THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 21, Issue of May 26, pp. 12355–12360, 1995
Printed in U.S.A.

The Role of the Lysyl Binding Site of Tissue-type Plasminogen Activator in the Interaction with a Forming Fibrin Clot*

(Received for publication, November 7, 1994, and in revised form, January 30, 1995)

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To describe the role of the lysyl binding site in the interaction of tissue-type plasminogen activator (t-PA, FGK1K2P) with a forming fibrin clot, we performed binding experiments with domain deletion mutants GK1K2P, K2P, and the corresponding point mutants lacking the lysyl binding site in the absence and the presence of ε-amino caproic acid (EACA). Occupation of the lysyl binding site in the K2 domain with EACA has a pronounced effect on the binding of FGK1K2P to a fibrin clot (C_{50} = 77 ± 11 nM *versus* 376 ± 45 nM with EACA). Deleting the lysyl binding site in the K2 domain (substitution D236N) also impairs fibrin binding but to a lesser extent (C_{50} = 169 ± 20 nM). Although the binding of K2P to a fibrin clot is weak (C_{50} = 1163 ± 490 nM), it still is 2 orders of magnitude stronger than the binding of EACA to K2P. Therefore it was surprising to find that deletion of the lysyl binding site in K2P completely abolishes fibrin binding. Even when both the F domain and the lysyl binding site were deleted, considerable fibrin binding is still observed (C_{50} = 557 ± 126 nM), suggesting other than F and K2-mediated interactions. The binding of FGK1K2P, FGK1K2P (D236N), GK1K2P, and GK1K2P (D236N) to fibrin could be competitively inhibited by FGK1K2P and K2P, indicating that all molecules recognize the same interaction sites on a fibrin clot. Based on these results, a new model for the interaction of t-PA with a forming fibrin clot is proposed. The fibrin binding sites in t-PA are not confined to the F and K2 domain. The main role of the lysyl binding site in the K2 domain of t-PA appears indirect rather than direct, most likely stabilizing a conformation favorable for fibrin binding.

Only in the presence of fibrin t-PA[1] efficiently converts its substrate plasminogen into the fibrin-degrading enzyme plasmin. The enzyme appears to play an essential role in dissolving fibrin rich clots in the bloodstream (Thorsen *et al.*, 1972; Collen, 1980; Carmeliet *et al.*, 1994). Fibrin binding of t-PA is thought to be a prerequisite for this enhanced plasminogen activation (Hoylaerts *et al.*, 1982; Rånby, 1982; Nieuwenhuizen *et al.*, 1985). This fibrin binding is localized in the heavy chain of t-PA (Rijken *et al.*, 1986). After the elucidation of the cDNA structure, it became apparent that t-PA is composed of several domains (Pennica *et al.*, 1983; Ny *et al.*, 1984). From the amino terminus, t-PA consists of a finger domain (F), an epidermal growth factor domain (G), two kringle domains (K1, K2), and a serine protease domain (P). Both the F and K2 domain were found to be involved in fibrin binding to a forming fibrin clot (Verheijen *et al.*, 1986; van Zonneveld *et al.*, 1986a). It was further shown that t-PA interacts with lysyl-Sepharose and arginyl-Sepharose (Radcliffe and Heinze, 1978; Wallen *et al.*, 1981). The interaction with lysyl-Sepharose can be disturbed by L-lysine, L-arginine, or the lysine analogue ε-amino caproic acid (EACA) (Radcliffe and Heinze, 1978; Allen and Pepper, 1981; de Munk *et al.*, 1989). The binding of t-PA with fibrin can be partially blocked with the lysine analogue EACA (van Zonneveld *et al.*, 1986b, de Munk *et al.*, 1989). Subsequently, isolated kringle 2 domains were shown to interact with EACA and lysyl-Sepharose (Byeon *et al.*, 1991; de Serrano and Castellino, 1993; de Vos *et al.*, 1992). This led to the view that t-PA binds to a forming fibrin clot via two modes: a lysyl binding site-mediated interaction and a non-lysyl-dependent interaction that requires the presence of the F domain (van Zonneveld *et al.*, 1986b).

Based on binding isotherms of t-PA to a forming fibrin clot, two independent nonidentical binding sites on the fibrin are proposed (Nesheim *et al.*, 1990). The high affinity interaction is F domain mediated, while a lower affinity interaction is K2 domain mediated. However, this model is questioned by the observation of one class of t-PA binding sites on a forming fibrin clot. Furthermore, the high affinity of t-PA for a forming fibrin clot could not be fully accounted for by the F-mediated and the K2-mediated interaction (Horrevoets *et al.*, 1994).

The role of the lysyl binding site of t-PA in fibrin binding is intricate. The K2 domain is thought to interact via an intrachain lysyl residue of the fibrin network, and therefore the binding site was called aminohexyl binding site. It was shown that increasing the amount of carboxyl-terminal lysyl residues in the fibrin network by partial degradation with plasmin results in new binding sites for t-PA (de Vries *et al.*, 1989). Although, the affinity of the K2 domain for aminohexyl-Sepharose differs from the affinity for lysyl-Sepharose, both interactions can be inhibited with EACA (de Munk *et al.*, 1989). Furthermore, deletion of the lysyl binding site in the K2 domain by the substitution of one amino acid residue (Asp^{236} replaced by Asn^{236}) abolishes binding to aminohexyl- and lysyl-Sepharose (Weening-Verhoeff *et al.*, 1990). Therefore, the structures in the K2 domain mediating lysyl binding and aminohexyl binding must be considered equivalent.

Substitution of one amino acid residue in the K2 domain (t-PA (D236N), t-PA (D236A) results in a t-PA analogue that no longer interacts with lysyl- or aminohexyl-Sepharose but still possesses high affinity for fibrin (Weening-Verhoeff *et al.*, 1990; Bennet *et al.*, 1991). This observation stands in clear contrast to the large effect of EACA on the fibrin binding of t-PA (van Zonneveld *et al.*, 1986b, de Munk *et al.*, 1989).

We studied the role of the lysyl binding site of t-PA in fibrin

* This work was supported by a grant from the Dutch Heart Foundation (project 89.081). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: t-PA, tissue-type plasminogen activator; EACA, ε-amino caproic acid; bp, base pair(s); S-2288, *H*-D-Ile-L-Pro-L-Arg-*p*-nitroanilide dihydrochloride; C_{50}, concentration of fibrin(ogen) at which binding is half-maximal; S-2251, *H*-D-Val-L-Leu-L-Lys-*p*-nitroanilide dihydrochloride; PPACK, Phe-Pro-Arg-CH_2Cl.

binding by performing fibrin binding experiments with domain deletion mutants lacking a functional lysyl binding site in the absence and presence of EACA. To describe the interaction site of t-PA and t-PA variants on a forming fibrin clot, we performed competition experiments with FGK1K2P and K2P.

We have found that for fibrin binding of t-PA, in addition to the F and the lysyl binding site-mediated interactions, other interactions must also exist. Furthermore, the binding sites of FGK1K2P (D236N) and the K2P on a forming fibrin clot appear to be in close proximity to each other. The lysyl binding site in the K2 domain appears not to interact directly with an aminohexyl group of the fibrin network, but it is probably involved in stabilizing a favorable conformation of t-PA needed for fibrin binding.

EXPERIMENTAL PROCEDURES

Proteins Used in this Report—Nomenclature and numbering of t-PA mutant proteins is according to Pannekoek *et al.* (1990). Recombinant t-PA (referred to as FGK1K2P), t-PA del (I5-H44) (referred to as GK1K2P), t-PA del (R7-C168) (referred to as K2P), and the point mutant t-PA D236N (referred to as FGK1K2P (D236N)) have been described before (Verheijen *et al.*, 1986; Weening-Verhoeff *et al.*, 1990). The construction of the corresponding domain deletion mutants t-PA D236N del (I5-H44) (referred to as GK1K2P (D236N)) and t-PA D236N del (R7- C168) (referred to as K2P (D236N)) are described below.

Construction of Mutant Proteins—The construction of the t-PA D236N del (I5-H44) was performed as follows. From the plasmids containing the reading frame for GK1K2P (peV2t-PA4) (Verheijen *et al.*, 1986), a 3835-bp *Nar*I-*Sac*I restriction fragment lacking the K1K2 and part of the P domain was isolated. From the plasmid containing the sequence coding for FGK1K2P (D236N) (Weening-Verhoeff *et al.*, 1990) a 900-bp *Nar*I-*Sac*I restriction fragment containing K1K2 (with the D236N substitution) and part of the P domain was isolated. This fragment was ligated into the above mentioned 3835-bp fragment according to Sambrook (Sambrook *et al.* 1989).

Construction of t-PA D236N del (R7-C168) was performed as follows. From the plasmid containing the sequence coding for FGK1K2P (D236N) (Weening-Verhoeff *et al.*, 1990), a *Pst*I partial fragment of 4249 bp (missing the FGK1 fragment of 486 bp) was isolated and ligated with itself according to Sambrook *et al.* (1989).

LB6 Cell Transfections—t-PA expression plasmids were used to transfect mouse L cells (LB6) by calcium phosphate co-precipitation with peV2/Neo, which contains the gene for aminoglycoside phosphotransferase 3′ (Graham and van der Eb, 1973). Cells that incorporated the plasmids and thus were Neo-resistant were selected in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (Boehringer Mannheim), L-glutamine (Life Technologies, Inc.), 100 units of penicillin/ml, 100 μg/ml streptomycin (Life Technologies, Inc.), and 1.2 mg/ml of the neomycin analogue geneticin (Life Technologies, Inc.). For purification of the recombinant proteins, cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 KIU/ml Trasylol (Bayer, Leverkusen, Germany) and 10 mM ε-amino caproic acid (Merck, Darmstadt, Germany) to prevent plasmin activity, 0.3 g/liter human serum albumin (CLB, Amsterdam, The Netherlands) L-glutamine, 100 units of penicillin/ml, 100 μg/ml streptomycin, and 1.2 mg/ml geneticin. Recombinant t-PA mutants were purified by immunoaffinity chromatography using a monoclonal antibody ESP-2 (Campro Scientific, The Netherlands) against the protease domain of t-PA coupled to agarose. A 0.5-ml aliquot of anti-t-PA-Sepharose suspension was placed on a disposable PD-10 gel filtration column (Pharmacia Biotech Inc.). The tandem column was equilibrated with 0.1 M Tris-HCl, 0.01% (v/v) Tween 80, pH 7.5. Approximately 50 ml of conditioned medium was loaded onto the column followed by washing with the buffer mentioned above. The column was then washed with 2 column volumes of a buffer containing 0.1 M Tris-HCl, pH 7.5, 1.0 M NaCl, and 0.01% (v/v) Tween 80. Subsequently, the column was reequilibrated with the same buffer without NaCl. The t-PA mutant was eluted from the column with a buffer containing 0.1 M Tris-HCl, 0.01% (v/v) Tween 80, and 3.0 M KSCN, pH 7.5 (Merck). Column fractions containing plasminogen activator activity were pooled for further characterization.

Gel Electrophoresis and Zymography—Polyacrylamide gel electrophoresis in the presence of SDS was performed under nonreducing conditions on 10% acrylamide gels with 5% stacking gels using the Laemmli system (Laemmli, 1970). After electrophoresis, gels were washed in 2.5% (v/v) Triton X-100 to remove SDS and placed on plasminogen-containing fibrin agarose layers (Granelli-Piperno and Reich, 1978). Upon incubation, the positions of plasminogen activators appear as clear lysis zones on an opaque background.

Conversion of the Single-chain to the Two-chain Form of t-PA and t-PA Analogues—Conversion of the single-chain form t-PA analogues to the two-chain form was performed as described previously (Wallén *et al.*, 1981). In short, plasmin-Sepharose slurry (200 μg plasmin/g of wet Sepharose-4B or Sepharose-4B (Pharmacia) was washed with 10 mM Tris-HCl, pH 7.5 and 0.01% (v/v) Tween 80. 50 μl of a 50% plasmin-Sepharose suspension or 50% Sepharose-4B suspension was added to 450 μl of t-PA analogue (10 pmol) in the same buffer. The reaction was carried out with constant mixing at 37 °C. Samples were removed from the incubation mixture at time intervals (t = 0, t = 10, t = 20, t = 30, t = 60, t = 90 min) centrifuged down, and 20 μl was transferred to wells of a microtiterplate containing 130 μl of 100 mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 80 and 20 KIU/ml Trasylol. Conversion of t-PA analogues from the single-chain to the two-chain form was confirmed by spectrophotometric activity determination with S-2288 (*H*-D-Ile-L-Pro-L-Arg-*p*-nitroanilide dihydrochloride, Chromogenix, Mölndal, Sweden). Spectrophotometric assays were performed as described previously (Verheijen *et al.*, 1985). Briefly, the reaction mixture (250 μl total volume) contained plasmin-Sepharose or Sepharose-treated t-PA analogues, 100 mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 80, Trasylol 20 KIU/ml, and 1 mM S-2288. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The absorbance at 405 nm was plotted against time ($\Delta A/\Delta t$) for six time points.

Labeling of t-PA and t-PA Analogues—For labeling of two-chain t-PA and t-PA analogues, an active site-directed inhibitor of t-PA was used. The inhibitor 4-aminobenzoyl-Gly-Arg-CH_2Cl (a kind gift of Dr. E. Shaw) was iodinated with ^{125}I and purified as described before (Rauber *et al.*, 1988). 1.5 pmol of t-PA analogues in 100 μl of 0.1 M Tris-HCl, pH 7.5, and 0.1% (v/v) Tween 80 were incubated with 2 μM iodinated inhibitor for 4 h at room temperature. Radiolabeled t-PA and t-PA analogues were bound on a 1-ml column of zinc chelate-Sepharose and extensively washed with 0.02 M Tris-HCl, pH 7.4, 1 M NaCl, and 0.01% (v/v) Tween 80, and then eluted with the same buffer containing 100 mM imidazole (Merck, Darmstadt, Germany). Specific activities of the labeled t-PA or t-PA analogues (final concentration, 5 nM) were approximately 2.6 10^4 cpm/pmol.

Binding of Two-chain t-PA or t-PA Analogues to Lysyl-Sepharose—Binding to a lysyl-Sepharose column was performed as described previously (de Munk *et al.*, 1989). In short, radiolabeled two-chain t-PA analogues (approximately 100 fmol in 500 μl) in buffer (0.1 M Tris-HCl, pH 7.4, 0.4 M NaCl, 0.01% (v/v) Tween 80) were applied to 1-ml lysyl-Sepharose columns (Pharmacia) equilibrated in the same buffer (flow rate, 50 ml/h at room temperature). These columns were washed with 2.5 ml of buffer. Specifically bound analogues (see below) were eluted with 2.5 ml of buffer containing 50 mM EACA. Radioactivity was assessed in the run-through, in washing fluid, in eluate, and in the column. The fraction of the total counts/min is given.

Binding of t-PA or t-PA Analogues to a Forming Fibrin Clot—Fibrin binding was performed as described previously (de Munk *et al.*, 1989). Radiolabeled t-PA analogues (0.06 nM, final concentration) were mixed with fibrinogen (Chromogenix, Mölndal, Sweden), which was depleted of plasminogen and plasmin as described before (de Munk *et al.*, 1989) in a buffer containing 15 mM Veronal, 140 mM NaCl, 0.5 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5 mM Tris-HCl, 0.005% Tween 80, and 500 Trasylol KIU/ml, pH 7.75. After 1 h of incubation at 37 °C, clots were centrifuged, and radioactivity in the supernatant was determined. t-PA bound was expressed as the fraction of the total amount of t-PA analogue added to the fibrinogen solution (F). The data were fitted to the Equation 1.

$$F = [\text{t-PA}_{\text{bound}}]/[\text{t-PA}_{\text{total}}] = [\text{fibrino(gen)}_{\text{total}}]/([\text{fibrino(gen)}_{\text{total}}] + C_{50}) \quad \text{(Eq. 1)}$$

with the nonlinear regression analysis algorithm of Slide Write 5.0 (Advanced Graphics Software, Inc. Carlsbad, CO). C_{50} is equal to the concentration of fibrin(ogen) where t-PA binding to fibrinogen is half-maximal. For n = 1 the standard error of the nonlinear regression estimate of the 10 data points is given. When $n > 1$, the mean C_{50} (with the standard error of the mean) of different binding experiments is given.

Determination of the t-PA and t-PA Analogue Concentration—Spectrophotometric assays were performed as described previously (Verheijen *et al.*, 1985). Briefly, the reaction mixture (250 μl total volume) contained plasmin-treated t-PA analogues in 100 mM Tris-HCl, pH 7.4,

0.1% (v/v) Tween 80, and 1.0 mM S-2288. The two-chain t-PA or t-PA analogue sample was tested at four different concentrations. The absorbance change at 405 nm ($\Delta A/\Delta t$) for each concentration was determined. These were plotted against the four different concentrations of t-PA or t-PA analogues, representing the absorbance change/concentration t-PA analogue. The absorbance change/concentration for a known amount of t-PA standard was compared with the absorbance change/concentration of the t-PA analogues. Since the amidolytic activity for the P domain of the t-PA standard is similar to the amidolytic activity of the P domain of the t-PA analogues, the concentration of t-PA was calculated (Bakker *et al.*, 1993).

Determination of the Stimulation Factor—Spectrophotometric assays were performed as described previously (Verheijen *et al.*, 1982). Briefly, the reaction mixture (250 μl of total volume) contained various amounts of plasmin-treated t-PA analogues, 100 mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 80, 0.12 μM Glu-plasminogen, and 0.7 mM S-2251. In certain cases, 120 μg/ml of CNBr-digested fibrinogen were included. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The t-PA analogue sample was tested at 10, 20, and 40 pM (final concentration) of active enzyme both for reaction mixtures containing fibrinogen fragments and for reactions mixtures without fragments. Fibrinogen fragments were prepared as described previously (Verheijen *et al.*, 1982). The enhancement factors were determined as follows. The change in absorbance was monitored over time for each t-PA analogue in the presence and in the absence of CNBr digest of fibrinogen. For each enzyme concentration, a slope was calculated, representing the absorbance change over time squared ($\Delta A/\Delta t^2$). These slopes, in turn, were plotted against enzyme concentration, representing the absorbance change/time squared/molar concentration of enzyme, and finally expressed as $\Delta A\ h^{-2}\ pmol^{-1}$. The ratio of the slope in the presence of fibrinogen fragments to the slope in the absence of fragments is the enhancement factor. This ratio reflects the extent to which fibrinogen fragments enhance the activity of the particular t-PA analogue preparation.

Competition Experiments—t-PA (final concentration, approximately 4 μM) and K2P (final concentration, approximately 8 μM) were inactivated with 50 μM PPACK in 0.1 M Tris-HCl, pH 7.5, 0.01% (v/v) Tween 80, and 1 M NaCl for 6 h at room temperature. PPACK-treated t-PA and K2P were separated from PPACK with a Sephadex G-50 fine column equilibrated in 0.1 M Tris-HCl, pH 7.5, 0.01% (v/v) Tween 80, and 1 M NaCl. The inhibition of t-PA and K2P was confirmed using a spectrophotometric assay (see "Determination of the t-PA Analogue Concentration.")

880 nM plasminogen-free fibrinogen was incubated with radiolabeled t-PA analogues (final concentration, 0.1 nM) and t-PA (final concentration, approximately 0.5 μM), K2P (final concentration, approximately 0.5 μM), or human serum albumin (final concentration, approximately 0.3 μM) in 15 mM Veronal, 140 mM NaCl, 0.5 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5 mM Tris-HCl, and 0.005% (v/v) Tween pH 7.75. Clotting was performed with 2 NIH units of thrombin/ml. After 1 h of incubation at 37 °C, clots were disrupted by vortexing. After centrifugation, radioactivity in the supernatant was determined. t-PA bound is expressed as the fraction of total added amount of t-PA analogue.

RESULTS

Characterization of the Recombinant Proteins—To study the role of the lysyl binding site in the K2 domain of t-PA in the interaction of t-PA with a forming fibrin clot, we constructed t-PA domain deletion analogues in which the lysyl binding site is impaired by a single amino acid substitution, FGK1K2P (D236N), GK1K2P (D236N), and K2P (D236N). The recombinant proteins show the expected molecular weight on a zymogram (Fig. 1).

All of the domain deletion variants specifically interact with lysyl-Sepharose. The mutation D236N in the different domain deletion mutants results in a loss of lysyl binding capacity (Table I). All molecules convert plasminogen to plasmin (Table II). In comparison with the t-PA variants, which show interaction with lysyl-Sepharose, the t-PA variants lacking the lysyl binding site activate plasminogen with lower efficiency (Table II, column 2). In the presence of CNBr-digested fibrinogen as a fibrin mimic, the plasminogen activation of all molecules was enhanced (Table II, column 3). The FGK1K2P and GK1K2P molecules showed a higher enhancement of plasminogen activation than the corresponding lysyl binding site mutants FGK1K2P (D236N) and GK1K2P (D236N). Interestingly K2P (D236N), which shows no interaction with a forming fibrin clot

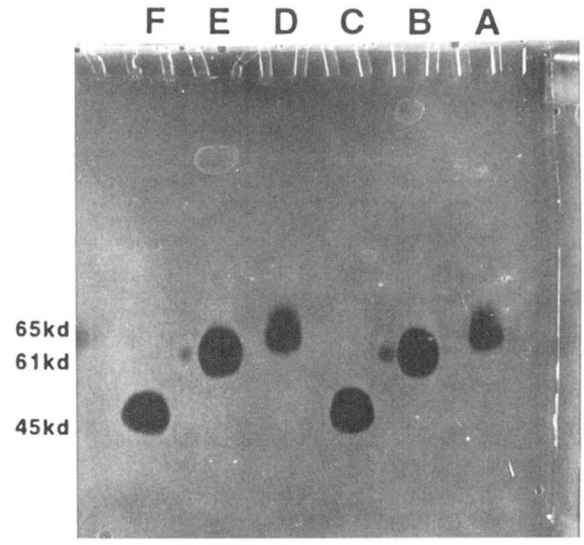

FIG. 1. **Fibrin zymography of t-PA analogues.** FGK1K2P (*lane A*), GK1K2P (*lane B*), K2P (*lane C*), FGK1K2P (D236N) (*lane D*), GK1K2P (D236N) (*lane E*), and K2P (D236N) (*lane F*) were isolated from culture media by affinity chromatography. Gel electrophoresis and zymography were performed as described under "Experimental Procedures." High molecular weight standards were run in a separate lane (not shown).

TABLE I
Binding to lysyl-Sepharose

Radiolabeled t-PA analogues were applied to a 1-ml lysyl-Sepharose column. Run-through was collected. The columns were washed with 2.5 ml of buffer and eluted with 2.5 ml of buffer containing 50 mM lysine analogue EACA. The radioactivity present in the flow-through, wash fraction, the elution fraction, and remaining on the columns was determined and expressed as a fraction of the total radioactivity. For details, see "Experimental Procedures."

t-PA or t-PA analogues studied	Fraction of total			
	Run-through	Wash	Eluate	Column
	cpm			
FGK1K2P	0	0.08	0.9	0.02
GK1K2P	0.2	0.1	0.7	0
K2P	0.05	0.05	0.9	0
FGK1K2P(D236N)	0.1	0.8	0.1	0
GK1K2P(D236N)	0.3	0.7	0	0
K2P(D236N)	0.2	0.8	0	0

TABLE II
Fibrin-dependent plasminogen activation

The amount of two-chain t-PA variant was determined using the amidolytic substrate S-2288 as described under "Experimental Procedures." Plasminogen activator activity of t-PA and the t-PA variants in the absence (column 2) and presence of CNBr fragments of fibrinogen (column 3) were determined as described under "Experimental Procedures" and expressed as $\Delta A\ h^{-2}\ pmol^{-1}$. Stimulation factors (column 4) were calculated as follows. The $\Delta A\ h^{-2}\ pmol^{-1}$ (in the presence of CNBr fragments of fibrinogen) divided by $\Delta A\ h^{-2}\ pmol^{-1}$ (in the absence of CNBr fragments of fibrinogen).

t-PA or t-PA analogue studied	Plasminogen activation		Stimulation factor
	No CNBr fragment of fibrinogen	CNBr fragments of fibrinogen	
	$\Delta A\ h^{-2}\ pmol^{-1}$		
FGK1K2P	0.24	30	125
GK1K2P	0.17	5.6	33
K2P	0.34	7.6	22
FGK1K2P(D236N)	0.22	11.1	50
GK1K2P(D236N)	0.09	1.1	12
K2P(D236N)	0.11	2.7	25

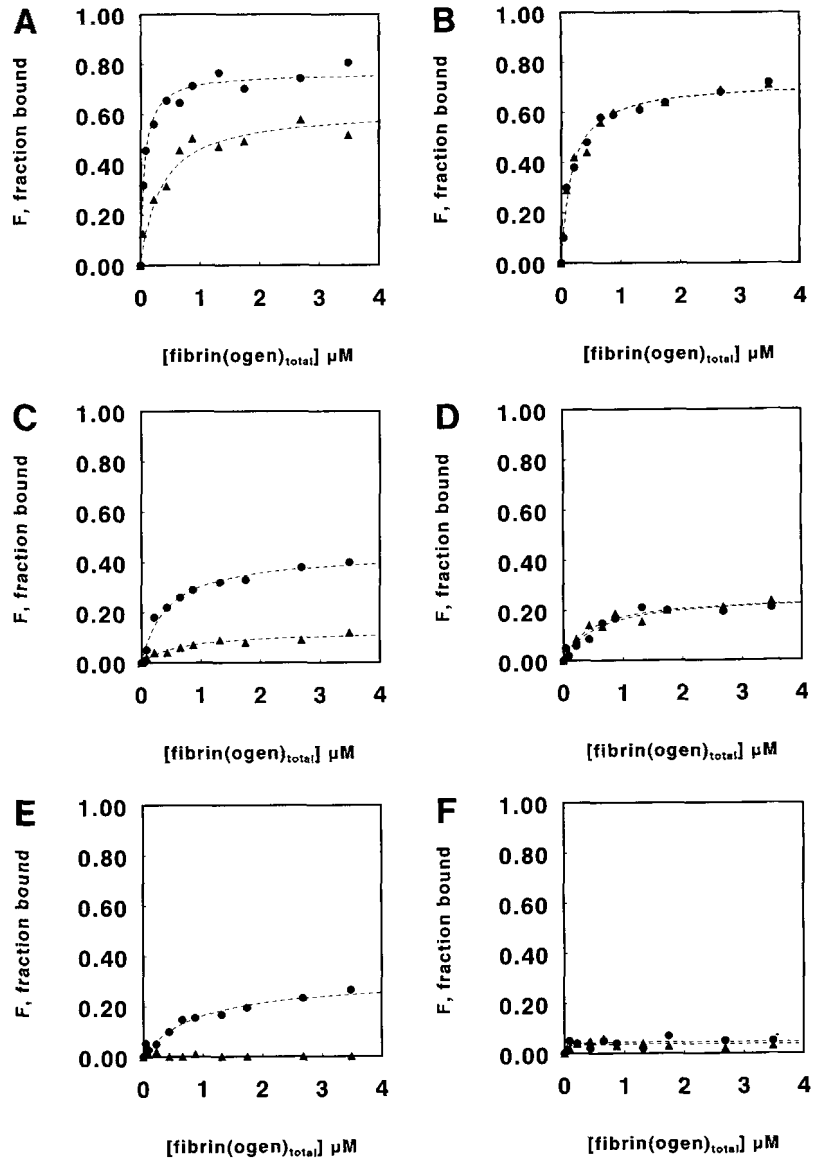

FIG. 2. **Experimental determination of the fraction of total t-PA bound (*F*) at various fibrin(ogen) concentrations.** Radiolabeled t-PA was incubated with various amounts of fibrinogen (0–3.4 μM) in the absence and presence of EACA. After clotting, the amount of radiolabeled t-PA or t-PA analogue bound to the fibrin clot was determined. On the *y* axis, *F* = the fraction of total t-PA bound to the fibrin clot; on the *x* axis is the amount of fibrin(ogen) present in the clot. *A*, FGK1K2P; *B*, FGK1K2P (D236N); *C*, GK1K2P; *D*, GK1K2P (D236N); *E*, K2P; *F*, K2P (D236N); ●, no addition; ▲, in the presence of 5 mM EACA (see "Experimental Procedures" and for further explanation see text).

(see below), still activates plasminogen in the presence of fibrin mimic as efficiently as K2P.

Fibrin Binding Sites within t-PA—Fibrin binding experiments were performed with low concentrations of t-PA or t-PA analogues (<0.1 nM), and the fraction of total t-PA or t-PA analogues bound at different fibrin(ogen) concentrations was assessed (Fig. 2). For t-PA, a high affinity interaction with a forming fibrin clot is found (Fig.2*A*). When the C_{50} of this interaction was determined, a value of 77 ± 11 nM was found (Table III). Fibrin binding of t-PA in the presence of 5 mM EACA is markedly reduced (Fig. 2*A*). Determination of the C_{50} for this interaction resulted in a value of 376 ± 46 nM (Table III). Deletion of the lysyl binding site (FGK1K2P (D236N)) also effects the binding to a forming fibrin clot (Fig. 2*B*), resulting in a C_{50} value of 169 ± 20 nM (Table III). The presence of 5 mM EACA had no influence on the fibrin binding of FGK1K2P (D236N), confirming the absence of a functional lysyl binding site in this molecule. Deletion of the F domain in t-PA (GK1K2P) reduces fibrin binding (Fig. 2*C*). Blocking the lysyl binding site in GK1K2P with 5 mM of EACA resulted in a lowered fibrin binding. Surprisingly, even when the F domain and the lysyl binding site in t-PA are lacking (GK1K2P (D236N)), considerable fibrin binding is still observed (Fig. 2*D*, C_{50} GK1K2P (D236N); 557 ± 126 nM (Table III)). As noticed with t-PA, occupying the lysyl binding site with EACA has a greater effect on fibrin binding than deleting the lysyl binding site. The K2P binding to fibrin is weak (Fig. 2*E*, C_{50} 1163 ± 490 nM (Table III)) and is completely inhibited in the presence of 5 mM EACA. Deleting the lysyl binding site in this molecule also abolishes fibrin binding (Fig. 2*F*).

t-PA Binding Sites on a Forming Fibrin Clot—To study the t-PA binding site on a forming fibrin clot, we performed competition experiments. Table IV shows the result of such a competition experiment. The binding of radiolabeled FGK1K2P is partially competed by FGK1K2P and K2P. The fibrin binding of FGK1K2P (D236N) lacking the lysyl binding site was only partially inhibited by FGK1K2P but also by K2P. This result suggests that the t-PA binding site in fibrin is also recognized by K2P. Binding of GK1K2P lacking the F domain is competed by K2P but also by FGK1K2P. Fibrin binding of GK1K2P (D236N) lacking both the F domain and the lysyl binding site in the K2 domain could be completely inhibited by FGK1K2P and K2P, indicating that the molecule GK_1K_2P (D236N) still recognizes the same t-PA binding site on fibrin. Competition with bovine serum albumin at competitor concentrations comparable with K2P and FGK1K2P did not occur (not shown).

TABLE III

Determination of C_{50} values of binding of t-PA and t-PA analogues to fibrin in the absence and presence of EACA

Column 1, t-PA analogues studied. Columns 2–5, C_{50} values for binding were determined from binding experiments of t-PA or t-PA analogues to a forming fibrin clot, comparable with the ones described (see Fig. 2). Using nonlinear regression analysis, the C_{50} of t-PA and t-PA domain deletion analogues to a forming fibrin clot in the absence or presence of 5 mM EACA and before and after deletion of the lysyl binding site (D236N) were determined. C_{50} values and the standard deviation are presented. NB, no binding observed. For details, see "Experimental Procedures."

t-PA or t-PA analogue studied	C_{50} nM			
		5 mM EACA	D236N	D236N (5 mM EACA)
FGK1K2P	77 ± 11 (n = 9)	376 ± 46 (n = 6)	169 ± 20 (n = 2)	186 ± 32 (n = 2)
GK1K2P	414 ± 26 (n = 1)	710 ± 189 (n = 1)	557 ± 126 (n = 2)	446 ± 126 (n = 2)
K2P	1,163 ± 490 (n = 2)	NB (n = 2)	NB (n = 2)	NB (n = 2)

TABLE IV

Competition experiments of radiolabeled t-PA and t-PA analogues with t-PA and K2P

Radiolabeled t-PA or t-PA analogue was incubated with 880 nM fibrin(ogen) in the absence (column 2) or in the presence of 0.5 μM FGK1K2P (column 3) or 0.5 μM K2P (column 4). After clotting, the fraction of total t-PA bound to fibrin was determined. The numbers in the table represent the fraction of total t-PA bound to the clot. The standard deviation was calculated from three data points. NB, no binding observed. For details, see "Experimental Procedures."

Labeled t-PA or t-PA analogue	Fibrin binding in the presence of competitor		
	None	FGK1K2P	K2P
FGK1K2P	0.73 ± 0.01	0.43 ± 0.01	0.23 ± 0.02
GK1K2P	0.29 ± 0.01	NB	0.08 ± 0.02
FG1K2P(D236N)	0.63 ± 0.01	0.26 ± 0.01	0.26 ± 0.01
GK1K2P(D236N)	0.14 ± 0.01	NB	NB

DISCUSSION

The first models describing the interaction of t-PA with a forming fibrin clot were based on the idea that t-PA not only consists of structurally autonomous domains but also of functionally autonomous domains (van Zonneveld *et al.*, 1986a). In these first models, there is a prominent role for the F and the K2 domain (van Zonneveld *et al.*, 1986b; Verheijen *et al.*, 1986; de Vries *et al.*, 1990; Nesheim *et al.*, 1990; Horrevoets *et al.*, 1994). However, a model in which the K1 domain plays an important role in the interaction of t-PA to preformed fibrin has been described (Kaczmarek *et al.*, 1993). The F/K2 models may be further subdivided into models in which the t-PA interaction sites on the fibrin are in juxtaposition (van Zonneveld *et al.*, 1986b; de Vries *et al.*, 1990; Horrevoets *et al.*, 1994) and a model in which the t-PA interaction sites are further apart (Nesheim *et al.*, 1990). All F/K2 models stress the importance of an direct interaction between the lysyl binding site in the K2 domain and a lysyl side chain of the fibrin network. Besides these models, in which the functional autonomy of domains is stressed, a model was presented in which the fibrin interaction sites in t-PA were spread over many domains, except the K2 domain (Bennet *et al.*, 1991).

Our results with the GK1K2P (D236N) a molecule that shows considerable interaction with a forming fibrin clot, suggest that besides the F and K2-mediated interaction, other fibrin interaction sites in t-PA exist. Interestingly, this molecule also shows enhanced plasminogen activation in the presence of fibrin. This indicates that besides the finger domain and lysyl binding site in the K2 domain, other domains of t-PA are involved in fibrin-dependent plasminogen activation. Remarkably, the fibrin binding of FGK1K2P (D236N) a molecule that no longer can interact via its lysyl binding site in the K2P part can be competitively inhibited by K2P. It seems therefore unlikely that the binding sites on fibrin for K2P and FGK1K2P (D236N) are far apart on the fibrin surface. This result questions the model of Nesheim (Nesheim *et al.*, 1990) in which no such competition would be expected.

The role of the lysyl binding site in binding to a forming fibrin clot is more complicated than expected. In the presence of 5 mM EACA, fibrin binding is more perturbed than after the deletion of the lysyl binding site. Such a result could be explained by steric hindrance. EACA binding to the lysyl binding site blocks the fibrin binding site and so reduces fibrin binding. Deletion of the lysyl binding site abolishes the interaction with EACA and therefore a possible inhibition of fibrin binding by EACA should no longer be possible. To test this steric hindrance hypothesis, we studied the fibrin binding of K2P in more detail. Fibrin binding of this molecule can be completely inhibited by 5 mM EACA. It is known that the dissociation constant of EACA for K2P is approximately 100 μM (Byeon *et al.*, 1991; de Munk *et al.*, 1989), 2 orders of magnitude higher than the dissociation constant of binding of K2P to fibrin (C_{50} 1163 ± 490 nM). Therefore, it seems reasonable to assume that fibrin binding is not solely aminohexyl-mediated and that the fibrin binding site comprises more than the lysyl binding site. However deletion of the lysyl binding site in the K2P molecule not only abolishes binding to EACA but also to fibrin. Therefore, steric hindrance does not seem to be a satisfying explanation for the observed difference in fibrin binding between t-PA in the presence of EACA and t-PA without a lysyl binding site.

A more likely explanation for this effect is to assume additional indirect effects of occupation of the lysyl binding site such as induction of a conformational changes in the t-PA molecule. Conformational changes upon occupation of a lysyl binding site in the closely related and structurally similar molecule plasminogen have been described (Markus *et al.*, 1978; Christensen and Molgaard, 1992). Plasminogen can occur in a closed conformation in the absence of EACA and an open conformation in the presence of EACA. The most likely explanation for this behavior is the occupancy of the lysyl binding site by an intramolecular lysyl or arginyl residue in the closed conformation (Ponting and Marshall, 1992). Circumstantial evidence suggests that a similar mechanism could be operating in t-PA since the solubility of t-PA increases considerably on addition of lysine or arginine (Hasegawa and Kondo, 1985; Ichimura, 1987). Furthermore electron microscopic studies suggest that the structure of the molecule is ellipsoidal with the domains folded toward each other (Margossian *et al.*, 1993). Based on differential scanning calorimetry experiments, an interaction between the FG domains and the P domain was predicted. Although no involvement of the lysyl binding site could be detected (Novokhatny *et al.*, 1991) a strong interdomain interaction involving lysyl binding sites has been observed in crystals of kringle 2 domain (de Vos *et al.*, 1992). Recently it was shown that replacement of stretches of charged amino acid residues containing lysyl residues or arginyl residues by alanyl residues influences the fibrin binding of the resulting t-PA analogue (Bennet *et al.*, 1991).

In view of these data, we propose an alternative model for the high affinity interaction of t-PA with fibrin and the role of the lysyl binding site in this (Fig. 3). In analogy to plasminogen, t-PA could occur in two conformations, an open conformation and a closed conformation. The interaction between the lysyl binding site in the K2 domain and a lysyl residue stabilizes the

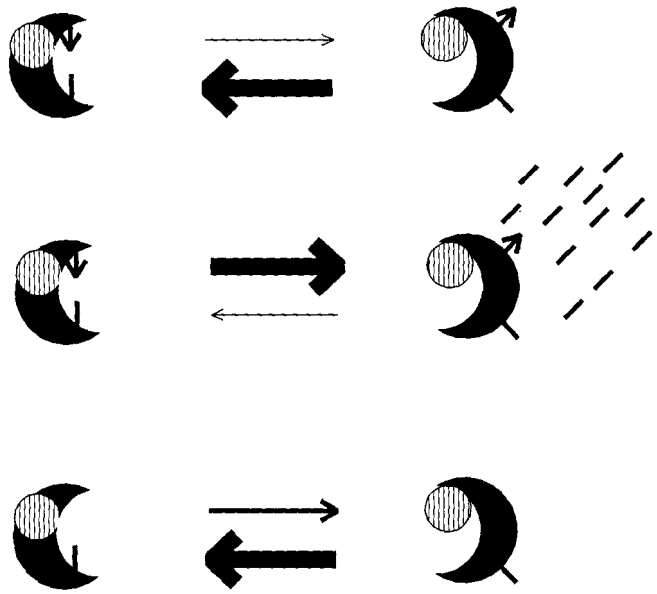

FIG. 3. **Presentation of a model to explain the effect of EACA and the deletion of the lysyl binding site in t-PA on the fibrin binding to a forming fibrin clot.** Within the t-PA molecule (⊖ = FGK1 part; ❨ = K2P part; ↓ = lysyl binding site; | = lysyl/arginyl residue from another t-PA domain or EACA) an intramolecular interaction probably between the lysyl binding site and a lysyl/arginyl residue of the P domain, results in an equilibrium between a closed and an open conformation. The closed conformation is hypothesized to possess the structural requirements for a high affinity interaction with a forming fibrin clot (*upper part*). In the presence of EACA the interaction between the lysyl binding site in K2 and the lysyl/arginyl residue is disturbed resulting in a shift in equilibrium toward the open conformation (*middle part*). The open conformation is hypothesized to possess a fibrin interaction site of lower affinity. Deletion of the lysyl binding site also results, although to a lesser extent, in a shift in equilibrium toward a more open conformation (*lower part*).

closed conformation. The addition of EACA or mutation of the lysyl binding site would free t-PA in a more open conformation. In the closed conformation, the affinity for fibrin is higher than in the open conformation. It has not escaped our notice that occupation of the lysyl binding site in t-PA by EACA could reflect a first step in the pathway of fibrin-dependent plasminogen activation. Occupation of the lysyl binding site by plasminogen would free the P domain of t-PA from the fibrin surface making it available for the hydrolysis of the Arg^{561}-Val^{562} bond of plasminogen. However, the enhancement of the plasminogen to plasmin conversion by K2P (D236N), that does not interact with a forming fibrin clot suggests that yet another mechanism of plasminogen activation exists, independent of the t-PA binding to fibrin.

Acknowledgments—We thank Drs. D. C. Rijken and W. Nieuwenhuizen for critically reading the manuscript and for helpful suggestions.

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