Optimal Subsite Occupancy and Design of a Selective Inhibitor of Urokinase*

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Human urokinase type plasminogen activator (u-PA) is a member of the chymotrypsin family of serine proteases that can play important roles in both health and disease. We have used substrate phage display techniques to characterize the specificity of this enzyme in detail and to identify peptides that are cleaved 840–5300 times more efficiently by u-PA than peptides containing the physiological target sequence of the enzyme. In addition, unlike peptides containing the physiological target sequence, the peptide substrates selected in this study were cleaved as much as 120 times more efficiently by u-PA than by tissue type plasminogen activator (t-PA), an intimately related enzyme. Analysis of the selected peptide substrates strongly suggested that the primary sequence SGRSA, from position P3 to P2, represents optimal subsite occupancy for substrates of u-PA. Insights gained in these investigations were used to design a variant of plasminogen activator inhibitor type 1, the primary physiological inhibitor of both u-PA and t-PA, that inhibited u-PA approximately 70 times more rapidly than it inhibited t-PA. These observations provide a solid foundation for the design of highly selective, high affinity inhibitors of u-PA and, consequently, may facilitate the development of novel therapeutic agents to inhibit the initiation and/or progression of selected human tumors.

Local activation and aggregation of platelets, followed by initiation of the blood coagulation cascade, assure that a fibrin clot will form rapidly in response to vascular injury (1). The presence of this thrombus, however, must be transient if the damaged tissue is to be remodeled and normal blood flow restored. The fibrinolytic system, which accomplishes the enzymatic degradation of fibrin, is therefore an essential component of the hemostatic system (1). The ultimate product of the fibrinolytic system is plasmin, a chymotrypsin family enzyme with relatively broad, trypsin-like primary specificity that is directly responsible for the efficient degradation of a fibrin clot (2). Production of this mature proteolytic enzyme from the inactive precursor, or zymogen, plasminogen is the rate-limiting step in the fibrinolytic cascade (2, 3). Catalysis of this key regulatory reaction is tightly controlled in vivo and is mediated by two enzymes present in human plasma, u-PA1 and t-PA (3–6).

u-PA and t-PA are very closely related members of the chymotrypsin gene family. These two proteases possess extremely high structural similarity (7, 8), share the same primary physiological substrate (plasminogen) and inhibitor (plasminogen activator inhibitor, type 1) (3), and, unlike plasmin, exhibit remarkably stringent substrate specificity (9–11). Despite their striking similarities, the physiological roles of t-PA and u-PA are distinct (5, 6), and many studies (5, 6, 12–18) suggest selective inhibition of either enzyme might have beneficial therapeutic effects. Mice lacking t-PA, for example, are resistant to specific excitotoxins that cause extensive neurodegeneration in wild type mice (13), and mice lacking u-PA exhibit defects in the proliferation and/or migration of smooth muscle cells in a model of restenosis following vascular injury (5, 6).

A large body of experimental evidence from studies involving both model systems and human patients suggests that u-PA may play an important role in tumor biology and provides a compelling rationale to pursue the development of u-PA inhibitors. For example, anti-u-PA antibodies inhibit metastasis of HEp3 human carcinoma cells to chick embryo lymph nodes, heart, and lung (19), and similar studies demonstrated that these antibodies inhibit lung metastasis in mice following injection of B16 melanoma cells into the tail vein (20). Anti-u-PA antibodies also inhibit both local invasiveness and lung metastasis in nude mice bearing subcutaneous MDA-MB-231 breast carcinoma tumors (21). In addition, a recent study indicated that u-PA-deficient mice are resistant to the induction and/or progression of several tumor types in a two-stage, chemical carcinogenesis model (18). Finally, high levels of tumor-associated u-PA correlate strongly with both a shortened disease-free interval and poor survival in several different human cancers (22–24).

Because mice lacking either u-PA or t-PA do not develop thrombotic disorders, selective inhibition of either of these two enzymes seems unlikely to create thrombotic complications in vivo. On the other hand, mice lacking both u-PA and t-PA suffer severe thrombosis in many organs and tissues, resulting in a significantly reduced life expectancy (5, 6). Nonselective inhibition of these two enzymes, therefore, seems almost certain to produce catastrophic consequences in the clinical setting. Consequently, significant interest exists in the development of inhibitors that are stringently specific for either t-PA or u-PA.

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The abbreviations used are: u-PA, urokinase type plasminogen activator; t-PA, tissue type plasminogen activator; FTC, fAFF1-tether C; HPLC, high pressure liquid chromatography; PAI-1, plasminogen activator inhibitor type 1.
or u-PA, which are expected to facilitate a detailed investigation of the precise roles of the two enzymes in several important pathological processes and may aid the development of novel therapeutic agents to combat these processes. Rational design of these selective inhibitors is greatly complicated, however, by the absence of obvious "lead compounds"; both their primary physiological substrate and inhibitors fail to discriminate between the two closely related proteases.

We have used substrate phage display (25, 26) to elucidate optimal subsite occupancy of u-PA. Peptide substrates that match the consensus sequence for substrates of u-PA derived from these studies are cleaved by u-PA 840–5300 times more efficiently than control peptides containing the physiological target sequence present in plasminogen. In addition, unlike the plasminogen-derived control peptides, the selected peptides exhibit substantial selectivity for cleavage by u-PA versus t-PA.

Information gained in these investigations was used to augment the u-PA/t-PA selectivity of PAI-1, the physiological inhibitor of both t-PA and u-PA (27, 28); suggests potential lead compounds for the design of selective, small molecule inhibitors of u-PA; and provides new insights into the divergent evolution of molecular recognition by intimately related enzymes.

MATERIALS AND METHODS

Reagents—Competent MC1061 (F') Escherichia coli and nitrocellulose were purchased from Bio-Rad Laboratories. Pansorbin (Protein A-bearing Staphylococcus aureus) cells were obtained from Calbiochem. K91 (F') and MC1061 (F') strains of E. coli were provided by Steve Cwirla (Affymax). mAb 3-E7 was purchased from Gramsch Laboratories (Schwabhausen, FRG). u-PA was obtained from Jack Henkin (Abbott Laboratories).

Construction of the phage vector fAFF1-tether C (fTC) and the random hexapeptide library fAFF-TC-LIB has been previously described (29). Control substrate phage fTC-PL, which contained the physiological target sequence for u-PA and t-PA, was constructed by hybridizing the single-stranded oligonucleotides 5'-TCGAGCCGGTGATTCGGTA-CTGGTGGATGTCTGACGTGC-3' and 5'-CGCCACCTAGG-CCAGGACCCAGACAAACCAACAGAC-3' and then ligating the annealed, double-stranded products into the Xhol/KpnI-cut vector fTC. All constructs were first transformed into MC1061 by electroporation and then transferred to K91.

Measurement of Enzyme Concentrations—Concentrations of functional t-PA and u-PA were measured by active site titration with 4-methylumbelliferyl p-guanidinobenzoate (29) using a Perkin-Elmer LS 50B Luminescence Fluorometer as described previously (9, 30). In addition, the enzymes were titrated with a standard PAI-1 preparation that had been previously titrated against a trypsin primary standard. Total enzyme concentrations were measured by enzyme-linked immunosorbent assay.

Phage Selection Using u-PA—Substrate phage display was originally developed by Matthews and Wells (25) using monovalent phage, and an adsorbed, double-stranded product into the Xhol/KpnI-cut vector fTC. All constructs were first transformed into MC1061 by electroporation and then transferred to K91.

Measurement of Enzyme Concentrations—Concentrations of functional t-PA and u-PA were measured by active site titration with 4-methylumbelliferyl p-guanidinobenzoate (29) using a Perkin-Elmer LS 50B Luminescence Fluorometer as described previously (9, 30). In addition, the enzymes were titrated with a standard PAI-1 preparation that had been previously titrated against a trypsin primary standard. Total enzyme concentrations were measured by enzyme-linked immunosorbent assay.

Phage Selection Using u-PA—Substrate phage display was originally developed by Matthews and Wells (25) using monovalent phage, and an alternative method that used multivalent phage was reported later by Smith and Navre (26). Multivalent substrate phage were screened with u-PA using reaction conditions identical to those previously reported for t-PA (31) except that digestion of the phage was performed using the dideoxy method.

Kinetics of Cleavage of Synthetic Peptides by t-PA and u-PA—Peptides were synthesized and purified as described (9). Kinetic data were obtained by incubating various concentrations of peptide with a constant enzyme concentration to achieve between 5 and 20% cleavage of the peptide in each reaction. For assays with u-PA, enzyme concentration was either 815 or 635 nM. For assays with t-PA enzyme, concentration was 700 nM. Peptide concentrations were chosen where possible to surround K_m and in all cases were between 0.5 and 32 mM. The buffer used in these assays has been described (9). Reactions were stored by the addition of trifluoroacetic acid to 0.33% or by freezing on dry ice. Cleavage of the 13- and 14-residue peptides was monitored by reverse phase HPLC as described (9). The 4–6-residue peptides were acetylated at their amino termini and amidated at their carboxyl termini. Cleavage of the 4–6-residue peptides was monitored by hydrophilic interaction HPLC chromatography (32) using a polyhydroxyaspartamine column from PolyLC (Columbia, MD). Buffer A was 50 mM triethylamine phosphate in 10% acetonitrile, and buffer B was 10 mM triethylamine phosphate in 80% acetonitrile. Peptides were eluted by a gradient that was varied from 100 buffer B to 100% buffer A during a 15-min interval. The percentage of cleaved peptide was calculated by dividing the area under the product peaks by the total area under substrate and product peaks. For all peptides containing multiple basic residues, mass spectral analysis of products confirmed that cleavage occurred at a single site and identified the scissile bond. Data were interpreted by Edie-Hofstee analysis. Errors were determined as described (33) and were <25%.

Site-directed Mutagenesis and Construction of an Expression Vector Encoding a Recombinant Variant of PAI-1—The expression vector pPAI7THS was derived from the plasmid pBR322 and contained a full-length cDNA encoding human PAI-1 that was transcribed from a T7 gene 10 promoter (34). The 300-base pair SalI-BamHI fragment of human PAI-1 was subcloned from pPAI7THS into bacteriophage M13mp18. Single-stranded DNA produced by the recombinant M13mp18 constructs was used as a template for site-specific mutagenesis according to the method of Zoller and Smith (35) as modified by Kunkel (36). The mutagenic oligonucleotide had the sequence 5'-CCACAGCTGTCTAGCCGGCGAAACAGCCCCGAGGAGA-TG-3'. Following mutagenesis, single-stranded DNA corresponding to the entire 300-base pair SalI-BamHI fragment was fully sequenced to ensure the presence of the desired mutations and the absence of any additional mutagenesis. The 300-base pair SalI-BamHI double-stranded DNA fragment from the mutated, replicative form DNA was used to replace the corresponding fragment in pPAI7THS to yield a full-length cDNA encoding PAI-1/U1K1, which contained the amino acid sequence GSGKSA from the P4 to P2' position of the reactive center loop.

Expression and Purification of Recombinant Wild Type PAI-1 and the Variant PAI-1/UK1—Expression of wild type and the mutated variant of PAI-1 was accomplished in E. coli strain BL21(DE3)pLysE (Novagen), which synthesizes T7 RNA polymerase in the presence of isopropyl-1-thio-β-D-galactopyranoside. Bacterial cultures were grown at 37 °C with vigorous shaking to an A_600 of 0.9-1.1, and isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM to induce the synthesis of T7 RNA polymerase and the production of PAI-1 proteins. Cultures were grown for an additional 1-2 h at 37 °C and then shifted to 30 °C for 2–6 h.

Cells were pelleted by centrifugation at 8000 × g for 20 min at 4 °C and resuspended in 40 ml of cold start buffer (20 mM sodium acetate, 200 mM NaCl and 0.1% Tween 20, pH 5.6). The cell suspension was disrupted in a French pressure cell (Aminoce), and cellular debris was removed by ultracentrifugation for 25 min at 320,000 × g.

Purification of soluble, active PAI-1 was performed as described previously (37). PAI-1 containing supernatants were injected onto an XK-26 column (Pharmacia Biotech Inc.) packed with CM-50 Sephadex (Pharmacia). The column was washed with 5 column volumes of start buffer (20 mM sodium acetate, 200 mM NaCl, and 0.01% Tween 20, pH 5.6), and PAI-1 proteins were eluted using a 0.2–1 g linear gradient of NaCl in the same buffer. Peak fractions were collected, pooled, and concentrated using a COULAC 30 concentrator (Amicon). Purified preparations were analyzed by activity measurements using standard, direct assays of t-PA, SDS-polyacrylamide gel electrophoresis, and measurement of optical density at 280 nm.

Measurement of Active PAI-1 in Purified Preparations—A primary standard of trypsin was prepared by active site titration using p-nitrophenyl guanidinoacetate HCl as described previously (38). Concentra-
Data were analyzed by plotting the reciprocal of the residual enzyme PAI-1 antibody, plasminogen, and Spec PL to the reaction mixture, which PAI-1 was added after preincubation and the addition of anti-standard, indirect chromogenic assay. These indirect, chromogenic assays were performed using a clearing anti-PAI-1 antibody (generously provided by Dr. David Loing et al.). Following preincubation, the mixtures were quenched with an excess of neutralizing anti-PAI-1 antibody (generously provided by Dr. David Loing et al.) and residual enzymatic activity was measured using a pseudo-first order reaction. The residual enzymatic activity varied between 20 and 80% of the initial activity. Reaction conditions and data analysis for pseudo-first order reactions were as described previously (40–43).

Second order rate constants (k<sub>i</sub>) were determined for inhibition of t-PA or u-PA were determined using pseudo-first order reactions. These second order rate constants were determined by titration of standardized trypsin as described by Olson et al. (39) and by titration of standardized t-PA preparations.

Kinetic Analysis of the Inhibition of t-PA and u-PA by Recombinant PAI-1 and PAI-1/UKI—Second order rate constants (k<sub>i</sub>) for inhibition of t-PA or u-PA were determined using pseudo-first order (k<sub>i</sub> < 2 × 10<sup>4</sup>) or second order (k<sub>i</sub> > 2 × 10<sup>6</sup>) conditions. For each reaction, 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. Reaction conditions and data analysis for pseudo-first order reactions were as described previously (40–43).

Optimal Subsite Occupancy for u-PA—For second order reactions, equimolar concentrations of u-PA and PAI-1 were mixed directly in microtiter plate wells and preincubated at room temperature for periods of time varying from 0 to 30 min. Following preincubation, the mixtures were quenched with an excess of neutralizing anti-PAI-1 antibody (generously provided by Dr. David Loing et al.), and residual enzymatic activity was measured using a standard, indirect chromogenic assay. These indirect, chromogenic assays were compared with control reactions containing no PAI-1 or to which PAI-1 was added after preincubation and the addition of anti-PAI-1 antibody, plasminogen, and Spec PL to the reaction mixture. Data were analyzed by plotting the reciprocal of the residual enzyme concentration versus the time of preincubation.

RESULTS

Construction and Use of Substrate Phage Libraries—A polyvalent fd phage library that displayed random hexapeptide sequences and contained 2 × 10<sup>8</sup> independent recombinants was prepared (25, 31). Each member of this library displayed an N-terminal extension from phage coat protein III that contained a randomized region of six amino acids, a six-residue linker sequence (SSGGSG), and the epitopes for mAbs 179 and 3-E7. Because u-PA did not digest the phage coat protein III sequence, the antibody epitopes, or the flexible linker sequence, the loss of antibody epitopes from the phage surface upon incubation with u-PA required cleavage of the randomized peptide insert. Incubation of the library with u-PA, followed by removal of phage retaining the antibody epitopes, therefore, accomplished a large enrichment of phage clones whose random hexamer sequence could be cleaved by u-PA.

Analysis of Selected Phage Clones and Identification of a Consensus Sequence—Following five rounds of selection to enrich and amplify phage that display sequences that are readily cleaved by u-PA, 100 phage clones were identified as u-PA substrates. DNA sequencing of these clones revealed the presence of 91 distinct hexamer sequences among the selected phage (Table I). As expected from the trypsin-like primary specificity of u-PA, each hexamer contained at least one basic residue, and 89 of the 91 hexamer sequences contained at least one arginine residue. 35 of the 91 substrate phage contained a single basic residue, and in 33 of these 35 cases the single basic residue was an arginine.

### Table I

| Clone number | Amino acid sequence          | Clone number | Amino acid sequence          |
|--------------|------------------------------|--------------|------------------------------|
| 1            | S G R A R Q                  | 47           | (S G) R K V P G S           |
| 2            | S K G S G R S (L)            | 48           | (S G) R K W I S G           |
| 3            | G S R N A D                  | 49           | (S G) R L A T K A           |
| 4            | T A R L R G                  | 50           | (S G) R M R K L D N         |
| 5            | T A R S D N                  | 51           | (S G) R N A Q V R           |
| 6            | T S R M G T                  | 52           | (S G) R N A V E P           |
| 7            | T S R Q A Q                  | 53           | (S G) R N D R L N           |
| 8            | T T R R N K                  | 54           | (S G) R N G K S R           |
| 9            | T T S R R S                  | 55           | (S G) R N M P L L           |
| 10           | W G G R S G                  | 56           | (S G) R N T G S H           |
| 11           | A I K R S A                  | 57           | (S G) R R M T M G           |
| 12           | G G G R G R G N R            | 58           | (S G) R R R L N M           |
| 13           | G G G R S V N N              | 59           | (S G) R R T L D F           |
| 14           | H T R R M K                  | 60           | (S G) R A V S N             |
| 15           | I S T A R M (L)              | 61           | (S G) R S A K V D           |
| 16           | (S G) K A A D V T            | 62           | (S G) R S A N A I           |
| 17           | K K R T N D                  | 63           | (S G) R S A T R D           |
| 18           | K M S A R (L)                | 64           | (S G) R S A V K             |
| 19           | (G K) R K D V A              | 65           | (S G) R S D Q P L           |
| 20           | (G K) R V S K N              | 66           | (S G) R S D N P N           |
| 21           | (S G) K S A D A A            | 67           | (S G) R S E R S L           |
| 22           | (S G) R A A M V              | 68           | (S G) R S G D P G           |
| 23           | (S G) R A G N I R            | 69           | (S G) R S G N T T           |
| 24           | (S G) R A H R D N            | 70           | (S G) R S G N M G           |
| 25           | (S G) R A R D D R            | 71           | (S G) R S N G V G           |
| 26           | (S G) R A R H M V            | 72           | (S G) R S P D G M           |
| 27           | (S G) R A R S P R            | 73           | (S G) R S R R L P           |
| 28           | (S G) R A V G H Q            | 74           | (S G) R S R V T S           |
| 29           | (S G) R A V V D S            | 75           | (S G) R S S H S             |
| 30           | (S G) R G G K G P            | 76           | (S G) R S S S O A A         |
| 31           | (S G) R G R S A V            | 77           | (S G) R S S S S H           |
| 32           | (S G) R G V D M N            | 78           | (S G) R S S S S T V         |
| 33           | (S G) R G V R M H            | 79           | (S G) R S T D L G           |
| 34           | (S G) R H R S D I            | 80           | (S G) R S T N V E           |
| 35           | (S G) R K G Q G G            | 81           | (S G) R S T R H K           |
| 36           | (S G) R K L H M N            | 82           | (S G) R S T Y N S           |
| 37           | (S G) R K M D M G            | 83           | (S G) R T S P S T           |
| 38           | (S G) R K M D R S            | 84           | (S G) R T S V N L           |
| 39           | (S G) R K M R G G            | 85           | S K R A S I                 |
| 40           | (S G) R K N Q R V            | 86           | S Q T C V R (L V)           |
| 41           | (S G) R K Q R D S            | 87           | T E R R V R (L V)           |
| 42           | (S G) R K R V G A            | 88           | T Q R S T G                 |
| 43           | (S G) R K S K V V            | 89           | T R R D R I                 |
| 44           | (S G) R K S T S S            | 90           | V A R N Y K                 |
| 45           | (S G) R K V G S L            | 91           | V S R R N M                 |
| 46           | (G G) K A S L S              |              |                             |
residue was an arginine. An additional 22 phage contained two basic residues but only a single arginine. Alignment and analysis of these hexamer sequences suggested that the consensus sequence for optimal subsite occupancy for substrates of u-PA, from P3 to P2', was SGR/S > R,K,A,I,X, where X represents a variety of amino acid residues but was most often alanine, glycine, serine, valine, or arginine.

Analysis of these data was complicated by the fact that approximately 72% of the selected substrate phage contained an arginine in the first position of the randomized hexamer and therefore utilized the amino-terminal flanking residues, Ser-Gly, to occupy the P3 and P2 subsites. While these results left no doubt that the P3–P1 SGR sequence created by the fusion was a very favorable recognition site for u-PA, this use of flanking residues necessitated a particularly careful examination of the P3 and P2 preferences of u-PA. Consequently, we altered our experimental protocol in two ways to address this issue. First, we isolated an unusually large collection of substrate phage (91 distinct substrates) to ensure that a reasonable number of these (23) would not utilize the flanking Ser-Gly to fill the P3 and P2 subsites. This allowed a meaningful comparison of the consensus sequence derived from the entire library with that derived from the non-fusion phage and the demonstration of good agreement between the two consensus sequences. Second, we performed a previously described dot blot analysis (26, 31) of the digestion of all 100 substrate phage by u-PA using a wide variety of stringencies of digestion. Although this semiquantitative assay cannot provide kinetic constants, it can provide an accurate rank ordering of the lability of the substrate phage clones.

Under the most stringent conditions examined, 11 of the 100 substrate phage, containing eight distinct randomized hexamer sequences, proved to be particularly labile u-PA substrates (Table II). All eight of the most labile substrate phage contained the P3–P1 SGR motif, demonstrating that this sequence is, in fact, a more labile u-PA site than related, selected substrates. Consequently, to test these hypotheses, we synthesized variants of the most labile u-PA-selective substrate (peptide II) that contained mutations in the P3 and/or P4 positions.

**Table II**

| Clone number | P3 | P2 | P1 | P1' | P2' | P3' | P4 | P5' |
|--------------|----|----|----|-----|-----|-----|----|----|
| 46           | (S) | G  | R  | K   | A   | S   | L  | S  |
| 51           | (S) | G  | R  | N   | A   | Q   | V  | R  |
| 60           | (S) | G  | R  | S   | R   | A   | V  | S  |
| 61           | (S) | G  | R  | S   | R   | A   | K  | V  |
| 62           | (S) | G  | R  | S   | A   | N   | A  | I  |
| 63           | (S) | G  | R  | S   | A   | T   | R  | D  |
| 64           | (S) | G  | R  | S   | A   | V   | V  | K  |
| 77           | (S) | G  | R  | S   | S   | S   | S  | H  |

**Optimal Subsite Occupancy for u-PA**

Recent investigations that explored optimal subsite occupancy for substrates of t-PA suggested that the P3 residue was the primary determinant of the ability of a substrate to discriminate between t-PA and u-PA and that this selectivity could be enhanced modestly by appropriate occupancy of P4 by guest on July 25, 2018http://www.jbc.org/Downloaded from
a tyrosine, and in peptide XI the P3 serine was replaced by arginine. As expected, these mutations substantially decreased the u-PA/t-PA selectivity of the peptide by a factor of 330 or 360, respectively, and actually converted the peptide into a t-PA-selective substrate. Moreover, mutation of both the P3 serine and P4 glycine of the most labile u-PA substrate to arginine and glutamine, respectively (peptide XII), decreased the u-PA/t-PA selectivity by a factor of 330 or 740, respectively. These data confirm the proposed status of the P3 and P4 residues as specificity determinants for substrates of t-PA and u-PA and suggest a particularly prominent role of the P3 residue in this capacity.

Design and Characterization of a Variant of PAI-1 That Is Selective for u-PA—To test the prediction that information gained from the study of peptide substrates could facilitate the design of selective, high affinity inhibitors of urokinase, we sought to augment the u-PA/t-PA selectivity of the serpin PAI-1, the primary physiological inhibitor of both t-PA and u-PA. We used oligonucleotide-directed, site-specific mutagenesis to construct a variant of PAI-1 that contained the primary cleavage sequence from plasminogen. As anticipated, the mutated serpin possessed a u-PA/t-PA selectivity that was approximately 7-fold greater than that of wild type PAI-1. Moreover, this similarity calls into question the hypothesis that highly selective inhibitors can be generated, since the specificities of the two enzymes appear so similar. We find, however, both in this study and in a previous study aimed at the design of t-PA selective substrates (11), that there are subtle but significant differences in optimal subsite occupancy between the two enzymes, and these distinctions can be elucidated by substrate phage display protocols.

Sequences Selected for Optimal Cleavage Do Not Resemble the Physiological Target Sequence—A key observation of this study is that the primary sequence SGRSA, from the P3–P2' positions of a peptide substrate, affords highly labile subsite occupancy for urokinase. This sequence differs at P3, P1', and P2' from the target sequence found in plasminogen (PGRVV) and is cleaved by u-PA greater than 5300 times more efficiently. This major discrepancy, in both primary sequence and lability, of the physiological target sequence and the consensus sequence derived using substrate phage display protocols suggests that a physiological target sequence is not necessarily a reasonable lead compound for the design of specific, small molecule substrates or inhibitors of highly selective serine proteases.

A major contribution to the discrepancy between the physiological and consensus target sequences of u-PA almost certainly arises from the highly conserved mechanism ofzymogen activation of chymotrypsin family enzymes (45). Following activation cleavage of a chymotrypsinogen-like zymogen, the P1' and P2' residues insert into the activation pocket, where they form a number of conserved hydrophobic interactions as well as a new, buried salt bridge with the aspartic acid residue adjacent to the active site serine (45–47). Because these interactions substantially stabilize the active conformation of the mature enzyme, this key role after activation cleavage places severe functional constraints on the P1' and P2' residues of a chymotrypsinogen-like zymogen and consequently prevents the two residues from evolving simply to interact optimally with the activating enzyme. Consistent with this hypothesis, the consensus and physiological target sequences for u-PA agree well on the unprimed side of the scissile bond; however, the two target sequences diverge dramatically at the P1' and P2' subsites.

Additional factors are also likely to contribute to the observed discrepancy between the consensus and physiological
target sequences for u-PA. For example, modeling studies reported by Lamba, Huber, Bode and co-workers (8) suggest the S1′ and/or the S2′ pockets utilized by u-PA when hydrolyzing plasminogen may actually differ from those used when hydrolyzing peptide substrates. Moreover, as the enzyme diverged from a trypsin-like precursor, u-PA may have evolved a strong dependence for efficient catalysis upon productive interactions with substrates at secondary sites that diminished the contribution of optimal interactions with primary substrates in the active site cleft. Although the location, role, and even the existence of such secondary contacts between u-PA and plasminogen remain obscure at the present time, previous studies of the interaction of u-PA and t-PA with PAI-1 have demonstrated very clearly that these two enzymes are capable of using specific, secondary contacts efficiently both to enhance selectivity and to dampen the influence of optimal primary substrate interactions (42, 48–50). Although the reactive center loop of PAI-1 has evolved to match optimal substrate occupancy for urokinase very closely, in the absence of productive contact with a single, strong secondary site of interaction between the two proteins, PAI-1 becomes a poor inhibitor of u-PA (50).

Implications Regarding the Possibility of Additional Physiological Substrates for u-PA—The identification of synthetic peptides that are cleaved up to 120 times more efficiently by u-PA than by t-PA raises the possibility that similar u-PA-selective (or t-PA-selective) physiological substrates may exist that are currently not appreciated. Differences in the phenotypes exhibited by mice lacking either of the two enzymes are consistent with this possibility (5, 6). This issue remains uncertain, however, because selective expression of t-PA or u-PA in particular microenvironments could also account for these distinct phenotypes.

Importance of the P3 Residue in Discriminating between u-PA and t-PA—By demonstrating that mutation of the P3 residue alone could alter the relative u-PA/t-PA selectivity of a peptide substrate by a factor of greater than 300 (Table III), this study provided strong support for the hypothesis that the P3 residue was the primary determinant of the ability of a substrate to discriminate between u-PA and t-PA. We have previously reported that occupancy of P3 by arginine or large aromatic or hydrophobic residues favored cleavage by t-PA (11), and this investigation showed that a P3 serine residue favored cleavage by u-PA. In addition, this study demonstrated that more modest alterations of specificity could be achieved by selective occupancy of the P4 and P1 substrates. These data indicated that PAI-1, which contains a P3 serine, has evolved to match optimal substrate occupancy of u-PA more closely than that of t-PA. This observation may explain why PAI-1 inhibits u-PA more rapidly than it inhibits t-PA (Table IV) and suggests that, during the evolution of the fibrinolytic system, there may have been a greater need to suppress the activity of u-PA in the circulation than to regulate t-PA activity. Consistent with this hypothesis, the circulating single chain form of u-PA is a true zymogen, while t-PA is secreted into the circulation as an active, single chain enzyme.

**Substrate Phage Display Can Aid Inhibitor Design**—Another implication of these studies is that information gained from the application of substrate phage display libraries can lead directly to the design of specific inhibitors. Although hydrolysis of the selective, small peptide substrates by u-PA is characterized by $K_{\text{cat}}$ values in the 0.6–3 mM range, it has been routinely observed that the introduction of a transition state bond geometry adjacent to the P1 residue of a protease substrate can create either a reversible inhibitor whose affinity for the target protease is enhanced by 3–6 orders of magnitude or an irreversible inhibitor with an impressive second order rate constant for inhibition of the target protease ($>10^9 \text{ M}^{-1} \text{s}^{-1}$) for a review, see Ref. 51). Similar results using the substrates identified in this study would create highly selective, small molecule u-PA inhibitors, with affinities in the low nanomolar range, that might be further improved by subsequent, systematic chemical modification.

**Conclusion**—The ability to identify subtle but significant specificity differences between enzymes that share the same physiological substrates and inhibitors, as demonstrated in this study, is a fundamental challenge both for basic enzymology and rational drug design. Advances in this area will significantly enhance understanding of the molecular determinants and mechanisms of specific catalysis and may facilitate the design of highly selective and therapeutically valuable new enzymes.

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