Construction and structure–activity relationships of chimeric prourokinase derivatives with intrinsic thrombin-inhibitory potential

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The blood clotting enzyme thrombin plays a central role in the aetiology of occlusive disorders such as stroke and acute myocardial infarction. During fibrinolytic therapy with plasminogen activators, thrombin is neutralized by anticoagulative drugs. In order to combine plasminogen-activating and thrombin-inhibitory activities we constructed chimeric derivatives of recombinant single-chain, urokinase-type plasminogen activator (rscu-PA) which comprise the kringle and protease domain of rscu-PA fused via a linker sequence to a thrombin-inhibitory domain. The inhibitory domain contains a sequence element directed to the active site of thrombin and a sequence taken from either hirudin or the human thrombin receptor both binding to the fibrinogen recognition site of thrombin. Analysing different sets of point mutants showed that the linker between the protease domain and the active site-directed sequence is contributing significantly to the thrombin-inhibitory potential. Kinetic analysis of thrombin inhibition revealed that most of the chimeras tested competitively inhibit the thrombin-mediated cleavage of a peptide substrate in a concentration-dependent manner; however, in two examples the insertion of one glycine residue into the active site directed-sequence abolished the blockade of the active site. This supports the conclusion that the chimeras with high thrombin-inhibitory potential interact with the active site and the fibrinogen recognition site of thrombin.

Keywords: fibrinolysis/hirudin/prourokinase/thrombin/thrombin receptor

Introduction

Plasminogen activators such as recombinant prourokinase (rscu-PA) induce the dissolution of fibrin clots by converting plasminogen into its active form, plasmin and are being used for the thrombolytic treatment of patients with acute myocardial infarction (AMI). A key enzyme leading to the formation of occlusive intra-arterial clots is the serine protease thrombin which has several procoagulant activities: it activates factors V, VIII, XI and III, converts fibrinogen to fibrin and induces platelet aggregation (Furie and Furie, 1992). In order to achieve reperfusion and to avoid reocclusion during fibrinolytic treatment of AMI it is necessary to neutralize the procoagulative activity of thrombin by co-medication with heparin or, at an experimental level, with hirudin, protein C or argatroban (Eisenberg, 1991; Gruber et al., 1991; Schneider, 1991; Tebbe et al., 1991; Yao et al., 1992; Martin et al., 1993). One important factor which induces reocclusion appears to be active thrombin released from clots undergoing lysis (Rapold, 1990; Stubbs and Bode, 1995). Therefore, it may be advantageous for fibrinolytic treatment of occlusive disorders to use a plasminogen activator with combined plasminogen-activating and thrombin-inhibitory potential. Our approach is different from that described by Phaneuf et al. (1994) who covalently linked hirudin and streptokinase with a synthetic heterobifunctional cross-linker. The streptokinase–hirudin complex bound to immobilized thrombin but displayed a slightly decreased thrombin-inhibitory potential and a significant decrease of the plasminogen-activating activity compared to the parent molecules.

We constructed a set of chimeric plasminogen activators comprising the kringle and protease domain of rscu-PA [amino acids Ser47 through to Leu411; Holmes et al., 1985] fused by a linker sequence to a thrombin-inhibitory domain. The thrombin-inhibitory domain contains a sequence which binds to the fibrinogen recognition site of thrombin (FRS). The FRS, also called anion exosite, is a patch of positively charged amino acids on the surface of thrombin which plays a central role in the interaction with fibrinogen, fibrin, thrombomodulin and the human thrombin receptor (Stubbs and Bode, 1995); however, hydrophobic interactions also significantly contribute to the interaction between the FRS and its binding partner (Rydel et al., 1990). The FRS-binding sequence of the chimeric rscu-PA derivatives described here was taken either from hirudin (corresponding to amino acids Asn53 to Leu64 or Gln65; Rydel et al., 1990) or the human thrombin receptor (corresponding to amino acids Phe43 to Glu63; Vu et al., 1991a). The human thrombin receptor which is activated by thrombin-mediated cleavage contains in its N-terminal extracellular extension a FRS-binding domain adjacent to a thrombin cleavage site (Vu et al., 1991a). Synthetic peptides comprising the FRS-binding domain of hirudin and the human thrombin receptor respectively inhibit thrombin-stimulated coagulation by competitively blocking the binding of fibrinogen to thrombin (Maraganore et al., 1990; Cadroy et al., 1991; Vu et al., 1991b). The thrombin-inhibitory potential can be significantly increased when the active site-directed thrombin-inhibitory peptide (Phe-Pro-Arg-Pro or, to a lower extent, Phe-Pro-Arg-Pro is added to the N-terminus of the FRS-binding domain of hirudin via a linker consisting of at least four glycine residues (Maraganore et al., 1990; Kelly et al., 1992; Yue et al., 1992). The active site-directed sequence Phe-Pro-Arg-Pro is derived from the synthetic thrombin inhibitor PPACK, (Phe-Pro-Arg-chloromethylketone, which binds to the active site of thrombin (Bode et al., 1989). The Pro-Arg motif represents the P2 and P1 residues respectively of the cleavage site of many physiological substrates of thrombin (Fenton, 1995). The linker of at least four glycine residues is necessary to span the distance between the catalytic site and the FRS (Yue et al., 1992). Thus, these bifunctional thrombin
inhibitors act by directly blocking the active site and by preventing binding of thrombin to fibrinogen or to the thrombin receptor. Recent data from a clinical trial (Théroux et al., 1995) with hirulog-S (Maraganore et al., 1990) as an adjunct in thrombolytic treatment of AMI indicated that this synthetic bifunctional thrombin inhibitor leads to higher early patency rates than achieved with heparin as anticoagulant. However, it was also found that hirulog-S is cleaved by thrombin and that high doses (1 mg/kg/h) are less effective than lower doses (0.5 mg/kg/h) which may be explained by impairment of thrombin/thrombomodulin-mediated activation of protein C. Obviously a very high degree of thrombin inhibition does not directly translate into clinical benefit.

The structure of synthetic bifunctional thrombin inhibitors based on a combination of active site-directed sequence Phen-Pro-Arg-Pro, glycine linker and FRS-binding domain was one of the starting points for the design of chimeric rescu-PA derivatives with both plasminogen-activating and thrombin-preventing binding of thrombin to fibrinogen or to the thrombin receptor. Recent data from a clinical trial (Théroux et al., 1995) with hirulog-S (Maraganore et al., 1990) as an adjunct in thrombolytic treatment of AMI indicated that this synthetic bifunctional thrombin inhibitor leads to higher early patency rates than achieved with heparin as anticoagulant. However, it was also found that hirulog-S is cleaved by thrombin and that high doses (1 mg/kg/h) are less effective than lower doses (0.5 mg/kg/h) which may be explained by impairment of thrombin/thrombomodulin-mediated activation of protein C. Obviously a very high degree of thrombin inhibition does not directly translate into clinical benefit.

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Material and methods

Cloning and expression of chimeric rescu-PA derivatives

All expression vectors were constructed by modifying pBF160 (Briegelius-Flohé et al., 1992) which carries a synthetic gene encoding rescu-PA under the control of a synthetic trp-promoter. The region of the gene encoding amino acids 1-46 of rescu-PA was removed by digestion of pBF160 with NdeI and Neol (New England Biolabs, Schwalbach, Germany) and inserting a synthetic DNA linker comprising a ribosomal binding site and a transcriptional start codon. The BamHI/HindIII fragment of the resultant plasmid pSJ41 was subcloned into pUC18 (Pharmacia, Freiburg, Germany) and modified by insertion of synthetic double-stranded oligonucleotides encoding the desired amino acid sequences. The modified BamHI/HindIII fragments were then inserted back into pSJ41 yielding the respective expression plasmid. All modifications and the flanking regions were verified by DNA sequencing. A detailed description of the plasmid construction is given by Steffens et al. (1994). Recombinant expression and purification of the chimeric proteins was performed as described for rescu-PA (Briegelius-Flohé et al., 1992). Escherichia coli JM103 cells carrying the respective plasmid were grown in 1 L standard-medium (Merck, Darmstadt, Germany) supplemented with 150 µg/ml ampicillin (Sigma, Deisenhofen, Germany) at 37°C up to an optical density at 578 nm of ~1. Expression of recombinant protein was induced by addition of isoleucine (final concentration 60 µM) and cultures were grown up to an optical density at 578 nm of ~5. Cells were harvested by centrifugation (15 000 × g), resuspended in 200 ml water and homogenized with a French press. The suspension was then centrifuged again and the pellet consisting of inclusion bodies was solubilized in 500 ml M guanidinium hydrochloride, 40 mM cysteine, 1 mM EDTA, pH 8. After dilution with 2000 ml 25 mM Tris-HCl buffer, pH 9, the solution was stirred overnight in order to allow a proper refolding of the recombinant polypeptides. The proteins were isolated by adding 8 g silica gel to the refolding solution. After sedimentation, the silica gel was transferred into a chromatographic column and washed with 3 volumes of 50 mM citrate buffer, pH 3.5. The proteins were eluted with 0.5 M trimethylammonium chloride in 10 mM acetate buffer, pH 3.5. The eluates were dialysed against 50 mM citrate buffer, pH 4 and protein concentrations were determined by using the BCA-assay (Pierce, Oud Beijerland, The Netherlands). All proteins were analysed by SDS-PAGE (10% polyacrylamide) under reducing conditions.

Determination of specific urokinase activity

For determination of specific activity the rescu-PA derivatives were first converted into the two-chain form by digestion with plasmin. Fifty microtites of the protein sample were diluted with 50 µl Tween-80-citrate buffer [14 mM sodium citrate, 78 mM NaCl, 0.2% Tween 80 (Merck), pH 5]. This dilution was then mixed with 400 µl plasmin-solution [0.025 CU plasmin (Sigma)/ml in 50 mM Tris–HCl, 12 mM NaCl, 0.02% Tween 80, pH 7.4] and incubated for 15 min at 37°C. To 100 µl of this mixture, 800 µl of 0.5 µM aprotinin (Merck) dissolved in 50 mM Tris–HCl, 12 mM NaCl, pH 8.8 and 100 µl 50 mM Tris–HCl, 12 mM NaCl, 0.02% Tween 80, pH 7.4 were added. After pre-incubation for 5 min at 37°C 100 µl of 3 mM S-2444 ([L-pyroglutamyl-L-phenylalanyl-L-lysine-p-nitroanilide; Chromogenix, Antwerp, Belgium] dissolved in water were added and the mixture was further incubated for 10 min at 37°C. The reaction was stopped by addition of 100 µl of 50% acetic acid and the absorption at 405 nm was determined. Urokinase activity was calculated by multiplication of the A405/ml values by a factor of 5400 yielding the activity in Ploug units per millilitre. Specific activity was then derived based on the protein concentration determined with the BCA assay. The content of two-chain uPA was determined by directly measuring the activity of the respective protein against S-2444 without plasmin pre-treatment.

Coagulation assay

The anticoagulative activity of the chimeric rescu-PA derivatives was determined using a standard thrombin time assay. Human citrate-plasma was diluted 10-fold with veronal buffer (Boehringer-Mannheim, Mannheim, Germany) and kept at 37°C for at least 5 min. Two hundred microtites of this dilution were then mixed with 50 µl of solution containing the rescu-PA sample diluted in veronal buffer. The reaction was started by adding 50 µl of solution containing 0.2 NIH units human thrombin (Boehringer-Mannheim) in veronal buffer and the coagulation time was measured automatically by a coagulometer (Sarstedt, Sarstedt, Germany). Each thrombin time determination was performed in duplicate.

Inhibition of thrombin-mediated cleavage of chromogenic substrates and determination of Ki values

For selection of a suitable substrate, S-2238 (S-phenylalanyl-
L-pippecolyl-L-arginine-p-nitroanilide; Chromogenix, Antwerp, Belgium) and L-1150 (benzoyl-L-phenylalanyl-L-valyl-L-arginine-p-nitroanilide; Bachem, Heidelberg, Germany) respectively were incubated in a volume of 200 µl with 0.04 IU/200 µl human thrombin (Sigma) in the presence of increasing concentrations of inhibitor (M23) up to 100 µg/ml. The final concentration of S-2238 was 140 µM and 200 µM in the case of L-1150. The reaction buffer was 100 mM Tris–HCl, 200 mM NaCl, 0.05% Triton-X-100, pH 8. After 10 min incubation at 37°C the reaction was stopped by addition of 50 µl 50% acetic acid and the absorption was determined by using a microplate
Chimeric prourokinase derivatives

reader (SLT, Crailsheim, Germany). The same experimental
design was used to assess whether S-hir54-65 [amino acids
Gly54 to Gln65 of hirudin (sulphated); Bachem, Heidelberg,
FRG] is able to alleviate the inhibitory activity of M23 in the
thrombin-mediated cleavage of L-1150.

Determination of the $K_i$ values was performed using L-1150
at concentrations of 20, 30, 40, 60, 80 and 200 nM and 0.04 IU
thrombin per 200 µl assay volume. The mean $K_m$ value of
human thrombin was 171 nM which is in good agreement
with the previously published value of 160 µM for thrombin-
mediated cleavage of this substrate (Svendsen et al., 1972).
The chimeric rsu-PA derivatives were tested in concentrations
of between 92 and 870 nM. Hirudin (American Diagnostics,
Loxo, Dossenheim, Germany) was tested in concentrations of
between 7.5 and 750 pM. The buffer conditions were 45 mM
Tris–HCl, 90 mM NaCl, 0.02% Triton-X-100, pH 8. Each
determination was performed in duplicate. The initial velocity
of the reaction was determined using a SLT-microplate reader
and evaluated with the EasyKin-software (SLT). Based on the
molar absorption coefficient of p-nitroanilide at 405 nm
(8800 M$^{-1}$×cm$^{-1}$; Erlanger et al., 1965) the enzyme velocities
were calculated and further analysed by the method of
Lineweaver and Burk (Segel, 1975). $K_i$ values and inhibition
types were determined from Dixon plots of the kinetic data
(Segel, 1975).

Results

A set of 20 different chimeric rsu-PA derivatives was con-
structed by modification of a synthetic gene encoding rsu-PA
(Brigelius-Flohé et al., 1992) using conventional cloning
techniques. The chimeric rsu-PA derivatives comprise three
functional modules: a kringle domain, the protease domain
and a thrombin-inhibitory sequence. As an example the com-
plete amino acid sequence of the chimeric rsu-PA derivative
M23 is given in Figure 1. The thrombin-inhibitory domain
fused to the C-terminus of the protease moiety contains four
sequence elements: linker 1, an active site-directed sequence,
linker 2 and a FRS-binding sequence. Figure 2 shows an
alignment of the thrombin-inhibitory domains of the chimeric
rsu-PA derivatives described in this report and the sequence
elements mentioned above.

Protein chemical characterization and specific activity

All chimeric rsu-PA derivatives ran as single bands in the
SDS-PAGE under reducing conditions which demonstrates
that the proteins were expressed and purified in the single-
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Fig. 2. Alignment of amino acid sequences from the C-terminus of the protease domain (Lys358-Leu365) and the thrombin-inhibitory domain of the chimeric rscu-PA derivatives. Amino acid homology is indicated by (:) and gaps are marked by (-).

<table>
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<tr>
<th>rscu-PA derivative</th>
<th>Specific activity (Ploug units/mg protein)</th>
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<tr>
<td>M4</td>
<td>136 000</td>
</tr>
<tr>
<td>M8</td>
<td>111 000</td>
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<td>98 560</td>
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<td>M11</td>
<td>99 960</td>
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<tr>
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<td>140 720</td>
</tr>
<tr>
<td>M13</td>
<td>150 000</td>
</tr>
<tr>
<td>M14</td>
<td>151 820</td>
</tr>
<tr>
<td>M15</td>
<td>153 840</td>
</tr>
<tr>
<td>M16</td>
<td>131 100</td>
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<td>M17</td>
<td>121 000</td>
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<td>101 000</td>
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<tr>
<td>M28</td>
<td>87 500</td>
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<tr>
<td>M29</td>
<td>122 460</td>
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<tr>
<td>M30</td>
<td>108 000</td>
</tr>
<tr>
<td>M31</td>
<td>97 780</td>
</tr>
<tr>
<td>M32</td>
<td>135 960</td>
</tr>
</tbody>
</table>

Table I. Specific activities of the chimeric rscu-PA derivatives determined with S-2444 after conversion into the two-chain form by digestion with plasmin

inhibitor. The FRS-binding sequence motif linker 2 from the thrombin receptor.

Choice of the proper sequence of linker 1

The influence of the length and sequence of the linker 1 on the anticoagulative activity was assessed by analysing chimeric
Chimeric prourokinase derivatives


rscu-PA derivatives M11, M12, M13, M14 and M15 (Figure 2). With respect to their anticoagulative activity the proteins can be listed in the following way: M12 (SPVK-A-FPRP)>M15 ((SPP)3GG-FGRP)>M14 (SPPGG-FPRP)>M11 ((SPP)2GG-FPRP) and M13 ((SPP)2GG-FPRP) (Figure 3A). Sequence SPVK-A originally stems from a cloning intermediate. The significant increase conferred by the introduction of the sequence SPVKA (M12) as linker 1 appears to be sequence dependent, because the sequence SPPGG (M14) was associated with a very much lower anticoagulative activity. The varying length of the linker sequence was without effect on the anticoagulative activity of M14, M11 and M13. However, when M13 was modified by introduction of a glycine residue into the active site-directed sequence FPRP between phenylalanine and proline the anticoagulative activity of the resultant protein M15 was significantly increased. In summary, the
sequence SPVKA as linker 1 and a glycine insertion into the active site-directed sequence FPRP in combination with a (SPP)3GG linker respectively increased the thrombin-inhibitory potential of chimeric rscu-PA derivatives.

Variation of the aromatic amino acid within the active site-directed sequence and effect of a glycine insertion

Another set of chimeric rscu-PA derivatives, M16, M17, M19, M22, M24 and M25 (Figure 2), was constructed in order to test whether (i) the glycine insertion into the active site-directed sequence in combination with the sequence SPVKA of linker 1 and (ii) a conservative substitution of the phenylalanine residue within the active site-directed sequence may further enhance the thrombin-inhibitory potential. The cloning strategy that was chosen to produce this set of proteins made it necessary to introduce a leucine residue into linker 2. In addition, we added a glutamine residue to the C-terminus of the hirudin FRS-binding sequence (corresponding to Asn53 to Leu64 of hirudin) which is also present in native hirudin (amino acid 65). These modifications appear to decrease the anticoagulative activity because M25 which differed by these two modifications from M12 had a somewhat lower anticoagulative activity than the parent molecule (Figure 3A and B).

The rscu-PA derivatives with conservative substitution of the aromatic amino acid and no glycine insertion in the active site-directed sequence showed an anticoagulative activity in the following rank order: M22 (YPRP) > M24 (WPRP) > M25 (FPRP). However, with a glycine residue inserted the bias for tyrosine was not observed. M16 (WGP) and M17 (FGP), both with nearly identical anticoagulative activity, had a significantly higher anticoagulative activity than M19 (YGPRP), which was the weakest thrombin inhibitor in this set of proteins. The insertion of the glycine residue into the active site-directed sequence was without major effect in combination with tryptophan [M24 (WPRP), M16 (WGPRP)] or phenylalanine [M25 (FPRP), M17 (FGPRP)], but led to a very strong reduction of the anticoagulative activity in combination with tyrosine [M22 (YPRP) > M19 (YGPRP)]. Summarizing the results obtained with this set of proteins it can be stated (i) that in general introduction of a glycine residue into the active site-directed sequence has no stimulatory effect on the anticoagulative activity in combination with the sequence SPVKA of linker 1 and (ii) that tyrosine as an aromatic amino acid within the active site-directed sequence YPRP confers an increase of the anticoagulative activity compared to FPRP or WPRP.

Point mutations and deletion of linker 1

The chimeric rscu-PA derivatives M21 (SPVRA), M23 (SPVVA) and M27 (SPVVEV) were constructed (Figure 2) in order to test whether the lysine residue in the sequence SPVKA of linker 1 has a crucial role in the inhibition of thrombin. Again, the anticoagulative activity of these proteins was compared to that of M25 (SPVKA). The ranking with respect to their thrombin-inhibitory potential was M23 (SPVVA) > M25 (SPVKA) > M21 (SPVRA) > M27 (SPVVEV) (Figure 3C). Although M27 is a double-point mutant it appears that substitution of the basic amino acid (lysine or arginine) by an acidic amino acid (glutamic acid) is inducing a drastic decrease of the anticoagulative activity. However, the highest anticoagulative activity was found with M23 which carries an uncharged amino acid, valine, at the relevant position. In order to test whether a further increase of the hydrophobicity of linker 1 and a Phe → Val substitution of the aromatic amino acid within the active site-directed sequence confers a higher anticoagulative activity the rscu-PA derivatives M26 (SPVVV-FPRP) and M28 (SPVV-VPRP) were constructed. However, as shown in Figure 3(D) these chimeric rscu-PA derivatives had a lower anticoagulative activity than M23 (SPVVA). Comparison of M26 and M28 also shows that a Phe → Val substitution in the active site-directed sequence leads to a significant decrease of the anticoagulative activity. In addition, the proline residue of linker 1 was substituted by leucine in M30 (SLVVA) and M31 (SLVKA). This substitution led to a lower anticoagulative activity of M30 and M31 compared to M23 (SPVVA). Comparison of M30 with M31 shows again that the presence of a charged amino acid within linker 1 reduces the anticoagulative activity. Finally, we constructed a chimeric rscu-PA derivative in which linker 1 is missing (M33). This derivative showed a very strong reduction of the anticoagulative activity in combination with SPVKA of linker 1.

Fig. 4. (A) Effect of M23 on thrombin-mediated cleavage of S-2238 (■) and L-1150 (▲). The substrate concentration was 200 μM for L-1150 and 140 μM in case of S-2238. Thrombin was used at a concentration of 0.2 NIH units/ml. The A405/ml in the absence of inhibitor (M23) (corresponding to 100% thrombin activity) was 0.431 for S-2238 and 0.182 with L-1150. Incubation of S-2238 or L-1150 with M23 alone led to an A405/ml of 0.056 and 0.066 respectively which was only slightly above the buffer only control (0.047 for each substrate). (B) Effect of S-hiru54-65 on inhibition of thrombin-mediated cleavage of L-1150 by M23. The concentration of L-1150 and thrombin respectively was same as in (A) (■) shows the dose-dependent inhibition by M23 alone. S-hiru54-65 was used at a final concentration of 0.5 μM (▲) and 1 μM (▼). The A405/ml in the absence of inhibitor (M23) (corresponding to 100% thrombin activity) was 0.204 in the absence of S-hiru54-65, 0.310 in presence of 0.5 μM S-hiru54-65 and 0.268 in presence of 1 μM S-hiru54-65. The data represent means of triplicates and the SD was below 10%.
protein displayed a significant lower anticoagulative effect than most of the chimeric rscu-PA derivatives and therefore confirms the importance of linker 1 (Figure 3D). In summary, linker 1 has a strong influence on the anticoagulative activity of the chimeric rscu-PA derivatives. The linker 1 sequence SPVVA is associated with a significantly higher anticoagulative activity than sequences which contain a charged amino acid or a higher degree of hydrophobicity.

**Variation of the FRS-binding sequence**

All chimeric rscu-PA derivatives described so far contain the C-terminal fragment of hirudin which binds to the FRS of thrombin (Rydel et al., 1990). The human thrombin receptor contains a sequence which is similar to the FRS-binding sequence of hirudin (Vu et al., 1991b). Therefore, we constructed a chimeric rscu-PA derivative, M32, containing the FRS-binding sequence (corresponding to amino acids Phe43 to Glu63) of the human thrombin receptor. Linker 1 and the active site-directed sequence were identical with that used in M25, but linker 2 comprised amino acids 43-47 of the thrombin receptor. M32 induced a concentration-dependent prolongation of thrombin time; however, the anticoagulative activity of M32 is lower than that of M23 and M25 (Figure 3E).

**Effect on the amidolytic activity of thrombin**

In addition to the coagulation assay of the anticoagulative effect of the chimeric rscu-PA derivatives, we tested whether M23 inhibits the thrombin-mediated cleavage of the synthetic low molecular weight substrates (d-Phe)-Pip-Arg-pNA (S-2238) and Bz-Phe-Val-Arg-pNA (L-1150) respectively. As shown in Figure 4(A) inhibition of thrombin was observed only with L-1150 but not with S-2238. While M23 induced a concentration-dependent inhibition of thrombin-mediated cleavage of L-1150, rscu-PA did not inhibit thrombin and displayed no amidolytic activity towards L-1150. Therefore L-1150 was used to determine the $K_i$ of hirudin and several chimeric rscu-PA derivatives derived from M25 (Table II). The $K_i$-value of hirudin (0.038 ± 0.012 nM) was >1000-fold lower than that of the strongest chimeric rscu-PA derivative (M22; 89 ± 7 nM). However, the $K_i$ values of the chimeric proteins generally reflect the structure–activity relations established by means of the coagulation assay. With the exception of two proteins, M17 and M19, all proteins tested are competitive inhibitors as indicated by the evaluation of Dixon plots of the kinetic data obtained at different substrate and inhibitor concentrations (see for example Figure 5).

Substitution of the aromatic amino acid of the active site-directed sequence led to drastic differences in the $K_i$ value, which again show a bias towards tyrosine, but are interchanged with respect to M24 and M25, respectively, compared to the data of the coagulation assay. M22 (YPRP; 89 ± 7 nM) < M25 (FPRP; 289 ± 77 nM) < M24 (WPRP; 486 ± 101 nM). The insertion of a glycine residue into the active site-directed sequence led to significant increases of the $K_i$ of M19 (YGPRP, 868 ± 107 nM) compared to M22 (Figure 3E). The same was observed with M17 (FGPRP, 562 ± 61 nM) and, in a less pronounced manner, with M16 (WGPRP, 345 ± 34 nM), which were derived from M25 and M24 respectively. In addition, for M17 and M19 the type of inhibition changes from a competitive to a non-competitive inhibition of thrombin, as indicated by the position of the common intercept of the $1/v$ versus inhibitor concentration curves (Figure 5): a common intercept at the x-axis is typical for non-competitive inhibitors.

**Table II. $K_i$ values (means ± SD) for chimeric rscu derivatives and hirudin determined with L-1150 and human thrombin**

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<thead>
<tr>
<th>Protein</th>
<th>$K_i$ (nM)</th>
<th>Type of inhibition</th>
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<tr>
<td>Hirudin</td>
<td>0.038 ± 0.012</td>
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<tr>
<td>M8</td>
<td>&gt;2500</td>
<td>Competitive</td>
</tr>
<tr>
<td>M9</td>
<td>&gt;2500</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>M16</td>
<td>345 ± 34</td>
<td>Competitive</td>
</tr>
<tr>
<td>M17</td>
<td>562 ± 61</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>M19</td>
<td>868 ± 107</td>
<td>Non-competitive</td>
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<tr>
<td>M28</td>
<td>1683 ± 117</td>
<td>Competitive</td>
</tr>
<tr>
<td>M31</td>
<td>256 ± 39</td>
<td>Competitive</td>
</tr>
<tr>
<td>M32</td>
<td>441 ± 101</td>
<td>Competitive</td>
</tr>
<tr>
<td>M33</td>
<td>536 ± 94</td>
<td>Competitive</td>
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**Fig. 5. Dixon plots of a pair of chimeras differing by a point mutation within the active site-directed peptide (glycine insertion). (A) Inhibition of thrombin-mediated cleavage of L-1150 in the presence of M22 (YPRP): substrate concentrations: (□) 20 μM, (△) 30 μM, (▲) 40 μM, (●) 60 μM, (▲) 80 μM and (●) 200 μM. (B) Inhibition by M19 (YGPRP): substrate concentrations (△) 30 μM, (▲) 40 μM, (●) 60 μM and (●) 200 μM.**
while in the case of non-competitive inhibitors the reduction of the reaction velocity can be neutralized by excess substrate leading to an intercept at \(1/v_{\text{max}}\) (Segel, 1975). Substitution of the aromatic residue of the active site-directed sequence by valine led to a drastic increase of the \(K_i\) (M28, SPVVV-VPRP; 1683 ± 117 nM) compared to M26 (SPVVV-FPRP; 402 ± 61 nM), which confirms the important role of the aromatic residue within the active site-directed sequence.

Point mutations within linker 1 directly influence the thrombin-inhibitory potential. The data confirm that the sequence SPVVVA of linker 1 of M23 is associated with a higher thrombin-inhibitory potential (\(K_i\) 159 ± 20 nM) than M25 (SPVKVA; \(K_i\) 289 ± 77 nM), M26 (SPVVV; \(K_i\) 402 ± 61 nM), M27 (SPVEV; \(K_i\) 661 ± 143 nM) and M31 (SLVKA; \(K_i\) 256 ± 39 nM). However, in contrast to the data from the coagulation assay no decrease of \(K_i\) induced by substitution of the proline residue in linker 1 against leucine was found (compare M25 and M31). In addition, the relatively low \(K_i\) of M21 (SPVRA; 169 ± 48 nM) was not expected on the basis of its relatively weak anticoagulative activity in the coagulation assay. Omission of linker 1 increased the \(K_i\) of M33 compared to the relatively good inhibitors M21, M22 and M23. Finally, the protein M32, carrying the FRS-binding sequence from the human thrombin receptor, was found to competitively inhibit the cleavage of L-1150 with a \(K_i\) value of 441 ± 101 nM.

**Effect of S-hir 54-65 on thrombin inhibition induced by M23**

In order to analyse how the FRS-binding sequences of the chimeras contribute to inhibition of thrombin we tested whether S-hir 54-65 which represents the FRS-binding sequence of hirudin can alleviate the inhibitory activity of M23 in the thrombin-mediated cleavage of L-1150. S-hir 54-65 itself did not inhibit the cleavage of L-1150 by thrombin but in contrast slightly increased thrombin's activity (Figure 4B, legend). These observations confirm previously published data on hirudin peptides (Maraganore et al., 1990; Naski et al., 1990; Kelly et al., 1992). Addition of hirugen in concentrations of 0.5 and 1 \(\mu\)M respectively to the amidolytic assay led to a reduction of the thrombin inhibition induced by increasing concentrations of M23 (Figure 4B). The concentration range of M23 (MW 43000) was between 0.46 \(\mu\)M (20 \(\mu\)g/ml) and 2.32 \(\mu\)M (100 \(\mu\)g/ml); therefore stoichiometric concentrations of S-hir 54-65 are sufficient to alleviate the thrombin inhibition mediated by M23.

**Discussion**

The rsu-PA derivatives described in this report were constructed by fusion of a thrombin-inhibitory domain to the C-terminus of a truncated rsu-PA comprising only the kringle and protease domain. The chimeras inhibited the thrombin-stimulated coagulation of human plasma with different efficacy and displayed different \(K_i\) values for thrombin-mediated cleavage of a chromogenic substrate depending on certain sequence variations within the thrombin-inhibitory domain. Two sequence elements within the thrombin-inhibitory domain, linker 1 and the active site-directed sequence respectively were individually modified in order to improve the thrombin-inhibitory potential of the chimeric proteins. The length of linker 1 appeared to be without effect on the anticoagulative activity; however, it was found that the sequence SPVKVA (M12 and M25) significantly increased the anticoagulative activity (Figure 3A). The role of single residues within this linker sequence was analysed by determining the effect of point mutations on the anticoagulative activity. The Pro → Leu (M31) and Lys → Arg (M21) point mutations and the Ala → Val, Val (M26) and Lys, Ala → Glu, Val (M27) double mutations were associated with a decrease of the anticoagulative effect compared to M25, carrying the parent sequence SPVK. However, when the lysine residue within linker 1 was substituted by valine the resultant protein M23 (SPVVA) displayed a significantly higher anticoagulative effect than any other variant of linker 1 suggesting that charged amino acids within linker 1 reduce the anticoagulative activity (Figure 3C and D). Complete omission of linker 1 in M33 was associated with a reduced anticoagulative activity compared to M12, M22, M23 and M25. The effects of modifications of linker 1 on the anticoagulative activity may be either explained by a direct interaction of linker 1 with thrombin or by an effect of these substitutions on the accessibility of the active site-directed and FRS-binding domain for interaction with thrombin. Introduction of the linker 1 sequence (SPP)GG in combination with the modified active site-directed sequence FGPRP (M15) induced an increase of the anticoagulative activity similar to that of M12 or M25. Based on this result several derivatives were constructed in order to find out whether a combination of the linker 1 sequence SPVK with the active site-directed sequences FGPRP, YGPRP and WQPRP respectively leads to an increased anticoagulative activity. However, as shown in Figure 3(B) the insertion of glycine did not generally increase the anticoagulative activity and, in combination with tyrosine even led to a dramatic decrease. Comparison of those proteins without glycine insertion revealed that the presence of tyrosine in the active site-directed sequence increases the anticoagulative activity of M22 over M25 and M24 which carry phenylalanine and tryptophan respectively. In addition, the important role of the aromatic residue is highlighted by the fact that substitution of phenylalanine by valine induced a significant decrease of the anticoagulative activity (compare M26 and M28, Figure 3D). These data support the conclusion that the aromatic residue directly interacts with the argyl-binding site of thrombin which belongs to the apolar-binding site and is located close to the catalytic cleft (Stubbs and Bode, 1995).

We also tested whether the FRS-binding domain from hirudin which was used in the chimeras (except M32) could be substituted by an analogous domain from the human thrombin receptor. Indeed, by using linker 1 and the active site-directed sequence from M25 it was shown that the FRS-binding domain from the human thrombin receptor can be used for the construction of thrombin-inhibitory domains (Figure 3E) although the anticoagulative activity was lower than that achieved with proteins containing the FRS-binding domain from hirudin.

As outlined above inhibition of thrombin by peptides can be achieved by blocking either the active site or the FRS of the enzyme. In order to find out whether the active site of thrombin is actually blocked by the chimeric rsu-PA derivatives the effect of M23 on the thrombin-mediated cleavage of two chromogenic peptide substrates, S-2238 and L-1150, was assessed. Interestingly, cleavage of S-2238 was unaffected by M23, but inhibition of thrombin was observed with L-1150 (Figure 4). This substrate dependence is probably a matter of affinity: S-2238 appears to have a much higher affinity for thrombin than L-1150 since the \(K_m\) values of human thrombin differ by two orders of magnitude for these two substrates (S-2238: 7 \(\mu\)M, supplier’s datasheet; L-1150: 160 \(\mu\)M).
L-1150 was used for kinetic analysis of thrombin inhibition by several chimeric rscu-PA derivatives (Table II). In general, the $K_i$ values nicely correlate with the relative anticoagulative activity determined in the coagulation assay. Dixon plot analysis of the kinetic data obtained at different substrate concentrations indicated that with exception of M17 and M19 all chimeric rscu-PA derivatives were competitive inhibitors of thrombin (see, for example, Figure 5). This indicates that the chimeras (except M17 and M19) directly interact with the active site, although the catalytic center still appears to be accessible for the interaction with substrates with higher affinity for thrombin than L-1150. Substitution of the aromatic amino acid within the active site-directed sequence (M25, FPRP) by tyrosine (M22, YPRP), tryptophan (M24, WPRP) and valine (M28, VPRP) was associated with significant changes of the $K_i$ values. The data indicate that an aliphatic amino acid strongly reduces the thrombin-inhibitory potential, while tyrosine is the best aromatic amino acid. The effect of these point mutations within the active site-directed sequence indicate that it is indeed this sequence element which blocks the catalytic center. This is further confirmed by the fact that M17 and M19 which carry one glycine residue inserted into the active site-directed sequence showed a non-competitive type of thrombin inhibition, in contrast to their respective counterparts without glycine insertion, which competitively inhibited the cleavage of L-1150 (Figure 5). The non-competitive inhibition can be explained by binding of M17 and M19 to the FRS of thrombin. Binding of the chimeras to thrombin might affect the proper positioning of the catalytic center. This type of inhibition will probably also contribute to the anticoagulative activity of the other chimeric rscu-PA derivatives which competitively inhibit the cleavage of L-1150 by thrombin. The observation that a peptide representing the FRS-binding sequence of hirudin can alleviate the inhibition of thrombin mediated by M23 indicates that the chimeric rscu-PA derivatives also contribute to the anticoagulative activity of the other chimeric rscu-PA derivatives which competitively inhibit the cleavage of L-1150 by thrombin. This may be also speculated that the clot specificity of prourokinase (Fleury et al., 1993; Husain, 1993) is retained in the chimeric rscu-PA derivatives leading to an anticoagulative effect localized on the surface of the clot. This may then contribute to a more efficient inhibition of fibrin deposition on the thrombus surface than with a standard anticoagulative and thrombolytic treatment. The advantage of fibrin-targeted anticoagulation has been recently demonstrated in vitro with hirudin linked to a fibrin-specific antibody (Bode et al., 1994).

However, further pre-clinical studies are necessary to analyse the mechanism of action leading to the high clot specificity of M23 and to evaluate the possible therapeutic advantage of this new fibrinolytic agent.

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