Mapping of the catalytic site of CHO-t-PA and the t-PA variant BM 06.022 by synthetic inhibitors and substrates

ARTICLE *in* PROTEIN SCIENCE · AUGUST 1992

Impact Factor: 2.85 · DOI: 10.1002/pro.5560010806 · Source: PubMed

5 AUTHORS, INCLUDING:

Ulf Neumann
Novartis Institutes for BioMedical Research
62 PUBLICATIONS **1,599** CITATIONS

SEE PROFILE

Protein Science (1992), *1*, 1007–1013. Cambridge University Press. Printed in the USA.

Mapping of the catalytic site of CHO-t-PA and the t-PA variant BM 06.022 by synthetic inhibitors and substrates

J. STÜRZEBECHER,[1] U. NEUMANN,[1,3] U. KOHNERT,[2] G.-B. KRESSE,[2] AND S. FISCHER[2]

[1] Institute of Pharmacology & Toxicology, Medical Academy Erfurt, D-(0)-5010 Erfurt, Germany
[2] Boehringer Mannheim GmbH, Biochemical Research Center Penzberg, D-8122 Penzberg, Germany

(RECEIVED December 24, 1991; REVISED MANUSCRIPT RECEIVED March 2, 1992)

Abstract

BM 06.022 is a t-PA deletion variant that is produced as inactive inclusion bodies in *Escherichia coli* and transformed into the native form by an in vitro refolding process. Until now, no X-ray and NMR structures of BM 06.022 were available. Therefore a detailed kinetic analysis of the hydrolysis of peptide substrates and of the inhibition by several benzamidine-derived inhibitors was carried out in order to assess that the active site region of the protease domain of BM 06.022 is correctly structured in comparison with t-PA. Our data reveal that the single-chain as well as the two-chain form of BM 06.022 and native t-PA are similar in catalytic and in inhibitor binding properties. This indicates that the active site and the highly complex rearrangement of t-PA upon cleavage of the Arg_{275}–Ile_{276} bond are maintained in BM 06.022.

Keywords: active site mapping; inhibitors; substrates; TPA variant

Tissue-type plasminogen activator (t-PA) converts the inactive proenzyme plasminogen to the active protease plasmin. A structural model was suggested from the comparison of the primary sequence of t-PA with known structures (Harris, 1987). According to this model t-PA consists of five domains: the finger domain, the epidermal growth factor domain, two kringle domains, and the protease domain with the catalytic triad Asp_{371}, His_{322}, and Ser_{478} (Pennica et al., 1983). Upon cleavage of the Arg_{275}–Ile_{276} bond, t-PA is transferred into the two-chain form. The two-chain form of t-PA has a higher catalytic efficiency against peptide substrates, and its inhibition by peptidyl chloromethyl ketones is much faster than with the single-chain form (Andreasen et al., 1991). t-PA turned out to be a potent therapeutic agent for treatment of myocardial infarction, which is caused by a fibrin clot obstructing the blood vessel. However, clinical trials revealed that t-PA had some major disadvantages, such as a very short half-life of 3 min in human plasma (Collen et al., 1984). Although in vitro t-PA activates plasminogen to plasmin only in the presence of fibrin, in vivo the high dose of t-PA used for the treatment of myocardial infarction may cause systemic activation of plasminogen, inducing the degradation of several plasma proteins like fibrinogen and α_2-antiplasmin.

During recent years, several variants of t-PA were developed in order to find a fibrinolytic agent with improved pharmacological properties (for a review see Higgins & Bennett [1990]). BM 06.022 is a t-PA deletion variant comprising only the kringle 2 and the protease domains. BM 06.022 is produced in *Escherichia coli* as inactive inclusion bodies and is transformed into the native structure by an in vitro refolding process (Kohnert et al., 1992). A detailed comparison between CHO-t-PA and BM 06.022 provided evidence that the biochemical properties of the protease domain and the kringle 2 domain are maintained in BM 06.022 (Kohnert et al., 1992). A kinetic analysis of the amidolytic and plasminogenolytic

Reprint requests to: Ulrich Kohnert, Boehringer Mannheim GmbH, Biochemical Research Center Penzberg, Department of Enzymology, Nonnenwald 2, D-8122 Penzberg, Germany.
[3] Present address: Friedrich-Miescher Institut, CH-4002 Basel, Switzerland.

Abbreviations: Cbo, carbobenzoxy; CHA, cyclohexylalanine; CHG, cyclohexylglycine; CHO-t-PA, recombinant tissue-type plasminogen activator from Chinese hamster ovary cells; ETI, erythrina trypsin inhibitor; HHT, hexahydrotyrosine; Tos, tosyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

activity yielded similar values for BM 06.022 and recombinant t-PA from Chinese hamster ovary cells. However, the in vitro plasminogenolytic activity of BM 06.022 in the presence of CNBr fragments of fibrinogen was reduced.

Until now no detailed information on the structure of CHO-t-PA and BM 06.022 was available by X-ray analysis and NMR studies. In order to analyze whether the protease moiety of the in vitro refolded BM 06.022 is correctly structured as compared to t-PA we asked whether a detailed analysis of the enzymatic activity could serve as a tool to assess the fine structure of the active site. t-PA and BM 06.022 are trypsinlike serine proteinases that cleave the peptide bond on the C-terminal side of Arg and Lys residues at the P_1 position (according to Schechter & Berger [1968]). Synthetic inhibitors for enzymes of the trypsin family have been found not only among Arg and Lys derivatives but also among structurally related benzamidines (Stürzebecher et al., 1976). In most cases benzamidine derivatives are not selective inhibitors. However, among derivatives of the $N\alpha$-arylsulfonyl-ω-amidinophenyl-α-aminoalkylcarboxylic acids, selective inhibitors of several proteases were found (Stürzebecher et al., 1981, 1982). There are remarkable differences between the structure–activity relationships for the inhibition of various enzymes. Inhibitors of the benzamidine type have not yet been analyzed for their effects on t-PA.

In this paper we present a detailed analysis comparing the K_i values for the inhibition of BM 06.022 and CHO-t-PA by several benzamidine-derived inhibitors and in addition by determination of the kinetic constants of the cleavage of a variety of Arg- and Lys-containing chromogenic peptide substrates differing in P_2 and P_3 positions.

Results

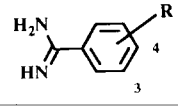
Inhibition of BM 06.022 and CHO-t-PA by benzamidines

We determined the K_i values for the inhibition of BM 06.022 and CHO-t-PA by benzamidine and benzamidine derivatives. Data presented in Table 1 indicate that benzamidine does not inhibit the single-chain forms of both enzymes. Benzamidine is also only a weak inhibitor for the two-chain forms of BM 06.022 (K_i = 690 μmol/L) and CHO-t-PA (K_i = 910 μmol/L). Furthermore, introduction of hydrophobic residues in positions 3 and 4, respectively, resulted also in rather poor inhibitors for the single-chain and the two-chain forms of both enzymes. Only 4-amidinophenylpyruvic acid exerts a certain affinity for BM 06.022 and CHO-t-PA. The K_i for the single-chain forms of BM 06.022 and CHO-t-PA was 700 and 710 μmol/L, respectively. The K_i values for the two-chain form of both BM 06.022 and CHO-t-PA were 90 μmol/L. Even though there is no strong inhibition of the molecules, one can observe that CHO-t-PA and BM 06.022 show similar affinities to the described inhibitors. This is true for the comparison of the single-chain and the two-chain forms as well as for the difference between the respective single-chain and two-chain form.

Inhibition by derivatives of amidinophenyl-α-aminoalkyl-carboxylic acids

Several tosylated anilides of the nonnatural amino acids amidinophenylalanine, amidinophenyl-α-aminobutyric acid, and amidinophenyl-α-aminovaleric acid exert only a moderate inhibitory activity against the single-chain

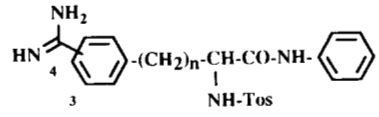
Table 1. *Inhibition of the single-chain and two-chain (tc) forms of BM 06.022 and CHO-t-PA by simple benzamidine derivatives*



No.	R	Position of R	K_i (μmol/L)[a] BM 06.022	CHO-t-PA	BM 06.022 (tc)	CHO-t-PA (tc)
1	H		>1,000	>1,000	690 ± 60	910 ± 170
2	CH_2-O-	3	950 ± 170	920 ± 90	290 ± 40	250 ± 40
3		4	930 ± 190	950 ± 70	470 ± 30	780 ± 100
4	CH_2-CO-COOH	3	>1,000	>1,000	480 ± 210	530 ± 60
5		4	700 ± 10	710 ± 210	92 ± 30	86 ± 15
6	CH_2-CO-	3	860 ± 220	>1,000	420 ± 120	440 ± 80
7		4	>1,000	>1,000	850 ± 60	>1,000

[a] Mean ± SD from at least three experiments.

Table 2. *Inhibition of the single-chain and two-chain (tc) forms of BM 06.022 and CHO-t-PA by anilides of tosylated ω-amidinophenyl-α-aminoalkylcarboxylic acids*



No.	n	Position of the amidino group	K_i (μmol/L)[a] BM 06.022	CHO-t-PA	BM 06.022 (tc)	CHO-t-PA (tc)
8	1	4	>1,000	>1,000	>1,000	>1,000
9	2	4	340 ± 60	440 ± 90	64 ± 3	67 ± 12
10	3	3	440 ± 90	350 ± 50	81 ± 12	71 ± 31
11	3	4	130 ± 20	130 ± 20	20 ± 4	15 ± 2

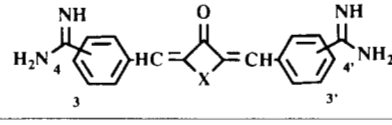
[a] Mean ± SD from at least three experiments.

forms of BM 06.022 and CHO-t-PA (Table 2). Like the simple benzamidines, the derivatives of ω-amidinophenyl-α-aminoalkylcarboxylic acids inhibited the two-chain form of BM 06.022 and CHO-t-PA better than the respective single-chain form. The K_i values for the inhibition of the two-chain form were lower by a factor of 5–10. Besides the length of the methylene bridge, the position of the amidino group is important for the potency of the inhibitor. The shift of the amidino group from the meta to the para position of the valeric acid derivative decreased K_i by a factor of 3–4. Other derivatives of the tosylated ω-amidinophenyl-α-aminoalkylcarboxylic acids, like piperides and morpholides, had an even lower affinity for the enzymes (data not shown).

Inhibition by bis-benzamidines

The bis-benzamidines containing a cycloalkanone linking bridge inhibited the two-chain forms of BM 06.022 and CHO-t-PA with K_i values between 1 and 10 μmol/L (Table 3). Again, the K_i values for the single-chain forms of both enzymes were higher by one order of magnitude as compared to the K_i value for the respective two-chain form. The analysis of several bis-benzamidine derivatives revealed that a cycloheptanone and cyclooctanone ring structure gave the highest affinity for the single-chain form and the two-chain form of both enzymes. In the case of the bis-benzamidines with a central cyclohexanone ring, the shift of the amidino group from the para to the

Table 3. *Inhibition of the single-chain and two-chain (tc) forms of BM 06.022 and CHO-t-PA by bis-benzamidines*



No.	X	Position of the amidino group	K_i (μmol/L)[a] BM 06.022	CHO-t-PA	BM 06.022 (tc)	CHO-t-PA (tc)
12	C_2H_4	4,4′	51 ± 11	34 ± 5	9.6 ± 0.9	6.5 ± 1.9
13	C_3H_6	4,4′	35 ± 9	33 ± 8	2.9 ± 0.6	2.4 ± 0.5
14	C_4H_8	4,4′	6.9 ± 3.1	1.9 ± 0.8	0.45 ± 0.16	0.60 ± 0.25
15	C_5H_{10}	4,4′	9.2 ± 1.7	8.6 ± 1.6	0.67 ± 0.19	0.40 ± 0.06
16	C_3H_6	3,3′	91 ± 17	99 ± 4	16 ± 1	18 ± 7
17	C_3H_6	4[b]	520 ± 90	363 ± 84	110 ± 25	109 ± 28

[a] Mean ± SD from at least three experiments.
[b] The amidino group at position 4′ is replaced by a hydrogen atom.

Table 4. *Inhibition of BM 06.022, CHO-t-PA, bovine trypsin, and bovine thrombin by selected benzamidine derivatives*

	K_i (μmol/L)			
No.[a]	BM 06.022	CHO-t-PA	Trypsin[b]	Thrombin[b]
1	>1,000	>1,000	35	220
2	950	920	9	66
3	930	950	9	58
6	860	>1,000	5	69
7	>1,000	>1,000	12	8
9	340	440	0.34	40
10	440	350	2	4
12	51	34	1	9
13	35	33	1	1

[a] Numbering according to Tables 1–3.
[b] Data from Stürzebecher et al. (1976, 1981, 1982) and Walsmann et al. (1976).

meta position resulted in 2–3-fold and 5–9-fold higher K_i values for the single-chain and the two-chain form of both enzymes, respectively.

Inhibition of other serine proteinases

The K_i values for the inhibition of BM 06.022 and CHO-t-PA by several inhibitors are compared with the data for trypsin and thrombin as published in the literature (Table 4). Although the inhibitors have a very similar affinity for BM 06.022 and CHO-t-PA, there are clear differences in the inhibition of trypsin and thrombin. As described for simple benzamidines (Stürzebecher et al., 1976) the K_i values for the inhibition of trypsin by benzamidine (1) and by the inhibitors 2, 3, 6, 9, and 12 are lower by an order of magnitude as compared to the respective data for the inhibition of thrombin. However, there are also some inhibitors, like structures 7, 10, and 13, that have a very similar affinity for both trypsin and thrombin.

Amidolytic activity of BM 06.022 and CHO-t-PA against various peptide substrates

We analyzed the catalytic properties of BM 06.022 and CHO-t-PA using several peptide substrates. The kinetic analysis of the hydrolysis of these substrates revealed that the substrate specificity of BM 06.022 is in agreement with the substrate specificity of CHO-t-PA. Both enzymes preferentially cleave the substrates $MeSO_2$-D-HHT-Gly-Arg-pNA and D-HHT-Gly-Arg-pNA (Table 5). The catalytic efficiency (k_{cat}/K_m) for the cleavage of $MeSO_2$-D-HHT-Gly-Arg is about twice the value measured for D-HHT-Gly-Arg-pNA, indicating that an N-terminal blocked peptide may be a better substrate than the respective free peptide. The urokinase-specific substrate pyroGlu-Gly-Arg-pNA, the glandular kallikrein-specific substrate D-Val-CHA-Arg-pNA (data not shown) and the plasmin-specific substrate Tos-Gly-Pro-Lys-pNA were only poor substrates for both BM 06.022 and CHO-t-PA. Furthermore, the catalytic efficiency of the two-chain form of BM 06.022 and CHO-t-PA was higher by a factor of 10 as compared to the respective single-chain form, which is due to a decrease of K_m (Table 6) and an increase of k_{cat} (Table 7) as compared to the respective single-chain form.

Discussion

BM 06.022, a t-PA deletion variant comprising only the kringle 2 and the protease domains, was produced by re-

Table 5. *Catalytic efficiencies (k_{cat}/K_m) for the hydrolysis of peptide substrates by the single-chain and the two-chain (tc) forms of BM 06.022 and CHO-t-PA*

	k_{cat}/K_m ($L \cdot s^{-1} \cdot mmol^{-1}$)			
Substrate	BM 06.022	CHO-t-PA	BM 06.022 (tc)	CHO-t-PA (tc)
$MeSO_2$-D-HHT-Gly-Arg-pNA	23.32	28.32	146.67	181.82
D-CHG-Gly-Arg-pNA	1.91[a]	3.40	24.81	30.28
Cbo-Gly-Val-Arg-pNA	0.73	0.92	8.78	9.20
D-HHT-Gly-Arg-pNA	9.48	12.11	40.96	80.80
Tos-Gly-Pro-Arg-pNA	1.56	1.68	12.50	18.22
D-Phe-Pip-Arg-pNA	1.70	2.08	21.00	24.94
D-Ile-Pro-Arg-pNA	3.68	5.87	38.42	36.51
Tos-Gly-Pro-Lys-pNA	0.02[a]	0.04[a]	0.19	0.26

[a] For these substrates k_{cat}/K_m was calculated from the slope of the linear part of the velocity versus substrate plot, which is $k_{cat} \cdot C_{enzyme}/K_m$.

Table 6. *K_m values for the hydrolysis of peptide substrates by the single-chain and the two-chain (tc) forms of BM 06.022 and CHO-t-PA*

	K_m (mmol/L)[a]			
Substrate	BM 06.022	CHO-t-PA	BM 06.022 (tc)	CHO-t-PA (tc)
$MeSO_2$-D-HHT-Gly-Arg-pNA	0.53 ± 0.03	0.43 ± 0.02	0.15 ± 0.01	0.11 ± 0.01
D-CHG-Gly-Arg-pNA	n.d.[b]	1.66 ± 0.13	0.84 ± 0.05	0.68 ± 0.03
Cbo-Gly-Val-Arg-pNA	2.08 ± 0.16	1.97 ± 0.15	0.87 ± 0.06	0.94 ± 0.07
D-HHT-Gly-Arg-pNA	1.51 ± 0.10	1.11 ± 0.13	0.29 ± 0.02	0.30 ± 0.02
Tos-Gly-Pro-Arg-pNA	0.87 ± 0.09	0.77 ± 0.09	0.16 ± 0.02	0.31 ± 0.02
D-Phe-Pip-Arg-pNA	0.87 ± 0.06	0.48 ± 0.04	0.32 ± 0.02	0.17 ± 0.02
D-Ile-Pro-Arg-pNA	1.36 ± 0.10	0.99 ± 0.04	0.33 ± 0.02	0.29 ± 0.02
Tos-Gly-Pro-Lys-pNA	n.d.[b]	n.d.[b]	0.62 ± 0.04	0.47 ± 0.03

[a] The data represent the mean and SD from at least three experiments.
[b] This value was not determined because no saturation of the enzyme was achieved at the highest substrate concentration.

combinant expression in *E. coli* as an inactive protein and transformed into its active form by an in vitro refolding process. The recombinant expression of a deletion variant and in particular the in vitro folding process raise the question of whether the molecule can be obtained with the fully functional structure. The determination of the structure by X-ray and NMR analysis may answer this question, but this type of analysis is either complex and labor intensive or limited if proper crystals for the X-ray analysis cannot be obtained. In order to get some information about the binding properties of the active site of the protease of BM 06.022 in comparison with CHO-t-PA, we analyzed substrate specificity and inhibition by several benzamidine-derived inhibitors.

The data presented in Tables 1–3 indicate that the inhibitors generally had almost identical affinities for the single-chain form of BM 06.022 and CHO-t-PA. The relatively large difference between the K_i value for the inhibition of BM 06.022 and CHO-t-PA by inhibitor 14 is due rather to some instability of the inhibitor in aqueous solutions than to differences in the active site of both enzymes. Furthermore, the analysis of the two-chain forms also gave similar K_i values for both enzymes, which were lower by a factor of about 10 than the K_i values for the respective single-chain form. The differences between the K_i values for BM 06.022 and CHO-t-PA were due rather to variations in the method than to differences in the structure around the substrate binding region. The K_i for the inhibition of the enzymes by bis-benzamidines were lower than the values measured for the inhibition by simple benzamidine derivatives and by the anilides of the tosylated amidinophenyl-α-aminoalkylcarboxylic acids.

Table 7. *k_{cat} values for the hydrolysis of peptide substrates by the single-chain and the two-chain (tc) forms of BM 06.022 and CHO-t-PA*

	k_{cat} (s^{-1})[a]			
Substrate	BM 06.022	CHO-t-PA	BM 06.022 (tc)	CHO-t-PA (tc)
$MeSO_2$-D-HHT-Gly-Arg-pNA	12.36 ± 0.24	12.18 ± 0.24	22.00 ± 0.68	20.00 ± 0.41
D-CHG-Gly-Arg-pNA	n.d.[b]	5.53 ± 0.24	20.84 ± 0.64	20.59 ± 0.35
Cbo-Gly-Val-Arg-pNA	1.52 ± 0.08	1.82 ± 0.12	7.64 ± 0.24	8.65 ± 0.29
D-HHT-Gly-Arg-pNA	14.32 ± 0.56	13.18 ± 0.82	11.88 ± 0.28	24.24 ± 0.65
Tos-Gly-Pro-Arg-pNA	1.36 ± 0.08	1.29 ± 0.06	2.00 ± 0.08	5.65 ± 0.18
D-Phe-Pip-Arg-pNA	1.48 ± 0.04	1.00 ± 0.06	6.72 ± 0.16	4.24 ± 0.12
D-Ile-Pro-Arg-pNA	5.00 ± 0.20	5.82 ± 0.24	12.68 ± 0.28	10.59 ± 0.18
Tos-Gly-Pro-Lys-pNA	n.d.[b]	0.047 ± 0.0024	0.12 ± 0.04	0.12 ± 0.029

[a] The data represent the mean and SD from at least three experiments.
[b] Not determined.

The significance of the results obtained becomes evident if one compares the K_i values for the inhibition of trypsin and thrombin with those of BM 06.022 and CHO-t-PA (Table 5). Trypsin and thrombin are highly homologous serine proteases. Both structures are resolved by X-ray analysis. From the structure of the complexes of trypsin and thrombin with several low molecular weight inhibitors it is evident that the binding sites near the active site are highly homologous (Bode et al., 1990; Brandstetter et al., 1992).

The catalytic efficiency for various substrates was similar for BM 06.022 and CHO-t-PA. As previously described for the amidolytic activity of BM 06.022 and CHO-t-PA against S-2288 (Kohnert et al., 1992), the activity of the two-chain form of both enzymes measured against the substrates analyzed was higher by a factor of 5–10 as compared to the respective single-chain form. This increase in the catalytic efficiency is due to a decrease in K_m and an increase in k_{cat}. Our data confirm the results of Rijken and Groeneveld (1991) concerning the substrate specificity of t-PA.

BM 06.022, like CHO-t-PA, shows a significant shift in activity upon cleavage of only one peptide bond (Arg_{275}–Ile_{276}), which induces a structural rearrangement of the molecule. t-PA shares this property with the members of the chymotrypsin family of serine proteases, even though the increase in catalytic efficiency of t-PA is lower by a factor of about one million as compared with chymotrypsinogen/chymotrypsin (Kraut, 1977). If one takes into account that the cleavage site is distant from the active site (as suggested by the model of Bennett et al. [1991]), this rearrangement has to be highly cooperative. This feature is maintained in BM 06.022.

The correspondence between the protease domain and kringle 2 of BM 06.022 and CHO-t-PA is documented by comparison of the inhibition of both enzymes by PAI-1 (Kohnert et al., 1992). Furthermore, the plasminogenolytic activity of BM 06.022 and CHO-t-PA in the absence of a stimulator is identical. However, in the presence of CNBr fragments of fibrinogen, the plasminogenolytic activity of BM 06.022 is lower by a factor of 2–3 as compared to CHO-t-PA. The data in this paper support the assumption that the lower in vitro plasminogenolytic activity of BM 06.022 is not due to impaired function of the protease domain but rather to the reduced fibrin binding of BM 06.022.

In conclusion, the active site mapping of BM 06.022 and CHO-t-PA with several benzamidine-derived inhibitors and chromogenic peptide substrates provided evidence that the deletion of the three N-terminal domains of t-PA, the production of BM 06.022 in *E. coli*, and its activation by an in vitro refolding process did not alter the catalytic center as well as the structural rearrangement upon cleavage of the Arg_{275}–Ile_{276} bond. In addition we confirmed that active site mapping with substrates and inhibitors as described in this paper provides a useful tool for the detailed comparative analysis of recombinant enzymes.

Materials and methods

Enzymes

CHO-t-PA was purified from CHO cells by affinity chromatography on red Sepharose® and lysine-Sepharose® (van Zonneveld et al., 1986; Macartney & Menhardt, 1989). BM 06.022 was expressed as inclusion bodies in *E. coli*. The inactive enzyme was transferred into its native structure by an in vitro refolding process and purified by affinity chromatography on ETI-Sepharose (Kohnert et al., 1992). The two-chain form of BM 06.022 and CHO-t-PA was produced by incubation with plasmin-Sepharose as described previously (Kohnert et al., 1992).

Substrates

$MeSO_2$-D-HHT-Gly-Arg-pNA and D-CHG-Gly-Arg-pNA were from Pentapharm Ltd., Basel, Switzerland. Cbo-Gly-Val-Arg-pNA (Chromozym® TRY), D-HHT-Gly-Arg-pNA (Chromozym® XII), Tos-Gly-Pro-Arg-pNA (Chromozym® TH), D-Val-CHA-Arg-pNA (Chromozym® GK), and Tos-Gly-Pro-Lys-pNA (Chromozym® PL) were from Boehringer Mannheim GmbH, Mannheim, Germany. D-Phe-Pip-Arg-pNA (S-2238), pyroGlu-Gly-Arg-pNA (S-2444), D-Ile-Pro-Arg-pNA (S-2288), and D-Val-Leu-Lys-pNA (S-2251) were obtained from Kabi Diagnostica, Mölndal, Sweden.

Inhibitors

The inhibitors were synthesized by Wagner and coworkers at the University of Leipzig (Wagner, 1982) and characterized as inhibitors of serine proteinases (Stürzebecher et al., 1976, 1982; Walsmann et al., 1976).

Inhibition studies were carried out with three different substrate concentrations in the presence of three concentrations of the inhibitor (without inhibitor and two different concentrations according to the potency of the inhibitor). A sample of 0.1 mL $MeSO_2$-D-HHT-Gly-Arg-pNA (0.5, 1, and 2 mmol/L, respectively) was mixed with 0.3 mL 0.1 mol/L HEPES, pH 8.0, 0.154 mol/L NaCl, 0.1% human serum albumin, 5% ethanol, and 0.05 mL enzyme solution. All enzymes were diluted with 0.1 mol/L HEPES, pH 8.0, 0.154 mol/L NaCl, 0.1% human serum albumin (Institut für Impfstoffe, Dessau, Germany) to a final concentration of 126 μg/mL (BM 06.022), 11 μg/mL (BM 06.022, two-chain form), 205 μg/mL (CHO-t-PA), and 11 μg/mL (CHO-t-PA, two-chain form). The K_i values were calculated graphically according to Dixon (1953) by plotting the reciprocal values of the initial velocities against the inhibitor concentrations.

Hydrolysis of the chromogenic substrates

The substrates were dissolved in 0.1 mol/L HEPES, pH 8.0, 0.154 mol/L NaCl, 0.1% human serum albumin. Fifty microliters of the substrate solutions (20, 15, 10, 7.5, 5, 4, 3.3, 2.85 mmol/L) were mixed with 0.4 mL 0.05 M Tris/HCl, pH 8.0, 0.154 mol/L NaCl, and prewarmed to 25 °C. The assays were started by the addition of 50 μL enzyme solution prewarmed to 25 °C. The following enzyme concentrations were used: BM 06.022: 126 μg/mL; BM 06.022 (tc): 75 μg/mL (11 μg/mL in the case of $MeSO_2$-D-HHT-Gly-Arg-pNA and D-HHT-Gly-Arg-pNA as substrate); CHO-t-PA: 205 μg/mL; CHO-t-PA (tc): 75 μg/mL (11 μg/mL in the case of $MeSO_2$-D-HHT-Gly-Arg-pNA and D-HHT-Gly-Arg-pNA as substrate). The amidolytic activity was calculated from the absorbance at 405 nm ($\epsilon_{pNA} = 7{,}850\ L \cdot mol^{-1} \cdot cm^{-1}$). The reaction time was varied so that a maximum of 10% of the respective substrate was cleaved (2–40 min). The velocity was plotted against the substrate concentration, and the data were fitted to the Michaelis–Menten equation with a nonlinear regression analysis program (Enzfitter, Elsevier Biosoft, Cambridge). The k_{cat} was calculated from V_{max}/enzyme concentration. The enzyme concentration (mg/mL) was determined from the absorbance at 280 nm with $\epsilon = 1.69$ and 1.81 $mL \cdot mg^{-1} \cdot cm^{-1}$ for BM 06.022 and CHO-t-PA, respectively. The molar concentration of BM 06.022 and CHO-t-PA was calculated with a molecular weight of 40 and 60 kDa, respectively.

References

Andreasen, P.A., Petersen, L.C., & Dano, K. (1991). Diversity in catalytic properties of single chain and two chain tissue-type plasminogen activator. *Fibrinolysis 5*, 207–215.

Bennett, W.F., Paoni, N.F., Keyt, B.A., Botstein, D., Jones, A.J.S., Presta, L., Wurm, F.M., & Ziller, M.J. (1991). High resolution analysis of functional determinants on human tissue-type plasminogen activator. *J. Biol. Chem. 266*, 5191–5201.

Bode, W., Turk, D., & Stürzebecher, J. (1990). Geometry of binding of the benzamidine- and arginine-based inhibitors N^{α}-(2-naphthylsulfonyl-glycyl)-DL-*p*-amidinophenylalanyl-piperidine (NAPAP) and (2R,4R)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolenesulphonyl)-L-arginyl]-2-piperidine carboxylic acid (MQPA) to human α-thrombin. X-ray crystallographic determination of the NAPAP–trypsin complex and modelling of NAPAP–thrombin and MQPA–thrombin. *Eur. J. Biochem. 193*, 175–182.

Brandstetter, H., Turk, D., Hoeffken, W., Grosse, D., Stürzebecher, J., Martin, P.D., Edwards, B.F.P., & Bode, W. (1992). X-ray crystal structure of thrombin complexes with synthetic thrombin inhibitors. *J. Mol. Biol. 226* (in press).

Collen, D., Stassen, J.M., Marafino, B.J., Builder, S., de Cock, F., Ogez, J., Tajiri, D., Pennica, D., Bennett, W.F., Salwa, J., & Hoyny, C.F. (1984). Biological properties of human tissue-type plasminogen activator obtained by expression of recombinant DNA in mammalian cells. *J. Pharmacol. Exp. Ther. 231*, 146–152.

Dixon, N. (1953). Determination of enzyme inhibitor constants. *Biochem. J. 55*, 170–171.

Harris, T.J.R. (1987). Second generation plasminogen activators. *Protein Eng. 1*, 449–458.

Higgins, D.L. & Bennett, W.F. (1990). Tissue plasminogen activator: The biochemistry and pharmacology of variants produced by mutagenesis. *Annu. Rev. Pharmacol. Toxicol. 30*, 91–121.

Kohnert, U., Rudolph, R., Verheijen, J.H., Weening-Verhoeff, E.J.D., Stern, A., Opitz, U., Martin, U., Lill, H., Prinz, H., Lechner, M., Kresse, G.-B., Buckel, P., & Fischer, S. (1992). Biochemical properties of the kringle 2 and protease domain are maintained in the refolded t-PA deletion variant BM 06.022. *Protein Eng. 5*, 93–100.

Kraut, J. (1977). Serine proteases: Structure and mechanism of catalysis. *Annu. Rev. Biochem. 46*, 331–358.

Macartney, H.W. & Menhardt, J. (1989). Verfahren zur Abtrennung und Reinigung von t-PA. European Patent Application 302503.

Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennett, W.F., Yelverton, E., Seeburg, P.H., Heynecker, H.L., & Goeddel, D.V. (1983). Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature 301*, 214–221.

Rijken, D.C. & Groeneveld, E. (1991). Substrate specificity of tissue-type and urokinase-type plasminogen activators. *Biochem. Biophys. Res. Commun. 174*, 432–438.

Schechter, J. & Berger, A. (1968). On the size of the active site in proteases. *Biochem. Biophys. Res. Commun. 27*, 157–162.

Stürzebecher, J., Horn, H., Markwardt, F., Wagner, G., & Walsmann, P. (1981). Synthetische Inhibitoren der Serinproteinasen. 25. Mitteilung: Hemmung von Trypsin, Plasmin und Thrombin durch Amide Nα-arylsulfonylierter Amidinophenylalanine und 3-Amidinophenyl-3-aminopropionsäuren. *Pharmazie 36*, 639–641.

Stürzebecher, J., Markwardt, F., Vieweg, H., Wagner, G., & Walsmann, P. (1982). Synthetische Inhibitoren der Serinproteinasen. 26. Mitteilung: Hemmung von Trypsin, Plasmin und Thrombin durch Amide Nα-arylsulfonylierter 2-Amino-4-(4-amidinophenyl)-buttersäure und 2-Amino-5-amidinophenylvaleriansäuren. *Pharmazie 37*, 281–283.

Stürzebecher, J., Markwardt, F., Wagner, G., & Walsmann, P. (1976). Synthetische Hemmstoffe der Serinproteinasen. 13. Quantitative Struktur-Wirkungs-Benziehungen bei der Hemmung von Trypsin, Plasmin und Thrombin durch 4-Amidinophenylverbindungen mit Ketonstruktur. *Acta Biol. Med. Ger. 35*, 1665–1676.

van Zonneveld, A.J., Veerman, H., & Pannekoek, H. (1986). On the interaction of the finger and the kringle-2 domain of tissue-type plasminogen activator with fibrin. *J. Biol. Chem. 261*, 14214–14218.

Wagner, G. (1982). Synthesis of benzamidine derivatives as inhibitors of clotting enzymes. *Folia Haematol. 109*, 67–74.

Walsmann, P., Horn, H., Markwardt, F., Richter, P., Stürzebecher, J., Vieweg, H., & Wagner, G. (1976). Synthetische Inhibitoren der Serinproteinasen. 11. Mitteilung: Über die Hemmung von Trypsin, Plasmin und Thrombin durch neue Bisamidinoverbindungen. *Acta Biol. Med. Ger. 35*, K1–K8.