The recent structure determination of the catalytic domain of tissue-type plasminogen activator (tPA) suggested that residue Arg^{174} could play a role in P3/P4 substrate specificity. Six synthetic chromogenic tPA substrates of the type R-Xaa-Gly-Arg-p-nitroanilide, in which R is an N-terminal protection group, were synthesized to test this property. Although changing the residue Xaa (in its i or d form) at position P3 from the hydrophobic Phe to an acidic residue, Asp or Glu, gave no improvement in catalytic efficiency, comparative analysis of the substrates indicated a preference for an acidic substituent occupying the S3 site when the S4 site contains a hydrophobic or basic moiety. The 2.9 Å structure determination of the catalytic domain of human tPA in complex with the bis-benzamidine inhibitor 2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one reveals a three-site interaction, salt bridge formation of the proximal amidino group of the inhibitor with Asp^{180} in the primary specificity pocket, extensive hydrophobic surface burial, and a weak electrostatic interaction between the distal amidino group of the inhibitor and two carbonyl oxygens of the protein. The latter position was previously occupied by the guanidino group of Arg^{174}, which swings out to form the western edge of the S3 pocket. These data suggest that the side chain of Arg^{174} is flexible, and does not play a major role in the S4 specificity of tPA. On the other hand, this residue would modulate S3 specificity, and may be exploited to fine tune the specificity and selectivity of tPA substrates and inhibitors.

The 60-kDa multidomain tissue-type plasminogen activator (tPA) catalyzes the conversion of the zymogen plasminogen into the active enzyme plasmin, the rate-limiting step in the endogenous fibrinolytic cascade (1). Recombinant tPA is used therapeutically as a fibrinolytic agent in the treatment of acute myocardial infarction and pulmonary embolism. Its fibrin dependent activity has attracted particular interest as it allows targeting of enzymatic activity to its natural substrate plasminogen (2, 3).

The recent structure determination of the catalytic domain of tPA (4) in complex with benzamidine (hereafter termed b-tPA) revealed a strong structural similarity to other trypsin-like serine proteases. 187 residues of tPA have topological equivalents in α-chymotrypsin, forming the basis for the chymotrypsinogen numbering of the tPA catalytic domain used in this report (4). Compared with chymotrypsin, tPA contains a deletion of three residues at the C terminus of the A-chain, two single residue deletions in the B-chain, and insertions at six different positions totaling 24 residues. Five of these insertions are noteworthy; one forms a helix, the “intermediate helix” (Pro^{165}–Leu^{171}), and four form surface loops, referred to as the 37-loop (Lys^{36}–Arg^{39}), 60-loop (Phe^{59}–His^{62}), 110-loop (Ser^{110}–Cys^{113}), and 186-loop (Asp^{185}–Ala^{186}) (see Fig. 1). Most of these surface loops cluster around the active site cleft and are involved in specific interactions of the molecule with its substrates and inhibitors.

tPA exhibits remarkable specificity, a single bond of plasminogen (Arg^{560}–Val^{561}) is the only known substrate cleavage site of tPA in vivo. While the role of the specificity pocket S1 of tPA (and trypsin-like serine proteinases in general) in determining its preference for P1 Arg residues is known, both the location and mechanism of additional specificity determinants remain uncertain (5). In contrast to its high in vivo activity and specificity toward plasminogen, tPA shows low activity toward small peptides, peptide substrates modelled according to the cleavage sequence of plasminogen are cleaved by trypsin with much higher activity (>10^4-fold) (5). Optimization of the most labile peptide using phage display (6) emphasized the limited reactivity of tPA compared with trypsin and its high sensitivity to changes in residues at distinct positions. This suggests that tPA recognizes complex or multiple elements on the surface of plasminogen distant from the cleavage site. This might not only represent an increased binding affinity, but also a subtle alteration of the catalytic machinery of the protease domain of tPA. On the other hand, tPA is able to cleave the more labile
amide bond of \( p \)-nitroanilide substrates, for example MeSO\(_2\)-D-HHT-Gly-Arg-pNA (spectrozyme tPA) and Boc-Leu-Gly-Arg-pNA with a catalytic efficiency (\( k_{\text{cat}}/K_m \)) only 6.5–18-fold lower than that of trypsin (5).

Few protein or synthetic tPA-specific inhibitors are known. Plasminogenolytic activity in vivo is controlled by the highly specific and fast acting serpin PAI-1 (7). Structural aspects of the interaction of PAI-1 and tPA have been described elsewhere (4, 8–11). One of the few non-serpin proteinase inhibitors that block tPA with high affinity is the \( E \)\( \text{rthrina} \) trypsin inhibitor (ETI) (12–14), for which docking studies have been described recently (4). Synthetic inhibitors for enzymes of the trypsin family such as tPA have largely been based on Arg and Lys derivatives and the structurally related benzamidinates. Remarkable variations are seen in the structure-activity relationships for the inhibition of trypsin family members with benzamidine-type inhibitors (15).

The three-dimensional structure of tPA (4) reveals some special features of the active site region, suggesting a strategy for obtaining more selective \( p \)-nitroanilide substrates and inhibitors. Adjacent to the catalytic triad, His\(^{377} \) (322), Asp\(^{102} \) (371), Ser\(^{195} \) (478) (the tPA numbering for the residues of the catalytic triad are given in parentheses), and the oxyanion hole, the specificity pocket S1 is bordered by the segment Ile\(^{213} \)–Cys\(^{220} \), including the residue Trp\(^{215} \) (the entrance of the hole, the specificity pocket S1 is bordered by the segment Ile\(^{213} \)–Cys\(^{220} \)), Asp\(^{189} \)–Ser\(^{195} \) (the base of the pocket), Pro\(^{225} \)–Trp\(^{215} \) (the back of the pocket), and the disulfide bridge Cys\(^{191} \)–Cys\(^{220} \) (the south of the pocket). Asp\(^{189} \) at the base of the S1 pocket allows salt bridge formation with basic residues. tPA shows a preference for S1 Arg over Lys, explained by the size of the S1 pocket. Asp\(^{189} \) at the base of the S1 pocket also permits hydrogen bonding with Arg and Lys derivatives that occupy the S4 site, while an amino-terminal substituent would occupy the S3 site.

The side chain of Arg\(^{174} \) in the b-tPA structure (4), which points toward the S4 pocket and partially occupies it, has drawn our attention. Its position suggested that it could influence the binding of substrates containing acidic P4 residues for all t-aminopeptide substrates, or acidic P3 residues when the P3 residue is in the d-configuration (18, 19). In the following, we shall use the terms "S3" and "S4" to denote the sites on tPA that would be occupied by a natural extended substrate. For a peptide of the form \( \text{Xaa-Arg} \), the S3 site would occupy the S4 site, while an amino-terminal substituent would occupy the S3 site. We have constructed and synthesized a series of substrates of the type R-Xaa-Gly-Arg-pNA. In addition, we have solved the structure of the catalytic domain of two-chain tPA (Fig. 1) in complex with 2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one, a bis-benzamidine derived inhibitor (see Fig. 2).

**EXPERIMENTAL PROCEDURES**

**Synthesis of the Inhibitor and Substrates**—The inhibitor 2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one (Pefabloc Xa/tPA, Fig. 2) was kindly provided by the Pentapharm Ltd., Basel, Switzerland. The inhibitory activity was characterized as described elsewhere (20–22). Enzyme assays were carried out following standard procedures. Six substrates of the type Xaa-Gly-Arg-pNA with t- and l-Phe, t- and l-Glu and t- and l-Asp as Xaa residue where synthesized as described previously (23). The substrates Boc-Gly-Gly-Arg-pNA, Boc-t-Val-Gly-Arg-pNA (chromozyme Try), Cmo-n-Val-Gly-Arg-pNA, Cmo-n-Nle-Gly-Arg-pNA (chromozyme X), Cmo-n-Leu-Gly-Arg-pNA, MeSO\(_2\)-D-Phe-Gly-Arg-pNA (chromozyme tPA), H-CHG-Gly-Arg-pNA, H-D-CHG-Gly-Arg-pNA, Cmo-n-CHG-Gly-Arg-pNA (spectrozyme Xa), H-D-HHT-Gly-Arg-pNA (chromozyme XII), Cmo-n-HHT-Gly-Arg-pNA (spectrozyme LAL), Cmo-n-CHA-Gly-Arg-pNA (pefachrome Xa) and H-D-Lys-Gly-Arg-pNA were kindly provided by Lars Svendsen, Pentapharm Ltd., Basel, Switzerland. Cho-Arg-Gly-Arg-pNA (S-2444) and pGlu-Gly-Gly-Arg-pNA (S-2765) were obtained from Hemochrom Diagnostic GmbH, Essen, Germany.

**Hydrolysis of the Chromogenic Substrates**—Single-chain CHO-tPA, used for kinetic studies, was purified from CHO cells by affinity chromatography on red Sepharose and lysine-Sepharose (24–26). The enzyme concentration (in mg/ml) was determined from the absorbance at 280 nm with \( \epsilon = 1.81 \text{ m}^2/\text{mg} \times \text{cm} \). The molar concentration was calculated with a molecular weight of 60 kDa.

The substrates were dissolved in distilled water or dimethyl sulfoxide as necessary. Fifty microliters of the substrate solutions (six concentrations between 20 and 1 mM) were mixed with 0.2 ml HEPES buffer, pH 8.0 (0.1 mM, pH 7.5, 0.1% human serum albumin), and prewarmed to 25°C. The assays were started by the addition of 25 \( \mu \)l of tPA (34 \( \mu \)g/ml) prewarmed to 25°C. Determination of the substrate cleavage was performed on microplates using a MR 5000 reader (Dynatech, Denkendorf, Germany). The amidiolytic activity was calculated from the absorbance at 405 nm with \( \epsilon = 6.480 \text{ m}^2/\text{mm} \times \text{cm} \). The velocity was calculated from the Michaelis-Menten equation with a nonlinear regression analysis program. The \( k_{\text{cat}}/K_m \) was calculated from \( V_{\text{max}}/\text{enzyme concentration} \).

**Cryocrystallization and Structure Analysis**—The des(Val\(^{1} \)-Cys\(^{2} \))tPA variant (27) used for crystallization studies was expressed as inclusion bodies in *Escherichia coli* (strain K12 C600\(^{2} \), containing the helper plasmid PUBS520 with the DNA Y gene). Isolation and solubilization of the inclusion bodies, derivatization and refolding were transformed as described elsewhere (28–30). Briefly, E. coli cells were lysed by the addition of lysozyme and subsequent sonication, and the inclusion bodies isolated by centrifugation. Solubilization was achieved by incubation with 6 M guanidium, HCl, and 0.1 M dithioerythritol at pH 8.5.

After dialysis against 6 M guanidium HCl, the thiol groups were derivatized with glutathione by incubating the solubilized protein in 0.05 M Tris/HCl (pH 7.5, 8 m guanidine, and 0.1 M ethanedithiol). After dialysis against 3 M guanidine, refolding was performed by stepwise addition of 300 ml of protein solution (mixed disulfide) to 10 liter of refolding buffer (0.7 M l-arginine/HCl, pH 8.6, 2 mM GSH, 1 mM EDTA) (31, 32). The refolded protein was purified by affinity chromatography on an \( E \)\( \text{rthrina} \) trypsin inhibitor-Sepharose column. The two-chain form was prepared from the refolded and purified tPA protein sample...
Crystal data and refinement parameters for the bis-benzamidine-tPA complex

| Spacegroup | C2 |
|------------|----|
| Cell constants (Å) | |
| a | 151.83 |
| b | 60.50 |
| c | 62.61 |
| β | 110.5^° |
| Significant measurements | 25355 |
| Independent reflections | 12586 |
| Limiting resolution (Å) | 15.0–3.5 Å |
| Completeness | 94.4% (≈2.8 Å) |
| outside shell | 86.2% (3.1–2.8 Å) |
| RMSB (Å) | 5.20 |

The structure of inhibited human tissue-type plasminogen activator (21715)

Crystal data and refinement parameters for the bis-benzamidine-tPA complex

| Independent reflections | 12586 |
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| Spacegroup | C2 |
| a | 151.83 Å |
| b | 60.50 Å |
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| β | 110.5° |
| Completeness | 94.4% (≈2.8 Å) |
| outside shell | 86.2% (3.1–2.8 Å) |
| RMSB (Å) | 5.20 |

Crystal data and refinement parameters for the bis-benzamidine-tPA complex

| Number of solvent molecules | 91 |
| Molecular weight | 55,244.4 (157,038 dalton) |

Kinetic data for the hydrolysis of p-nitroanilide substrates of the type R-Xaa-Gly-Arg-pNA, where R is an N-terminal protection group, by full-length single-chain tPA

| R | P3 | K_m | k_cat | k_cat/K_m |
|---|----|-----|------|---------|
| Ac | - | - | - | - |
| Boc | Gly | 3.50 | 1.29 | 0.37 |
| Cbo | Val | 1.36 | 1.69 | 1.24 |
| Cmo | d-Val | 0.80 | 7.57 | 7.22 |
| Cmo | d-Met | 0.99 | 6.82 | 6.89 |
| Cmo | d-Leu | 0.62 | 9.62 | 11.2 |
| H | Phe | 2.00 | 2.79 | 1.40 |
| H | p-Phe | 0.70 | 7.46 | 10.7 |
| Cbo | p-Phe | 0.30 | 8.96 | 19.9 |
| MeSO_2 | p-Phe | 0.10 | 7.36 | 73.6 |
| H | CHG | 5.32 | 0.66 | 0.26 |
| H | p-CHG | 1.53 | 6.74 | 4.40 |
| Cmo | p-CHG | 0.42 | 5.02 | 11.9 |
| H | p-HHT | 0.83 | 10.6 | 12.8 |
| Cmo | p-HHT | 0.29 | 8.31 | 21.3 |
| Cmo | p-CHA | 0.34 | 7.76 | 22.8 |
| H | Asp | 0.86 | 0.28 | 0.32 |
| Cbo | Asp | 0.72 | 0.35 | 0.49 |
| Cbo | Asp(O-t-BU) | 2.30 | 1.84 | 0.80 |
| H | d-Asp | 1.70 | 0.43 | 0.25 |
| Cbo | Asp(O-t-BU) | 1.50 | 4.98 | 3.27 |
| H | Glu | 3.20 | 0.13 | 0.041 |
| Cbo | Glu | 2.20 | 0.75 | 0.34 |
| H | Glu(O-t-BU) | 0.90 | 1.29 | 1.43 |
| H | p-Glu | 2.90 | 3.98 | 1.37 |
| Cbo | p-Glu | 2.60 | 7.46 | 2.87 |
| Cbo | p-Glu(O-t-BU) | 1.20 | 4.48 | 3.73 |
| H | p-Lys | 2.10 | 6.32 | 3.01 |
| Cbo | d-Arg | 0.52 | 8.53 | 16.4 |

* k_cat values < 1, k_cat/K_m values > 5.0, and k_cat/K_m values > 10 are emphasized in bold.

Substrate Design—Kinetic analysis of the hydrolysis of peptide substrates (5, 6, 16, 42) and the three-dimensional structure of b-tPA (4) have shown that Arg and Gly are the preferred P1 and P2 residues for tPA substrates, respectively. One striking feature of b-tPA’s active site region is Arg174, which suggests that acidic residues at P3 or P4 might provide additional stabilizing energies. To test this hypothesis, the P3 residue of b-tPA was varied, and the kinetic constants for their cleavage by tPA were determined and compared with known chromogenic substrates of this type (see Table II). In general, a clear preference is observed for d-configured amino acids at P3. Substitution of Xaa for the acidic residues d-Asp or d-Glu failed to produce the desired improvement in catalytic efficiency; indeed, these substrates were among the worst tested. On the other hand, the P3 residue l-Asp exhibited the lowest K_m value of all l-configured P3 residues. In this case, however, catalysis by tPA was impaired, suggesting that l-Asp-Gly-Arg-pNA does not interact with the active site region of tPA in a suitable conformation for cleavage.
Thr1A and carboxyl-terminal to Tyr6 are disordered, as are the bisb-tPA. Thus, residues of the A-chain amino-terminal to (b-tPA) structure (4).

tPA/bis-benzamidine complex. The Kᵢ values for the inhibition of full length sc-tPA and for factor Xa are 0.5 μM and 0.013 μM respectively.

The substrates with the highest specificity constant (kᵢ/Kᵢ) were of the form R-D-Xaa-Gly-Arg-pNA, where Xaa is a hydrophobic (phenylalanine or hexahydroxyrosine) or basic (Arg or Lys) residue. Among these substrates, a further gradient in catalytic efficiency was observed according to the protection group of the amino terminus (R-). In each case, a free amino terminus was suboptimal. The kinetic constants of all measured chromogenic substrates of the type R-Xaa-Gly-Arg-pNA are given in Table II.

Structure of tPA in Complex with Bis-benzamidine—Many specific features of tPA involve interaction with surface elements of the catalytic domain, in particular the surface loops (see Fig. 1). Their role in substrate recognition, cofactor binding and inhibition under physiological conditions has been discussed comprehensively elsewhere (4). In that study, some of the surface loops were not defined by electron density and therefore termed flexible. The crystal packing of the structure described here is quite different, leading to different environments of the molecular surface and allowing possible visualization of labile parts of the structure. Although the two molecules in the asymmetric unit are related by noncrystallographic symmetry, i.e. they are not identical, no significant differences are observed between them. The overall structure of the bis-benzamidine liganded tPA (bis-b-tPA) (Figs. 1 and 2) shows almost complete structural identity with the benzamidine liganded (b-tPA) structure (4).

Most regions that were undefined in b-tPA remain so in bisb-tPA. Thus, residues of the A-chain amino-terminal to Thr1A and carboxyl-terminal to Tyr6 are disordered, as are the six residues Lys43, His52, Arg71A, Arg71B, Ser77, Pro78 of the 37 insertion loop. The latter has been shown to be of fundamental importance for the interaction with PAI-1 (8–11) and for fibrin specificity and stimulation (43, 44). In contrast to b-tPA, the three residues Asp110A, Ser110B, and Ser110C of the 110 insertion loop are undefined in bis-b-tPA; the equivalent loop in uPA (45) also exhibits high mobility. The last two C-terminal residues Arg243 and Pro244, which are in structural proximity to the 110 loop, are also undefined in bisb-tPA.

The “autolysis” loop Tyr141 to Tyr151 to the surface of the active site cleft is well defined by electron density, with the same overall conformation as observed in b-tPA. However, the side chain of Glu145 forms a salt bridge with NZ Lys 17 in bisb-tPA instead of the H-bond to N Leu186 in b-tPA. This is probably a result of different crystal contacts.

The 186-loop to the south of the active site cleft consists of 8 additional residues compared with α-chymotrypsinogen. In contrast to b-tPA, all residues are defined by electron density (see Fig. 3). The extended loop projects out from the molecular surface into the solvent, and is stabilized by crystal contacts. The cluster of three residues Arg166A, Gln166F, His188, which point toward the main body of the molecule, might also stabilize this arrangement. It is not clear which is the main determinant for the stabilization of this loop in this structure. It is possible that the different pH conditions used for the crystallization of b-tPA (pH 7.5) and bisb-tPA (pH 5) could have lead to this conformational difference. Thus, the deprotonation of His188 in b-tPA might disfavor formation of the stabilizing cluster Arg166A, Gln166F, His188.

The exact conformation of this loop might not be important, as position 186G is deleted in mouse and rat tPA. Its function is not clear, but it may be involved in stimulation of the plasminogen activator by its cofactor fibrin (46). It is bordered on its eastern side by the N-terminal part of the B-chain, with the side chain of His188 approaching Gln19 and Gly19. In this conformation, the 186-loop is close to the entrance frame of the S1 pocket (Ser214–Gln221A) and the following residues Lys222, Asp223, Val224, which form a turn.

Interaction of Bis-benzamidine with the Active Site of tPA—The inhibitor is well defined by electron density (see Fig. 4). The binding of the bis-benzamidine to tPA is determined by two major interaction sites. The first amidino group, the “proximal” amidino group, binds in the specificity pocket while the second amidino group, the “distal” amidino group, fits in an hydrophobic groove, resulting in an extended binding of the inhibitor. The inhibitor exhibits a trans/trans conformation (see Fig. 2). The proximal amidino group is sandwiched in the S1 pocket between main-chain segments Trp215–Gly216 and Cys219, Gln221 in the same way as amidine in b-tPA. Its amidino function forms a symmetric two O-two N salt bridge with Asp189 and a N-O hydrogen bond with the carbonyl oxygen of Gly219. The proximal amidino group is almost coplanar and coincident with the superimposed amidine from b-tPA, yet its amidino group appears to be more out of the aromatic ring plane than in the latter structure.

The heptane ring system, which acts as a spacer between the two benzamidino groups, partially occupies the S2 pocket, resulting in a close contact between one carbon atom of the cycloheptane ring and the OH-group of the Tyr99 (3 Å distance). The restriction of the S2 pocket by the side chain of Tyr99 (see above) does not allow more bulky spacers in bisbenzamidine inhibitors, which correlates with previous published results (42). The rigid nature of the cycloheptanone ring results in a significant distance (3.8 Å) between the carbonyl oxygen of the inhibitor and the backbone of the segment Ile213, Cys220. This distance would be smaller for a cyclopentanone and cyclohexanone spacer, explaining their weaker inhibition of tPA. Gly216 does not contribute to binding by hydrogen bond formation, as seen in some synthetic inhibitor complexes with the related serine proteinase thrombin (47).

The distal benzamidino group penetrates into the hydrophobic groove, displacing the side chain of Arg174. The aliphatic portion of the Arg side chain now makes up the western border of the S4 pocket, making it more hydrophobic and flanking the distal benzamidine. The aromatic benzene ring is parallel to the indole moiety of the Trp215 and edge to face to the phenolic side chain of Tyr99, which may confer additional weak binding energy. Indeed, a complex of the same inhibitor in trypsin, 3 indicates a similar overall geometry of the inhibitor, but with the distal amidino group rotated 50° around its molecular axis; the aliphatic side chain of Leu99 in trypsin is unable to achieve the favorable aromatic stacking arrangements seen in tPA. The basic amidino group occupies the same position as the guanidino group of Arg174 in b-tPA (see Fig. 4), approaching an electronegative cavity on the surface of tPA formed by the carbonyl groups of Asp97 and Thr98.

3 M. Renatus, W. Bode, R. Huber, J. Stürzebecher, D. Prasa, and M. T. Stubbs, manuscript in preparation.
A series of \( p \)-nitroanilide substrates were synthesized on the premise that the guanidino group of Arg174 partially occupies the S4 site of tPA (4). Substrates containing an acidic D-amino acid at P3, which could form a stable salt bridge to this residue, failed to give the desired improvement in catalytic efficiency. On the other hand, substrates containing an L-Asp residue at P3 exhibited the lowest \( K_m \) values of all the L-P3 residues, comparable to the \( K_m \) values of the most specific substrates. In this case, however, cleavage of the substrate is compromised, indicating that binding of P3 L-Asp produces an incorrect peptide conformation at the cleavage site. These results suggest that the conformation of Arg174 as seen in benzamidine-ligated tPA is not fixed.

The 2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one tPA structure shows that the side chain of this residue can indeed adopt multiple conformations. While the proximal amidino group occupies the specificity pocket, and the distal benzamidine reaches toward an electrophilic pocket formed by the side chain carbonyl atoms of Asp97, Thr98, and Arg174. The benzyl ring of the inhibitor is perpendicular to the side chain of Tyr99; the side chain of Arg174 is displaced by the amidino group. A, experimental density (2\( F_o \) - \( F_c \)) of the active site region in complex with the bis-benzamidine inhibitor. B, active site region of human tPA in complex with bis-benzamidine. Arg174 from b-tPA is superimposed (thin lines).

**DISCUSSION**

A series of \( p \)-nitroanilide substrates were synthesized on the premise that the guanidino group of Arg174 partially occupies the S4 site of tPA (4). Substrates containing an acidic \( \beta \)-amino acid at P3, which could form a stable salt bridge to this residue, failed to give the desired improvement in catalytic efficiency. On the other hand, substrates containing an L-Asp residue at P3 exhibited the lowest \( K_m \) values of all the L-P3 residues, comparable to the \( K_m \) values of the most specific substrates. In this case, however, cleavage of the substrate is compromised, indicating that binding of P3 L-Asp produces an incorrect peptide conformation at the cleavage site. These results suggest that the conformation of Arg174 as seen in benzamidine-ligated tPA is not fixed.

The 2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one tPA structure shows that the side chain of this residue can indeed adopt multiple conformations. While the proximal amidino group occupies the S1 pocket as expected, the distal aromatic ring displaces the side chain of Arg174 from its position in b-tPA (see Fig. 4). The aliphatic side chain now rests against that of Thr98, resulting in an hydrophobic western edge to the S3/S4 pocket. The distal amidino group of the inhibitor occupies an electronegative cavity formed by the carbonyl groups of Asp97, Thr98, and Thr175, formerly the location of the guanidino group.
moieties were modeled into the active site of bisb-tPA according to the binding of D-Phe-Pro-Arg chloromethylketone to thrombin (18, 19). The positions of the Trp215 the phenylalanyl side chain of MeSO2-D-Phe-Gly-Arg-pNA (thick black connections) is stabilized by contacts to the aromatic moieties of Tyr99, Trp215, and the aliphatic portion of Arg174. An oxygen atom of the MeSO2 group could make an hydrogen bond to the amide nitrogen of Gly219. The P3 guanidino group of Cbo-D-Arg-Gly-Arg-pNA occupies the electrophilic cavity determined for bisb-tPA. Additional stabilization is afforded by the Cbo group, which covers the hydrophobic surface provided by residues Leu217, Val224, and Leu172.

The peptide moieties of Cbo-D-Arg-Gly-Arg-pNA and MeSO2-D-Phe-Gly-Arg-pNA were modeled into the active site of bisb-tPA assuming a “canonical conformation” (48) of the peptides. Although small substrates are in general more flexible than protein substrates and inhibitors, a survey of structures of serine proteinase containing short peptide chloromethyl ketone inhibitors (such as D-Phe-Pro-Arg-chloromethylketone in thrombin) (18) suggests that also small substrates in the Michaelis complex adopt canonical conformation. For the substrates discussed here, we might expect more flexibility associated with the P2 glycylic residues; recent structures of uPA in complex with Glu-Gly-Arg-chloromethylketone (45), single chain tPA in complex with dansyl-Glu-Gly-Arg-chloromethylketone (49), and vampire bat plasminogen activator in complex with Glu-Gly-Arg-chloromethylketone (50) reveal, however, that the P2 Gly residue adopts canonical $\phi$-$\psi$ angles in the bound state.

MeSO2-D-Phe-Gly-Arg-pNA—Assuming a short antiparallel $\beta$-sheet between the substrate Gly and Phe residues and the enzyme Trp215 and Gly216, the side chain of P3 D-Phe fits perfectly into the hydrophobic entrance of the S4 pocket (see Fig. 5). The Phe aromatic ring stacks over the indole system of Trp215, with concomitant edge to plane stacking to Tyr99. One oxygen atom of the methylsulfonyl group can form a hydrogen bond with the amide nitrogen of Gly219, while the methyl group could rest against the side chain of Leu217, providing an explanation for the sevenfold decrease in $K_m$ for MeSO2-D-Phe-Gly-Arg-pNA compared with H-D-Phe-Gly-Arg-pNA (see Table II).

Cbo-D-Arg-Gly-Arg-pNA—Assuming the same antiparallel $\beta$-sheet as above, the side chain of P3 D-Arg extends deeply into the S4 pocket to reach the electronegative cavity (see Fig. 5). A possible stacking of the aliphatic portions of P3 D-Arg and Arg174 could make a further contribution to the binding energy. The Cbo protecting group points in an extended conformation toward an hydrophilic patch formed by the residues Leu172, Leu217, Gln221A, and Val224, allowing partial burial of this hydrophobic by the benzyloxy group.

These models suggest possible modifications for improved substrates. Superposition of the two substrates indicate coincidence of P3 D-Phe C* with P3 D-Arg N* (or C6) with P3 D-Arg N*. A P3 amidinophenyl group could make dual use of the hydrophilic environment at the entrance at the pocket and the electrophilic environment at the base of the pocket for stabilizing interac-
tions. The large influence of a protection group at the N terminus on catalytic efficiency suggests that an additional hydrophobic residue at position P4 could allow additional stabilization through burial of tPA’s hydrophobic surface to the west of the active site.

Our results concerning the specificity sites of tPA are summarized in Fig. 6. Outside the primary specificity pocket, the major determinants for interaction with substrates and inhibitors would appear to be binding in the hydrophobic S4 pocket, together with weak electrostatic interactions at the electronegative cavity. Such an extended binding mode has also been observed for factor Xa specific inhibitors (51, 52). Indeed, the 99-loop forming the north-eastern edge of the S4 pocket is almost identical in factor Xa (Glu97–Thr98–Tyr99). This strongly suggests that both tPA and factor Xa could be inhibited by similar ligands. This raises the possibility that use of strong inhibitors; in this case, this could be achieved by tPA’s hydrophobic surface to the west of the active site.

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