Variants of Tissue-type Plasminogen Activator That Display Extraordinary Resistance to Inhibition by the Serpin Plasminogen Activator Inhibitor Type 1*

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Fibrinolysis is regulated in part by the interaction of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1). Previous investigations suggest that three specific arginine residues, Arg-298, Arg-299, and Arg-304 of t-PA, play a critical role in this important regulatory interaction. Our earlier studies have demonstrated that conversion of any of these three residues to a glutamic acid residue reduced the rate of inhibition of t-PA by PAI-1 by factors varying from 58–64. In addition, we have reported that the second order rate constant for inhibition by PAI-1 of the variant t-PA/K296E,R298E,R299E is reduced by a factor of approximately 2800 compared with that of wild type t-PA. In this study, we have significantly extended our earlier observations by identifying t-PA variants that are substantially more resistant to inhibition by PAI-1 than any previously reported variants of t-PA or urokinase-type plasminogen activator. Single-chain t-PA/ R275E,R298E,R299E,R304E, for example, is inhibited by PAI-1 approximately 120,000 times less rapidly than single-chain, wild type t-PA. We also report the first direct comparison of the effects of charge reversal mutations of Arg-298, Arg-299, and/or Arg-304 on the properties of the single- and two-chain forms of t-PA. While these mutations confer extraordinary resistance to inhibition by PAI-1 to both forms of the enzyme, our observations reveal that the single-chain enzyme is affected to a greater extent than the two-chain enzyme. Two-chain, wild type t-PA is inhibited by PAI-1 approximately 1.4 times more rapidly than single-chain t-PA. The corresponding ratio increases to 7.6 or 6.7, respectively, for variants of t-PA containing the R298E,R299E or R298E,R299E,R304E mutations.

Tissue-type plasminogen activator (t-PA),1 a 68-kDa member of the (chymo)trypsin family of serine proteases, catalyzes the rate-limiting step in the endogenous fibrinolytic cascade, activation of the circulating zymogen plasminogen into the active enzyme plasmin (1–3). This fibrinolytic activity of t-PA led investigators to examine use of the enzyme as a potential therapeutic agent for the treatment of thrombotic disorders (4–7), and administration of wild type human t-PA has now become a standard therapy for the treatment of acute myocardial infarction (3, 8–10). Use of the wild type enzyme as a therapeutic agent, however, is constrained by two mechanisms that normally serve to regulate the activity of endogenous t-PA (3, 11, 12). First, the activity of t-PA is rapidly inhibited by the serpin plasminogen activator inhibitor type 1 (PAI-1), and, second, the enzyme is rapidly cleared from the liver by at least two hepatic receptor systems (13, 14). The impact of these constraints is reduced in current thrombolytic regimens by using very high doses of the wild type enzyme (approximately 100 mg) and by administering the enzyme as a bolus followed by an infusion that lasts between 1.5 and 3 h (3, 8–10). While many clinical studies have demonstrated that these protocols are both safe and efficacious, there is substantial room for improvement in fibrinolytic therapy. Even when the most effective, current regimen is utilized, approximately 20% of patients fail to achieve patency of the infarct-related artery, and normal blood flow is established in this vessel for less than half of the patients (9, 10).

One approach to enhancing thrombolytic therapy is to develop improved variants of t-PA. Consequently, tremendous efforts aimed at altering the properties of human t-PA have been expended by a large number of investigators during the last 10 years (11–14). Recent attempts to improve t-PA as a thrombolytic agent have focused primarily on overcoming the two natural constraints mentioned above, rapid clearance of the wild type enzyme from the circulation by hepatic receptors and rapid inhibition of the enzyme by PAI-1, a serpin that is present at high levels in platelet rich arterial thrombi. We and others have reported the design and characterization of variants of t-PA that exhibit resistance to both of these natural constraints (11–14). Mutations mapping to the finger, growth factor, or kringle 1 domain can significantly prolong the circulating half-life of the enzyme while mutations mapping to residues 296–3042 of t-PA can endow the enzyme with resistance to PAI-1 (13, 14). In addition, Keyt et al. (15) have demonstrated that mutations of these two types can be combined into one variant to create a single enzyme that exhibits both new properties. These investigators and their colleagues have also demonstrated that one such variant, TNK-t-PA, possesses significantly enhanced potency in several animal models of thrombolytic therapy and, unlike the wild type enzyme, can be efficaciously administered as a single bolus (15–19). Consequently, TNK-t-PA is currently being tested in clinical trials as a therapeutic agent for treatment of acute myocardial infarction (20).

We have previously demonstrated that individual, charge reversal mutations of Arg-298, Arg-299, or Arg-304 of t-PA reduced the second order rate constant for inhibition of the

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‡ The abbreviations used are: t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1.

1 Positions 275, 296, 298, 299, and 304 of the t-PA numbering system correspond to positions 15, 36, 37A, 37B, and 39, respectively, in the chymotrypsin numbering system.
enzyme by PAI-1 by factors of 58–64 (21, 22). We have also reported that the second order rate constant for inhibition by PAI-1 of a variant containing three charge reversal mutations, t-PA/R298E,R299E,R304E, was reduced, compared with wild type t-PA, by a factor of approximately 2800 (22). The current study significantly extends earlier investigations by identifying t-PA variants that are substantially more resistant to inhibition by PAI-1 than any previously reported variant of t-PA or u-PA. Single-chain t-PA/R275,E298E,R299E,R304E, for example, is inhibited by PAI-1 approximately 120,000 times less rapidly than single-chain, wild type t-PA. In addition, unlike earlier reports, this study directly compares the effects of charge reversal mutations of Arg-298, Arg-299, and/or Arg-304 on the single- and two-chain forms of t-PA. While these mutations confer extraordinary resistance to inhibition by PAI-1 to both forms of the enzyme, our observations reveal that the single-chain enzyme is affected to a greater extent than the two-chain enzyme.

MATERIALS AND METHODS

Site-directed Mutagenesis and Construction of Expression Vectors

Encoding Variants of t-PA—Oligonucleotide-directed site-specific mutagenesis was performed by the method of Zoller and Smith (23) as modified by Kunkel (24). The R298E,R299E and R298E,R299E,R304E mutations were introduced into the 472-base pair EcoRI fragment of cDNAs encoding t-PA or t-PA/R275E that had been previously subcloned into bacteriophage M13mp18. The mutagenic primers had the following nucleotide sequences: R298E,R299E, 5'-TCTTTGCGAACACAGGAGAGGACC-3'; R298E,R299E,R304E, 5'-TTTTGGACAAGACGAGGAGGACTCCTGTGCGCGGGG-3'.

Following mutagenesis, single-stranded DNA from each new variant corresponding to the entire 472-base pair EcoRI fragment was fully sequenced to assure the presence of the desired mutations and the absence of any additional mutations. Replicitive form DNA was prepared for appropriate phage, and the mutated 472-base pair EcoRI fragment was recovered after digestion of replicative form DNA with EcoRI and electrophoresis of the digestion products on an agarose gel. The isolated, mutated EcoRI fragments were used to replace the corresponding fragment in full-length cDNAs encoding wild type t-PA to yield four new full-length cDNAs, encoding t-PA/R298E,R299E, t-PA/R298E,R299E,R304E, t-PA/R275E,R298E,R299E, and t-PA/R275E,R298E,R299E,R304E. Expression of Enzymes by Transient Transfection of COS Cells—cDNAs encoding t-PA, t-PA/R275E, t-PA/R275E,R298E,R299E, t-PA/R275E,R298E,R299E,R304E, and t-PA/R275E,R298E,R299E,R304E were ligated into the transient expression vector EcoRI fragment of plasmid pEBV-521. Following mutagenesis, single-stranded DNA from each new variant corresponding to the entire 472-base pair EcoRI fragment was fully sequenced to assure the presence of the desired mutations and the absence of any additional mutations. Replicitive form DNA was prepared for appropriate phage, and the mutated 472-base pair EcoRI fragment was recovered after digestion of replicative form DNA with EcoRI and electrophoresis of the digestion products on an agarose gel. The isolated, mutated EcoRI fragments were used to replace the corresponding fragment in full-length cDNAs encoding wild type t-PA to yield four new full-length cDNAs, encoding t-PA/R298E,R299E, t-PA/R298E,R299E,R304E, t-PA/R275E,R298E,R299E, and t-PA/R275E,R298E,R299E,R304E. The catalytic activity of single- and two-chain forms of wild type t-PA and the corresponding wild type enzyme.

Kinetic Analysis of t-PA Activity Using a Small, Synthetic Substrate—The direct chromogenic assay utilized the substrate methylsulfonyl-o-cyclohexylthieryl-gly-cygly-arg-p-nitroaniline (Spectrozyme t-PA, American Diagnostica) and was performed as described previously (25–27). Kinetic Analysis of Plasminogen Activation Using Indirect Chromogenic Assays—Indirect chromogenic assays of t-PA utilized the substrates lys-plasminogen (American Diagnostica) and Spectrozyme PL (American Diagnostica) and were performed as described previously (25–27).

Measurement of Second Order Rate Constants for Inhibition by PAI-1—Second order rate constants for the inhibition of wild type and mutated t-PAs were measured under pseudo-first order conditions as described previously (22, 27, 30, 31). Briefly, enzyme and inhibitor were preincubated at 23 °C for periods of time varying from 0 to 30 min. Following preincubation, the mixtures were diluted, and the residual enzymatic activity was measured in a standard indirect chromogenic assay. For each enzyme, the concentrations of enzyme and inhibitor and the times of preincubation were chosen to yield several data points for which the residual enzymatic activity varied between 20 and 80% of the initial activity. Data were analyzed by plotting in (residual activity/ initial activity) versus time of preincubation and measuring the resulting slopes. Division of this slope by [I], with [I] representing the concentration of PAI-1 during the preincubation, yielded the second order rate constants shown.

RESULTS

We used oligonucleotide-directed site-specific mutagenesis to construct cDNAs encoding t-PA/R298E,R299E and t-PA/R298E,R299E,R304E. Each variant contained multiple charge reversal mutations of Arg-298, Arg-299, and/or Arg-304 had little effect on the single- and two-chain forms of these mutated enzymes proved difficult, however, because plasmin formed during the assay rapidly and efficiently converted the enzymes into their mature, two-chain form by cleaving the Arg-275 ↓ Ile-276 bond of the single-chain t-PAs. Consequently, to overcome this technical obstacle, we also constructed non-cleavable forms of the two enzymes by introducing the mutation R275E into the existing variants.

Wild type t-PA, t-PA/R275E, and all four variants containing multiple charge reversal mutations were expressed by transient expression in COS cells. Because this procedure yielded predominantly single-chain enzyme, two-chain t-PAs were generated by treatment the enzyme preparations with plasmin-Sepharose (27). Quantitative conversion of the enzymes into their mature two-chain form was verified by SDS-PAGE. As previously demonstrated, variants containing the R275E mutation were synthesized and secreted exclusively as a single-chain enzyme and were not cleaved by plasmin-Sepharose (35).

The catalytic activity of single- and two-chain forms of wild type and mutated t-PAs toward a small synthetic substrate, Spectrozyme t-PA, is listed in Table I. Charge reversal mutations of Arg-298, Arg-299, and/or Arg-304 had little effect on the enzymatic activity of either single or two-chain t-PA in this assay. The two-chain variants retained 85–100% of the activity displayed by wild type two-chain t-PA while the single-chain variants possessed 81–95% of the activity exhibited by the corresponding wild type enzyme.

The introduction of multiple charge reversal mutations into the 296–304 region of t-PA had little effect on the catalytic activity of plasminogen in the presence of a fibrin co-factor (Table II). Two-chain t-PA/R298E,R299E,R304E actually exhibited slightly greater activity in this assay than the wild type two-chain t-PA while t-PA/R298E,R299E,R304E displayed 84% of the activity of the wild type control enzyme. A slightly greater reduction of catalytic activity in this assay was observed with the single-chain forms of these two variants. Both single-chain variants behaved similarly and showed an approximately 2-fold reduction in catalytic activity compared with wild type single-chain t-PA. These data strongly support our previous suggestion.
that, in the presence of fibrin, the 296–304 region of t-PA does not form important interactions with the natural substrate plasminogen (21, 22).

All four variants containing multiple, charge reversal mutations in the 296–304 region of t-PA displayed significantly reduced catalytic efficiency toward plasminogen in the absence of a co-factor (Table II). The reduction in catalytic activity observed in this assay was similar for both the single- and two-chain forms of the variants and varied from 12- to 29-fold compared with the corresponding wild type enzyme. These data are consistent with previous reports from Bennett, Eastman, Paoni, and their co-workers (36, 37) and suggest that, in the absence of fibrin, the 296–304 region of t-PA may form kinetically relevant interactions with plasminogen.

The combination of reduced catalytic activity toward plasminogen in the absence of fibrin and high activity in the presence of co-factor endowed the mutated enzymes with enhanced fibrin stimulation compared with wild type t-PA (Table II). The fibrin stimulation factor, defined for an individual enzyme as the ratio of the catalytic efficiency toward plasminogen in the presence and absence of fibrin, was approximately 300 for single-chain wild type t-PA and approximately 5800 for single-chain t-PA/R298E,R299E, or t-PA/R275E,R298E,R299E,R304E. These rate constants are reduced, compared with that of two-chain wild type t-PA, by factors of approximately 1600 or 25,000, respectively. The resistance to inhibition by PAI-1 displayed by the single-chain variants is even more dramatic. Single-chain t-PA/R275E,R298E,R299E or t-PA/R275E,R298E,R299E,R304E or t-PA/R298E,R299E, or t-PA/R298E,R299E,R304E were inhibited by PAI-1 with a second order rate constant of 210 or 15 M⁻¹ s⁻¹, respectively. These rates of inhibition are reduced, compared with that of two-chain wild type t-PA, by factors of approximately 8,600 or 120,000, respectively. The uncertainty regarding whether activated platelets secrete PAI-1 into a thrombus in an active or latent form and the fact that the concentrations of t-PA achieved during thrombolytic therapy substantially exceed the concentration of PAI-1 in the circula-

### Table I

| Enzyme                          | $k_{cat}$ (s⁻¹) | $K_m$ (μM) | $k_{cat}/K_m$ (s⁻¹ μM⁻¹) |
|---------------------------------|-----------------|------------|--------------------------|
| Two-chain form                  |                 |            |                          |
| t-PA                            | 56              | 0.42       | $1.3 \times 10^6$        |
| t-PA/R298E,R299E                | 54              | 0.48       | $1.1 \times 10^6$        |
| t-PA/R298E,R299E,R304E          | 69              | 0.53       | $1.3 \times 10^6$        |
| Single-chain form               |                 |            |                          |
| t-PA/R275E                      | 23              | 0.63       | $3.7 \times 10^5$        |
| t-PA/R275E,R298E,R299E          | 22              | 0.63       | $3.5 \times 10^5$        |
| t-PA/R275E,R298E,R299E,R304E    | 20              | 0.67       | $3.0 \times 10^5$        |

### Table II

| Enzyme                          | Fibrin | $k_{cat}$ (s⁻¹) | $K_m$ (μM) | $k_{cat}/K_m$ (s⁻¹ μM⁻¹) | Fibrin stimulation factor |
|---------------------------------|--------|-----------------|------------|--------------------------|---------------------------|
| Two-chain form                  |        |                 |            |                          |                           |
| t-PA                            |        | 0.10            | 6.5        | $1.5 \times 10^6$        |                           |
| t-PA/R298E,R299E                |        | 0.005           | 6.4        | $7.8 \times 10^2$        |                           |
| t-PA/R298E,R299E,R304E          |        | 0.003           | 5.8        | $5.2 \times 10^2$        |                           |
| t-PA                            | +      | 0.09            | 0.02       | $4.5 \times 10^5$        | 300                       |
| t-PA/R298E,R299E                | +      | 0.28            | 0.05       | $5.6 \times 10^5$        | 7,200                     |
| t-PA/R298E,R299E,R304E          | +      | 0.23            | 0.06       | $3.8 \times 10^5$        | 7,300                     |
| Single-chain form               |        |                 |            |                          |                           |
| t-PA/R275E                      |        | 0.02            | 15.0       | $1.3 \times 10^6$        |                           |
| t-PA/R275E,R298E,R299E          |        | 0.0006          | 7.0        | $8.6 \times 10^1$        |                           |
| t-PA/R275E,R298E,R299E,R304E    |        | 0.001           | 9.1        | $1.1 \times 10^2$        |                           |
| t-PA/R275E                      |        | 0.15            | 0.02       | $7.5 \times 10^5$        | 5,800                     |
| t-PA/R275E,R298E,R299E          | +      | 0.26            | 0.08       | $3.3 \times 10^5$        | 38,400                    |
| t-PA/R275E,R298E,R299E,R304E    | +      | 0.24            | 0.07       | $3.4 \times 10^5$        | 31,000                    |
tion, the role of platelet PAI-1 in mediating the resistance to lysis exhibited by arterial clots has been an extremely controversial issue. However, recent demonstrations that, compared with wild type t-PA, PAI-1-resistant variants possess enhanced potency toward platelet-rich thrombi but normal potency toward platelet-poor clots, both in vitro and in vivo, strongly suggest an important role for platelet derived PAI-1 during thrombolytic therapy (15, 38, 39).

We have previously suggested that the recruitment of residues in surface loops mapping near the active site of an enzyme to form specific enzyme-inhibitor and/or enzyme-substrate interactions was likely to be an important and recurring theme during the evolution of enzyme specificity (13, 21, 22, 40). The properties of variants of t-PA carrying mutations in the 296–304 region clearly demonstrate that this mechanism played an essential role during the co-evolution of specificity between t-PA and PAI-1, individual members of two very large and medically relevant gene families. We demonstrate in this study that mutations in the 296–304 region of t-PA, which maps near the edge of the t-PA active site juxtaposed with the primed side of the reactive center loop of PAI-1 in a t-PA-PAI-1 complex, can diminish the rate of the specific inhibition of t-PA by PAI-1 by more than 5 orders of magnitude. Loss of specific interactions with the 296–304 region of t-PA, then, converts PAI-1 from a

![Graph showing OD05 vs Time (min2)]

Fig. 1. Standard indirect chromogenic assay of plasminogen activation the the presence of buffer ( ), DESAFIB ( ), fibrinogen ( ), cyanogen bromide fragments of fibrinogen ( ), or the stimulatory peptide P368 ( ). WT, wild type.
highly selective to a very poor inhibitor of t-PA. By contrast, mutations in the 296–304 region of t-PA do not significantly alter the interaction of the enzyme with small chromogenic substrates or with plasminogen in the presence of fibrin.

Interactions between PAI-1 and two distinct regions of t-PA, the active site and an exosite formed by residues 296–304, apparently mediate the rapid inhibition of t-PA by PAI-1. Interactions between the inhibitor and the active site of the enzyme, however, appear to be highly conserved not only between other serine protease-serpin pairs but also between serine protease-substrate pairs. Consequently, mutations that affect the active site of t-PA, such as t-PA/D477N, diminish interaction of the enzyme not only with PAI-1 but also with plasminogen and synthetic chromogenic substrates (27). Specificity between t-PA and PAI-1, therefore, appears to have been achieved through the evolution of a single strong secondary site of interaction.

Although the molecular basis of the stringent substrate specificity of t-PA remains obscure, it seems almost certain that the interaction between t-PA and plasminogen is significantly more complex than that between t-PA and PAI-1. In the presence of fibrin, plasminogen activation is a very efficient ($k_{cat}/K_m \approx 10^7 \text{M}^{-1} \text{s}^{-1}$) and extremely complex reaction that apparently proceeds after formation of a ternary complex involving multiple sites of contact between enzyme, inhibitor, and co-factor. Interactions between t-PA and fibrin in the ternary complex appear to be particularly complex; regions within all five structural domains of t-PA have been implicated (13, 14, 41, 42).

Similarly, the interaction between fibrin and plasminogen appears to involve multiple sites of contact between the two proteins and, in addition, a major conformational transition of plasminogen (43–46). By contrast, the importance, and even the existence, of direct, secondary contacts between t-PA and plasminogen in the ternary complex remains obscure.

In the absence of fibrin, plasminogen activation by t-PA is a significantly less complex, but also less efficient ($k_{cat}/K_m \approx 10^6 \text{M}^{-1} \text{s}^{-1}$), reaction (47, 48). Nevertheless, even in the absence of a co-factor, t-PA retains stringent specificity for plasminogen (49). Since the target sequence present in plasminogen appears to be a very poor match to optimal primary substrate occupancy for t-PA (49), the specificity of t-PA for plasminogen under these conditions seems likely to be mediated by specific secondary sites of interaction. We have previously demonstrated that residues 420–423 of t-PA participate in important secondary sites of interaction. We have previously demonstrated that residues 296–304 of t-PA, an observation that is consistent with both our earlier studies (21, 22) and previous reports from Bennett, Eastman, Paoni, and their co-workers (36, 37, 41).

Mutations within either of the two putative secondary sites of interaction between t-PA and plasminogen compromise the interaction of the enzyme with plasminogen (36, 37, 41) to a much lesser extent than mutations in the 296–304 region of t-PA diminish the rate of interaction with PAI-1 (21, 22). Moreover, as we have demonstrated previously, these mutations in t-PA have no significant effect on the interaction with plasminogen in the presence of co-factor (21, 22). The stringent specificity of t-PA toward substrates and toward serpins, therefore, appears to have evolved through fundamentally distinct mechanisms. The specificity of t-PA for its major physiological inhibitor is apparently mediated by optimal interactions with both the primed and unprimed primary subsites of the active site as well as selective interactions with a single strong exosite (21, 22, 49). By contrast, the specificity of t-PA for its major physiological substrate is apparently mediated by optimal interactions with the unprimed, but not the primed, primary subsites (49), very complex interactions with a macromolecular co-factor (50), and, to a lesser extent, by multiple weak secondary contacts between t-PA and plasminogen (36, 40). It is, of course, possible that a strong exosite that contributes substantially to the substrate specificity of t-PA exists but has not yet been described. It is also possible, and perhaps even likely, that the unprimed primary subsites of t-PA for plasminogen are distinct from those utilized by PAI-1 and peptide substrates of t-PA (51).

Both the specific findings and the more general implications of this study raise intriguing issues. Keyt et al. (15) have demonstrated that the variant of t-PA, TNK-t-PA, possesses greater potency toward platelet-rich thrombi than wild type t-PA, and these investigators speculate that this enhanced potency may be a result of the resistance of TNK-t-PA to inhibition by PAI-1. Since t-PA/R275E,R298E,R299E,R304E is more resistant to inhibition by PAI-1 than TNK-t-PA by a factor of greater than 1200, it will be interesting to evaluate whether the new variant has higher thrombolytic potency toward platelet-rich thrombi than TNK-t-PA, a variant that is currently being examined in clinical trials. It will also be of great interest to investigate the similarities and distinctions between mechanisms used by other (chymo)trypsin family enzymes to achieve specificity and those utilized by t-PA.

### TABLE III

| Enzyme                        | Second order rate constant $M^{-1}s^{-1}$ |
|-------------------------------|------------------------------------------|
| Two-chain form                |                                          |
| t-PA                          | $2.5 \times 10^6$                        |
| t-PA/R298E,R299E              | $1.6 \times 10^6$                        |
| Single-chain form             |                                          |
| t-PA/R275E                    | $1.8 \times 10^6$                        |
| t-PA/R275E,R298E,R299E        | $2.1 \times 10^6$                        |
| t-PA/R275E,R298E,R299E,R304E  | $1.5 \times 10^5$                        |

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