Distinct Contributions of Residue 192 to the Specificity of Coagulation and Fibrinolytic Serine Proteases*

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Archetypal members of the chymotrypsin family of serine proteases, such as trypsin, chymotrypsin, and elastase, exhibit relatively broad substrate specificity. However, the successful development of efficient proteolytic cascades, such as the blood coagulation and fibrinolytic systems, required the evolution of proteases that displayed restricted specificity. Tissue-type plasminogen activator (t-PA), for example, possesses exquisitely stringent substrate specificity, and the molecular basis of this important biochemical property of t-PA remains obscure. Previous investigations of related serine proteases, which participate in the blood coagulation cascade, have focused attention on the residue that occupies position 192 (chymotrypsin numbering system), which plays a pivotal role in determining both the inhibitor and substrate specificity of these enzymes. Consequently, we created and characterized the kinetic properties of new variants of t-PA that contained point mutations at position 192. These studies demonstrated that, unlike in coagulation serine proteases, Glu-192 does not contribute significantly to the substrate or inhibitor specificity of t-PA in physiologically relevant reactions. Replacement of Gln-192 with a glutamic acid residue did, however, decrease the catalytic efficiency of mature, two-chain t-PA toward plasminogen in the absence of a fibrin co-factor.

Appreciation that thrombotic disorders are the major cause of morbidity and mortality in many countries sparked intense interest in the human fibrinolytic system, which normally provides a counterbalance to the blood coagulation cascade (1–5). The rate-limiting step in the fibrinolytic cascade, conversion of the circulating zymogen plasminogen into the active protease plasmin, is catalyzed by t-PA,† a member of the chymotrypsin family of serine proteases (4, 6, 7). Discovery of this important biochemical property of t-PA is the highly stringent substrate and inhibitor specificity (6, 7), which is in stark contrast to that of archetypal chymotrypsin family enzymes such as chymotrypsin, trypsin, and elastase (16). The striking substrate specificity of t-PA is mediated in part by interactions of both the enzyme and its substrate with the co-factor fibrin (6, 7). However, even in the absence of a co-factor, t-PA maintains strict specificity for plasminogen, the primary physiological substrate, and we have shown that this specificity is an inherent property of the isolated protease domain of t-PA (17). Recent structural investigations of the protease domain of t-PA have confirmed the expected close similarity of the three-dimensional structure of the enzyme to that of trypsin, a nonspecific protease (18, 19). The molecular basis of the strong, fibrin-independent specificity of t-PA for plasminogen therefore remains poorly understood.

Like t-PA, the chymotrypsin family enzymes forming the blood coagulation cascade display significantly restricted specificity compared with archetypal family members (16). Esmon, Rezaie, Morrissey, and their co-workers (20–25) have reported a number of interesting studies that suggest the residue at position 192 (chymotrypsin numbering) of the coagulation proteases plays a pivotal role in mediating the inhibitor and substrate specificity of these enzymes. For example, these investigators have shown that the variant thrombin/E192Q activates protein C approximately 20 times more rapidly than wild type thrombin (21), and the variants factor Xa/Q192E and factor Xa/Q192M activate prethrombin 1 approximately 10 times less efficiently than factor Xa (21). In addition, replacement of Glu-192 of activated protein C with a glutamine residue increased the rate of inhibition of the enzyme by the serpin α1-protease inhibitor by a factor of approximately 300 (22).

By contrast to the coagulation proteases, the role of residue 192 in mediating specificity of the fibrinolytic proteases has not been explored. Consequently, we created new variants of the fibrinolytic protease t-PA in which Gln-192 was replaced either with a glutamic acid residue, as in thrombin and protein C, or a methionine, as in chymotrypsin. We examined the effect of these mutations on the reactivity of the single- and two-chain forms of t-PA toward four distinct synthetic substrates, the physiological substrate plasminogen both in the presence and absence of a fibrin co-factor, and the physiological inhibitor PAI-1. Unlike with the coagulation proteases, mutation of residue 192 did not dramatically alter the reactivity of t-PA toward physiological inhibitors. Furthermore, in the presence of fibrin, both the single- and two-chain forms of the mutated enzymes retained high catalytic activity toward plasminogen.

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‡ The abbreviations used are: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor, type 1.
These results suggest that residue 192 does not contribute significantly to the specificity of fibrinolytic proteases in physiologically relevant reactions and consequently that subtle but clear distinctions may exist between the molecular determinants of specificity for coagulation and fibrinolytic proteases.

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 (26) as modified by Kunkel (27). The Q192E and Q192M mutations were introduced into the 290-base pair SacI-Smal fragment of cDNA encoding t-PA that had been previously subcloned into bacteriophage M13mp18. The mutagenic primers had the following nucleotide sequences: Q192E, 5'-GAGCCTCGGAGGCGATT-3'; and Q192M, 5'-CGACCCGTGCATTGGCAGTC-3'.

Following mutagenesis, single-stranded DNA corresponding to the entire 290-base pair SacI-Smal fragment was fully sequenced to assure the presence of the desired mutation and the absence of any additional mutations. Double-stranded, replicative form DNA was prepared for the appropriate phage, and the mutated 290-base pair SacI-Smal fragment was recovered after digestion of replicative form DNA with SacI and SmaI and electrophoresis of the digestion products on an agarose gel. The isolated, mutated SacI-Smal fragments were used to replace the corresponding fragment in full-length cDNAs encoding wild type t-PA or t-PA/R15E to yield new, full-length cDNAs encoding t-PA/Q192E, t-PA/Q192M, t-PA/R15E/Q192E, and t-PA/R15E/Q192M.

Expression of Enzymes by Transient Transfection of COS Cells—cDNAs encoding t-PA, t-PA/Q192E, t-PA/Q192M, t-PA/R15E, t-PA/R15E/Q192E, and t-PA/R15E/Q192M were ligated into the transient expression vector pSPV17 (28) and then introduced into COS cells by electroporation using a Bio-Rad gene pulser as described previously (29, 30). 20 

| Enzyme | hcat | Km | hcat/Km |
|--------|------|----|--------|
| Spectrozyme t-PA | 54 | 0.38 | 1.4 x 10^3 |
| t-PA-Q192M | 32 | 0.26 | 1.3 x 10^3 |
| t-PA-Q192E | 60 | 0.61 | 1.0 x 10^3 |
| Single-chain form | 15 | 0.60 | 2.6 x 10^4 |
| t-PA/R15E, Q192M | 13 | 0.56 | 2.4 x 10^4 |
| t-PA/R15E, Q192E | 14 | 0.70 | 2.1 x 10^4 |
| Spectrozyme UK | 28 | 0.9 | 3.2 x 10^4 |
| t-PA-Q192M | 10 | 0.53 | 2.0 x 10^4 |
| t-PA-Q192E | 14 | 0.66 | 2.2 x 10^4 |
| Single-chain form | 7.1 | 0.95 | 7.5 x 10^3 |
| t-PA/R15E, Q192M | 2.7 | 0.87 | 3.3 x 10^3 |
| t-PA/R15E, Q192E | 2.8 | 0.4 | 7.5 x 10^3 |
| S-2675 | 22.4 | 1.2 | 1.9 x 10^4 |
| t-PA-Q192M | 6.9 | 0.46 | 1.5 x 10^4 |
| t-PA-Q192E | 17.1 | 1.4 | 1.2 x 10^4 |
| Single-chain form | 14 | 1.1 | 1.2 x 10^4 |
| t-PA/R15E, Q192M | 16 | 1.6 | 9.6 x 10^3 |
| t-PA/R15E, Q192E | 8.5 | 1.4 | 6.5 x 10^3 |
| S-2444 | 30 | 2.1 | 1.5 x 10^4 |
| t-PA-Q192M | 22 | 1.6 | 1.4 x 10^4 |
| t-PA-Q192E | 23 | 2.2 | 1.1 x 10^4 |
| Single-chain form | 14 | 2.3 | 3.0 x 10^3 |
| t-PA/R15E, Q192M | 4.9 | 2.6 | 2.0 x 10^3 |
| t-PA/R15E, Q192E | 7.1 | 2.9 | 2.5 x 10^3 |

PAI-1 during the preincubation, yielded the second order rate constants shown.

RESULTS AND DISCUSSION

To investigate the functional significance of Gln-192 of t-PA, we used oligonucleotide-directed site-specific mutagenesis to replace this residue with either the isosteric residue glutamic acid, which is present at position 192 of the related enzyme thrombin, or methionine, which occupies the analogous position in chymotrypsin. Accurate measurement of the catalytic activity of the single-chain form of these variants toward the physiological substrate plasminogen was difficult, however, because plasmin produced during this assay rapidly and efficiently converted the variants into their mature, two-chain forms by cleaving the Arg-15–Ile-16 bond of the single-chain t-PA. Consequently, to overcome this technical difficulty, we also constructed noncleavable forms of the two mutated enzymes by introducing the additional mutation R15E into the mature variants, a strategy that was first described by Tate et al. (37).

The activity of the two-chain form of t-PA, t-PA-Q192E, and t-PA-Q192M toward the small chromogenic substrate Spectrozyme t-PA varied by less than 30% (Table I). Individual Km values toward the synthetic substrate varied by less than a factor of 1.7 compared with the wild type enzyme for both variants. Gln-192 of t-PA, therefore, does not significantly modulate the activity of the enzyme toward the chromogenic substrate. However, because Spectrozyme t-PA contains a d-rather than an l-amino acid residue at the P3 position, this result is consistent with expectations.
The activities of single-chain t-PA/R15E, t-PA/R15E,Q192M, and t-PA/R15E,Q192M in the direct assay are even more similar than those of the corresponding two-chain enzymes (Table I). Individual $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ values for the two variants carrying point mutations at position 192 are all within 20% of the corresponding values for single-chain t-PA/R15E.

Similar data were obtained using three additional, small chromogenic substrates (Table I). Compared with the corresponding wild type enzyme, two-chain t-PA/Q192M maintained 93, 75, or 63% activity toward the substrates S-2444, S-2765, and Spectrozyme UK, respectively. The catalytic activity of two-chain t-PA/Q192E toward these same substrates was 73, 60, or 69%, respectively, of two-chain t-PA. The single-chain t-PA variants also maintained high activity toward these chromogenic substrates, ranging from 100% activity for single-chain t-PA/Q192E toward Spectrozyme UK to 44% activity for single-chain t-PA/Q192M toward the same substrate. Mutation of residue 192 of t-PA, therefore, did not substantially alter the activity of either the single- or two-chain enzyme toward synthetic substrates containing a D-, L-, or unnatural amino acid at the P3 position.

In contrast to Spectrozyme t-PA, plasminogen, the primary physiological substrate of t-PA, contains an L-amino acid at the P3 position. Because Le Bonniec et al. (9) have suggested that residue 192 of the coagulation proteases may interact directly with the P3 and/or the P3' residue of natural substrates, the effects of mutations at position 192 of t-PA might be expected to be more pronounced in indirect assays of plasminogen activation than in the direct assays described above. The results of indirect assays, in the absence of a fibrin co-factor, of wild type t-PA and the four mutated enzymes confirmed this expectation. Compared with the corresponding wild type enzyme, the catalytic efficiency toward plasminogen of the single-chain form of the variants was reduced by a factor of 2.4 (Q192M) or 5.2 (Q192E), and plasminogen activation by the two-chain form of the variants was reduced by a factor of 3.4 (Q192M) or 15 (Q192E) (Table II). For all four mutated enzymes, the observed decrease in catalytic efficiency resulted exclusively from a reduced $k_{\text{cat}}$, $K_m$ values of all four variants were actually improved by factors of approximately 2–5.

Mutation of Gln-192 of t-PA compromised plasminogen activation in the presence of the fibrin monomer DESAFIB significantly less than in the absence of a co-factor (Table I). For example, in the presence of fibrin, the activity of both the single- and two-chain forms of the Q192M variant varies by less than 30% from that of the corresponding wild type enzyme (Table II). Consistent with the results of both assays described above, the Q192E mutation had a larger effect in this assay than the Q192M mutation. However, even with the Q192E mutation, the observed effects were still modest. The catalytic activity of two-chain t-PA/Q192E was approximately 60% of two-chain t-PA, whereas the activity of single-chain t-PA/R15E,Q192E was approximately 40% of single-chain t-PA/R15E (Table II).

In the presence of a fibrin co-factor, plasminogen activation is an efficient and complicated reaction that apparently proceeds following formation of a ternary complex involving multiple sites of interaction between enzyme, substrate, and co-factor (17, 38, 39). Interactions between t-PA and fibrin in the tertiary complex apparently appear to be particularly complex; regions within all five structural domains of t-PA may participate in this interaction (6, 7, 40, 41). Similarly, the interaction between fibrin and plasminogen apparently involves multiple sites of contact between the two proteins and, in addition, a large conformational transition of plasminogen (42–45). By contrast, the importance and precise role of direct contacts between t-PA and plasminogen in the ternary complex, other than that of the catalytic triad of t-PA and the scissile bond of plasminogen, remain uncertain.

In the absence of fibrin, the stringent specificity of t-PA for plasminogen must be dictated by direct contacts between the two proteins (17). Therefore, consistent with our experimental observations, the loss of a single interaction between t-PA and plasminogen is expected to have a more significant effect on plasminogen activation in the absence rather than the presence of fibrin. However, because plasminogen activation by t-PA in vivo presumably occurs almost exclusively in the presence of a fibrinogen co-factor, our observations suggest that Gln-192 of the enzyme does not contribute significantly to the substrate specificity of physiologically relevant catalysis by t-PA.

Because the presence of a fibrin co-factor can overcome the modest catalytic defect of the t-PA variants in which Gln-192 was replaced by either methionine or glutamate, these mutated enzymes are stimulated by fibrin to a greater extent than wild type t-PA. The fibrin stimulation factor, defined as the ratio of the catalytic efficiencies toward plasminogen in the presence and absence of fibrin, is approximately 610 for two-chain, wild type t-PA (Table II). This stimulation factor increases to a value of 1800 or 5500 for two-chain t-PA/Q192M or t-PA/R15E,Q192E, respectively (Table II).

Fibrin stimulation of two-chain t-PA is believed to result primarily from a catalytically productive juxtaposition of enzyme and substrate as both proteins bind the co-factor. Single-chain, wild type t-PA, with a fibrin stimulation factor of 5700
The catalytic activity of t-PA/R15E,Q192M is stimulated by fibrin to a significantly greater extent than wild type t-PA, this enzyme would be expected to exhibit enhanced "crot selectivity" in vivo because of reduced activity in the circulation but full thrombolytic potency at sites of fibrin deposition. Whether enhanced clot selectivity would improve t-PA as a thrombolytic agent remains an extremely controversial issue that merits additional investigation. Moreover, the clot selectivity of t-PA may assume additional importance as variants with a prolonged circulating half-life are administered in high concentrations as a single bolus. The availability of variants of t-PA with enhanced fibrin specificity, such as t-PA/R15E,Q192E, should facilitate examination of the relationship, if any, between the clot selectivity and the potency and/or safety of the enzyme as a thrombolytic agent.

Replacement of Gln-192 of t-PA with a methionine residue had little effect upon the interaction of t-PA and the serpin PAI-1, the primary physiological inhibitor of this enzyme (Table III). As observed in activity assays toward both synthetic and natural substrates (Tables I and II), however, the Q192E mutation was more disruptive than the Q192M mutation. Nevertheless, enzymes containing the Q192E mutation were rapidly inhibited by PAI-1. The second order rate constant for inhibition of two-chain t-PA/Q192E by PAI-1 was reduced by a factor of approximately 4 compared with that of two-chain t-PA, and single-chain t-PA/R15E,Q192E was inhibited approximately 5-fold less rapidly than single-chain t-PA/R15E (Table III). These data are in striking contrast to those obtained by Rezaie and Esmon (21) for similar variants of prothrombinase A, the primary physiological inhibitor of this enzyme (Table III).

## Table III

| Enzyme                  | Second order rate constant | $\mu^{-1} s^{-1}$ |
|-------------------------|----------------------------|-----------------|
| Two-chain form          |                            |                 |
| t-PA                    | $2 \times 10^7$            |                 |
| t-PA/Q192M              | $2 \times 10^7$            |                 |
| t-PA/Q192E              | $5 \times 10^6$            |                 |
| Single-chain form       |                            |                 |
| t-PA/R15E               | $1 \times 10^6$            |                 |
| t-PA/R15E,Q192M         | $1 \times 10^5$            |                 |
| t-PA/R15E,Q192E         | $2 \times 10^5$            |                 |

Understanding of these distinct specificity determinants will provide new insights into the divergent evolution of coagulation and fibrinolytic proteases and may facilitate the rational design of new serine proteases with novel, highly stringent specificities.

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13. Gln-192, the catalytic efficiency of plasminogen activation stimulation of single-chain t-PA is further enhanced by mutation of Gln-192. The catalytic efficiency of plasminogen activation increased by fibrin by a factor of 17,000 or 12,000, respectively (Table II).

Because the catalytic activity of t-PA/R15E,Q192M is stimulated by fibrin to a significantly greater extent than wild type t-PA, this enzyme would be expected to exhibit enhanced "crot selectivity" in vivo because of reduced activity in the circulation but full thrombolytic potency at sites of fibrin deposition. Whether enhanced clot selectivity would improve t-PA as a thrombolytic agent remains an extremely controversial issue that merits additional investigation. Moreover, the clot selectivity of t-PA may assume additional importance as variants with a prolonged circulating half-life are administered in high concentrations as a single bolus. The availability of variants of t-PA with enhanced fibrin specificity, such as t-PA/R15E,Q192E, should facilitate examination of the relationship, if any, between the clot selectivity and the potency and/or safety of the enzyme as a thrombolytic agent.

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