Identification of a Hydrophobic Exosite on Tissue Type Plasminogen Activator That Modulates Specificity for Plasminogen*  

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A wide variety of important biological processes, including both the formation and dissolution of blood clots, depend on specific cleavage of individual target proteins by serine proteases. For example, tissue type plasminogen activator (t-PA), a trypsin-like enzyme that catalyzes the rate-limiting step of the endogenous fibrinolytic cascade, has only one known substrate in vivo, a single peptide bond (Arg\textsuperscript{561}–Val\textsuperscript{562}) in the proenzyme plasminogen. We have previously suggested that the specificity of t-PA for plasminogen is mediated in part by direct protein-protein interactions between the protease domain of t-PA and plasminogen that are distinct from those occurring within t-PA’s active site. We demonstrate in this study that residues 420–423 of t-PA, which form a fully solvent-exposed, hydrophobic region of a surface loop mapping near one edge of the active site of t-PA, form, or are essential for the integrity of, an important, secondary site of interaction between t-PA and plasminogen that significantly modulates the rate of plasminogen activation in the absence, but not the presence, of fibrin. Identification of this secondary site of interaction between t-PA and plasminogen provides new insight into molecular details of the evolution of stringent substrate specificity by t-PA and suggests a novel strategy to enhance the fibrin dependence of plasminogen activation by t-PA. While the activity of wild type t-PA is stimulated by fibrin by a factor of approximately 650, the activity of two variants characterized in this study, t-PA/R275E,P422G and t-PA/R275E,P422E, is stimulated by a factor of approximately 39,000 or 61,000, respectively. It is therefore possible that, compared with wild type t-PA, the two variants would display enhanced “clot selectivity” in vivo due to reduced activity in the circulation but full activity at a site of fibrin deposition.

Tissue type plasminogen activator (t-PA)\textsuperscript{1} has been widely and successfully used as a therapeutic agent to treat acute myocardial infarction (1). t-PA does not, however, dissolve blood clots directly; instead, the enzyme catalyzes conversion of the zymogen plasminogen into the active protease plasmin, which efficiently degrades the fibrin mesh forming the core of a thrombus (2). Both of these critical fibrinolytic enzymes, t-PA and plasmin, are members of the chymotrypsin family of serine proteases (3, 4). Unlike plasmin, however, t-PA is a remarkably specific enzyme. Because such highly restricted substrate specificity is in striking contrast to the broad specificity of well studied serine proteases such as trypsin, chymotrypsin, and elastase (5), the molecular basis of the selectivity of t-PA for plasminogen is of considerable interest. A detailed understanding of the mechanisms employed by t-PA to ensure selectivity would provide new insight into the evolution of the endogenous fibrinolytic cascade and might suggest effective new strategies for the rational design of novel, highly selective proteases with unique specificities as well as novel plasminogen activators.

The specificity of t-PA for plasminogen is enhanced by the co-factor fibrin. Fibrin, t-PA, and plasminogen form a specific, ternary complex, which serves to reduce the $K_m$ of t-PA for plasminogen by a factor of greater than 400 (6–8). Even in the absence of fibrin, however, t-PA maintains stringent specificity for plasminogen, and this specificity is an inherent property of the protease domain of t-PA (8).

Although it is the only known efficient substrate for t-PA in vivo, we have presented evidence that the primary sequence surrounding the cleavage site in plasminogen is actually a poor match to optimal subsite occupancy for t-PA (9). When placed into synthetic linear or cyclic peptides or into a trypsin-accessible site in an unrelated protein, this target sequence is cleaved extremely inefficiently by t-PA (10). By contrast, target sequences that do represent optimal subsite occupancy for t-PA, which were identified by screening a large peptide library containing random hexapeptide sequences, can be efficiently cleaved by t-PA in all three of these structural contexts (10). Catalysis of plasminogen by t-PA, therefore, appears to be accelerated not only by the co-factor fibrin but also by productive, protein-protein interactions between t-PA and plasminogen that occur at a site or sites distinct from the enzyme’s active site.

We have previously observed that disruption of a surface loop that maps to one edge of the active site of t-PA and is homologous to the “autolysis loop” of trypsin dramatically reduces the catalytic efficiency of t-PA for plasminogen.\textsuperscript{2} Examination of this region of the protease domain of t-PA in the crystal structure revealed the presence of three hydrophobic residues, Leu\textsuperscript{420}, Pro\textsuperscript{422}, and Phe\textsuperscript{423}, that were fully exposed to solvent, and therefore candidates for interaction with substrates, inhibitors, and/or co-factors (Fig. 1) (11). Consequently, we hypothesized that this region of the “autolysis loop” of t-PA, residues 420–423, may form an important secondary site of interaction between t-PA and plasminogen. To test this hypothesis, we used site-specific mutagenesis to construct a series of nine single-chain and nine two-chain variants of t-PA containing point mutations at positions 420–423.

\textsuperscript{1} The abbreviations used are: t-PA, tissue type plasminogen activator; PAI-1, plasminogen activator inhibitor, type 1.

\textsuperscript{2} E. L. Madison, unpublished observations.

\textsuperscript{3} Positions 275 and 420–423 of t-PA correspond to positions 15 and 147–150, respectively, in the standard chymotrypsin numbering system.

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Point mutations mapping to residues 420–423 of t-PA significantly decreased the catalytic efficiency of t-PA for plasminogen activation in the absence of fibrin. The activity of all nine single-chain variants was reduced in this assay by factors of approximately 2–7 compared with wild type t-PA. These data suggest that residues 420–423, particularly Leu\textsuperscript{420}, Pro\textsuperscript{422}, and Phe\textsuperscript{423}, form, or are critical for the integrity of, an important, secondary site of interaction between t-PA and plasminogen in the absence of fibrin.

**MATERIALS AND METHODS**

Site-directed Mutagenesis and Construction of Full-length cDNAs Encoding Variants of t-PA—Oligonucleotide-directed site-specific mutagenesis was performed by the method of Zoller and Smith (12) as modified by Kunkel (13). Mutations were introduced into the 290-base pair cDNA encoding t-PA that had been previously subcloned into bacteriophage M13mp18. The nine point mutations introduced into t-PA and the corresponding mutagenic oligonucleotides were as follows: L420A, 5′-AAGCATGAGGCCGATCTCCTTGTCTC-3′; S421G, 5′-CATGAGGCCTTTGGGACCTTTCTATT-3′; S421E, 5′-CATGAGGGCTTGGACCTTTCTATTGGG-3′; P422A, 5′-GAGGCCGTTGTCTGCATTCTATTCGGAG-3′; P422E, 5′-GAGGCCGTTGTCTGCATTCTATTCGGAGCGG-3′; P422G, 5′-GAGGCCGTTGTCTGCATTCTATTCGGAGCGG-3′; P422E, 5′-GAGGCCGTTGTCTGCATTCTATTCGGAGCGG-3′; P422A, 5′-GAGCCCTGTTGTCTGATTCGGAGCGG-3′; F423A, 5′-GAGCCCTGTTGTCTGATTCGGAGCGG-3′; F423E, 5′-GAGCCCTGTTGTCTGATTCGGAGCGG-3′.

Following mutagenesis, ssDNA corresponding to the entire 290-base fragment was fully sequenced to assure the presence of the desired mutation and the absence of any additional mutations. Replicative form DNA was prepared for appropriate phage, and the desired mutation and the absence of any additional mutations.

**Expression of Wild Type and Mutated Enzymes by Transient Transfection of COS Cells—**cDNAs encoding all 18 variants described above were ligated into the transient expression vector pSVT7 (14) and then introduced into COS 1 cells by electroporation using a Bio-Rad Gene Pulser. 20 μg of cDNA, 100 μg of carrier DNA, and approximately 10^7 COS cells were placed into a 0.4-cm cuvette, and electroporation was performed at 320 V, 960 microfarads, and Ω = ∞. Following electroporation, cells were incubated overnight at 37 °C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 5 mM sodium butyrate. Cells were then washed with serum-free medium and incubated in Dulbecco's modified Eagle's medium for 48 h at 37 °C. After the incubation with serum-free media, conditioned media were collected, and enzyme concentrations were determined by enzyme-linked immunosorbent assay.

**Kinetic Analysis of t-PA Activity Using Small Synthetic Substrates—**The direct chromogenic assay utilized the substrate methylsulfonyl-d-cyclohexyl-tirosyl-glucyl-arginine-p-nitroaniline (Spectrozyme t-PA; American Diagnostica) and was performed as described previously (15–18). Assays were performed both in the presence and absence of the co-factor DESAFIB (American Diagnostica). The concentration of Lys-plasminogen (American Diagnostica) and H-D-norleucyl-cyclohexyl-tyrosyl-glycyl-arginine-p-nitroaniline (Spectrozyme t-PA; American Diagnostica) were performed as described previously (18–20). Assays were performed both in the presence and absence of the co-factor DESAFIB (American Diagnostica). The concentration of Lys-
plasminogen was varied from 0.0125–0.2 μM in the presence of DESA-FIB and from 0.9 to 15 μM in the absence of the co-factor.

**Inhibition of Wild Type and Mutated Variants of t-PA by PAI-1—** Approximately 0.25–1 ng of wild type or mutated variant of t-PA was preincubated at 25 °C for 15 min with varying concentrations of highly purified active PAI-1. Following this preincubation, each mixture was diluted, and the residual enzymatic activity was measured using a standard indirect chromogenic assay as described previously (14–16).

**Kinetics of the Inhibition of t-PA by PAI-1—** Second order rate constants for inhibition of t-PA by PAI-1 were measured under pseudo-first order conditions as described previously (15, 16, 19–22). Briefly, enzyme and inhibitor were preincubated at 25 °C for periods of time varying from 0 to 30 min. Following preincubation, the mixtures were diluted, and residual enzymatic activity was measured in the indirect chromogenic assay and compared with control reactions in which PAI-1 was either omitted from the reaction or added after preincubation, dilution, and the addition of fibrin, plasminogen, and Spectrozyme PL to the reaction mixture. For each enzyme, the concentrations of enzyme and inhibitor were chosen to yield several data points for which the residual enzymatic activity varied between 20 and 80% of the initial activity, and the molar excess of PAI-1 over t-PA was always more than 20-fold. Data were analyzed by plotting ln(residual activity/initial activity) versus time of preincubation and calculating the resulting slope. Division of this slope by [PAI-1] produced the second order rate constant (mM⋅s⁻¹⋅s⁻¹).

**RESULTS**

**Design, Construction, and Production of t-PA Mutants—** To investigate the functional significance of residues 420–423 in the “autolytic loop” of t-PA, we used oligonucleotide-directed site-specific mutagenesis to introduce point mutations at each of these four positions. A total of nine variants were constructed; Leu₄₂⁰ was replaced by alanine or glutamic acid, Ser₄₂¹ was replaced by glycine or glutamic acid, Pro₄₂² was replaced by glycine, alanine, or glutamic acid, and Phe₄₂³ was replaced by alanine or glutamic acid. Because it has been demonstrated that activation cleavage of both trypsinogen and chymotrypsinogen resulted in a striking rearrangement of the autolysis loop of these enzymes, it is quite possible that the interactions formed by residues 420–423 will differ for single- and two-chain t-PA (23–26). It was therefore essential to assay both the single- and two-chain form of each variant of t-PA containing a mutation in the autolytic loop. Accurate measurement of the enzymatic activity toward plasminogen of the single-chain form of these variants proved difficult, however, because plasmin produced during the assay rapidly and efficiently converted the enzymes into their mature, two-chain form by cleaving the Arg²⁷⁵–Ile²⁷⁶ bond of the single-chain t-PA. Consequently, to overcome this technical difficulty, we also constructed noncleavable forms of the nine mutated enzymes by introducing the additional mutation R²⁷⁵E into the new variants, a strategy that was first described by Tate and co-workers (27).

Wild type t-PA, t-PA/R²⁷⁵E, and all 18 variants containing mutations in the autolysis loop were cloned into the expression vector pSTV7 and produced by transient expression in COS cells. Since this procedure yielded predominantly single-chain enzymes, two-chain t-PAs were generated by treating the enzyme preparations with plasmin-Sepharose. Quantitative conversion of the enzymes into their two-chain forms was confirmed by SDS-PAGE and Western blotting. As previously demonstrated (27), variants containing the mutation R²⁷⁵E were not cleaved by plasmin-Sepharose (data not shown).

**Kinetic Analysis of Catalytic Activity toward a Small, Synthetic Substrate—** None of the nine point mutations in the autolysis loop of t-PA significantly affected the catalytic activity of the mature, two-chain form of the enzyme toward a small, synthetic substrate (Table I). Six of the two-chain variants possessed at least 92% of the activity of two-chain t-PA in this assay, and all nine of these variants displayed at least 75% of the activity of the corresponding wild type enzyme.

The nine point mutants mapping to residues 420–423 had slightly larger effects on the activity of the single-chain form of t-PA toward the synthetic substrate than the wild type enzyme. Although six of the single-chain variants maintained at least 82% of the activity of single-chain t-PA, one of the variants, t-PA/S₄₂¹G, displayed only 15% of the activity of the wild type enzyme in this assay. The catalytic activity of the remaining two variants, t-PA/R²⁷⁵E,P₄₂²G and t-PA/R²⁷⁵E,S₄₂¹E, was 25 or 33%, respectively, that of single-chain t-PA.

Fibrin did not stimulate the activity of t-PA, t-PA/R²⁷⁵E, or any of the 18 new variants toward the synthetic substrate Spectrozyme t-PA (data not shown). Interaction of these enzymes with fibrin, therefore, does not appear to significantly influence the conformation of the active site.

**Kinetic Analysis of Catalytic Activity toward the Physiological Substrate Plasminogen—** In the presence of the co-factor fibrin, point mutations in residues 420–423 have very small effects on plasminogen activation by either single- or two-chain t-PA (Table II). Six of the nine two-chain variants possessed at least 86% of the activity of two-chain, wild type t-PA, and the catalytic efficiency of the least active two-chain variant, t-PA/P₄₂²E, was reduced by a factor of less than 2 compared with the wild type enzyme. Similarly, five of the nine single-chain variants had at least 70% of the activity of single-chain t-PA in this assay, and the catalytic efficiency of the least active single-chain variant was reduced by a factor of approximately 2 compared with the single-chain wild type enzyme (Table II). As observed with the two-chain variants, the least active single-chain variant contained the P₄₂²E mutation.

The catalytic efficiency for plasminogen activation in the absence of fibrin of the two-chain form of variants containing point mutations in residues 420–423 varied from 15 to 100% that of two-chain, wild type t-PA (Table III). Eight of the nine variants possessed higher affinity for plasminogen than wild type t-PA. This property was particularly evident for t-PA/L₄₂⁰E, t-PA/P₄₂²E, and t-PA/P₄₂²G, whose Kᵢ for plasminogen was reduced by factors of 7–8 compared with that of the wild type enzyme. k₉ values for the two-chain variants, however, were all reduced, by factors varying from 2.4 to 45, com-

| t-PA variant | Kᵢ (nM) | k₉ (s⁻¹) | k₉/Kᵢ | Relative k₉/Kᵢ |
|--------------|---------|---------|-------|---------------|
| Wild type t-PA | 0.4 | 47 | 1.2 × 10⁴ | 1.00 |
| t-PA/L₄₂⁰E | 0.4 | 45 | 1.1 × 10⁵ | 0.92 |
| t-PA/S₄₂¹G | 0.4 | 36 | 9.0 × 10⁴ | 0.75 |
| t-PA/S₄₂¹E | 0.4 | 46 | 1.2 × 10⁵ | 1.00 |
| t-PA/P₄₂²A | 0.4 | 47 | 1.2 × 10⁵ | 1.00 |
| t-PA/P₄₂²G | 0.6 | 60 | 1.0 × 10⁵ | 0.83 |
| t-PA/P₄₂²E | 0.4 | 49 | 1.2 × 10⁵ | 1.00 |
| t-PA/F₄₂³A | 0.5 | 49 | 9.8 × 10⁴ | 0.82 |
| t-PA/F₄₂³E | 0.5 | 57 | 1.1 × 10⁵ | 0.92 |

Table I: Kinetic constants for the cleavage of the chromogenic substrate Spectrozyme t-PA by single- and two-chain t-PA variants

Relative k₉/Kᵢ is calculated by comparison to t-PA for the two-chain variants and to t-PA/R²⁷⁵E for the single-chain variants.
t-PA/R275E, for the single-chain variants.

Table II

| t-PA variant       | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ | Relative $k_{cat}/K_m$ |
|--------------------|------------|----------------------|---------------|------------------------|
| Two-chain form     |            |                      |               |                        |
| Wild-type t-PA     | 0.011      | 0.10                 | 9.1 × 10$^{-6}$ | 1.00                   |
| t-PA/L420E         | 0.019      | 0.17                 | 8.9 × 10$^{-6}$ | 0.98                   |
| t-PA/S421G         | 0.021      | 0.17                 | 8.1 × 10$^{-6}$ | 0.89                   |
| t-PA/S421E         | 0.016      | 0.11                 | 6.9 × 10$^{-6}$ | 0.76                   |
| t-PA/P422A         | 0.013      | 0.11                 | 8.5 × 10$^{-6}$ | 0.91                   |
| t-PA/P422G         | 0.026      | 0.15                 | 5.8 × 10$^{-6}$ | 0.64                   |
| t-PA/P422E         | 0.029      | 0.15                 | 5.2 × 10$^{-6}$ | 0.57                   |
| t-PA/F423A         | 0.014      | 0.12                 | 8.6 × 10$^{-6}$ | 0.95                   |
| t-PA/F423E         | 0.023      | 0.18                 | 7.8 × 10$^{-6}$ | 0.86                   |
| Single-chain form  |            |                      |               |                        |
| t-PA/R275E         | 0.010      | 0.13                 | 1.3 × 10$^{-6}$ | 1.00                   |
| t-PA/L420A,R275E   | 0.014      | 0.18                 | 1.3 × 10$^{-6}$ | 1.00                   |
| t-PA/L420E,R275E   | 0.018      | 0.15                 | 8.3 × 10$^{-6}$ | 0.64                   |
| t-PA/S421G,R275E   | 0.026      | 0.19                 | 7.3 × 10$^{-6}$ | 0.58                   |
| t-PA/S421E,R275E   | 0.023      | 0.21                 | 9.1 × 10$^{-6}$ | 0.70                   |
| t-PA/P422A,R275E   | 0.010      | 0.15                 | 1.5 × 10$^{-6}$ | 1.15                   |
| t-PA/P422G,R275E   | 0.027      | 0.21                 | 7.8 × 10$^{-6}$ | 0.60                   |
| t-PA/P422E,R275E   | 0.028      | 0.17                 | 6.1 × 10$^{-6}$ | 0.47                   |
| t-PA/F423A,R275E   | 0.022      | 0.20                 | 9.1 × 10$^{-6}$ | 0.70                   |
| t-PA/F423E,R275E   | 0.015      | 0.15                 | 1.0 × 10$^{-6}$ | 0.77                   |

Table III

| t-PA variant       | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ | Relative $k_{cat}/K_m$ |
|--------------------|------------|----------------------|---------------|------------------------|
| Two-chain form     |            |                      |               |                        |
| Wild-type t-PA     | 0.068      | 1.4 × 10$^4$         | 1.00          |                        |
| t-PA/L420A         | 0.014      | 1.4 × 10$^4$         | 1.00          |                        |
| t-PA/L420E         | 0.005      | 7.1 × 10$^3$         | 0.51          |                        |
| t-PA/S421G         | 0.018      | 4.5 × 10$^3$         | 0.32          |                        |
| t-PA/S421E         | 0.016      | 5.3 × 10$^3$         | 0.38          |                        |
| t-PA/P422A         | 0.028      | 7.0 × 10$^3$         | 0.50          |                        |
| t-PA/P422G         | 0.0028     | 4.7 × 10$^3$         | 0.34          |                        |
| t-PA/P422E         | 0.0015     | 2.1 × 10$^3$         | 0.15          |                        |
| t-PA/F423A         | 0.020      | 5.0 × 10$^3$         | 0.36          |                        |
| t-PA/F423E         | 0.017      | 3.4 × 10$^3$         | 0.24          |                        |
| Single-chain form  |            |                      |               |                        |
| t-PA/R275E         | 0.024      | 1.4 × 10$^4$         | 1.00          |                        |
| t-PA/L420A,R275E   | 0.008      | 8.9 × 10$^3$         | 0.63          |                        |
| t-PA/L420E,R275E   | 0.002      | 4.0 × 10$^3$         | 0.29          |                        |
| t-PA/S421G,R275E   | 0.001      | 2.5 × 10$^3$         | 0.18          |                        |
| t-PA/S421E,R275E   | 0.002      | 2.9 × 10$^3$         | 0.21          |                        |
| t-PA/P422A,R275E   | 0.007      | 5.4 × 10$^3$         | 0.39          |                        |
| t-PA/P422G,R275E   | 0.0008     | 2.0 × 10$^3$         | 0.14          |                        |
| t-PA/P422E,R275E   | 0.0005     | 1.0 × 10$^3$         | 0.07          |                        |
| t-PA/F423A,R275E   | 0.004      | 4.0 × 10$^3$         | 0.29          |                        |
| t-PA/F423E,R275E   | 0.003      | 3.8 × 10$^3$         | 0.27          |                        |
chain variants in this assay was indistinguishable from that of two-chain t-PA (data not shown). We conclude, therefore, that the solvent-exposed, hydrophobic region of the autolysis loop of two-chain t-PA does not form significant interactions with PAI-1.

Similar results were obtained when the nine single-chain variants were subjected to this assay. None of the single-chain variants behaved significantly differently from single-chain wild type t-PA (data not shown); however, these data did suggest that two of the single-chain variants, t-PA/R275E, S421G and t-PA/R275E,P422G, might be inhibited by PAI-1 slightly less rapidly than single-chain t-PA. To examine this possibility, we measured the rate of inhibition by PAI-1 for t-PA/R275E, t-PA/R275E,S421G, and t-PA/R275E,P422G, and these data indicated that the second order rate constants for inhibition of the single-chain form of t-PA/R275E,S421G and t-PA/R275E,P422G were, respectively, 3.4 $\times 10^5$ and 5.0 $\times 10^5$ M$^{-1}$ s$^{-1}$, or approximately 2–3-fold lower than that observed with single-chain t-PA (Table IV).

### DISCUSSION

The chymotrypsin family has evolved to contain many members functioning in the same milieu (e.g. human plasma) that are exclusive in their reactivity toward both substrates and inhibitors. While the role of the “specificity pocket” of a serine protease in determining the “primary” or P1 specificity of the enzyme has long been appreciated (43–45), additional determinants of protease specificity remain largely obscure. Although both the location and mechanism of action of these additional determinants of enzyme specificity remain unclear, their existence and critical importance are well established. For example, t-PA and almost all other proteases that participate in the fibrinolytic cascade, the coagulation cascade, and complement activation possess virtually identical, trypsin-like primary specificity pockets and exhibit identical P1 specificity (i.e. they cleave COOH-terminal to arginine and lysine residues). Nevertheless, most of these enzymes display very different substrate and inhibitor specificity.

Previous studies have strongly suggested that the stringent specificity of t-PA for plasminogen is mediated in part by productive protein-protein interactions that are distinct from those occurring in the enzyme’s active site (8–10). In the presence of fibrin, plasminogen activation by t-PA is an extremely complex reaction that appears to occur after formation of a ternary complex involving multiple sites of contact among enzyme, substrate, and co-factor (6). While sites within all five structural domains of t-PA have been implicated in interaction of the enzyme with fibrin (29, 30, 46), the importance, and even the existence, of direct, secondary contacts between t-PA and plasminogen in the ternary complex remain obscure.

In the absence of fibrin, plasminogen activation by t-PA is a significantly less complex but also less efficient reaction. Nevertheless, even in the absence of co-factor, t-PA retains stringent specificity for plasminogen (8). Since the target sequence present in plasminogen appears to be a very poor match to optimal subsite occupancy for t-PA (9), the specificity of t-PA for plasminogen under these conditions seemed very likely to be mediated by a direct, secondary interaction or interactions between t-PA and plasminogen. The goal of this investigation was to identify regions of t-PA that participated in these interactions, and our major finding is that residues 420–423 of t-PA, a solvent-exposed, hydrophobic region of the “autolysis loop” of t-PA, forms, or is critical for the integrity of, an important, secondary site of interaction between t-PA and plasminogen.

During their evolution from a trypsin-like progenitor, serine proteases have acquired amino acid substitutions, deletions, and insertions most often at the protein surface, and these mutations frequently map to surface loops located near the enzyme’s active site (47). We have previously suggested that the recruitment of these substituted or inserted residues, located in surface loops near the active site, to participate in specific enzyme-substrate and enzyme-inhibitor interactions is an important and recurring theme in the evolution of specificity by chymotrypsin family enzymes (14–16, 18, 29). Our studies of the determinants of specificity for t-PA have proved consistent with this hypothesis. We have previously reported that residues located on one surface loop near the enzyme’s active site modulate the interaction of t-PA with its primary endogenous inhibitor PAI-1 (14, 16), and this study demonstrated that residues mapping to another surface loop near the active site can modulate the interaction of the enzyme with substrate.

The elucidation of a secondary site of interaction between t-PA and plasminogen that exerts a substantial effect in the absence, but not the presence, of fibrin provides new insight into molecular details of the evolution of stringent substrate specificity by t-PA and also suggests a novel strategy to enhance the co-factor dependence of t-PA. In fact, several of the variants described in this initial study already possess this property. While plasminogen activation by wild type t-PA is stimulated by a factor of approximately 650, the corresponding fibrin stimulation factors for t-PA/R275E,S421G; t-PA/R275E,S421E; t-PA/R275E,P422E; and t-PA/R275E,P422G, for example, are 29,200, 31,400, 39,000, and 61,000, respectively (Table V). It is therefore possible that, compared with wild type t-PA, these variants would display enhanced “clot selectivity” in vivo due to reduced activity in the circulation but full activity at a site of fibrin deposition. Whether enhanced clot selectivity would improve the enzyme as a thrombolytic agent remains extremely controversial; however, this property may assume increased importance as variants of t-PA with a prolonged circulating half-life are administered as a single bolus.

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