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A Steady-state Template Model That Describes the Kinetics of Fibrin-stimulated [Glu\(^1\)]- and [Lys\(^{78}\)]Plasminogen Activation by Native tissue-type Plasminogen Activator and Variants That Lack Either the Finger or Kringle-2 Domain*

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The kinetics of activation of both [Glu\(^1\)]- and [Lys\(^{78}\)]Plg(S741C-fluorescein) by native (recombinant) tissue-type plasminogen activator and its deletion variants lacking either the finger or kringle-2 domain were measured by fluorescence within fully polymerized fibrin clots. The kinetics conform to the Michaelis-Menten equation at any fixed fibrin concentration so long as the plasminogen concentration is expressed as either the free or fibrin-bound, but not the total. The apparent \(k_{cat}\) and \(K_m\) values both vary systematically with the concentration of fibrin. Competition kinetics disclosed an active site-dependent interaction between t-PA and [Glu\(^1\)]Plg(S741C-fluorescein) in the presence, but not the absence, of fibrin. A steady-state template model having the rate equation \(v/(A_0) = k_{cat,app}(1/[P][K_{m,app}]+[P])\) was derived and used to interpret the data. The model indicates that catalytic efficiency is determined by the stability of the ternary activator-fibrin-plasminogen complex rather than the binding of the activator or plasminogen to fibrin. This implies that efforts to improve the enzymatic properties of t-PA might be more fruitfully directed at enhancing the stability of the ternary complex rather than fibrin binding.

The major protein constituent of blood clots, fibrin, is degraded to soluble products by plasmin, which displays a broad trypsin-like specificity. The activity of plasmin is restricted as a result of the fibrin-specific way in which its inactive precursor plasminogen is activated by tissue-type plasminogen activator (t-PA)\(^1\) (1). Fibrin, therefore, not only serves as a substrate for plasmin, it is also required for its production. Both t-PA and plasminogen are built from autonomous domains, and those involved in binding to fibrin have been identified (2, 3). Native [Glu\(^1\)]plasminogen, however, exhibits a very tight spiral structure (4), thereby shielding the activation cleavage site (Arg\(^{561}\)-Val\(^{562}\)) from easy access by plasminogen activators (5) and attenuating the interaction of the kringle domains with fibrin (2, 6, 7). A change in hydrodynamic properties is observed when the NH\(_2\)-terminal acidic domain of native [Glu\(^1\)]plasminogen is removed by limited plasmin-catalyzed proteolysis, resulting in a truncated form of plasminogen having Lys\(^{78}\) as the new NH\(_2\)-terminal residue (5, 8). [Lys\(^{78}\)]plasminogen has been shown to be a significant, although not essential, intermediate in the activation of [Glu\(^1\)]plasminogen during in vitro fibrinolysis (9–11), where its formation is strictly dependent on the presence of polymerized fibrin (10, 12). The work of Violand et al. (5) suggested that [Glu\(^1\)]plasminogen bound to fibrin adopts a [Lys\(^{78}\)]plasminogen-like conformation.

The kinetics of fibrin-stimulated plasminogen activation by native t-PA are currently described by an ordered sequential mechanism (cyclic ternary complex model), in which solution phase plasminogen interacts with high affinity only with fibrin-bound t-PA (13). Subsequent studies employing [Glu\(^1\)]- and especially [Lys\(^{78}\)]plasminogen or variants of t-PA have shown deviations from this kinetic model (14–17).

A detailed kinetic analysis of plasminogen activation necessitates the performance of steady-state measurements during the different stages of fibrin degradation. Since the reaction generates active plasmin, however, the structure of the cofactor fibrin is subject to continuous change. In the accompanying paper (18), we describe the expression of a variant of recombinant human [Glu\(^1\)]plasminogen, in which serine of the active site of plasmin has been replaced by cysteine and labeled with fluorescein: Plg(S741C-fluorescein). Employing this plasminogen variant and the Lys\(^{78}\) form of it, we quantified by fluorescence the activation rate of these zymogens without generating active plasmin. The impact of fibrin affinity of the activator on these kinetics was studied by employing deletion variants (del.K2 and del.F) that show altered affinity for fibrin (17). In the present paper we describe a detailed study of the kinetics of t-PA-mediated plasminogen activation in a fully polymerized, intact fibrin clot. Analysis of the kinetics led to a new, modified model for fibrin-stimulated plasminogen activation within fully intact, polymerized fibrin.

**EXPERIMENTAL PROCEDURES**

**Materials**

Native (recombinant) t-PA was Activase, a generous gift of Dr. G. Vehar (Genentech, San Francisco, CA). SDS-polyacrylamide gel electrophoresis indicated that the material is predominantly in the single-chain form. Single-chain t-PA and its deletion variants were obtained as described previously (17). The production and purification of recombi-
niant Plg(S741C-fluorescein) and the human proteins α-thrombin and fibrinogen are described in the accompanying paper (18). The chromogenic substrate n-Glu-Gly-Arg-p-nitroanilide (S2444) was obtained from Chromogenics (Molndal, Sweden).

Fluorescence Activation Assay of [Glu1]- and [Lys308]Plg(S741C-fluorescein)

Activation experiments were performed in 96-well plates (Dynatech) at 20°C, using a Perkin Elmer LS50B Luminescence Spectrometer equipped with a fluorescence plate reader. Fluorescence intensities were measured at excitation and emission wavelengths of 495 and 535, respectively, employing a 550-nm emission cut-off filter. In this instrument the sample is excited from above and emitted light is collected from above. Wells were pre-equilibrated with 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl (HBS) plus 1% Tween 80 (w/v) for 1 h to prevent adsorption of proteins to the plastic. Subsequently, wells were loaded with 90 μl of HBS, 0.02% Tween 80 (w/v) (HBST) containing various concentrations of [Glu1]- or [Lys308]Plg(S741C-fluorescein) and fibrinogen and equilibrated at 20°C. Stability of the fluorescence intensity was verified for 5 min. The reaction was initiated by adding 10 μl of HBST, 100 mM CaCl2 containing 60 mM human α-thrombin and various concentrations of t-PA (1–10 nM final concentration). Data were collected every 60 s and stored as print files for each individual well using a data acquisition program written by Dr. W. K. Stevens in our laboratory. Initial rates of fluorescence decrease were determined by linear regression analysis and converted to rates of zymogen activation according to Equation 1, in which dP/du is the rate of cleavage of the zymogen, P, into initial [Glu1]- or [Lys308]Plg(S741C-fluorescein) concentration, Lc is initial fluorescence intensity, and r represents the ratio of the fluorescence intensities of the fluorescent analogues of plasmin and plasminogen. The values of r for the Glu1- and Lys308 forms of Plg(S741C-fluorescein) are 0.5 and 0.4, respectively (18).

\[
\frac{dP}{du} = \frac{P(Lc/r)}{(Lc/r)(1-r)} \quad (\text{Eq. 1})
\]

**Competition Kinetics**

The impact of the presence of [Glu1]Plg(S741C-fluorescein) as a competing substrate on the t-PA-catalyzed hydrolysis of S2444 was measured in microtiter wells in a Titertek Twinreader at 37°C. In brief, 10.0-μl aliquots of 200 nM t-PA were added to 90.0 μl of HBST containing 1 mM S2444 and various concentrations of [Glu1]Plg(S741C-fluorescein), and hydrolysis was monitored at 1-min intervals at 405 nm. The influence of fibrin was measured by adding 10.0-μl aliquots containing 200 nM t-PA, 60 nM α-thrombin, and 100 mM CaCl2 to 90 μl of HBST containing 1 mM S2444, 3.3 μM fibrinogen, and various concentrations of [Glu1]Plg(S741C-fluorescein). Clotting was complete within 1 min, as seen from the rapid increase in turbidity, which remained constant during the remainder of the experiment. The apparent Keq for the interaction of t-PA and fibrinogen with [Glu1]Plg(S741C-fluorescein) was deduced from Equation 2, which describes the relationship between activity toward a chromogenic substrate in the presence of increasing concentrations of a competing substrate, and in which v and v are reaction rates of S2444 hydrolysis in the absence and presence of [Glu1]Plg(S741C-fluorescein), F is the fibrin concentration, K1 is the dissociation constant for t-PA and fibrin, P is the [Glu1]Plg(S741C-fluorescein) concentration, and K2 is the apparent Keq for the t-PA-[Glu1]Plg(S741C-fluorescein) interaction.

\[
v = v_0 \cdot \frac{(1 + F/K1)(1 + (F/K2))}{(1 + F/K1) + (1 + F/K2)} \quad (\text{Eq. 2})
\]

**Models of Plasminogen Activation**

In order to interpret the dependences of initial rates of plasminogen activation on fibrin and plasminogen concentrations, a model of the reaction was sought. The model, in order to accurately reflect the data, would need to predict Michaelis-Menten behavior with respect to the substrate concentration at all fibrin concentrations; saturation in kcat with respect to the fibrin concentration; template-like, biphasic behavior with respect to the fibrin concentration at all fixed substrate concentrations; and a very high Keq in the absence of fibrin. In addition, it would need to fit all accumulated data accurately, with randomly distributed residuals over the entire data set, and not predict values for parameters such as dissociation constants for binding interactions, that are inconsistent with independently measured values. In an attempt to find a model that satisfies the above criteria, all possible equilibrium models that describe three component systems were constructed and examined with respect to their ability to predict the experimental data. In addition, a steady-state model was constructed. These models and the derivations of their corresponding rate equations are shown below.

**Equilibrium Models**

Since general experience has shown that significant plasminogen activation occurs only when all three components are present (activator, A, plasminogen (P) and fibrin (F)), the assumption is made that catalysis occurs from a ternary complex of the three components (AFP) which turns over into product. The rate constant for the turnover step is designated kcat. In addition, the assumption is made that the ternary complex is assembled through binary interactions among the individual components and subsequent interactions of the binary complexes with the third component. Furthermore, all interactions are assumed to be essentially at equilibrium. The three components can form three individual binary complexes (AF, FP, PA). Thus, seven equilibrium models of the assembly of AFP are possible, as indicated by Boskovic et al. (19). Three models involve only one of each of the binary complexes, three involve the three possible pairs of components, and one involves all three components. Expressions relating the equilibrium among components and the conservation of fibrin, concepts of the seven models, and derivations of their rate equations are as follows.

Equilibrium interactions are shown by Equations 3–8.

\[
[A][F] = K_{FDA}(F) \quad (\text{Eq. 3})
\]

\[
[F][P] = K_{FPD}(P) \quad (\text{Eq. 4})
\]

\[
[P][A] = K_{APD}(P) \quad (\text{Eq. 5})
\]

\[
[A][F][P] = K_{AFP}\text{(AFP)} \quad (\text{Eq. 6})
\]

\[
[F][P][A] = K_{APF}\text{(AFP)} \quad (\text{Eq. 7})
\]

\[
[P][A][F] = K_{FAP}\text{(AFP)} \quad (\text{Eq. 8})
\]

From Equations 3–8, Equation 9 results.

\[
K_{FAP}K_{APF} = K_{AFP}K_{APF} \quad (\text{Eq. 9})
\]

Conservation of fibrin is given by Equation 10.

\[
[F] = [F] + [AF] + [FP] + [AFP] \quad (\text{Eq. 10})
\]

Since [A] ≪ [F], we get Equation 11.

\[
[F] = [F] + [FP] = [F](1 + [P]/K_{FP}) \quad (\text{Eq. 11})
\]

All rate equations below are expressed as the ratio v/[A], where v is the rate and [A] is the total concentration of activator (t-PA). P is the free concentration of plasminogen (when [FP] exists in the model) and [P] is the total concentration of plasminogen (when [FP] does not exist in the model). [F] is the total concentration of fibrin. The rate equations were obtained using the expressions for v/[A] given below, the equilibrium expressions of Equations 3–8 and the conservation equation for fibrin (Equation 11).

\[
\frac{v}{[A]} = \frac{K_{FAP}}{K_{FAP} + [F]} \quad (\text{Eq. 12})
\]

\[
\frac{v}{[A]} = \frac{K_{APF}}{K_{APF} + [F]} \quad (\text{Eq. 13})
\]

\[
\frac{v}{[A]} = \frac{K_{FAP}}{K_{FAP} + [P]} \quad (\text{Eq. 14})
\]

\[
\frac{v}{[A]} = \frac{K_{APF}}{K_{APF} + [P]} \quad (\text{Eq. 15})
\]

\[
\frac{v}{[A]} = \frac{K_{FAP}}{K_{FAP} + [P]} \quad (\text{Eq. 16})
\]

\[
\frac{v}{[A]} = \frac{K_{APF}}{K_{APF} + [P]} \quad (\text{Eq. 17})
\]
complexes, activator-fibrin (AF) and plasminogen-fibrin (PF). These bi-
plasminogen and can interact with each other to form the respective binary
follows. Protomers of fibrin possess unique binding site(s) for t-PA and
The concepts and equations of this model are as follows.

Model IV

\[
K_{A} + P \leftrightarrow A \cdot P + K_{r} \quad K_{s} + P \rightarrow A \cdot P + K_{r} \\
F + P \rightarrow A \cdot F + P + K_{s} \\
A \cdot F \rightarrow A + F + P \\
\]

(Eq. 88)

\[
\nu[A] = k_{cat} \cdot [AF]/([A] + [AF] + [AFP]) \\
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
A + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 20)

\[
K_{s} = K_{s}K_{n} + K_{n}K_{s} \\
\]

(Eq. 18)

\[
\nu[A] = k_{cat} \cdot [AF]/([A] + [AF]) \\
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
F + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 19)

\[
K_{s} = K_{s}K_{n} + K_{n}K_{s} \\
\]

(Eq. 21)

\[
\nu[A] = k_{cat} \cdot [AF]/([A] + [AF]) \\
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
F + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 22)

\[
\nu[A] = k_{cat} \cdot [AFP]/([A] + [AF] + [AFP]) \\
\]

(Eq. 23)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
A + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 24)

\[
\nu[A] = k_{cat} \cdot [AF]/([A] + [AF]) \\
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
A + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 25)

\[
\nu[A] = k_{cat} \cdot [AFP]/([A] + [AF] + [AFP]) \\
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
A + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 26)

\[
K_{s} = K_{s}K_{n} + K_{n}K_{s} \\
\]

(Eq. 27)

\[
\nu[A] = k_{cat} \cdot [AF]/([A] + [AF]) \\
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
A + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 28)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
A + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 29)

\[
K_{s} = K_{s}K_{n} + K_{n}K_{s} \\
\]

(Eq. 29)

\[
\nu[A] = k_{cat} \cdot [AF]/([A] + [AF]) \\
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
A + P \rightarrow A \cdot P + K_{s} \\
P + A \rightarrow A \cdot P + K_{s} \\
\]

(Eq. 30)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 31)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 32)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 33)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 34)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 35)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 36)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 37)

\[
\nu[A] = \frac{k_{cat} \cdot [P][F]/(K_{n} + [F])}{K_{n}[A] + [F] + [F]_{0} + [P]} \\
\]

(Eq. 38)

This equation predicts that at high levels of [P], the reaction kinetics
with respect to [P] will deviate from the Michaelis-Menten relationship
because of the terms containing [P]². Since this was not the case over
the range of [P] concentrations used presently, terms in [P]² were
considered negligible and therefore not included in the rate equation.
This approximation and with Equation 35 used to substitute for [F]
in Equation 38, the rate Equation 39 results, which is written in a
simpler format in Equation 40. This later equation shows that, at any
fixed concentration of fibrin, plasminogen activation conforms to the
Michaelis-Menten equation when the plasminogen concentration is
expressed as the free concentration. The values depend on the fibrin
concentration as indicated in Equations 41 and 42.

\[
V = \frac{k_{cat} \cdot [P]}{K_{s}K_{n} + K_{n}K_{s}} \\
\]

(Eq. 40)

\[
\nu[A] = \frac{k_{cat} \cdot [P]}{K_{s}K_{n} + K_{n}K_{s}} \\
\]

(Eq. 41)

\[
K_{on} = \frac{K_{0}K_{n}}{K_{s}K_{n}} \\
\]

(Eq. 42)

\[
K_{on} = \frac{K_{0}K_{n}}{K_{s}K_{n}} \\
\]

(Eq. 43)

\[
K_{on} = \frac{K_{0}K_{n}}{K_{s}K_{n}} \\
\]

(Eq. 44)

\[
K_{on} = \frac{K_{0}K_{n}}{K_{s}K_{n}} \\
\]

(Eq. 45)

The rate of the reaction is given by Equation 30.

\[
v = k_{cat}[AFP] \\
\]

The steady-state equations for [AF] and [AFP] are given by Equations
31 and 32.

\[
d[AF]/dt = 0 = k_{cat}[A] - (k_{1} + k_{4}[P])[AF] + (k_{2} + k_{3}[P])[AFP] \\
\]

(Eq. 31)

\[
d[AFP]/dt = 0 = k_{cat}[A] - (k_{1} + k_{4}[P])[AF] + (k_{2} + k_{3}[P])[AFP] \\
\]

(Eq. 32)
Kinetics of Plasminogen Activation on Fibrin

Equations 41 and 42 predict that $k_{cat,app}$ and $K_{cat,app}$ approach the true $k_{cat}$ and $K_{cat}$ (Equations 43 and 45) at high fibrin concentrations. Equation 39 predicted well all experimental data and therefore was used to calculate, by nonlinear regression analyses (SYSTAT, NONLIN module), best values for the parameters $k_{cat}$, $K_m$, and $K_v$. Free concentrations of plasminogen were calculated from the total concentrations of plasminogen and input fibrinogen by using the binding parameters reported previously: $[\text{Lys}^78]\text{Plg}(S741C\text{-fluorescein}): K_p = 1.2 \times 10^3 \text{M}^{-1} \text{nM}^{-1}, K_v = 30 \text{mM}$, $n = 2$ (17, 18), and the quadratic equation $[P] = [P]_0 - 0.5(k_p + [P]_0 + [F])^2 - 4(P)[F]_0(n^2) + (k_v + [P]_0 + [F])^2 - 4(P)[F]_0(n^2)$.

RESULTS

Activation of [Glu$^1$]- and [Lys$^78$]Plg(S741C-fluorescein) in a Fully Polymerized Fibrin Clot—The fibrin-stimulated substrate activation by t-PA was studied by measuring rates of activation of the recombinant, fluorescently labeled Glu$^1$ and Lys$^78$ forms of variant plasminogen Plg(S741C-fluorescein). This system allows steady-state measurements of plasminogen activation without the concurrent formation of active plasmin, thereby preventing any possible feedback by plasin-mediated proteolytic alterations of t-PA, plasminogen, and fibrin. The turbidity of the fibrin clot does not change under this experimental setup, allowing the performance of the fluorimetric analysis within a fully polymerized fibrin clot. This precludes the introduction of potential artifacts by chemical and/or physical treatment of the fibrin stimulator, and allows determination of the role of fibrin polymer formation, which has been shown to be essential for full stimulatory activity (20).

Large data sets were obtained at fibrin concentrations ranging from 20 nM to 10 mM, [Glu$^1$]Plg(S741C-fluorescein) concentrations ranging from 50 nM to 2.5 mM, and [Lys$^78$]Plg(S741C-fluorescein) concentrations ranging from 25 nM to 1 mM. The concentration of native t-PA and deletion variants was constant within each data set (1–10 nM), although the turnover number was shown to be independent of t-PA concentrations ranging from 0.5 to 50 nM (not shown). Representative sets of the kinetic data for native t-PA and its variants and [Glu$^1$]Plg(S741C-fluorescein) are shown in Fig. 1. At any given fibrin concentration, the rate of activation by native t-PA (Activase and sct-PA) as a function of plasminogen concentration approximates a Michaelis-Menten rectangular hyperbola as shown previously (13). Similar profiles are observed for del.K2, whereas no saturation was evident in the case of del.F (not shown). Further analysis of the data with respect to the substrate concentration is exhibited at any fixed fibrin and plasminogen dependence of rates as calculated from Equa-

![Fig. 1. Kinetics of [Glu$^1$]Plg(S741C-fluorescein) activation in a fibrin clot. Rates of [Glu$^1$]Plg(S741C-fluorescein) activation at the indicated t-PA or t-PA variant and fibrin (F) concentrations were determined in a fluorescent plate reader at 20 °C as described under “Experimental Procedures.” Results are expressed as rates per unit concentration of t-PA or t-PA variant (s$^{-1}$). Panel A: Activase (t-PA); panel B, sct-PA; panel C, del.K2; panel D, del.F. The lines represent the fibrin and plasminogen dependences of rates as calculated from Equation 39 (see “Experimental Procedures”) and derived parameter values (Table I).](https://www.jbc.org/content/281/7/2186)
Since [Lys78]Plg(S741C-fluorescein) has a high affinity for fibrin (1.2 μM) and [Glu1]Plg(S741C-fluorescein) a low one (30 μM), this behavior would be expected for a template mechanism in this range of fibrin concentrations (21). Notably, both $k_{cat}$ and $K_m$ values at saturating fibrin are approximately 3-fold higher for del.K2 than for t-PA, making their catalytic efficiencies very similar. The individual $k_{cat}$ and $K_m$ values for del.F were not determined due to linear activity/substrate concentration relationships. $k_{cat}/K_m$ values, however, were 20-fold lower for this variant and did saturate with fibrin (Fig. 3E).

In order to determine whether fibrin facilitates an active site-dependent association between t-PA and [Glu1]Plg(S741C-fluorescein), we performed competition kinetics with the amidolytic substrate S2444 in the absence and presence of fibrin (Fig. 4). [Glu1]Plg(S741C-fluorescein) does not compete for the t-PA-catalyzed hydrolysis of the amidolytic substrate S2444 in the absence of fibrin, therefore precluding the presence of an active site-dependent high affinity interaction between these proteins. The presence of a fibrin clot results in a modest increase in activity of the fibrin-bound single-chain t-PA as a result of a decreased $K_m$ for the amidolytic substrate in accordance with published data (22). In contrast to the lack of interaction between t-PA and plasminogen in the absence of fibrin, an interaction between [Glu1]Plg(S741C-fluorescein) and t-PA with a $K_a$ value of approximately 0.5 μM can be inferred in the presence of a 3 μM fibrin clot (Fig. 4). Thus, we conclude that fibrin promotes the association between the enzyme and the substrate. The value of the apparent $K_m$ (0.5 μM) indicates in this three-component system the substrate concentra-

**Fig. 2.** **Kinetics of [Lys78]Plg(S741C-fluorescein) activation in a fibrin clot.** Rates per unit concentration of t-PA or t-PA variants (s⁻¹) were determined as in Fig. 1. Panel A, Activase (t-PA); rate as a function of the [Lys78]Plg(S741C-fluorescein) concentration at various fibrin concentrations (F). panel B, Activase (t-PA); rate as a function of log [F], at various [Lys78]Plg(S741C-fluorescein) concentrations (P, μM). The lines represent the dependences of rates on fibrin and [Lys78]Plg(S741C-fluorescein) concentrations as calculated from Equation 39 (see “Experimental Procedures”) and derived parameter values (Table I).

**Fig. 3.** **Fibrin dependence of the kinetic parameters for [Glu1]- and [Lys78]Plg(S741C-fluorescein) activation.** A and B, t-PA (Activase); C and D, del.K2; E, del.F. The open symbols represent [Glu1]Plg(S741C-fluorescein), and the closed symbols represent [Lys78]Plg(S741C-fluorescein). The experimentally determined $k_{cat}$ and $K_m$ values, plus and minus standard errors, for each fibrin concentration were calculated by fitting rate data versus the free Plg(S741C-fluorescein) concentration to the Michaelis-Menten equation. The values represented by solid lines for $k_{cat}$ and $K_m$ were calculated by Equations 41 and 42 and the parameters of Table I. For symbols not accompanied by error bars, the magnitude of the error was less than the size of the symbol.

In order to determine whether fibrin facilitates an active site-dependent association between t-PA and [Glu1]Plg(S741C-fluorescein), we performed competition kinetics with the amidolytic substrate S2444 in the absence and presence of fibrin (Fig. 4). [Glu1]Plg(S741C-fluorescein) does not compete for the t-PA-catalyzed hydrolysis of the amidolytic substrate S2444 in the absence of fibrin, therefore precluding the presence of an active site-dependent high affinity interaction between these proteins. The presence of a fibrin clot results in a modest increase in activity of the fibrin-bound single-chain t-PA as a result of a decreased $K_m$ for the amidolytic substrate in accordance with published data (22). In contrast to the lack of interaction between t-PA and plasminogen in the absence of fibrin, an interaction between [Glu1]Plg(S741C-fluorescein) and t-PA with a $K_a$ value of approximately 0.5 μM can be inferred in the presence of a 3 μM fibrin clot (Fig. 4). Thus, we conclude that fibrin promotes the association between the enzyme and the substrate. The value of the apparent $K_m$ (0.5 μM) indicates in this three-component system the substrate concentra-

**Data Analysis and Selection of a Steady-state Template**
very well. The physical meanings of the constants in the two models are different, however. In addition, Model IV has the restraint \( K_A K_m A/K_m F = K_p \) (see Equation 9 in “Experimental Procedures”), which implies that the fit parameters \( K_A, K_m A \) and \( K_m F \) should predict the dissociation constant for the binding of fibrin to plasminogen (\( K_p \)). When the data were fit to this equation, predicted values of \( K_p \) were 1.6 \( \mu M \) and 0.15 \( \mu M \) for the Glu1 and Lys78 forms of Plg(S741C-fluorescein), which are smaller than the experimentally measured values by factors of 19 and 8.0, respectively. Thus, in spite of the excellent fit of the rate equation of Model IV to the data, the model was excluded because of the latter difficulty.

Model V includes the AF and AP binary complexes and has the rate equation \( V/A_o = k_{cat}(P)/K_{an}(1 + K_m K_{an}/K_m A) + [P]). \) This equation is similar to that of Model IV, except that the concentration of plasminogen is expressed as the nominal ([P]) rather than free ([P]) concentration. This is because Model V does not include the binary plasminogen-fibrin interaction. The data, especially those with the Glu1 form of the substrate (which only binds fibrin weakly, such that \([P] \approx [P_o]\)), fit this equation well. Because of the restraint \( K_A K_m A/K_m F = K_p \); however, values of \( K_p = 1.2 \mu M \) and \( K_p = 0.03 \mu M \) were predicted for the dissociation constants for solution phase interactions between t-PA and the Glu1 and Lys78 forms of the substrate. These high affinity interactions are not consistent with the high \( K_p \) values (\( \geq 50 \mu M \)) reported for the solution phase reaction (13) or the lack of saturation of rates of reactions in the absence of fibrin (18).

Model VI includes the FP and AP binary complexes and has the rate equation \( V/A_o = k_{cat}(P)/K_{an}[K_{an}(1 + K_m K_{an} + [P]) + [P]). \) This does not predict a variation in \( k_{cat} \) with the concentration of fibrin, which is consistent with the data of Fig. 3 and Equation 39. It does, however, fit the data reasonably well at fibrin concentrations in excess of 1.0 \( \mu M \), where the activator is completely bound and \( k_{cat} \) no longer varies with the fibrin concentration.

Model II includes only the FP binary complex and has the rate equation \( V/A_o = k_{cat}(P)/K_{an}[1 + K_{an} + [P]). \) This equation predicts the expected behavior of \( k_{cat(app)} \) as a function of \([F]\), but the \( k_{cat(app)} = K_m K_{an}/K_m A + [F] \) term does not conform to the data or to the equation above.

Model III includes only the AP binary complex and has the rate equation \( V/A_o = k_{cat}(P)/K_{an}[1 + K_{an} + [F] + [P]). \) It has a problem very similar to that of Model II; it predicts the proper variation in \( k_{cat(app)} \) but not in \( k_{cat(app)} \) with the fibrin concentration.

Model IV, like the steady-state template model, includes both the AF and PF binary complexes and has the rate equation: \( V/A_o = k_{cat}(P)/K_{an}[1 + K_{an} + [F] + [P]). \) This equation is identical in form to the Equation 39 for the steady-state template model; thus, it fits the data very well. The physical meanings of the constants in the two
The values of the parameters were obtained by fitting the data by nonlinear regression analyses to Equation 39 under "Experimental Procedures." $k_{\text{cat}}$ is the turnover number of the ternary fibrin-Plg(S741C-fluorescein)/activator complex, $K_m$ is the Michaelis constant at saturating fibrin, $K_A$ is the dissociation constant for the binding of the activator to fibrin, and $K$ is a parameter which indicates the fibrin concentration at which the apparent $k_{\text{cat}}$ is equal to one-half the turnover number.

| Substrate | $k_{\text{cat}}$ | $k_{\text{cat}}$ | $K_m$ | $K$ | $k_{\text{cat}}/K_m$ |
|-----------|-----------------|-----------------|------|-----|---------------------|
| [Glu]$^1$[Plg(S741C-fluorescein)] | 0.058 ± 0.001 | 4.41 ± 0.03 | 0.30 ± 0.04 | 0.077 ± 0.012 | 1.42 ± 0.11 |
| act-PA | 0.075 ± 0.004 | 0.78 ± 0.05 | 0.59 ± 0.18 | 0.059 ± 0.004 | 1.00 ± 0.08 |
| del.K2 | 0.176 ± 0.019 | 1.23 ± 0.26 | 0.71 ± 0.22 | 0.131 ± 0.090 | 1.43 ± 0.34 |
| del.F | 0.041 ± 0.010 | 2.46 ± 0.92 | 2.88 ± 0.85 | 1.010 ± 0.970 | 0.17 ± 0.06 |
| [Lys]$^78$[Plg(S741C-fluorescein)] | 0.066 ± 0.002 | 0.023 ± 0.002 | 0.125 ± 0.026 | 0.027 ± 0.005 | 26.9 ± 2.6 |
| t-PA | 0.329 ± 0.020 | 0.159 ± 0.022 | 0.530 ± 0.090 | 0.114 ± 0.028 | 20.7 ± 3.1 |
| del.K2 | 0.107 ± 0.015 | 0.353 ± 0.088 | 1.610 ± 0.270 | 3.2 ± 0.9 |

$^a$ These values were obtained by fitting experimentally measured apparent $k_{\text{cat}}$ values to $k_{\text{cat(app)}} = k_{\text{cat}}[F]/[K + [F]]$, Equation 41.

consistent with observations.

Since the steady-state model, unlike the equilibrium models, both fit the data well and did not predict unrealistic properties of the binary interactions, it was chosen to represent and interpret the data.

**Parameters of Kinetics of Plasminogen Activation**—The steady-state model predicts the behavior of the reaction over the entire data set and provides a means of summarizing the results through the four parameters $k_{\text{cat}}$, $K_m$, $K_A$, and $K$. The ability of the model to reflect the data with reasonable accuracy is attested by the fact that the *solid lines* indicated in Figs. 1–3 all are regression lines obtained upon fitting the data globally to a single rate equation (Equation 39). The values of the parameters of kinetics obtained by nonlinear regression analysis over the entire range of fibrin and plasminogen concentrations for each type of activator and either [Glu]$^1$- or [Lys]$^78$[Plg(S741C-fluorescein)] (up to 200 points/analysis) are provided in Table 1.

With [Glu]$^1$[Plg(S741C-fluorescein)] as the substrate, the respective $k_{\text{cat}}$ values (s$^{-1}$) for t-PA, act-PA, del.K2, and del.F were 0.058, 0.078, 0.78, and 0.041. The corresponding $K_m$ values (μM) were 0.41, 0.78, 1.23, and 2.46. These values can be interpreted as those which are obtained at saturating levels of fibrin. With [Lys]$^78$[Plg(S741C-fluorescein)] as substrate, $k_{\text{cat}}$ values of t-PA, del.K2, and del.F were greater than those with [Glu]$^1$[Plg(S741C-fluorescein)] by factors of 1.5, 1.9, and 2.6, respectively. Corresponding $K_m$ values were smaller by factors of 13, 8, and 7. $K_A$ values (μM, dissociation constant for the activator-fibrin interaction) for t-PA, del.K2, and del.F, inferred from the kinetics of cleavage of [Glu]$^1$[Plg(S741C-fluorescein)] were 0.30, 0.71, and 2.88, respectively. The corresponding values obtained with [Lys]$^78$[Plg(S741C-fluorescein)] were 0.13, 0.53, and 1.61. These values for each form of the activator are relatively insensitive to the identity of the substrate, as expected, and are consistent with the values obtained previously in independent measurements of binding ($K_A$ t-PA = 0.36 μM; $K_A$ del.K2 = 1.1 μM; and $K_A$ del.F = 1.4 μM), as reported by Horrevoets et al. (17).

The values for the catalytic efficiency ($k_{\text{cat}}/K_m$, M$^{-1}$s$^{-1}$) of t-PA, del.K2, and del.F in [Glu]$^1$[Plg(S741C-fluorescein)] activation were: 1.42 × 10$^3$, 1.43 × 10$^3$, and 0.17 × 10$^3$. When [Lys]$^78$[Plg(S741C-fluorescein)] is the substrate the corresponding values for t-PA, del.K2, and del.F are: 26.9 × 10$^3$, 20.7 × 10$^3$, and 3.2 × 10$^3$. Thus, the catalytic efficiencies of t-PA and del.K2 are very similar to one another with either form of the substrate, whereas those of t-PA are larger than those of del.F by a factor of 8.4 with either form of the substrate. The $k_{\text{cat}}/K_m$ values of t-PA, del.K2, and del.F with [Lys]$^78$[Plg(S741C-fluorescein)] were, respectively, 19-, 12-, and 19-fold greater than those with [Glu]$^1$[Plg(S741C-fluorescein)].

The effect of fibrin on the catalytic efficiency of t-PA in catalysis of cleavage of the two forms of the substrate can be quantified by comparing $k_{\text{cat}}/K_m$ values obtained in the presence and absence of fibrin. In the absence of fibrin, the relationship between rate and the concentration of substrate is strictly linear and values of the slopes ($k_{\text{cat}}/K_m$, M$^{-1}$s$^{-1}$) are 3.44 × 10$^2$ and 1.43 × 10$^3$ for the Glu$^1$ and Lys$^78$ forms of the substrate, respectively (18). The corresponding values in fibrin for Glu$^1$ and Lys$^78$[Plg(S741C-fluorescein)] thus exceed those obtained in the absence of fibrin by factors of 413 and 1,881, respectively.

**DISCUSSION**

Previous efforts to elucidate the kinetics of fibrin-dependent activation of plasminogen by t-PA have been complicated by two features of the reaction. The first is that the reaction occurs within a clot, thereby complicating the measurement of the time course of the generation of plasmin. The other is that plasmin can modify the properties of fibrin, plasminogen, and plasmin-catalyzed feedback reactions, thereby simplifying the interpretation of data.

The results of these studies indicate that at any particular input concentration of fibrin, the kinetics of plasminogen activation conform to the Michaelis-Menten equation, $v = k_{\text{cat(app)}} [S]/(K_{m(app)} + [S])$, so long as the substrate (plasminogen) concentration is expressed as the free, rather than total, concentration. Although not shown here, conformity to the Michaelis-Menten equation also is achieved if the substrate concentration is taken as that bound. The $K_{m(app)}$ values vary with the input concentration of fibrin, such that $k_{\text{cat(app)}} = k_{\text{cat}}[F]/[K + [F]]$ and $K_{m(app)} = K_{m} + [F]o/([K + [F]]o)$. Thus, the rate equation that describes the kinetics of fibrin-dependent plasminogen activation is given by Equation 47.

$$r = k_{\text{cat}}[F]/[K + [F]]/([F]) + [Plg]$$

The four characteristic parameters of this equation are $k_{\text{cat}}$, $K$, $K_{m}$, and $K_A$. Except for $K_A$, which is the dissociation constant for the activator-fibrin interaction, these parameters are re-
lated in somewhat complex ways to the individual rate constants for assembly and turnover of the ternary activator-plasminogen-fibrin complex, as indicated by Equations 43–46 (see “Experimental Procedures”). In the absence of experimentally determined values for the individual rate constants, the values of \( k_{cat}, K_m, k_{cat(app)}, \) and \( K_m(app) \) unfortunately cannot be interpreted with mechanistic rigor. Nonetheless, they have the conventional meanings in that \( k_{cat(app)} \) at any particular fibrin concentration yields the maximum rate at saturating substrate and \( k_{cat} \) yields the rate at both saturating substrate and fibrin. \( K_m \) and \( K_m(app) \) are both numerically equal to the concentration of free substrate when the rate is one-half the maximum, and \( K_m(app) \) is the limiting value of \( K_m(app) \) at saturating fibrin. The parameter \( K \) provides the value of the fibrin concentration at which \( k_{cat(app)} \) is one-half of the \( k_{cat} \) value at saturating fibrin.

As the data of Table I indicate, \( k_{cat} \) values are fairly insensitive to the identity of either the activator or the substrate. Thus, because the affinities of the various forms of the activators and the two substrates for fibrin vary quite substantially, \( k_{cat} \) is not very sensitive to the affinities of the activators and substrates for fibrin. In contrast, \( K_m \) values increase and \( k_{cat}/K_m \) values decrease, with decreasing affinity of either the substrate or the activator for fibrin. Equations 43 and 45 of the model, described under “Experimental Procedures” provide a reasonable explanation for these behaviors. According to those equations, \( k_{cat} \) and \( K_m \) are defined as shown below in Equations 48 and 49.

\[
h_{cat} = \frac{k_3(K_fk_5 + K_hk_5)}{K_fk_5 + K_hk_5 + k_3(k_{-2} + k_{-3})k_1}
\]  
(Eq. 48)

\[
K_m = \frac{k_3(k_{-2} + k_{-3})}{K_fk_5 + K_hk_5 + k_3(k_{-2} + k_{-3})k_1}/K_fk_5 + K_hk_5 + k_3(k_{-2} + k_{-3})k_1/k_3
\]  
(Eq. 49)

Therefore,

\[
k_{cat}/K_m = k_3(K_fk_5 + K_hk_5)/(k_{-2} + k_{-3})K_fk_5 + K_hk_5 + k_3(k_{-2} + k_{-3})k_1/k_3
\]  
(Eq. 50)

The relative invariance of \( k_{cat} \) can be explained if the denominator of the equation for \( k_{cat} \) is dominated by the term \( K_fk_5 + K_hk_5 \). Since the denominator also can be written as \( K_fk_5 + k_3(k_{-2} + k_{-3})k_1 \), domination of the denominator by \( K_fk_5 + K_hk_5 \) implies that \( k_{-2} \approx k_{-3} \), i.e. the rate constant for dissociation of t-PA from fibrin greatly exceeds those for dissociation of t-PA from the ternary complex and substrate turnover. In this case \( k_{cat} \) is approximately equal to \( k_{cat} \), the turnover number of the ternary complex. \( k_{cat} \) therefore is not sensitive in this case to the affinity of either the activator or substrate for fibrin, as observed experimentally. Similarly, under these conditions the term for \( K_m \) would approach as \( K_m \approx (k_{-2} + k_{-3})K_fk_5/K_fk_5 + K_hk_5 + k_3(k_{-2} + k_{-3})k_1/k_3 \). This equation predicts an increase in \( K_m \) with an increase in \( K_f \), i.e. the dissociation for the plasminogen-fibrin interaction), which is consistent with observations (\( K_f \) for [Glu\(^1\)Plg(S741C-fluorescein)] = 30 \( \mu M \), \( K_f \) for [Lys\(^8\)Plg(S741C-fluorescein)] = 1.2 \( \mu M \); and \( K_m \) for [Glu\(^1\)Plg(S741C-fluorescein)] = 0.41 \( \mu M \), \( K_m \) for [Lys\(^8\)Plg(S741C-fluorescein)] = 0.0032 \( \mu M \)). It also predicts, however, a decrease in \( K_m \) with an increase in \( K_f \), which is not consistent with observations (i.e. the \( K_m \) do not decrease when the affinity of the activator for fibrin decreases). However, if \( k_3 \) and \( k_{cat} \) are approximately equal to one another \( (k_3 \approx k_{cat} = k) \), then \( k_{cat} \approx k_3 \), which is much larger than \( K_m(app) \), especially with [Glu\(^1\)Plg(S741C-fluorescein)], because \( K_m(app) \) exceeds \( K_m \) by a factor of 10–100 depending on the activator). Under these circumstances, \( K_m(app) \) approximates as \( K_m(app) \approx (k_{-2} + k_{-3} + k_{-5})/k_5 \). Thus, the magnitude of \( K_m(app) \) depends on the values of \( k_{-2} \) and \( k_{-5} \), which are the off-rate constants for dissociation of t-PA or plasminogen from the ternary plasminogen-fibrin-t-PA complex, rather than on values of the dissociation constants for binary interactions between fibrin and the substrate or the activator. Thus, the value of the \( K_m(app) \) is predicted to change linearly with changes in either \( k_{-2} \) or \( k_{-5} \), that is, with the stability of the ternary complex. That \( k_{-2} \) and \( k_{-5} \) are equal is reasonable, because these are the on-rate constants for the binding of similar proteins (t-PA and plasminogen) to similar structures (binary complexes between a fibrin protoomer and plasminogen or t-PA). Similarly, the catalytic efficiency is given by \( k_{cat}/K_m(app) \approx k_3/(1 + k_{-2} + k_{-5}/K_m(app)) \), which therefore changes reciprocally with changes in either \( k_{-2} \) or \( k_{-5} \). Since \( k_{-2} \) and \( k_{-5} \) are the off-rate constants for the dissociation of t-PA and plasminogen from the ternary complex, their magnitudes are directly related to the stability of complex. When these rate constants are small, such that the complex is stable, the \( K_m(app) \) value is small, the \( k_{cat}/K_m(app) \) value (catalytic efficiency) is high, and vice versa.

According to this interpretation, the \( K_m \) and catalytic efficiency \( (k_{cat}/K_m) \) values are not determined directly by the affinities of the binary interactions between the activator and substrate with fibrin, but rather by the stability of the ternary plasminogen-fibrin-activation complex. This uncoupling of fibrin binding affinity from catalytic properties is illustrated by comparing the results with t-PA or the variant del.K2 with those of del.F. They have very similar \( K_m \) values in fibrin binding (17) but substantially different \( k_{cat} \), \( K_m \), and \( k_{cat}/K_m \) values (Table I).

A representation of the steady-state model is presented in Fig. 5. The figure depicts a fibrin protoomer with a binding site \((a)\) for the activator \((A)\) and another \((p)\) for plasminogen \((P)\). The activator and substrate can add in either order to form the respective binary species. Further interactions produce the ternary complex, from which plasmin is generated. Also depicted is an interaction between the activator and plasminogen within the ternary complex. This interaction would contribute to the stability of the complex. The plasminogen-fibrin interaction is assumed to be essentially at equilibrium because of the high plasminogen and fibrin concentrations, relative to the activator concentration. The analysis presented in the preceding paragraph applies to conditions in which the rate constants \( k_{-2} \) and \( k_{-5} \) are much smaller than \( k_{-1} \), i.e. where the rate constant for dissociation of the activator from fibrin is large compared to those for dissociation of the activator from the ternary complex \((k_{-3})\) and substrate turnover \((k_{-4})\). Under these circumstances \( k_{cat} \) is approximately equal to \( k_{cat} \), and \( K_m(app) \) and \( k_{cat}/K_m(app) \) are determined by the values of \( k_{-2} \) and \( k_{-5} \), i.e. by the stability of the ternary complex. Thus, according to the model, catalytic efficiency, expressed through \( k_{cat}/K_m \), would be approximately equal to \( k_{cat}/K_m(app) \).
can be dissociated from the binding of activator \((k_1,k_2)\) and substrate \((k_3,k_{-3})\) to fibrin. Essentially, the potential exists that the catalytic efficiency of fibrin-potentiated plasminogen activation is determined by the stability of the three component complex rather than the affinities of the binding interactions of the substrate and activator with fibrin.

The above considerations are relevant to efforts to use recombinant technologies to produce modified forms of plasminogen activators with catalytic properties that are improved compared to those of the wild type form of the activator. An intuitively obvious change includes producing variants with enhanced fibrin binding, an effect that presumably would improve both fibrin specificity and enhanced catalysis. The possibility exists, however, that changes in the structure of t-PA could be introduced through protein engineering that greatly amplify fibrin binding but do not contribute to enhanced catalytic efficiency. Conversely, changes in structure could potentially be incorporated, which, although not appreciably altering the affinity of the activator for fibrin, could substantially increase or decrease the catalytic efficiency, depending on whether such changes enhance or diminish the stability of the ternary activator-fibrin-plasminogen complex. This distinction between fibrin binding and the stability of the ternary complex, and the elements of structure that contribute to them, may rationalize the frequently observed lack of correlation between the fibrin binding affinities of variants of t-PA and their potentials in plasminogen activation or thrombolysis \((23, 24, 25)\). Perhaps those structural modifications that will prove efficacious in optimizing the catalytic efficiency of variant forms of the activator will be those that maximize the stability of the ternary complex rather than the fibrin binding properties of the variants.

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