Distinct Mechanisms Contribute to Stringent Substrate Specificity of Tissue-type Plasminogen Activator*

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Tissue-type plasminogen activator (t-PA) has evolved to optimize cleavage of plasminogen (Plg) while minimizing cleavage of other potential protein and peptide substrates. We find that the S2 and S2′ subsites of t-PA are important determinants of specificity, and occupancy of the S3 subsite is essential for catalysis. t-PA efficiently hydrolyzes a protein substrate which incorporates an optimized substrate sequence within a peptide. This reduction of Km for hydrolysis of the same target sequence within a peptide. This reduction of Km suggests that binding is facilitated by interactions between protein substrate and protease that are distant from the P4-P2′ residues. We use this kinetic data to derive a model in which several distinct mechanisms contribute to the remarkable specificity of t-PA.

Highly specific proteases efficiently hydrolyze target proteins while leaving other proteins intact. To achieve selectivity, these enzymes must possess mechanisms to solve a challenging problem of molecular recognition—the same chemical reaction, peptide bond hydrolysis, must be favored within one protein context while being disfavored in all others. During the evolution of chymotrypsin-like serine proteases such stringent substrate specificity has been achieved, and this evolutionary feat has provided the basis for both the blood-clotting cascade and the fibrinolytic system. Understanding the molecular basis for specificity would facilitate rational design of proteases for the selective hydrolysis of peptide bonds, affording valuable reagents for peptide mapping and the isolation of protein domains. In addition, the ability to direct proteolytic activity to desired targets, and thereby selectively activate or inactivate extracellular proteins, would be likely to have wide ranging therapeutic applications.

Tissue-type plasminogen activator (t-PA) is an attractive model for the study of the evolution of highly specific proteolysis (1). t-PA is a chymotrypsin-like serine protease that initiates the fibrinolytic cascade by cleaving a single bond (Arg560-Val561) in the circulating zymogen plasminogen (Plg). This bond is the only known substrate for t-PA in vivo, a remarkably stringent specificity given that t-PA shares 40% sequence identity with trypsin, a nonspecific protease (2). Part of this specificity is due to the formation of a ternary complex involving t-PA, Plg, and fibrin, which serves to reduce the Km of t-PA for plasminogen by more than 400-fold (2, 3). However, even in the absence of fibrin, t-PA retains stringent specificity for Plg, and this specificity is an inherent property of the protease domain of t-PA (2). A detailed understanding of the mechanisms employed by t-PA to ensure selectivity would provide new insight into the evolution of the endogenous fibrinolytic system, and might suggest effective, knowledge-based strategies for design of novel proteases with unique substrate specificities. In this study we characterize the restriction of t-PA's substrate specificity by enhanced discrimination against suboptimal occupancy of the P4-P2′ subsites. We also show that interactions between t-PA and protein substrates that are distant from the active site play an important role in the binding of substrate. t-PA utilizes these distinct mechanisms concomitantly to efficiently recognize Plg while remaining inert to other potential substrates.

MATERIALS AND METHODS

Enzyme Preparations—Purified t-PA (Activase™) was provided by Genentech (San Francisco, CA). Isolated protease domain of t-PA was prepared as described previously (2). Bovine trypsin was purchased from Sigma. Plasminogen was purchased from American Diagnostica (Greenwich, CT). Enzyme concentrations were determined by titration with 4-methylumbelliferyl p-guanidinobenzoate using a Perkin-Elmer LS 50B luminescence fluorometer (4). Titrations of t-PA were performed in 100 mM NaCl, 20 mM CaCl2, 50 mM Tris-HCl (pH 8.0). Titrations of t-PA were performed in 150 mM NaCl, 10 mM Tris-HCl (pH 7.5).

Kinetics of Cleavage of Synthetic Peptides by Trypsin and t-PA—Substrate peptides were synthesized and purified as described (2). Kinetic data for cleavage of synthetic peptides by bovine trypsin was obtained by incubating each substrate at concentrations ranging from 0.01 mM to 2.0 mM at 37°C in the presence of 1 mM to 1.5 μM trypsin. Trypsin concentrations were held constant for each individual peptide assay and were at least 10-fold lower than the lowest substrate concentration in the assay. Peptide concentrations used to assay t-PA activity ranged from 0.1 mM to 20 mM. The concentration of t-PA for each assay was 640 nM. The buffer used for both trypsin and t-PA assays was as described previously (2). Reactions were stopped by the addition of trifluoroacetic acid to 0.33% after the conversion of between 3 and 20% of substrate to product. Enzymatic cleavage of the peptides

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The abbreviations used are: t-PA, tissue-type plasminogen activator; Plg, plasminogen; ODC, ornithine decarboxylase; BPTI, bovine pancreatic trypsin inhibitor.
was monitored at 220 nm by reverse phase high performance liquid chromatography (Rainin, Woburn, MA) using a microsorb 5-mm 300 Å gradient of 0.1% trifluoroacetic acid in doubly distilled water, and 0.08% trifluoroacetic acid in acetonitrile. The gradient varied from 5 to 15 min depending on the resolution of substrate and product peaks. The percentage of proteolyzed peptide was determined by comparing the calculated area beneath the product peaks to the total area beneath the peptide and uncleaved substrate peaks. Data was interpreted by Eadie-Hofstee analysis, and errors were calculated as described previously (5).

The identity of hydrolyzed peptide fragments was determined by mass spectral analysis.

### Analysis of Peptide Additivity—

Treating the sequence of a 14-amino acid peptide containing the P3-P4′ sequence of plasminogen, YKKGSPGRVGGSKY, and a 15-amino acid peptide containing the best selected substrate from a substrate phage display, GGSGPFSALVPEE, as parent substrates, we calculated the differences in free energy of transition state binding between these sequences and a set of 16 peptides containing 1-, 2-, or 4-amino acid changes from one of the parent peptides. Calculation of these free energy terms was done by fitting $k_{cat}/K_m$ values for the above-mentioned peptides to Equation 1:

$$
\Delta \Delta G = - RT \ln \frac{k_{cat}/K_m}{[\text{Substituent peptide}]} \frac{[\text{Parent peptide}]}{K_m}
$$

(Eq. 1)

as described previously by Wells (6). $\Delta \Delta G$ represents the difference between two substrates in the free energy required to reach the transition state complex during hydrolysis. The degree of substrate interdependence was investigated by plotting the sums of free energy changes for single substitutions against free energy changes for multiple substitutions at the same sites.

### Construction of Protein Substrates for t-PA

The plasmids pOCD29, encoding ornithine decarboxylase (ODC) of Trypanosoma brucei, and plOD11, encoding ODC of Leishmania donovani, were generously provided by Dr. Margaret Phillips (7). These plasmids express fusions of pLOD11, encoding ODC of *L. donovani* (8), with 10 nM trypsin, or t-PA concentrations ranging from 25 nM to 1.3 μM, and five to seven substrate concentrations were used within the range listed above. Proteolytic digests were terminated between 10 and 20% completion by addition of 0.33% trifluoroacetic acid. Samples of each digest were separated on 12% or on 7–14% SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue. After digesting, substrate to proteolytic product ratios were determined by densitometric scanning on a model 300A scanning densitometer from Molecular Dynamics operating with Image Quant 3.0 software. The ratios obtained were used to determine initial velocities of cleavage. The site of cleavage was confirmed by amino-terminal amino acid sequencing of the carboxyl-terminal proteolytic product. $k_{cat}$ and $k_{cat}/K_m$ were derived from Eadie-Hofstee analysis.

### RESULTS AND DISCUSSION

#### Effect of P2 and P2′ Alterations on Catalysis by t-PA

We have previously employed substrate phage display screening to identify peptides that are efficiently hydrolyzed by t-PA and have defined a consensus sequence for the P3, P2, P1, P1, and P2′ substrate residues (10). This consensus was X-G (A)-X-X′ (A-G) where X was usually either a hydrophobic residue or arginine, and X′ was most often arginine, but could be other residues as well. The most labile peptide characterized was GGSGPFSALVPEE, in which PFGRSA was a hexapeptide sequence derived from the phage display screen, and GGSG and LVPEE were flanking sequences derived from the phage display vector. This peptide was hydrolyzed 5000-fold more efficiently than a peptide of similar length derived from the native cleavage site in plasminogen, and an understanding of this enhancement would afford insights into the origins of sequence-specific proteolysis by t-PA.

To begin to gain additional insights into the determinants of specificity of t-PA, we altered the highly conserved P2 and P2′ residues of this peptide and examined the effects on catalysis of the amino acid substitutions. We synthesized two sets of peptides containing sequences GGSGPFXRSALVPEE or GGSGPFGRSLVPEE, where X is Gly, Ala, Ile, Phe, Lys, or Asp. Each peptide was kinetically characterized as a substrate for both trypsin and t-PA (Table I) to determine whether the conservation of residue identity observed in the phage display screen is predictive of optimal substrate occupancy, to quantitate contributions of substrate preferences to selectivity, and to identify properties of substrate residues which account for enhanced discrimination.

### For occupancy of the S2 subsite in t-PA, the order of preferences is Gly > Ala > Lys > Ile > Phe > Asp. The preferences of trypsin (Ala > Gly > Lys > Phe > Ile > Asp) are qualitatively similar to those of t-PA (Table I); both enzymes prefer alanine or glycine, and cleave peptides with negatively charged side chains poorly. Quantitatively, t-PA is more discriminating than trypsin toward nonpreferred residues at this subsite, displaying an 11-fold greater difference between its most efficiently cleaved and least efficiently cleaved substrates. At the S2′ subsite, the order of preferences in subsite occupancy is Ala > Gly > Phe > Ile > Asp > Lys for t-PA, and Ile > Ala > Phe > Lys > Gly > Asp for trypsin. Again, t-PA exhibits greater specificity for its preferred S2′ subsite.

### Table I

| Substrate | Pn, P3, P2, P1 | P1-P2′ P3 Pn′ | kcat | K_m | kcat/K_m | kcat | K_m | kcat/K_m |
|-----------|---------------|---------------|------|-----|---------|------|-----|---------|
| I         | GSSGPFGR     | SALVPEE       | 4.20 | 3500| 1200    | 220  | 110 | 2.0 10^6 |
| II        | GSGDFGR     | SALVPEE       | 2.60 | 3100| 840     | 200  | 58  | 3.4 10^5  |
| III       | GSGDFGR     | SALVPEE       | 0.73 | 1800| 410     | 86   | 76  | 1.1 10^5  |
| IV        | GSGDFGR     | SALVPEE       | 0.17 | 1100| 150     | 130  | 88  | 1.5 10^4  |
| V         | GSGDFGR     | SALVPEE       | 2.10 | 4500| 470     | 98   | 54  | 1.8 10^3  |
| VI        | GSGDFGR     | SALVPEE       | 0.02 | 2900| 5.2     | 39   | 310 | 1.3 10^2  |
| VII       | GSGDFGR     | SALVPEE       | 2.20 | 3200| 690     | 180  | 240 | 7.5 10^5  |
| VIII      | GSGDFGR     | SALVPEE       | 0.51 | 2200| 230     | 240  | 93  | 2.6 10^5  |
| IX        | GSGDFGR     | SALVPEE       | 0.67 | 2300| 290     | 96   | 58  | 1.7 10^4  |
| X         | GSGDFGR     | SALVPEE       | 0.10 | 2000| 50      | 81   | 62  | 1.3 10^4  |
| XI        | GSGDFGR     | SALVPEE       | 0.31 | 2500| 124     | 2.6  | 1700| 1.5 10^4  |

*a Positional nomenclature of subsite (22); † represents site of scissile bond. Errors are less than 30%. Residues that are underlined and in bold face represent substitutions at subsites P2 (I–VI) or P2′ (VII–XI).
discrimination (by 7-fold) against suboptimal interactions at this subsite with the exception of occupation of the subsite by aspartic acid, which reduces the catalytic efficiency of trypsin by 1700-fold while reducing the catalytically efficiency of t-PA only 10-fold. Similar preferences for occupancy of the S2 subtitles of trypsin have been mapped previously by monitoring acyl transfer rates of varied peptide nucleophiles (11, 12).

The greater preference of t-PA for optimal occupancy at the S2 and S2' subtitles relative to trypsin strongly suggests that one mechanism for narrowing the specificity of t-PA is enhanced discrimination against suboptimal subsite occupancy. Kinetic differences among substrates with variations at individual residues are not dramatic but cumulatively become a major barrier to nonselective hydrolysis. The preferences for subsite occupancy correlate well with the frequency of occurrence of residues during the phage display screen (10). For example, glycine was found at P2 three times as often as alanine during the screen, and the peptide containing P2 glycine was cleaved approximately twice as efficiently as the analogous peptide containing alanine.

Disruption of Catalysis by Substrate Binding—The binding of amino acid residues of peptide substrates to subsites on the surface of serine proteases can inhibit peptide bond hydrolysis by interfering with ionic or electrostatic interactions necessary for optimal catalysis. Where such negative effects involve interactions with highly conserved residues critical to catalytic function, the enzyme cannot solve the problem directly by evolving to remove the source of the unfavorable interaction. Such negative interactions therefore present obstacles to catalysis relevant to a whole family of enzymes. In examining P2 and P2' subsite preferences, we have observed effects on catalysis which suggest that negative interactions with catalytically important residues can influence peptide bond hydrolysis by t-PA and trypsin.

A substrate containing aspartic acid at P2 is inefficiently cleaved by both trypsin and t-PA (Table I). Based on the position of the P2 residue in a complex of trypsin and bovine pancreatic trypsin inhibitor (BPTI) (13), a P2 aspartic acid may be within 2.6–3 Å from His57 of the catalytic triad and may interfere with its functions during catalysis. For t-PA, whose catalytic efficiency is expressed entirely as a reduction in kcat, decreased catalysis may result from a direct negative influence on the catalytic machinery. An alternate possibility is that the presence of aspartic acid induces nonproductive binding which subtly alters the positioning of the scissile bond.

Aspartic acid at P2 also reduces catalytic efficiency of both t-PA and trypsin, but the negative effect is 170-fold greater for trypsin (Table I). Modeling of an aspartic acid at the P2' residue in the trypsin-BPTI complex suggests a possible candidate for interaction with its side chain carbonyl may be the backbone amide of Gly193. Since the backbone amide of Gly193 forms part of the oxyanion hole, interaction of P2' with this amide is likely to reduce transition state stabilization. An aspartic acid at the P2' position of substrate may also produce a buried negative charge in the enzyme/substrate complex which trypsin possesses no apparent means of neutralizing. t-PA, by contrast, may be able to at least partially neutralize this charge; modeling based on the BPTI-trypsin complex suggests that a P2' aspartic acid would approach within ±4 Å of arginine 304 of t-PA, a residue which is analogous to tyrosine 39 of trypsin. Neutralization of unfavorable electrostatic interactions that disturb catalytic function or substrate binding may be one way to modulate the specificity of serine proteases. A likely example of a protease employing this mechanism is enteropeptidase (14), which possesses the basic sequence, RRRK, at residues 886–889 (96–99 in chymotrypsin) lining the binding cleft. These positive charges have been proposed to neutralize negatively charged substrate residues and account for the strong selectivity of enteropeptidase for aspartic acid at P4, P3, and P2.

Subsite Occupancy and Catalysis—The binding energy gained through interactions between substrates and proteases at non-S1 substrates can contribute to stabilization of the transition state for peptide bond hydrolysis (15, 16). As a result, hydrolysis of peptide substrates that lack P3, P2, or P2' can be less efficient than hydrolysis of longer substrates that contain these residues. Varying subsite occupancy affords a sensitive probe for enzyme-substrate interactions and allows identification of the position of residues whose absence reduces catalytic efficiency, confirming the importance of occupancy of particular subsites. The requirements for subsite occupancy of t-PA were determined by kinetically assaying the peptide PFGR-SALV, containing residues P4 through P4', and a set of smaller peptides truncated by one or more amino acids at either the amino or carboxyl termini (Table II). For comparison, the kinetics of hydrolysis of these peptides by trypsin were also determined. Both trypsin and t-PA require occupation of subsites spanning S2–S3', as the absence of either P2' or P' yields substrates XV and XIII that are hydrolyzed 7,500–100,000-fold less efficiently than the full-length reference peptide PFGR-SALV. A significant difference is found between the two enzymes in their relative requirement for occupation of their S3 subsites. Peptide XVI, which lacks a P3 residue, is cleaved by trypsin at 1% of maximal efficiency, and by t-PA at 0.026% of its maximal efficiency. This 40-fold difference relative to trypsin may play a significant role in limiting the reactivity of t-PA.
toward protein substrates.

To further probe the subsite occupancy requirements of t-PA and trypsin we assayed the hydrolysis of peptides that were acetylated at the amino terminus and amidated at the carboxyl terminus. These modifications remove potential electrostatic effects on subsite affinity that might be produced by the charged termini of peptides XII–XIX and allow effects of steric occupancy of substrates to be probed more directly. Peptides based on the parent sequence FGRSAL spanning P3 to P3′, P3′, and XX, which contained charged and uncharged termini, respectively, were assayed for hydrolysis by trypsin and t-PA and were observed to yield similar kinetic constants (Table I). Shorter uncharged peptides XXI and XXII which lack P2′ or P3′ residues continued to be cleaved with relatively high efficiency, results that are in sharp contrast to the greatly impaired hydrolysis observed for peptides XIII and XIV. This observation suggests that unfavorable electrostatic interactions within the S2′ or S3′ pockets may prevent efficient catalysis of peptides that contain charged termini. Occupation of these subsites, however, does not appear to be necessary for efficient catalysis, implying that binding energy derived from the S2′ and S3′ pockets does not contribute to lowering the activation barrier for the rate-limiting step for peptide bond hydrolysis, nor is subsite occupation necessary to properly position a substrate.

By contrast to the lack of effect observed from removal of P2′ and P3′ residues, uncharged peptides XXIII and XXIV lacking P3 and P2 residues are hydrolyzed significantly less efficiently by trypsin and t-PA, although the effect is smaller than that observed for charged peptides. Removal of P2 affects trypsin and t-PA to a similar extent, with both enzymes exhibiting approximately 150-fold reduction in catalytic efficiency. The P1 terminal amine of substrate XXIII is within 3.5 Å of Ser195 and Ser214. Acylation of this amine removes a charge which may interfere with catalysis or proper substrate geometry. Analysis of the hydrolysis of peptide XXIV shows that the removal of the P3 residue has a much greater effect on the $K_m$ for t-PA than for trypsin, again suggesting that t-PA is more dependent on occupancy of P3 than is trypsin.

Additivity of Subsite Contributions to Catalysis—We examined the additivity of alterations of substrate residues to gain insight into the relationship between subsite occupancy and stabilization of the transition state (6). We synthesized peptide substrates which differed at one, two, or four positions from reference peptides derived from either the optimal sequence from the substrate phage display screen or from the native cleavage site in PIg. Altered residues were located at positions P4, P3, P2, P1′, and/or P2′. Kinetic analysis of cleavage of these peptide substrates by t-PA and trypsin was performed, and the changes in free energy for binding of the transition state were calculated (Table III). Changes in free energy values observed with peptides containing multiple exchanged residues were plotted versus the sums of the corresponding values for combinations of peptides containing matching single residue exchanges (Fig. 1). These linear plots yielded slopes of 0.76 ± 0.11 (R² = 0.86) for trypsin (Fig. 1B) and of 0.98 ± 0.07 (R² = 0.96) for t-PA (Fig. 1A). The range of ΔGₜ values plotted for t-PA is 2.3-fold broader than that for trypsin due to the much smaller effects of amino acid substitutions on trypsin’s catalytic efficiency relative to t-PA. This is likely to account for most of the difference in the slope of the plots for the two enzymes, and for trypsin’s reduced R² value relative to that for t-PA. The correlation between the effects of single and multiple substitutions suggests that the altered residues do not influence each other significantly upon binding to the enzymes and that they do not produce conformational changes in the proteases which alter

| Substrate | ΔΔGᵥ t-PA | ΔΔGᵥ trypsin |
|-----------|----------|-------------|
| YKKSPPGRVVGGSKY | -0.00 | -0.00 |
| YKPPPGGRVVGGSKY | -0.88 | -0.83 |
| YKKSPPGRVVGGSKY | -1.48 | -1.57 |
| YKKSPPGRVVGGSKY | -2.03 | -1.27 |
| YKFPGRVGGSKY | -1.38 | -0.45 |
| YKFPGRVGGSKY | -3.19 | -1.10 |
| YKFPGRVGGSKY | -3.00 | -1.59 |
| YKFPGRVGGSKY | -4.67 | -1.85 |
| GGGSPGRVGLVPEE | 0.00 | 0.00 |
| GGGSPGRVGLVPEE | 0.31 | 0.14 |
| GGGSPGRVGLVPEE | 1.65 | 0.66 |
| GGGSPGRVGLVPEE | 0.66 | 0.75 |
| GGGSPGRVGLVPEE | 0.62 | -0.03 |
| GGGSPGRVGLVPEE | 0.66 | 0.37 |
| GGGSPGRVGLVPEE | 1.28 | 0.17 |
| GGGSPGRVGLVPEE | 2.64 | 0.75 |
| GGGSPGRVGLVPEE | 3.22 | 1.31 |
| GGGSPGRVGLVPEE | 1.86 | 1.45 |
| GGGSPGRVGLVPEE | 2.44 | 0.71 |
| GGGSPGRVGLVPEE | 1.47 | -0.23 |
| GGGSPGRVGLVPEE | 2.79 | 1.06 |
| GGGSPGRVGLVPEE | 3.07 | 2.37 |

*Parent peptide sequences. Underlined boldfaced residues indicate substitutions from the parent sequence above them. ΔΔGᵥ values were calculated using kinetic data reported in Ding et al. (10) in addition to peptides subsequently analyzed for this work.

other subsites.

The observed additivity is consistent with the possibility that substrate residue side chains contact separate surfaces of the enzyme. This is also consistent with the β-sheet like conformation of substrate residues evaluated here within the three dimensional structures of many serine protease-inhibitor complexes, where side chains alternately extend from the backbone in opposite directions (17). Mutagenesis of residues forming the protein surface and further kinetic analysis will be necessary to fully establish the predictability and independence of changes within non-S1 subsites. True independences of substrate interactions may allow the engineering of unique specificities through sequential mutation of subsites. Manipulation of non-S1 subsites would maintain the S1 site and the catalytic triad intact, thus retaining structural features necessary for efficient catalysis. The retention of efficient catalysis might be combined with the potential for significant diversity of substrate specificity, as alterations of the four sites examined here could theoretically allow as many as 1.3 × 10⁴ distinct specificities.

Proteolysis of Protein Substrates by t-PA—Our initial assays employed peptide substrates because, unlike substrates which contain p-nitroanilide or coumarin-leaving groups, peptides contain amide linkages that do not possess chemically labile aromatic leaving groups. In addition, peptide substrates occupy subsites on both sides of the scissile bond, allowing evaluation of the contribution of S and S′ subsites to catalysis. Peptide substrates are not, however, a substitute for proteinaceous substrates, particularly for enzymes like t-PA that have evolved to discriminate among folded proteins. To investigate the behavior of t-PA toward potential substrate sequences within the context of a folded protein, we inserted a P4-P2′ sequence, PFGRSA, of an efficiently cleaved peptide substrate derived from substrate phage display between a 20 residue amino-terminal extension and the mature form of T. brucei ODC (7) to yield the variant ODC-PFGRSA. For comparison, the native cleavage site within PIg, SPGRVV, was introduced into the same location to yield variant ODC-SPGRVV.
The recognition of PFGRSA as a labile site and a reduction of $K_m$ mediated by enzyme-substrate interactions which are independent of the binding cleft surrounding P1 combined to yield efficient hydrolysis of ODC-PFGRSA. These data demonstrate the importance of distal interactions in the recognition of substrates by t-PA, a feature that may characterize other chymotrypsin-family proteases. Cleavage of proteins also demonstrates the ability to adapt t-PA as a highly selective protease, a property which may prove valuable for peptide mapping and the hydrolysis of protein fusions.

The kinetics of hydrolysis of ODC-PFGRSA were remarkably similar to those reported for activation cleavage of Lys-plasminogen by t-PA (2, 3) (Table IV). ODC-SPGRVV, however, was cleaved with a $k_{cat}$ 2000-fold lower than that for Plg, in spite of the identity of primary sequence with Plg at P4 to P2. This observation suggests that t-PA utilizes productive secondary interactions with Plg which are distinct from those at P4-P2 to overcome unfavorable subsite contacts in the vicinity of the scissile bond and to promote efficient cleavage of Plg. ODC-SPGRVV does not possess the potential for such interactions, so that insertion of SPGRVV into ODC is not sufficient to overcome the extremely low $k_{cat}$ observed for catalysis of peptide substrates containing this P4-P2 sequence. Interactions with fibrin, which lower the apparent $K_m$ of t-PA for Plg by 400-fold also appear to be unique to Plg. No significant effect on catalysis for any ODC variant was observed when fibrin was present, suggesting that the mechanism of fibrin activation does not make a general contribution to the binding and cleavage of proteins, including substrates like ODC-PFGRSA which are as susceptible as Plg to cleavage by t-PA in the absence of fibrin.

At least two explanations could account for the reduction in $K_m$ for the hydrolysis of sequences within proteins relative to the same sequences within peptides. Insertion of these sequences into a protein context may reduce the number of conformations available to the amino acid sequence and thus reduce the entropic cost of binding to the protease. Because a large reduction in $K_m$ is observed for hydrolysis of substrate sequences within a flexible amino-terminal extension which would not be expected to be significantly constrained, however, this argument does not seem compelling. Alternatively, the protein may possess additional sites for interaction with t-PA that are distant from the active site. The ability of t-PA to cleave two entirely unrelated proteins, Plg and ODC, with nearly identical kinetic parameters strongly suggests that at least one determinant of these interactions with t-PA is non-specific. Trypsin has also been observed to exhibit a significant
decrease in $K_m$ for cleavage of protein relative to peptide substrates (2), and the favorable influence of tertiary structure on binding of proteinaceous substrates may be a general characteristic of serine proteases and an essential part of the physiological mechanism of protein cleavage.

The sequence PFGRSA was placed into L. donovani ODC at an internal site at which the wild-type ODC was known to be readily cleaved by a catalytically impaired variant of trypsin, H57A trypsin (8). H57A trypsin hydrolyzes peptide substrates 100-1000-fold less efficiently than does trypsin, which is similar to the 1000-fold difference between t-PA and trypsin. It was surprising, therefore, that the ODC variant containing PFGRSA at the trypsin-accessible internal site was not detectably cleaved by 100 nM t-PA within 32 h of incubation time. Recent studies at the trypsin-accessible internal site was not detectably cleaved by 100 nM t-PA within 32 h of incubation time. Recent studies of sites of trypsin-mediated proteolysis in proteins suggest that, to productively bind substrate, trypsin must be able to disrupt the local structure of 10–12 residues surrounding the cleavage site (18), and comparison of B factors of labile sequences with their solvent accessible surface area suggests that a target sequence's accessibility is more important than its flexibility (19). The inability of t-PA to cleave this substrate suggests that, relative to trypsin, t-PA may require that substrate sequences within proteins be more flexible and achieve greater localized distortions of protein structure to participate in productive binding. The more stringent dependence of t-PA on occupancy of the S3 subsite may be one reason for this requirement, as precise binding of the P3 residue of the substrate protein may be necessary for catalysis. The ability of a protein surface to form favorable interactions with diverse protein partners is reminiscent of the serine protease inhibitor ecotin (20) which inhibits a broad spectrum of proteases by deemphasizing the importance of interactions at the S1 site in favor of extensive complementarity with more distant surface residues. We speculate that surfaces interacting with ecotin from widely variant serine proteases may be conserved to facilitate binding and catalysis of protein substrates.

A Multilevel Mechanism for t-PA Specificity—t-PA utilizes mechanisms which produce negative and positive effects on catalysis to discriminate among potential substrates. Similarly to trypsin, the S1 binding pocket of t-PA recognizes basic residues at P1. Relative to trypsin (21), however, t-PA shows an enhanced preference for arginine over lysine, thus effectively narrowing its P1 specificity (10). Specificity is further restricted by the added importance of substrate occupancy at S3 and through enhanced discrimination among residues occupying the S4–S2 and S1′–S2′ subsites. Since t-PA is generally less active than trypsin, even toward substrates which are optimized for hydrolysis by t-PA, narrowed specificity reduces catalysis of many sequences to levels which are barely detectable. Acting together, these negative mechanisms effectively curtail nonspecific hydrolysis by t-PA relative to trypsin.

The existence of general mechanisms to reduce and restrict catalysis by t-PA suggests that mechanisms must also be present to enhance the specific hydrolysis of Plg. Such positive contributions to peptide bond cleavage seem particularly likely since the P4 to P2′ residues surrounding the labile bond in Plg are apparently not optimized to yield efficient catalysis. t-PA and Plg are therefore likely to interact productively at secondary sites distant from the S4 to S2′ subsites. At least one of these secondary sites may be able to interact with many protein substrates, as they reduce the $K_m$ for cleavage of a protein, ODC, that is unrelated to Plg. These interactions, however, are not sufficient to permit efficient cleavage of the ODC variant containing the native cleavage site from plasminogen. Overcoming the negative effect on catalysis by the P4 to P2′ residues of Plg apparently requires secondary interactions that are specific to t-PA and Plg. The exact nature of these specific interactions is unclear but may involve, (i) conformational changes in t-PA upon binding Plg which enhance its activity, (ii) adoption by the cleavage site in Plg of a conformation that has particularly favorable substrate occupancy and that lowers the energy of the resultant transition state, or (iii) utilization of interactions other than those involving Plg's P4-P2′ residues to achieve a productive interaction in spite of weak or inefficient substrate interactions. Finally, the efficiency of Plg activation by t-PA is specifically enhanced by formation of a ternary complex which includes fibrin and lowers $K_m$ by a factor of 440 (2, 3). Cumulatively, these effects greatly reduce the potential of the trypsin-like protease domain of t-PA for proteolysis of virtually all proteins and then effectively restore efficient proteolysis of one selected substrate. As the exact molecular details of this impressive natural engineering become more clear, the ability to rationally design proteases combining novel specificities with high activity will be greatly enhanced.

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