_{fge}, E_{fgl}, E_2, E_3, D_1, and D_{EDTA} showed affinity for Lys-Pg–Sepharose, and the bound proteins were specifically eluted with buffer containing a low concentration (10 mM) of ϵ-ACA. The specificity of the elution of both types of fragments with ϵ-ACA is emphasized by our observation that the bound proteins (fragments E_3 and D_{EDTA}) are not eluted with buffer containing high salt (300 mM NaCl). The bound proteins were fully recovered in the ϵ-ACA-eluted fraction, suggesting that nonspecific binding was not significant in our experiments. Furthermore, when buffer containing 6 M urea was passed through the column after ϵ-ACA elution, no protein material was eluted. The fact that complexes of affinant and fibrin(ogen) derivatives can be specifically disrupted by 10 mM ϵ-ACA indicates that the "lysine-binding sites" known to be involved in the fibrin–plasminogen interaction (Thorsen, 1975) are responsible for the observed binding.

Although it cannot be excluded that Lys-Pg has undergone a conformational alteration upon immobilization,[2] the sensitivity of its fibrin-specific site(s) to ϵ-ACA remained un-

[2] Immobilized Lys-Pg could be activated with urokinase. Active site titration of the urokinase-treated Lys-Pg–Sepharose showed that 39% of the immobilized molecules were converted to plasmin, suggesting that at least this portion of the immobilized molecules preserved the native conformation and remained accessible to urokinase.

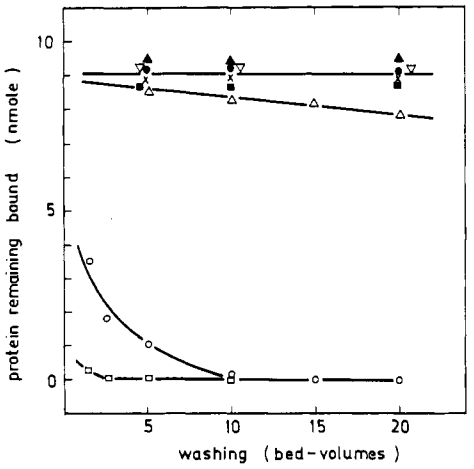

FIGURE 1: Influence of washing on amount of fibrinogen and fibrin(ogen) fragments remaining bound to Lys-plasminogen–Sepharose. The Lys-Pg–Sepharose 4B column (1 mL) was overloaded with the protein in question, and after the column was washed with different bed volumes of buffer, the protein remaining bound was eluted with buffer containing 10 mM ϵ-ACA and quantitated. Proteins studied were fibrinogen (○), fragment D_1 (△), fragment D_{EDTA} (▽), fragment E_2 (×), fragment E_3 (■), fragment E_{fge} (▲), fragment E_{fgl} (●), and bovine serum albumin (□).

Table I: Capacity of Lys-plasminogen-Sepharose 4B for Various Fibrin(ogen) Fragments[a]

fragment	capacity (nmol/mL of settled gel)
E_{fge}	9.4
E_{fgl}	9.1
E_2	9.0
E_3	8.9
D_1	8.6
D_{EDTA}	9.3

[a] The capacities were measured as described under Experimental Procedures.

changed. Therefore, the data obtained by affinity chromatography on Lys-Pg–Sepharose seem to be suitable to detect specific interactions of fibrin(ogen) fragments with Lys-plasminogen.

Plasminogen Binding Site of E Domain. Plasmic fragments E_{fge} and E_{fgl} derived from the central nodule of fibrinogen and fragments E_2 and E_3 derived from fibrin are all bound to Lys-Pg–Sepharose 4B and can be specifically eluted with ϵ-ACA. As shown in Figure 1, the amount of fragment E species bound to the affinity column is practically independent of the extent of washing, suggesting strong interaction with the affinant. The capacity of the column for all E species is about identical (Table I), probably because they bind to the same sites of the affinant.

A survey of the primary structure of these fragments (Table II) reveals that $\alpha(Val_{20}–Lys_{78})$, $\beta(Lys_{54}–Leu_{120})$, and γ-$(Tyr_1–Lys_{53})$ are the residues present in all E fragments; therefore, the region essential for plasminogen binding must be within these boundaries.

The amino-terminal disulfide knot, prepared by cyanogen bromide cleavage of fibrinogen, corresponds to the central nodule of the molecule (Kowalska-Loth et al., 1973) and is thus closely related to E fragments. This fragment was not bound to Lys-Pg–Sepharose, and neither thrombin-catalyzed removal of fibrinopeptides nor plasminolysis could evoke binding to affinant. This suggests that an intact plasminogen-binding site is not preserved in this fragment. From Table II, one can conclude that residues $\alpha(52–78)$ and $\beta(119–120)$ present in all the above E fragments are missing from NDSK.

Table II: NH_2- and COOH-Terminal Amino Acids of Fragments Derived from the Central Nodule of Fibrin(ogen)

polypeptide chain	fragment	terminal amino acid: NH_2	terminal amino acid: COOH	ref
Aα	E_{fge}[a]	Ala-1		*c*
	E_{fgl}	Ala-1	Lys-81	*d*
		Gly-17	Lys-78	*c, e*
		Val-20		*c*
	E_2	Gly-17	Lys-78	*f*
	E_3	Val-20	Lys-78	*f*
	NDSK[b]	Ala-1	Met-51	*g*
Bβ	E_{fge}[a]	Lys-54		*c*
	E_{fgl}	Lys-54	Leu-121	*c, e*
		Lys-54	Leu-122	*c, e*
	E_2	Gly-15	Leu-121	*f*
		Lys-54		
	E_3	Lys-54	Leu-120	*f*
	NDSK[b]	<Glu-1	Met-115	*g*
γ	E_{fge}	Tyr-1		*c*
	E_{fgl}	Tyr-1	Lys-53	*c*
		Tyr-1	Lys-58	*g*
	E_2	Tyr-1	Lys-62	*f*
	E_3	Tyr-1	Lys-53	*f*
	NDSK	Tyr-1	Met-78	*g*

[a] Fibrinopeptide analysis of fragment E_{fge}, used in the present study, confirmed the presence of fibrinopeptide A (residues 1–16 of the Aα-chain) but absence of fibrinopeptide B (residues 1–14 of the Bβ-chain). [b] Fibrinopeptide analysis of NDSK, used in the present study, confirmed the presence of both fibrinopeptides A and B, indicating that the amino terminal of Aα- and Bβ-chains is intact. [c] Takagi & Doolittle, 1975a. [d] Gårlund, 1977. [e] Takagi & Doolittle, 1975b. [f] Olexa et al., 1981. [g] Kowalska-Loth et al., 1973.

It seems thus probable that these peptide segments are involved in maintaining an intact structure of the plasminogen-binding site of the central nodule.[3]

Plasminogen-Binding Site of D Domain. Two different fragments, derived from the terminal globular domain of fibrinogen, were studied in the present investigation. Fragment D_1 obtained by exhasutive proteolysis of fibrinogen in the presence of calcium ions resists further proteolysis by plasmin (Haverkate & Timan, 1977). Incubation of this fragment D_1 with plasmin, however, leads to further proteolysis if the reaction mixture contains EDTA (yielding D_{EDTA}); the cleavage sites are in the carboxy-terminal region of the D γ-chain (Olexa & Budzynski, 1981).

Both fragments D_1 and D_{EDTA} are bound to Lys-Pg–Sepharose and can be specifically eluted with ϵ-ACA. As shown in Figure 1, the amount of fragment D_{EDTA} bound to the column is independent of the extent of washing whereas the amount of fragment D_1 bound decreases slightly upon prolonged washing, suggesting somewhat weaker interaction of fragment D_1 with the affinant. The binding capacity of the column for the two kinds of fragments, however, is practically the same when extrapolated to zero washing (Figure 1). Moreover, the capacity of the column is essentially the same for all E and D species, probably because the same sites of the affinant are involved in binding of the fragments derived from the two, sequentially nonidentical globular domains of fibrin(ogen).

To obtain quantitative information about the difference in the affinity of the two D species for Lys-Pg–Sepharose, we

[3] Lack of affinity of NDSK for Lys-Pg–Sepharose cannot be due to the relatively harsh conditions used in the preparation of NDSK as exposing fragment E_2 to the same conditions (but omitting cyanogen bromide) did not abolish its affinity for immobilized plasminogen.

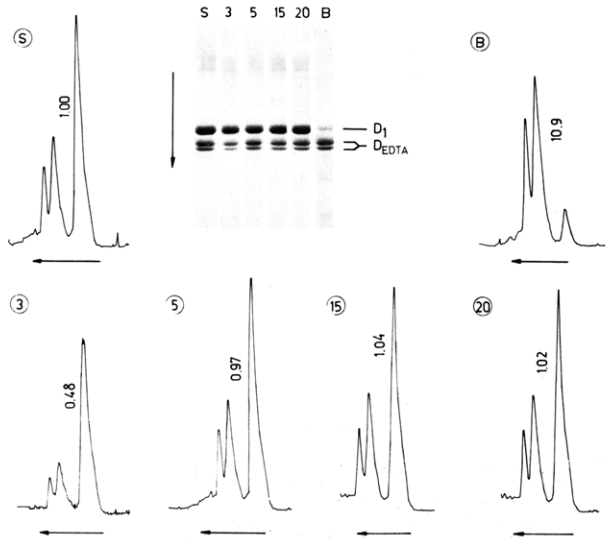

FIGURE 2: Competitive affinity chromatography of fragments D_1 and D_{EDTA} on Lys-plasminogen–Sepharose. An equimolar mixture (9:9 μM) of fragments D_1 and D_{EDTA} (4 mL) was continuously loaded onto the affinity column (0.4 mL), and fractions of 0.2 mL were collected. After passage of the sample through the column, the affinant was washed with 5 bed volumes of buffer, and the bound proteins were eluted with buffer containing 10 mM ϵ-ACA. The composition of the sample (S), the different fractions (3, 5, 15, and 20) of the effluent, and the bound proteins (B) was analyzed by densitometric evaluation of the $NaDodSO_4$ gel electrophoretic pattern. The numbers included in the densitograms represent the molar ratios of D_{EDTA}:D_1. The arrows indicate the direction of the electrophoretic migration of the proteins.

have subjected an equimolar mixture of fragments D_1 and D_{EDTA} (9:9 μM) to competitive affinity chromatography as described under Experimental Procedures. The results of this experiment are shown in Figure 2. The sample (S) was continuously loaded onto the affinity column; the start of overloading was indicated by the appearance of protein in the effluent. The composition of the effluent was monitored by $NaDodSO_4$ gel electrophoresis and densitometry. The amount of fragment D_{EDTA} was decreased in the early fractions of the effluent relative to fragment D_1 (Figure 2, fraction 3), as compared with the starting sample, due to the more efficient competition of fragment D_{EDTA} for the binding sites of the affinant. In later fractions of the effluent, the composition was already identical with that of the sample (Figure 2, fractions 15 and 20). At this stage, the loading of sample was stopped, and the column was washed free of nonadsorbed proteins with 5 bed volumes of buffer, and the bound proteins were eluted with buffer containing 10 mM ϵ-ACA. Densitometric evaluation of the gels showed that in the bound fraction (Figure 2, fraction B) there is a large shift in favor of D_{EDTA} relative to D_1 although the free concentrations of the two fragments in the column were equal when sample loading was finished (Figure 2, fraction 20).[4] Under these conditions, eq 2 is valid: i.e., the ratio of the two proteins in the bound fraction reflects the ratio of the association constants of their interactions with the affinant. The densitometric data presented in Figure 2 indicate that the molar ratio of D_{EDTA}:D_1 in the bound fraction is 10.9, whereas the free concentrations of the two proteins were equal. This shift thus indicates that the association constant of the fragment D_{EDTA}–Lys-Pg–Sepharose interaction is about 11-fold higher than that in the case of fragment D_1. The two variants of fragment D_{EDTA} (D_2 and D_3) apparently have similar affinity for Lys-Pg–Sepharose as there is no detectable shift in their molar ratio during competitive affinity chromatography.

[4] Since similar washing of a column saturated with fragment D_1 or fragment D_{EDTA} removes less than 10% of the bound fragments (cf. Figure 1), the inevitable washing step in the above experiment will not cause a significant error in the estimation of the bound proteins.

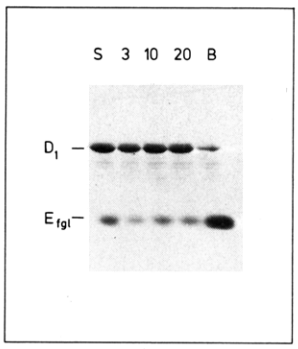

FIGURE 3: Competitive affinity chromatography of fragments D_1 and E_{fgl} on Lys-plasminogen–Sepharose. An equimolar mixture of fragments D_1 and E_{fgl} (9:9 μM; 4 mL) was continuously loaded onto the affinity column (0.4 mL), and fractions of 0.2 mL were collected. After passage of the sample through the column, the affinant was washed with 5 bed volumes of buffer containing 10 mM ϵ-ACA. Aliquots of the sample (S), the different fractions (3, 10, and 20) of the effluent, and the bound proteins (B) were subjected to $NaDodSO_4$ gel electrophoresis.

Our finding that the capacities of Lys-Pg–Sepharose for fragments E and D species are identical (see Table I) raised the possibility that the E and D species bind to the same sites of the affinant. The competitive affinity chromatographic technique is able to demonstrate whether different proteins compete for the binding sites of the affinant or not. Therefore, an equimolar mixture of fragments D_1 and E_{fgl} (9:9 μM) was subjected to competitive affinity chromatography under the same conditions as described for the competition of D_1 and D_{EDTA} (cf. Figure 2). The gel electrophoretic pattern of the experiment is presented in Figure 3. The fact that fragment E_{fgl} competes off fragment D_1 from the affinant suggests that they bind to the same sites of the affinant and proves that the affinity of E_{fgl} for these sites is significantly higher than that of fragment D_1. From densitometry of the gels, it was calculated that the association constants of the two interactions differ by a factor of 18.

Interaction of Fibrinogen with Lys-plasminogen–Sepharose. Lys-Pg–Sepharose was overloaded with a high excess of fibrinogen, and the column was washed with various volumes of buffer. Fibrinogen remaining associated with the affinant was determined after elution with ϵ-ACA containing buffer. As shown in Figure 1, no fibrinogen remained associated with the affinant after washing with 10 bed volumes of buffer. If less extensive washing (2–5 bed volumes) was used, low quantities of fibrinogen were found in the ϵ-ACA-eluted fractions. This seems to be due to a weak but definite interaction with the affinant since bovine serum albumin was not retained by the column under these conditions (Figure 1). The ability of ϵ-ACA to eliminate this weak binding of fibrinogen to Lys-Pg–Sepharose argues for the specificity of the interaction.

Discussion

Two Types of Plasminogen-Binding Sites Are Present on Fibrin(ogen). In the present work, we studied the affinity of different fibrin and fibrinogen fragments for Lys-Pg–Sepharose 4B in order to identify those regions that are complementary to the fibrin-specific site of plasminogen. Both nonoverlapping and sequentially distinct terminal plasmic fragments of fibrin(ogen), fragments D and E, were found to carry plasminogen-binding sites. Despite the sequential nonidentity of

plasminogen-binding sites of domains D and E, they are similar to each other in that their complexes with plasminogen are disrupted by ϵ-ACA, indicating that in both cases the ω-aminocarboxylic acid binding site(s) of plasminogen is (are) involved in binding.

Our result that there are two sequentially nonidentical regions of fibrin(ogen) that carry plasminogen-binding site is at variance with the conclusion of Cederholm-Williams & Swain (1979) that only the E domain has a plasminogen-binding site. These authors studied the effect of fragments D and E on the incorporation of Lys-plasminogen into fibrin clots and found that whereas fragment E could inhibit binding, fragment D was without effect. In our view, lack of inhibition by fragment D in this assay system does not prove the absence of a plasminogen-binding site from this domain. First, fragment D possessing the regions participating in fibrin polymerization is also incorporated into fibrin polymer (Haverkate et al., 1979; Williams et al., 1981), and this could interfere with the assay. Second, since fragment D was shown in the present paper to have lower affinity than fragment E (Figure 3), its inhibitory action may have escaped detection in their assay system.

Plasminogen-Binding Sites Are Buried in Fibrinogen. The plasminogen-binding sites are not fully functional in fibrinogen: whereas fragments D and E species are strongly bound to Lys-Pg–Sepharose, fibrinogen is removed from the affinant by washing (Figure 1). The absence of strong plasminogen-binding sites from fibrinogen explains the observation of Landmann (1973) that the ϵ-aminocarboxylic acid sensitive interaction, important for binding of plasmin to fibrin, does not play a significant role in the case of the fibrinogen–plasmin interaction. The presence of strong plasminogen-binding sites in fibrin but their absence from fibrinogen also explains the large difference in the K_M values of the plasmin–fibrin and plasmin–fibrinogen interactions (Shen et al., 1977; Kanai et al., 1979).

We suggest that the structural basis of the absence of strong plasminogen-binding sites from fibrinogen but their presence in isolated fragments is that these sites are buried in fibrinogen but are exposed by proteolysis, since protease removes peptide regions masking the binding sites. In the present work, we have found that proteolytic modification of the carboxy-terminal region of the D γ-chain causes a further large (11-fold) increase in the affinity of the D domain for Lys-Pg–Sepharose; i.e., this region may be involved in masking the plasminogen-binding site of the D domain.

The ϵ-ACA-sensitive binding sites absent from fibrinogen are present in fibrin (Landmann, 1973); i.e., the thrombin-catalyzed conversion of fibrinogen to fibrin polymer somehow exposes these sites. The mechanism of this exposure is not known, but in principle, thrombin cleavage of fibrinopeptides A and B may have a direct effect on the plasminogen-binding sites or may induce a change in their exposure through the conformational changes accompanying fibrin polymerization.

A direct role of thrombin cannot be invoked to explain the exposure of the binding site of the D domain since the covalent structure of this domain is unaltered by thrombin treatment. Unmasking of this binding site in fibrin therefore is probably due to a conformational change accompanying fibrin polymerization. Thrombin is known to expose two pairs of polymerization sites in the E domain (Laudano & Doolittle, 1978; Olexa & Budzynski, 1980), and these sites specifically and strongly interact with the D domains of two neighboring fibrin units. The interaction between D and E globules causes gross changes in the structure of the D domain as detected by immunochemical techniques (Cierniewski et al., 1977); the conformational change is manifested in a large increase in the accessibility of the antigenic sites of the D γ-chain. Since the carboxy-terminal 38 residue long portion of the D γ-chain is directly involved in the binding of D domain to E domain in the fibrin polymer (Olexa & Budzynski, 1981), it seems possible that this interaction is responsible for perturbing the structure of the remainder of the D γ-chain, exposing its antigenic sites. Considering the large structural changes occurring in the carboxy-terminal region of the D γ-chain upon fibrin polymerization, it seems possible that the plasminogen-binding sites of the D domain become exposed by this very conformational change. It is noteworthy in this respect that proteolytic modification of the same γ-chain region was found in the present study to unmask the plasminogen-binding site located in the D domain.

The obvious physiological significance of masking plasminogen-binding sites in fibrinogen but exposing them upon conversion to fibrin is that by this mechanism plasmin activity may be directed against fibrin polymer while leaving fibrinogen unharmed. Appearance of plasminogen-binding sites permits recruitment of plasminogen onto fibrin polymer, activation to plasmin, and finally destruction of the fibrin polymer.

Registry No. Plasminogen, 9001-91-6.

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Structural Organization of Filamentous Proteins in Postsynaptic Density†

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ABSTRACT: Actin is one of the major protein constituents of the postsynaptic density (PSD), a characteristic structural entity subjacent to the postsynaptic membrane in excitatory synapses of the vertebrate central nervous system. In isolated purified PSD preparations, it is present to the extent of 29 ± 2 μg/mg of total protein, 90% of which is in the filamentous (F-actin) form. Iodination by a discriminatory labeling technique demonstrates that actin is located on the surface of the PSD from which it can be stripped by treatment with a mixture of strong anionic detergents, leaving behind an insoluble core held together by disulfide bridges, consisting in part of tubulin and "PSD protein".

Although the function of the postsynaptic density (PSD),[1] the filamentous organelle that underlies the postsynaptic membrane at excitatory synapses in the central nervous system, is unknown, it is becoming increasingly well characterized structurally. In common with many cytoskeletal elements, it may be isolated by virtue of its insolubility in detergents. Organelles isolated in this fashion are enriched in both actin and tubulin (Banker et al., 1974; Walters & Matus, 1975; Cohen et al., 1977; Matus & Taff-Jones, 1978; Kelly & Cotman, 1978a; Carlin et al., 1980) and have been shown to contain microtubule-associated proteins (Matus et al., 1981) and fodrin (Carlin et al., 1983) in lesser amounts. The major constituent of the PSD is a protein with an apparent molecular weight (M_r) of 50 000 ± 2000 called the postsynaptic density protein (PSD protein) (Banker et al., 1974; Blomberg et al., 1977; Kelly & Cotman, 1978a). This protein appears to be a major structural element, since, following treatments that remove many of the approximately 30 proteins composing or associated with the PSD, it is the major protein that remains in a detergent-insoluble residue that retains some of the structural attributes of the intact PSD (Blomberg et al., 1977; Matus & Taff-Jones, 1978).

The presence of filamentous proteins such as actin and tubulin in the PSD have led to speculation that the function of the density might be a structural one; membrane proteins could be anchored at the synapse by linkage to the density. The presence of Ca^{2+} and calmodulin binding proteins (Grab et al., 1979; Wood et al., 1980; Carlin et al., 1982, 1983) and of elements of a protein phosphorylation system (Florendo et al., 1971; Ariano & Appleman, 1979; Ng & Matus, 1979; Carlin et al., 1980; Grab et al., 1981a,b; Mahler et al., 1982) suggests the possibility of dynamic interactions among density proteins, leading to modification in protein–protein interactions perhaps linked to long- or short-term changes in a synapse's response to depolarization.

Freeze–fracture and deep etching (Gulley & Reese, 1981) of synapses in situ and careful observation of thin sections (Grey & Guillery, 1966; LeBeaux, 1973; Hansson & Hyden, 1974; Grey, 1975; Fifkova & Delay, 1982), as well as replicas made from isolated PSDs (Cohen et al., 1977), show filaments emerging from the main, mostly compact, body of the PSD, which itself may consist of particles linked by filaments (Cohen et al., 1977; Blomberg et al., 1977). The particles (Blomberg et al., 1977; Matus & Taff-Jones, 1978) that are observed by ethanolic phosphotungstic acid staining of PSDs in critical point dried preparations appear to be rodlike and may represent bundles of 10–20-nm filaments seen in cross section (Blomberg et al., 1977).

In order to begin to analyze the organization of the PSD and the interaction between it and the overlying postsynaptic

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47405. *Received October 5, 1982; revised manuscript received February 15, 1983.* This investigation was supported by National Institutes of Health Grant NS 08309; H.R.M. holds a Research Career Award (K06 05060) from this source.

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[1] Abbreviations: ADF, actin depolymerizing factor; DOC, deoxycholate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DDT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; M_r, apparent molecular weight; βME, β-mercaptoethanol; NEM, *N*-ethylmaleimide; PSD, postsynaptic density; $NaDodSO_4$, sodium dodecyl sulfate; SLS, sodium lauroyl sarcosinate; Temed, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, CBB, Coomassie Brilliant Blue; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/83/0422-2446$01.50/0