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Interaction of One-Chain and Two-Chain Tissue Plasminogen Activator with Intact and Plasmin-Degraded Fibrin

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ABSTRACT: Tissue-type plasminogen activator (t-PA) plays a central role in fibrinolysis in vivo. Although it is known to bind to fibrin, the dissociation constant (K_d) and number of moles bound per mole of fibrin monomer (n) have never been measured directly. In this study, the binding of both the one-chain form and the two-chain form of recombinant, human t-PA to fibrin was measured. Although more one-chain t-PA than two-chain t-PA is bound to fibrin, the K_d's and n's were within experimental error of each other. Significantly more t-PA is bound to clots made from fibrinogen which has been digested with plasmin than to clots made from intact fibrinogen. The additional binding was shown to be due to the formation of new set(s) of binding site(s) with dissociation constants that are 2–4 orders of magnitude tighter than the binding site present on clots made from intact fibrinogen. ϵ-Aminocaproic acid was capable of competing for the loose binding site present on both intact and degraded fibrin but had little effect on the binding of t-PA to the new site(s) formed by plasmin digestion. This increase in binding caused by plasmin-mediated proteolysis of fibrin suggests a possible mechanism for a positive regulation capable of accelerating fibrinolysis.

Human tissue-type plasminogen activator (t-PA) is a serine protease that plays a key role in the in vivo dissolution of fibrin (Collen, 1980; Verstraete & Collen, 1986). t-PA is initially synthesized as a single-chain form (Pennica et al., 1983). This species can be hydrolyzed at the Arg-275–Ile-276 peptide bond to form a two-chain form of t-PA in which the two chains are connected by a single disulfide bond (Pennica et al., 1983; Rijken & Collen, 1981). The hydrolysis at Arg-275 has been shown to be catalyzed by plasmin (Wallen et al., 1981), factor Xa, and tissue kallikrein (Ichinose et al., 1984). Two-chain t-PA is more active than one-chain t-PA with small molecular weight substrates (Ranby et al., 1982; Rijken et al., 1982; Tate et al., 1987) and with plasminogen in the absence of fibrin (Tate et al., 1987). At physiological concentrations of plas-

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minogen and fibrin, however, both forms of t-PA are equally effective in converting plasminogen to plasmin (Rijken et al., 1982; Tate et al., 1987). Both t-PA and plasminogen interact with fibrin, and these interactions are believed to be responsible in part for the enhancement of the rate of plasminogen activation in the presence of fibrin (Hoylaerts et al., 1982). Several groups have published studies of the binding of plasminogen to intact fibrin and fibrin which was partially degraded by plasmin (Suenson et al., 1984; Tran-Thang et al., 1984, 1986; Bok & Mangel, 1985; Harpel et al., 1985). Few studies, however, have quantitatively studied the binding of t-PA to fibrin although binding was first demonstrated 15 years ago (Thorsen et al., 1972). A kinetic analysis of the activation of plasminogen by t-PA (Hoylaerts et al., 1982) suggested that the binding constant was 0.14 μM. Direct binding studies using a plasmin inhibitor to prevent the conversion of one-chain t-PA to two-chain t-PA have demonstrated that about 5% more one-chain t-PA than two-chain t-PA binds to fibrin (Rijken et al., 1982). More recently, we have demonstrated that at least 20% more one-chain t-PA than two-chain t-PA binds to plasminogen-free fibrin (Tate et al., 1987). This paper extends these observations and reports the first quantitative analysis of a direct binding study. In addition, it shows differences in the binding of one-chain t-PA and two-chain t-PA to intact fibrin and in the binding of t-PA to fibrin that has been partially digested with plasmin.

Experimental Procedures

Purification of t-PA. Recombinant, human tissue-type plasminogen activator was isolated from the supernatants of Chinese hamster ovary cells which express t-PA following transfection with a plasmid containing the human t-PA gene (Pennica et al., 1983; Goeddel et al., 1983). The protein was purified by using chromatography on lysine–agarose (Radcliffe & Heinze, 1978). The resulting protein was a mixture of the one-chain and two-chain forms. One-chain t-PA was isolated from a mixture of purified one-chain and two-chain t-PA by chromatography on a monoclonal antibody column which preferentially binds the one-chain form of t-PA (PAM-1 from American Diagnostica, Greenwich, CT). Approximately 500 μg of t-PA was applied to 1 mL of the monoclonal antibody–Sepharose. Following a wash with 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 0.12 M NaCl, and 0.01% Tween 80, pH 7.4, the one-chain material was eluted with 0.1 M acetic acid/0.15 M NaCl, pH 2.9. The pH of the eluate was immediately raised to 7 by the addition of 0.5 M Na_2HPO_4, and it was dialyzed against 0.05 M Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4. Homogeneous two-chain material was prepared by incubating $\sim$500 μg of one-chain t-PA with 0.05 mL of plasmin–agarose (see below) for 90 min at 37 °C. The tube was rotated end-over-end to ensure adequate mixing, and the reaction was terminated by centrifugation and the removal of the supernatant containing two-chain t-PA. The final products were analyzed for homogeneity by gel electrophoresis on 10% acrylamide gels in the presence of sodium dodecyl sulfate (SDS–PAGE) (Laemmli, 1970). Dithiothreitol (10 mM final concentration) was added to the samples in order to verify that the t-PA was in the one-chain or two-chain form. The silver-staining technique of Morrissey (1981) was used to visualize the proteins. The one-chain t-PA used in these experiments contained less than 10% contaminating two-chain material. The two-chain t-PA appeared to be free from contaminating one-chain material.

Preparation of Plasmin–Agarose. Human plasmin (25 casein units from Sigma) was coupled to 5 mL of CNBr-activated Sepharose 4B (Pharmacia) in a buffer composed of 0.1 M sodium bicarbonate, pH 8.0. After incubation for 2 h at room temperature and overnight at 4 °C, centrifugation was used to remove the unreacted plasmin. The remaining reactive groups were blocked by incubation with 2 M ethanolamine, pH 8.0, for 2 h at room temperature. The unreacted material was then removed by extensive washing of the resin with 0.1 M ammonium bicarbonate/1.0 M sodium chloride, pH 8.0, and 0.1 M ammonium acetate/1.0 M sodium chloride, pH 4.0. The resin was stored at 4 °C as a 50% slurry in 0.05 M Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4. The specific activity of this resin as measured with a small synthetic substrate is $\sim$3 casein units/mL.

FIGURE 1: SDS–PAGE of intact and degraded fibrinogen. Lane A, degraded fibrinogen; lane B, molecular weight standards of 92K, 68K, 43K, 31K, 21K, and 14K; lane C, intact fibrinogen.

Fibrinogen. Human fibrinogen (fraction I, 98.6% clottable) was obtained from Calbiochem. The lyophilized protein was dissolved to a concentration of 10 mg/mL and dialyzed versus 0.05 M Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4. To remove any contaminating plasminogen, the solution was passed over a lysine–Sepharose column (Deutsch & Mertz, 1970) which had been equilibrated with 0.05 M Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4. The removal of plasminogen was monitored by incubating a sample of the fibrinogen with 2 units/mL urokinase (Calbiochem). After 48 h, the sample was reduced and subjected to SDS–PAGE. The lack of degradation of fibrinogen indicated sufficient removal of plasminogen. The plasminogen-free fibrinogen was then diluted to 1.5 mg/mL and stored frozen until use. Plasmin-degraded fibrinogen was produced by incubating 10 mL of fibrinogen with 1.0 mL of the plasmin–Sepharose slurry. The tube containing the mixture was rotated end-over-end for 1 h at 37 °C. The reaction was stopped by centrifugation and the supernatant removed. The clottability of the partially degraded fibrinogen was determined by using a modification of the method of Mosesson and Sherry (1966) and of Laki (1951). Fibrinogen at 1.5 mg/mL was clotted by the addition of 1 unit/mL thrombin. As the clot formed, it was wound around a glass rod. After 1 h at 37 °C, the clot was removed, and the absorbance of the supernatant at 280 nm was measured and compared to the absorbance of the original fibrinogen solution. SDS–PAGE analysis on reduced samples of intact and degraded (50% clottable) fibrinogen (Figure 1) indicated that prior to incubation with plasmin, it consisted of intact Bβ and γ chains with a small amount of heterogeneity in the Aα chain bands. Following plasmin digestion, Aα chains had been significantly degraded, and new, lower molecular weight bands were present. This result agrees with the ac-

cepted pattern of fibrinogen degradation by plasmin (Castellino, 1981; Lucas et al., 1983).

Fibrin Binding. The binding of t-PA to fibrin was monitored by using a modification of the method of Rijken et al., (1982). Samples of one-chain and two-chain t-PA (5 ng/mL final concentration) were mixed with various concentrations of human plasminogen-free fibrinogen which either was intact or had been partially degraded with plasmin–Sepharose. The total volume was 1 mL, and the buffer was 0.05 M Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4, containing 1 mg/mL human serum albumin. One unit of human thrombin was added, and the mixtures were incubated for 1 h at 37 °C. The clots were physically removed by centrifugation for 5 min at 10000 rpm. An aliquot of the supernatant was then assayed for t-PA content by an ELISA assay. In all cases, duplicate tubes, containing all of the assay components except for the fibrinogen, were used to determine the total amount t-PA added. No t-PA was nonspecifically removed from the solution by experimental conditions, as indicated by the complete recovery of t-PA when the experiments were performed in the absence of thrombin or in the absence of fibrinogen. The t-PA bound to fibrin was calculated by comparing the total t-PA added with the amount of t-PA free in the supernatant. As a control to measure protein nonspecifically trapped in the fibrin matrix, t-PA was replaced with ^{125}I-labeled tumor necrosis factor.

Experiments to determine the dissociation constant (K_d) and the moles of t-PA bound per mole of fibrin monomer (n) were done as described above except that the final concentration of intact or degraded fibrinogen was held constant at 0.1 mg/mL and the t-PA concentration was varied from 0.005 to 50 μg/mL. In some cases, either 0.1 M ϵ-aminocaproic acid (ϵ-ACA) or 0.1 M glycine (final concentration) was added to the binding mixtures, so that the ability of ϵ-ACA to compete for t-PA binding could be measured. As a negative control in experiments to measure the dissociation constant, yeast alcohol dehydrogenase was used to replace t-PA in the binding assay. After the clots were removed, an aliquot of the supernatant was assayed for alcohol dehydrogenase activity using the rate at which NAD is reduced in the presence of 0.015 M sodium pyrophosphate, pH 8.8, 0.32 M ethanol, and 8 nM NAD (Vallee & Hoch, 1955).

Analysis of Binding Constants. Dissociation constant(s) (K_d) and the moles of t-PA bound per mole of fibrin monomer (n) were determined by using a computer fit to a Scatchard analysis based on LIGAND (Munson & Rodbard, 1980).

Preparation and Reaggregation of Fibrin Monomers. Fibrin monomers were prepared by clotting either intact or plasmin-digested plasminogen-free fibrinogen with 1 unit of thrombin /mL. As the clots formed, they were wound around glass rods, rinsed, and dissolved in 0.02 M acetic acid. The concentration of the monomer solution was determined by measuring the absorbance at 280 nm and using the extinction coefficient for fibrinogen. Reaggregation of monomers was started by adding a small portion of the monomer solution to 50 mM Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4.

RESULTS

At physiological concentrations of t-PA, 5 ng/mL (Bergsdorf et al., 1983; Rijken et al., 1983), significantly more one-chain t-PA than two-chain t-PA binds to all concentrations of fibrin (Figure 2). Nonspecific binding as measured by the binding of ^{125}I-labeled tumor necrosis factor (Aggarwal et al., 1985) was less than 5% in this assay. The binding was also shown to be reversible. The binding of t-PA remained constant

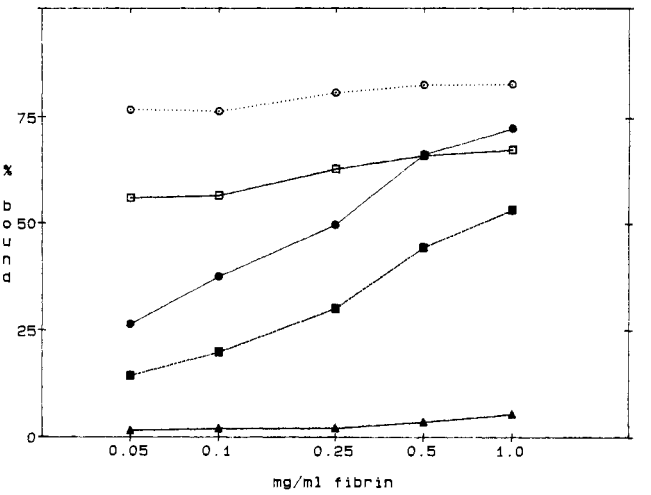

FIGURE 2: Binding of one-chain and two-chain t-PA to fibrin. Samples of one-chain (○, ●) and two-chain (□, ■) t-PA (5 ng/mL final concentration) were incubated with fibrinogen which was either intact and 95% clottable (closed symbols) or partially degraded with plasmin–agarose and 50% clottable (open symbols). Samples were clotted as described under Experimental Procedures, and an ELISA was used to determine the amount of free t-PA in the supernatant. (▲) Nonspecific binding as determined by replacing t-PA with ^{125}I-labeled tumor necrosis factor. The milligrams per milliliter fibrin was not corrected to account for the nonclottable fibrinogen.

over an incubation period of 15 min to at least 8 h, indicating that equilibrium had been reached at the normal 1-h incubation. Changing the thrombin concentration from 0.1 unit/mL to 10 units/mL also had no effect on the binding. When fibrinogen is degraded slightly with plasmin and then clotted, a significantly higher amount of both one-chain and two-chain t-PA is bound (Figure 2). This is most evident at lower concentrations of fibrin. When the portion of t-PA which failed to bind to 1 mg/mL fibrin was incubated with a fresh sample of fibrinogen (1 mg/mL) and thrombin, over 50% of the t-PA was bound, indicating that the plateau observed is primarily a function of the binding affinity and stoichiometry and not indicative of the inability of the portion of the t-PA to bind to fibrin. When fibrin monomers prepared from clots of intact fibrinogen were allowed to polymerize in the presence of t-PA, the monomers (0.1 mg/mL) made from intact fibrinogen bound the same amount of t-PA (27%) as clots made directly from the fibrinogen. When a sample of two-chain t-PA was bound to monomers (0.1 mg/mL) made from degraded fibrin, 59% of the t-PA was bound. This compares well to the 45% of t-PA bound to plasmin-degraded fibrinogen (0.1 mg/mL) following clotting. The increased binding to the monomers reflects the higher concentration of "clottable" monomers in that sample. The supernatants obtained from the clotting of intact and degraded fibrin had no effect on the ability of t-PA to bind fibrin (data not shown). Thus, the change required for the observed increased binding was in the clot itself as opposed to in the supernatant, and t-PA does not interact to any significant extent with the soluble fibrinogen degradation products in the supernatant. Ca^{2+} and ethylenediaminetetraacetic acid (EDTA) had little effect on the interaction of t-PA with fibrin, although SDS–PAGE analysis demonstrated that the fibrin prepared in the presence of Ca^{2+} was cross-linked but the fibrin with EDTA was not. Thus, the binding appeared to be independent of the amount of cross-linking in the fibrin.

The increased binding of one-chain t-PA over two-chain t-PA and the increased binding observed when fibrinogen is partially degraded by plasmin could be due to either a tighter dissociation constant or a higher number of binding sites. To

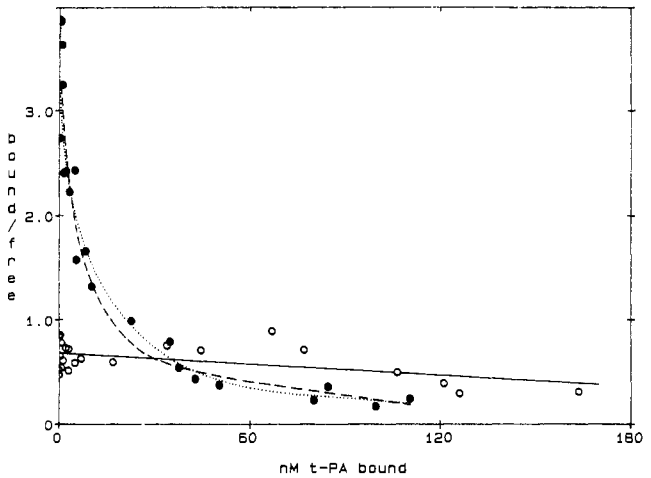

FIGURE 3: Scatchard analysis of the binding of one-chain t-PA to intact (O) and degraded (●) fibrin (0.1 mg/mL final concentration). The degraded fibrin was 50% clottable. (—) is a computer fit of the data with intact fibrin to a single binding site. The computer fit of the data with degraded fibrin to a two-site (- - -) and three-site (···) model is shown.

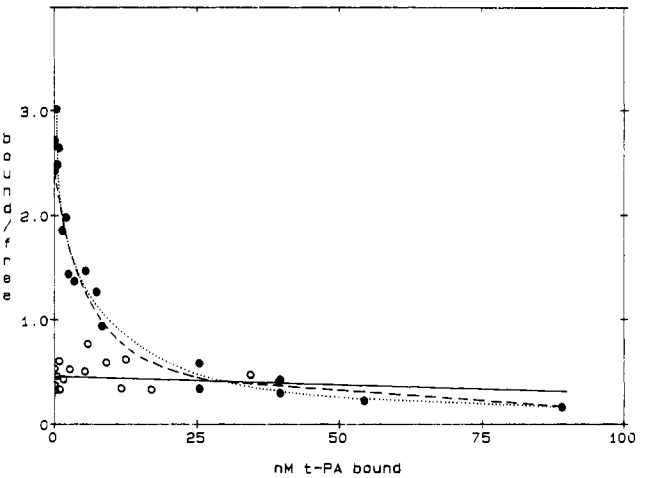

FIGURE 4: Scatchard analysis of the binding of two-chain t-PA to intact (O) and degraded (●) fibrin (0.1 mg/mL final concentration). The degraded fibrin was 50% clottable. (—) is the computer fit of the data with intact fibrin to a single binding site. The computer fit of the data with degraded fibrin to a two-site (- - -) and three-site (···) model is shown.

Table I: Binding Constants for the t-PA–Fibrin Interaction

	one-chain t-PA		two-chain t-PA	
	K_d (M)	n	K_d (M)	n
intact fibrin	3.8×10^{-7}	1.1	5.2×10^{-7}	0.91
degraded fibrin				
2-site model	3.2×10^{-9}	0.06	3.6×10^{-9}	0.03
	5.5×10^{-6}	0.86	1.2×10^{-6}	1.2
3-site model	5.9×10^{-10}	0.02	2.0×10^{-9}	0.02
	2.2×10^{-8}	0.16	1.1×10^{-7}	0.11
	1.2×10^{-6}	1.1	1.2×10^{-6}	1.0
+0.1 M ϵ-ACA	5.4×10^{-10}	0.01	n.d.[a]	n.d.
	2.9×10^{-8}	0.06	n.d.	n.d.

[a] Not determined.

discern between these possibilities, the binding of various amounts of t-PA to a constant concentration (0.1 mg/mL) of fibrin was investigated. Figures 3 and 4 show the results of these experiments. A single set of loose binding sites exists for both one-chain and two-chain t-PA to fibrin formed from intact fibrinogen. Values for the dissociation constant (K_d) and stoichiometry of the binding (n) are shown in Table I. The experimental error in these numbers makes it impossible to discern whether the differences observed between the binding of one- and two-chain t-PA result from changes in K_d or n. The high dissociation constant and the limited solubility of t-PA under the conditions of the experiments contribute to the error in these values. The small amount (<10%) of contaminating two-chain t-PA in the one-chain material does not significantly alter the K_d and n values obtained with one-chain t-PA since an identical experiment with Arg-275 → Glu t-PA (Tate et al., 1987) gave similar results (data not shown).

The increased binding of t-PA observed when fibrinogen is partially degraded with plasmin and then clotted is due to the formation of new binding site(s) (Figures 3 and 4). When a Scatchard analysis is performed on these data, it can be fit to either a two-site or a three-site model, with the three-site model giving a statistically better fit to the data. The K_d's and n's obtained from such an analysis are listed in Table I. The new set or set(s) of binding sites formed exhibit a K_d which is up to 4 orders of magnitude higher affinity than the K_d for the site on intact fibrin. The observed differences in K_d's and n's are not statistically significant between one-chain and two-chain t-PA although the data seem to favor that the enhanced binding of one-chain t-PA is due to a lower K_d.

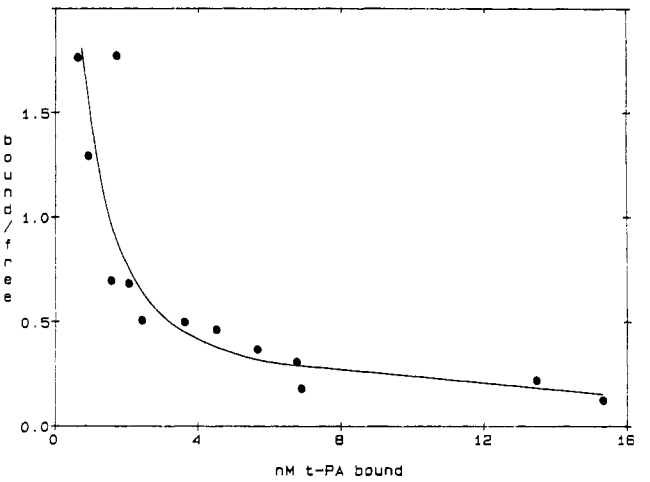

FIGURE 5: Effect of ϵ-aminocaproic acid (ϵ-ACA) on the binding of one-chain t-PA to degraded fibrin (0.1 mg/mL final concentration). One-chain t-PA was bound to degraded fibrin in the presence of 0.1 M ϵ-ACA (●). (—) shows a computer fit of the data to a two-site model.

To investigate the relationship between these t-PA binding sites and the "lysine-binding" site, the effect of the addition of 0.1 M ϵ-ACA on the binding of t-PA to intact and degraded fibrin was monitored. These results are shown in Figure 5. Virtually no t-PA bound in the presence of 0.1 M ϵ-ACA to clots made from intact fibrinogen. When the clots were made from degraded fibrinogen, the tight sites were retained while the weak sites were lost. These data could be fit most accurately to a model containing two binding sites ($K_{d_1} = 5.4 \times 10^{-10}$ M, $n_1 = 0.01$; $K_{d_2} = 2.9 \times 10^{-8}$ M, $n_2 = 0.06$).

Discussion

The interaction of t-PA with fibrin is central to its function as a fibrin-specific fibrinolytic agent. The direct binding studies reported here suggest several possible regulatory controls caused by the binding of t-PA to fibrin. Both one-chain t-PA and two-chain t-PA interact with intact fibrin with a K_d of approximately 10^{-7} M. This dissociation constant agrees well with that of 1.4×10^{-7} M (Hoylaerts et al., 1982) determined by a kinetic method. Significantly more (>20%) one-chain t-PA than two-chain t-PA binds to fibrin, which disagrees somewhat with the results of Rijken et al., (1982),

who found only a very small difference. Although the methods used in these studies are quite similar, Rijken et al. (1982) did not remove the plasminogen from their fibrinogen preparations but added 10 KIU/mL aprotinin to prevent the conversion of one-chain t-PA to two-chain t-PA. It is possible that these levels of aprotinin were not sufficient to completely prevent the conversion of one-chain t-PA to two-chain t-PA at the clot surface. When clots are formed in the presence of t-PA, the stoichiometry of binding is about 1 mol of t-PA/mol of fibrin monomer. Although there are 2 mol each of α, β, and γ chains in each fibrin monomer, this stoichiometry has also been shown by some workers in the interaction of plasminogen with fibrin (Bok & Mangel, 1985; Cederholm-Williams, 1977).

The differential binding of t-PA to intact and degraded fibrin may be a physiologically significant regulatory mechanism. When fibrinogen is degraded with plasmin prior to clot formation, or presumably when fibrin is degraded by plasmin in vivo, new sites with significantly lower (2–4 orders of magnitude) dissociation constant(s) for t-PA are formed. These data, along with evidence for a similar increase in plasminogen binding to degraded fibrin (Suenson et al., 1984; Tran-Thang et al., 1984, 1986; Harpel et al., 1985; Bok & Mangel, 1985), suggest a mechanism whereby early fibrin cleavage by plasmin could accelerate fibrinolysis by increasing the amount of t-PA and plasminogen bound and thereby enhancing the rate of plasminogen conversion. Alternatively, the formation of new, tighter binding sites could counteract the effect of the decreased binding caused by the conversion of one-chain t-PA to two-chain t-PA allowing for constant lysis.

The results reported here closely parallel those of Tran-Thang et al. (1986) for the interaction of plasminogen with fibrin. They have shown that plasmin digestion of fibrin creates a new binding site ($K_d = 3.5 \times 10^{-7}$ M, $n = 0.05$) for Glu-plasminogen with an affinity which is 100-fold stronger than the low-affinity binding site. The newly formed site(s) created for the binding to t-PA reported here also has (have) significantly lower dissociation constants, and $n \ll 1$. The very low n observed may reflect heterogeneity in the plasmin degradation and that the new sites which are formed upon degradation are very specific. Bok and Mangel (1985) found that the dissociation constant of the new site which is formed for plasminogen is 10^{-5} M, $n = 1$. In addition, they found a similar binding constant for the binding of plasminogen to lysine–Sepharose and suggested that newly exposed carboxy-terminal lysine residues comprise the new binding site. It seems unlikely that the K_d of t-PA and lysine would be 4 orders of magnitude lower as is the K_d for t-PA and fibrin. A more plausible explanation for the increased binding and low K_d may be that t-PA first binds through one domain and the additional interaction contributed by a second domain causes a significant drop in the K_d as proposed by Jencks (1981). Alternatively, the new sites for interaction which are exposed may independently account for the very tight K_d. It is also impossible to rule out that a protein cofactor for the binding of t-PA to fibrin, present in the fibrinogen preparations, is somehow involved.

Our results using ϵ-aminocaproic acid to compete for the binding of t-PA to both intact and degraded fibrin suggest that the lysine side chain is actually more intimately involved in the binding of t-PA to intact fibrin than to degraded fibrin. This result is contradictory to the hypothesis of van Zonneveld et al. (1986) that initial binding causing the low rate of plasminogen activation was governed by the finger domain and that upon degradation of fibrin, the newly exposed carboxyl-terminal lysines or arginines cause an enhanced binding and enhanced rate of activation due to the additional interaction of the lysine binding domains (kringle 2).

Although previous work demonstrated that more t-PA could bind to plasmin-digested fibrin (Suenson et al., 1984), this study represents the first observation of a distinct new binding site with a significantly lower K_d. Thus, it contains the first quantitative evidence to support the hypothesis (van Zonneveld et al., 1986; Suenson & Peterson, 1986) that the kinetic transition in the activation of plasminogen in the presence of fibrin (Norrman et al., 1985) is due to increased binding of t-PA to degraded fibrin. Plasminogen activation by t-PA in the presence of fibrin undoubtedly has a very complex mechanism. The binding of both t-PA and plasminogen to fibrin (both of which are increased upon degradation of the fibrin), the conformational changes in plasminogen when bound to fibrin (Castellino, 1981), and also possible conformational changes in t-PA when bound to fibrin all could have a significant impact on the rate of catalysis. Additional kinetic analyses of both intact and mutant forms of t-PA, when combined with the quantitative measurement of the binding interactions of the components, should help elucidate the contributions of the concentration effect of enzyme and substrate upon binding, and the conformational changes which alter catalysis.

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Neutron Scattering Determination of the Binding of Prothrombin to Lipid Vesicles†

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ABSTRACT: Low-angle neutron scattering is used to study the binding of human prothrombin to small single-bilayer vesicles consisting of phosphatidylcholine and phosphatidylserine (1/1 w/w). The radius of gyration of prothrombin indicates that it is an elongated molecule. The vesicles alone were not observed to coalesce, and their molecular weight, outer radius, and average surface area per lipid were respectively $(1.6 \pm 0.32) \times 10^6$, 114 ± 4 Å, and 110 ± 18 Å^2. These values were independent of the presence of calcium and were not altered significantly by prothrombin, which binds reversibly to the vesicle outer surface with its long axis projecting approximately radially forming a 90-Å thick protein shell. From the titration of the protein–vesicle interaction, the apparent dissociation constant of the binding of prothrombin to these vesicles is estimated to be 0.8 ± 0.4 μM. At saturation, 57 ± 7 prothrombin molecules bind, giving 25 ± 6 lipid residues and an area of 2900 ± 400 Å^2 per prothrombin molecule on the vesicle outer surface. This area is about twice that calculated from a prolate ellipsoid model for prothrombin. However, it is close to the maximum cross-sectional area of fragment 1, the lipid binding region of prothrombin, which is coin-shaped in the high-resolution X-ray structure [Park, C. H., & Tulinsky, A. (1986) *Biochemistry 25*, 3977–3982]. This similarity suggests that prothrombin binding could be sterically limited.

Blood coagulation involves a series of zymogen–enzyme conversions that require protein cofactors, negatively charged phospholipid surfaces, and calcium ions (Jackson & Nemerson, 1980). Thrombin (M_r 37 000), the enzyme that activates fibrinogen prior to its polymerization into fibrin, is produced by proteolytic cleavages of the zymogen prothrombin (M_r 72 000) in the prothrombinase enzymatic complex that includes factor X_a (the enzyme), factor V_a (protein cofactor), phospholipids, and calcium ions (Nesheim & Mann, 1983; Mann, 1984). Prothrombin is a single-chain vitamin K dependent glycoprotein containing approximately 10% carbohydrate and 10 γ-carboxyglutamic acid residues. The latter are involved in the binding of calcium ions, and most if not all are required for complete prothrombin attachment to the membrane surface (Nelsestuen, 1976; Borowski et al., 1985). No specific function for the carbohydrate has been identified. The prothrombin structure can be subdivided into three domains designated fragments 1 and 2 and prethrombin 2 with molecular weights respectively of approximately 21 000, 13 000, and 38 000 (Heldebrant et al., 1973; Owen et al., 1974). The γ-carboxyglutamate residues are on fragment 1 to which are also attached two of the three carbohydrate chains, the third being bound to prethrombin 2. Fragment 1 binds to phospholipid, fragment 2 appears to contain sites involved in interactions with factor V_a and factor X_a, and prethrombin 2 is converted into thrombin by cleavage of a single peptide bond (Mann &

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