Variants of Tissue-type Plasminogen Activator with Substantially Enhanced Response and Selectivity toward Fibrin Co-factors*

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Unlike most proteases, tissue-type plasminogen activator (t-PA) is not synthesized as an inactive precursor or zymogen. Instead, the single-chain “proenzyme” form of t-PA possesses very significant catalytic activity. Recent investigations of the molecular basis of the unusually high enzymatic activity of single-chain t-PA have focused attention upon Asp-194, a residue that is invariant among chymotrypsin-like enzymes. The critical role of this residue in stabilizing the active conformation of mature chymotrypsin-like enzymes has been discussed extensively. Subsequent work, however, has indicated that this conserved residue can also form interactions that dramatically influence the catalytic activity of serine protease zymogens. While Asp-194 forms interactions that suppress the activity of the zymogen chymotrypsinogen, it may, by contrast, directly promote the catalytically active conformation of single-chain t-PA. To test the hypothesis that Asp-194 promotes the activity of both single- and two-chain t-PA and therefore plays opposing roles in single-chain t-PA and chymotrypsinogen, and also to examine whether this invariant residue plays an essential role in the stimulation of t-PA by fibrin, we used site-directed mutagenesis to construct the following variants of t-PA: t-PA/D194E, t-PA/D194N, t-PA/R15E,D194E, and t-PA/R15E,D194N. In the absence of fibrin, the activity of enzymes carrying a mutation at position 194 was reduced by factors of 1000-2000 compared to wild-type t-PA. Similar reductions in activity were observed for both single- and two-chain variants, suggesting an important role for Asp-194 in both forms of the enzyme. The mutated enzymes, however, displayed a dramatically enhanced response to fibrin monomers. While the activity of wild type t-PA was stimulated by fibrin monomers by a factor of 960, the corresponding stimulation factor for the mutated enzymes varied from 498,000–1,050,000.

Many critical biological processes (1–3) depend on specific cleavage of individual target proteins by serine proteases. One important example is the dissolution of blood clots in which the initiating and rate-limiting step is the activation of the circulating zymogen plasminogen by tissue-type plasminogen activator (t-PA).¹ (4, 5).

Unlike typical chymotrypsin-like enzymes, the single-chain or “proenzyme” form of t-PA possesses high catalytic activity (6–12). In the absence of the co-factor fibrin, single-chain t-PA is approximately 8% as active as two-chain t-PA. In the presence of fibrin, however, single- and two-chain t-PA display equivalent enzymatic activity. “Zymogen activation” of single-chain t-PA, therefore, can be accomplished either by activation cleavage or by binding to the co-factor fibrin.

The unusually high, intrinsic enzymatic activity of single-chain t-PA may reflect both the absence of interactions, present in typical proenzymes, that suppress activity of the zymogen and the presence of interactions, absent in typical zymogens, that stabilize an active conformation of the single-chain enzyme. Recent studies suggest that the zymogen triad (Ser-32-His-40-Asp-194), which exists in strong zymogens like chymotrypsinogen but not in t-PA, is an example of the former type of interaction, and that Asp-194,² together with either Lys-143 or Lys-156, may participate in the latter interactions (12–15).

Asp-194, a residue that is invariant among enzymes of the chymotrypsin family, may therefore play a very different role in single-chain t-PA from the one it plays in typical serine protease zymogens.

Asp-194 forms critical interactions that profoundly influence the catalytic activity not only of serine protease zymogens but also of the corresponding mature enzymes. Upon activation cleavage of chymotrypsinogen, for example, the interaction between the side chains of His-40 and Asp-194 described above is broken as the side chain of Asp-194 rotates approximately 170° to form a strong, buried salt bridge with the new amino terminus at Ile-16 (16). This new salt bridge, which promotes enzymatic activity by securing the active conformation of the oxyanion hole and the P1 binding pocket, is present in every mature chymotrypsin-like enzyme whose structure has been determined (16–19). It seems very likely, therefore, that this salt bridge will also be present in mature two-chain t-PA, and, consequently, that interactions involving Asp-194 will influence the activity of two-chain t-PA.

To test the hypothesis that Asp-194 participates in interactions which influence the activity of both single- and two-chain t-PA and also to examine whether this invariant residue plays an essential role in the stimulation of t-PA by fibrin, we used site-specific mutagenesis to construct the following variants of t-PA: t-PA/D194E, t-PA/D194N, t-PA/R15E,D194E, and t-PA/R15E,D194N. Unlike wild type t-PA or the two single mutants, enzymes containing the R15E mutation were resistant to activation cleavage by plasmin and therefore remained in the

Techniques used include PAGE, polyacrylamide gel electrophoresis; PAI-1, plasminogen activator inhibitor 1; Spec-PA, methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-para-nitroaniline; Spec PL, H-o-norleucyl-hexahydrotrypsinyl-lysine-p-nitroaniline diacetate salt.

¹ These abbreviations are as follows: t-PA, tissue-type plasminogen activator; CAPS, 3-(cyclohexylamino)propanesulfonic acid; DESAFIB, soluble fibrin; PAGE, polyacrylamide gel electrophoresis; PAI-1, plasminogen activator inhibitor 1; Spec-PA, methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-para-nitroaniline; Spec PL, H-o-norleucyl-hexahydrotrypsinyl-lysine-p-nitroaniline diacetate salt.

² To avoid confusion, we used the chymotrypsin numbering system to designate particular residues of the protease domain of t-PA. Positions 15, 32, 40, 143, 156, and 194 of chymotrypsin correspond to residues 275, 292, 305, 416, 429, and 477, respectively, in the t-PA numbering system.
single-chain form during assays of plasminogen activation. In the presence of fibrin, the activity of enzymes carrying a mutation at position 194, both towards plasminogen and towards small synthetic substrates, was reduced by factors varying from 1000–2000. Similar reductions in activity were observed for both the single- and two-chain forms of t-PA/D194E and t-PA/D194N. The mutated enzymes, however, displayed a dramatically enhanced response and selectivity toward fibrin co-factors.

EXPERIMENTAL PROCEDURES

Reagents—The chromogenic substrates methylsulfonyl-p-cyclohexy-1,4-lyrosyl-glycyl-argnine-para-nitroanilide (Spec t-PA), H-norleucyl-hexahydroxylysyl-lysine-p-nitroanilide diacetate salt (Spec PL), soluble fibrin (DESAFIB), Lys-plasminogen, CNBr-digested fibrinogen, and fibrinogen were purchased from American Diagnostica (Greenwich, CT). CAPS, amiloride, and imidazole were purchased from Sigma. Active plasminogen activator inhibitor type 1 (PAI-1) was a kind gift from Drs. Jee Shore, J An Kvaasman, and co-workers. Aprotinin was purchased from Boehringer Mannheim (Mannheim, Germany) and lysine-Sepharose from Pharmacia (Uppsala, Sweden).

Recombinant DNA Techniques—Buffer and reaction conditions for restriction enzymes and T4 DNA ligase were as recommended by the commercial source, New England Biolabs or Boehringer Mannheim (Mannheim, Germany). Other standard recombinant DNA techniques were performed as described (20).

Site-directed mutagenesis was performed as described previously (21, 22) using single-stranded M13mp18 DNA containing cDNA encoding t-PA as a template. The synthetic oligonucleotides used as mutagenic primers were as follows: t-PA(D194E), 5'-CATGGAATTCGGAGGAGGACGAGTCCGAGG-3', t-PA(D194N), 5'-GCCAGCAATTCGGGAGG-3'. Following mutagenesis, single-stranded DNA was prepared from several independent plasmid isolates. The 290 base constructs of t-PA cDNA in these templates were sequenced completely to verify the presence of the desired mutation and the absence of any additional mutation. Doubly recombinant active form DNA from plasmid containing confirmed mutations was prepared and used to isolate the 290-base pair SacI-Smal fragment of t-PA cDNA. The mutated 290-base pair SacI-Smal DNA fragments were then used to replace the corresponding region of cDNA encoding wild type t-PA in the eucaryotic expression vector pSV77R-tPA (23, 24). Vectors that direct the expression of non-cleavable, single-chain variants of t-PA containing mutations at both positions 15 and 194 were constructed from the mutated plasmid described above by replacing the internal 472-base pair EcoRI fragment of t-PA cDNA with a corresponding fragment encoding the mutation R15E. All new plasmid DNAs were isolated, rechecked for the presence of the correct mutation(s), and purified by CsCl equilibrium centrifugation.

Transient Expression and Purification of Mutant t-PA—Expression vectors encoding t-PA(D194E), t-PA(D194N), t-PA(R15E,D194E), or t-PA(R15E,D194N) were introduced into COS-1 cells by electroporation using a Bio-Rad Gene Pulser. 20 μg of carrier DNA, and 0.5 ml of lysine-Sepharose (Pharmacia), using a separate Bio-Rad centrifugation. Since this procedure yielded almost exclusively single-chain material, two-chain t-PAs were generated by treatment with plasmin-Sepharose as described previously (9), and quantitative cleavage was confirmed by SDS-polyacrylamide gel electrophoresis.

General Methods with Recombinant Proteins—Concentration of conditioned media or purified enzyme was carried out in Amicon Centriprep® 30 or Centricon® 30 concentrators according to the manufacturer's instructions. Measurement of enzyme concentrations was accomplished by enzyme-linked immunosorbent assay as described previously (25). SDS-PAGE was performed on 12% polyacrylamide gels using the buffer system of Laemmli (26). Proteins were visualized either by Coomassie Blue staining or by Western blotting, using the primary antibody 3748 (American Diagnostica) and the ECL Western detection system from Amersham (United Kingdom).

Indirect Chromogenic Assays of Plasminogen Activation in the Presence of Various Stimulators—Standard indirect chromogenic assays were performed as described previously (23, 24, 27). Assays were performed in the presence and absence of the co-factor DESAFIB. The concentration of Lys-plasminogen was varied from 0.01 to 2.0 μg/ml, and the concentration of DESAFIB from 0.1 to 0.5 μg/ml in the absence of the co-factor. kcat and km values were calculated as described previously (24, 27, 29, 30). The Km (0.062 μM) and kcat (24 s⁻¹) of plasmin for Spec PL were determined under our experimental conditions using a preparation of plasmin whose activity had been measured by active site titration.

Direct Chromogenic Assays of t-PA Activity Using Activated Substrates—Direct assays of t-PA activity utilized the substrate Spec-PL A500 and were performed as described previously (22) except that 0.5 μM aprotinin, 0.3 μM amiloride, and 0.02% Tween 80 were included in the assay.

Measurement of Second Order Rate Constants for Inhibition 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 (24, 27). 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 4–40-fold, depending on the enzyme and PAI-1 concentrations in a particular reaction, and residual enzymatic activity was measured in the indirect chromogenic assay and compared to control reactions in which PAI-1 was added after dilution and addition of fibrin, plasminogen, and Spec PL. 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 (M⁻¹ s⁻¹).

RESULTS

Design, Construction, and Production of t-PA Mutants—To investigate the functional significance of Asp-194 of t-PA, we used site-directed mutagenesis to replace this residue with either a glutamic acid or an asparagine residue. The resulting variants were denoted t-PA(D194E) and t-PA(D194N), respectively. 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 Arg-15–Ile-16 bond of the single-chain t-PAs. Consequently, to overcome this technical difficulty, we also constructed noncleavable forms of the two mutated enzymes by introducing the additional mutation R15E into the existing variants.

Wild type t-PA, t-PA/R15E, and all four variants containing a mutation at position 194 were expressed by transient expression in COS-1 cells and purified by lysine-Sepharose affinity chromatography. Since this procedure yielded almost exclusively single-chain material, 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. As expected, variants containing the mutation R15E were not cleaved by plasmin-Sepharose (data not shown).

Kinetic Analysis of Catalytic Activity toward a Low Molecular Weight Substrate—The enzymatic activity of the single- and two-chain form of wild type t-PA and each variant toward a small synthetic substrate is listed in Table I. Compared to wild type, two-chain t-PA, the catalytic efficiency of two-chain t-PA/D194E and t-PA/D194N is decreased by a factor of approximately 1400. Single-chain t-PA/R15E,D194E and t-PA/R15E,D194N displayed approximately a 850-fold reduction in catalytic efficiency compared to t-PA/R15E. In all cases, the observed decrease in activity was due primarily to a large decrease in $k_{cat}$. $K_m$ increased by factors of only 1–3. Mutations at position 194 of t-PA, therefore, apparently affect the catalytic machinery of the enzyme, both in the single- and two-chain form, much more dramatically than they affect binding determinants for the ground state, low molecular weight substrate. It is also interesting that the mutated enzymes were activated to the same extent as wild type t-PA by conversion into the mature, two-chain form. This activation occurs mainly by decreasing $K_m$, suggesting that activation cleavage of the variants does not alleviate the distortion(s) of their catalytic machinery caused by the mutations at position 194.

Kinetic Analysis of Catalytic Activity toward the Physiological Substrate Plasminogen—The enzymatic activity of wild type and mutated variants of t-PA toward the natural substrate plasminogen was measured both in the presence and the absence of the co-factor fibrin. In the absence of soluble fibrin, $k_{cat}/K_m$ for plasminogen activation by the two mutants was approximately 2000-fold lower than that of wild type t-PA, and similar decreases in activity were observed for the noncleavable, single-chain form of the mutants compared to t-PA/R15E (Table II).

The noncleavable, single-chain mutants retained a $K_m$ value similar to that of single-chain t-PA/R15E, and the decrease in activity toward plasminogen was due to a reduced $k_{cat}$. The basis of decreased activity by the two-chain forms of the mutants was more complicated and included both a large decrease in $k_{cat}$ and at least a 10-fold increase in $K_m$. While the $k_{cat}/K_m$ values reported in Table II for these two-chain variants are very reliable, precise partitioning of the catalytic efficiency of these two variants should be interpreted with caution since the high $K_m$ made it impossible to carry out assays at sufficiently high plasminogen concentrations to ensure optimal accuracy of individual $K_m$ and $k_{cat}$ values. Comparison of $k_{cat}/K_m$ values for the single- and two-chain form of enzymes carrying mutations at position 194 indicated that activation cleavage of these variants, as with the wild type enzyme, produced an approximately 10-fold enhancement of enzymatic activity.

The activity toward plasminogen of all six enzymes utilized in this study was substantially enhanced by soluble fibrin monomers (Tables II, III, and IV). Fibrin increased the catalytic efficiency of two-chain t-PA by a factor of 960, and this stimulation was due primarily to a decreased $K_m$ for plasminogen. The response of the single-chain enzyme t-PA/R15E to fibrin was even more impressive; in this case, a 9-fold increase in $K_m$, and a 1400-fold decrease in $K_{cat}$, combined to yield a stimulation factor of approximately 12,000. Even when compared to these large stimulation factors, however, the response of the enzymes containing mutations at residue 194 to fibrin is dramatic. In all cases, fibrin stimulation of these mutated enzymes includes substantial contributions to both $K_m$ and $k_{cat}$, and fibrin stimulation factors vary from 116,000–1,050,000. For example, with t-PA/R15E,D194N, the presence of soluble fibrin increases $k_{cat}$ by a factor of 4710 and decreases $K_m$ by a factor of 219, resulting in a fibrin stimulation factor of 1,050,000 which is significantly larger than that reported for any other plasminogen activator.

The Effect of Different Stimulators on the Activity of t-PA Variants toward Plasminogen—The four enzymes carrying a mutation at residue 194 are not only stimulated to a much greater extent by soluble fibrin than t-PA (Table IV), but they are also significantly more selective toward fibrin co-factors than the wild type enzyme (Fig. 1). Two-chain t-PA is strongly stimulated by soluble fibrin monomers, fibrinogen, and CNBr fragments of fibrinogen, and single-chain t-PA/R15E is stimulated strongly by soluble fibrin and fibrinogen and moderately by the CNBr fragments. By contrast, although dramatically stimulated by fibrin monomers, the variants containing an asparagine residue at position 194 are virtually nonresponsive to either fibrinogen or CNBr fragments of fibrinogen, and variants containing glutamate at position 194 are only poorly (t-PA/D194E) or moderately (t-PA/R15E,D194E) stimulated by fibrinogen and nonresponsive toward the CNBr fragments.

The ratio of the specific activity of a plasminogen activator in the presence of fibrin to that in the presence of fibrinogen, or "fibrin selectivity factor," may be one indication of the "dot selectivity" an enzyme will demonstrate in vivo. An enzyme with enhanced fibrin selectivity may be able to accomplish efficient thrombolysis while displaying decreased systemic activity. Under the conditions of the assays reported here, the fibrin selectivity factor is 1.2 for two-chain t-PA and 1.0 for single-chain t-PA/R15E. All four mutated enzymes exhibit enhanced selectivity toward fibrin, having fibrin selectivity ratios of 30 (t-PA/R15E,D194E), 50 (t-PA/R15E,D194N), 60 (t-PA/D194E), or 190 (t-PA/D194N).

Inhibition of Enzymatic Activity by PAI-1—As expected from their reduced enzymatic activity, all variants containing a mutation at position 194 were less reactive toward PAI-1, the
primary endogenous inhibitor of t-PA (31, 32), than the wild type enzyme (Table V). By contrast to their similar activities toward the substrate plasminogen, there was a clear difference in reactivity toward PAI-1 between the two mutants. Mature t-PA/D194E was inhibited by PAI-1 approximately 30-fold more rapidly than two-chain t-PA/D194N; similarly, single-chain t-PA/R15E,D194E was inhibited by PAI-1 approximately 7 times more rapidly than single-chain t-PA/R15E,D194N. The single-chain form of both variants was less reactive toward PAI-1, by a factor of 3 (D194N) or 13 (D194E), than the corresponding two-chain enzyme.

**DISCUSSION**

All chymotrypsin-like serine proteases that have been crystallized to date possess a salt bridge formed by the new amino-terminal residue, created by activation cleavage, and a conserved aspartic acid residue, Asp-194, which is believed to be necessary for maintenance of the active conformation of the mature enzyme (18). Although no crystal structure of the t-PA molecule is available, it seems likely that this important ionic interaction is also present in two-chain t-PA. In this report, we describe the construction and characterization of t-PA mutants in which Asp-194 is replaced by either a glutamic acid or an asparagine. Assuming formation of the new salt bridge in two-chain t-PA, the Asp to Glu mutation is expected to introduce steric constraints on the salt bridge, while the isosteric Asp to Asn replacement would eliminate the salt bridge. The structural effect of these mutations on single-chain t-PA, however, is less predictable because both the molecular basis of the enzyme's unusually high catalytic activity and the interactions formed by Asp-194 in single-chain t-PA remain obscure.

Our data support the hypothesis that Asp-194 forms key interactions that promote the enzymatic activity of both single- and two-chain t-PA and provide the first direct evidence that this invariant residue may play opposing roles in single-chain t-PA and chymotrypsinogen. Both single- and two-chain variants of t-PA containing a mutation at position 194 displayed similarly reduced enzymatic activity toward synthetic and natural substrates. In each case, the reduced activity of the mutated enzymes resulted primarily from a decreased $k_{cat}$, suggesting that the mutations retard the enzyme's catalytic capacity more than it's ability to bind substrate. Because it is known that interactions involving Asp-194 can dramatically influence the position and conformation of Gly-193, one possible explanation for this observation is that our mutations have disrupted a structure known as the oxyanion hole (33). After formation of the tetrahedral intermediate, the oxyanion hole, which is formed by the main chain amide groups of Gly-193 and Ser-195, donates two hydrogen bonds to the carbonyl group of the P1 residue of the substrate (17). This key structure, therefore, promotes catalysis, presumably enhancing $k_{cat}$ by stabi-
lizing the oxygenation of the transition state formed during proteolysis.

Comparison of the structures of the mature and precursor forms of trypsinogen and chymotrypsinogen revealed that a surprisingly large segment of these proteins, which was designated the activation domain (17) and contained portions of the primary specificity pocket, the oxyanion hole, and the autodigestive loop, was reorganized upon activation cleavage (16, 34–36). This large conformational change involved 16% of the enzyme, provided the structural basis underlying enhanced activity in the mature enzyme compared to thezymogen, and was initiated by insertion of the first two residues of the newly created amino terminus into the activation pocket, where they formed the salt bridge with Asp-194 and at least seven additional, primarily hydrophobic interactions with other residues (17, 34). Because single-chain t-PA/D194E and t-PA/D194N display an increase in activity upon activation cleavage which is similar to that observed with wild type t-PA, it is likely that insertion of the mature amino terminus, along with at least a significant part of the subsequent structural reorganization of the activation domain, occurs in both variants as well as in wild type t-PA.

Molecular details of the stimulation of t-PA by fibrin, a complex event that almost certainly involves multiple points of contact between the two proteins, remain obscure (37–39). For single-chain t-PA, stimulation by fibrin appears to involve at least two distinct mechanisms. First, fibrin apparently stimulates both single- and chain t-PA through a “templating” mechanism, or the formation of a ternary complex which greatly augments the local concentration of the enzyme and substrate (40, 41). Second, because single- and two-chain t-PA have equivalent catalytic activity in the presence but not the absence of fibrin, it seems likely that binding to fibrin induces a conformational change in the activation domain of single-chain t-PA (7, 8). Induction of such a conformational change in the absence of activation cleavage and concomitant generation of the mature amino terminus is particularly intriguing but not unprecedented. Similar activation of plasminogen by binding to streptokinase (42–47) as well as activation of prothrombin by binding to staphylocoagulase (48, 49) has been described previously. Although the mechanism of this nonclassical, nonproteolytic activation of serine protease zymogens remains completely unclear, the behavior of single-chain t-PA/R15E,D194E and t-PA/R15E,D194N suggests that Asp-194 does not play an essential role in the process.

Stimulation of mature t-PA is primarily a result of a drastically reduced $K_{\text{m}}$, an observation which is consistent with the templating mechanism described above. It is uncertain, therefore, whether binding to fibrin alters the conformation of two-chain, wild type t-PA. The two-chain variants of t-PA with mutations at residue 194, however, are stimulated by fibrin to a much greater extent than the wild type enzyme, and this stimulation includes very large improvements in both $K_{\text{m}}$ and $K_{\text{cat}}$. Consequently, while the variants possess only 0.003–0.05% of the activity of two-chain t-PA in the absence of a co-factor, they develop 3.3–6.2% of the activity of the mature wild type enzyme in the presence of fibrin. It seems likely, therefore, that fibrin does induce a conformational change in both the single- and two-chain form of the variants and that this conformational change may partially compensate for the deleterious effect of the mutations.

Data in Tables II, III, and IV establish that the variants of t-PA examined in this study are stimulated by fibrin monomers to a greater extent than any previously described plasminogen activator; moreover, in Fig. 1 indicate that these variants also exhibit substantially greater selectivity toward fibrin.

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