Autocatalytic Peptide Bond Cleavages in Prothrombin and Meizothrombin†

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ABSTRACT: During factor Xa-catalyzed prothrombin activation, several other reaction products accumulate as a result of proteolysis of prothrombin and its activation products by thrombin and meizothrombin. Gel electrophoretic analysis and N-terminal sequencing of reaction products showed that in the absence of Ca2+ ions thrombin cleaved the following peptide bonds: Arg51—Thr52/Arg54—Asp55 in the fragment 1 (F1) domain (k = 0.4 × 10^4 M^-1 s^-1), Arg155—Ser156 in prothrombin (k = 2 × 10^4 M^-1 s^-1), and Arg284—Thr285 in prethrombin 1 (k = 0.02 × 10^4 M^-1 s^-1). In the presence of 2.5 mM CaCl2, cleavage in fragment 1 (Arg51—Thr52/Arg54—Asp55) was not detectable, whereas cleavage at Arg155—Ser156 (i.e., removal of F1) was inhibited 25-fold. Cleavage at Arg284—Thr285 (formation of prethrombin 2 des-1—13) was not affected by the presence of Ca2+ ions. Meizothrombin rapidly converted itself into meizothrombin des-F1. The half-life (t1/2 = ~30 s) of this reaction was independent of the meizothrombin concentration (0.1—1 μM meizothrombin), which is indicative for intramolecular autocatalysis (k = 0.02 s^-1 in the presence of 2.5 mM Ca2+ ions). Since the rapid removal of fragment 1 precludes investigations of the cleavage at Arg284—Thr285 in intact meizothrombin, we analyzed the cleavage of this peptide bond in R155A-meizothrombin, a recombinant product that is resistant to autocatalytic removal of the fragment 1 domain. In the absence of phospholipids, R155A-meizothrombin converted itself into thrombin des-1—13 by a combination of intramolecular (k = 0.8 × 10^4 s^-1) and intermolecular autocatalysis (k = 0.2 × 10^2 M^-1 s^-1). Intramolecular autocatalytic conversion of R155A-meizothrombin into thrombin was not affected by the presence of phospholipids (k = 0.8 × 10^-4 s^-1), whereas intermolecular autocatalysis was accelerated 25-fold (k = 5.6 × 10^3 M^-1 s^-1) by phospholipid vesicles. Since factor Xa/Va-catalyzed conversion of meizothrombin into thrombin occurs with k = 5.5 × 10^8 M^-1 s^-1, we conclude that in reaction systems containing purified proteins autocatalysis of meizothrombin hardly contributes to thrombin formation during factor Xa-catalyzed prothrombin activation.

The conversion of the zymogen prothrombin into its active form thrombin is one of the central reactions of blood coagulation. Thrombin formation is the result of cleavage of two peptide bonds at Arg271 and Arg270 in prothrombin1 by blood coagulation factor Xa (Suttie & Jackson, 1977; Heldebrant et al., 1973; Downing et al., 1975). Dependent on the order of cleavage, two activation pathways are possible and both intermediates, prethrombin 2 (Esmon et al., 1974; Rosing et al., 1980) and meizothrombin (Krishnaswamy et al., 1986; Rosing et al., 1986) are detected during prothrombin activation. Prethrombin 2 has no catalytic activity, while meizothrombin, a two-chain molecule with the same molecular weight as thrombin, exhibits full esterolytic and amidolytic activity toward small substrates. Compared with thrombin, meizothrombin has reduced activity on macromolecular thrombin substrates (Rhee et al., 1982; Krishnaswamy et al., 1986; Doyle & Mann, 1990), which may be enhanced, however, by the presence of negatively charged phospholipids (Tans et al., 1994).

A clear description of product generation during prothrombin activation is difficult because of very effective autocatalytic degradation of prothrombin and prothrombin activation products. Thrombin and meizothrombin are capable of a feedback reaction at Arg155—Ser156 bond in prothrombin or meizothrombin. This results in the removal of fragment 1 (F1)2 and generation of prethrombin 1 or meizothrombin des-F1. The half-life (t1/2 = ~30 s) of this reaction was independent of the meizothrombin concentration (0.1—1 μM meizothrombin), which is indicative for intramolecular autocatalysis (k = 0.02 s^-1 in the presence of 2.5 mM Ca2+ ions). Since the rapid removal of fragment 1 precludes investigations of the cleavage at Arg284—Thr285 in intact meizothrombin, we analyzed the cleavage of this peptide bond in R155A-meizothrombin, a recombinant product that is resistant to autocatalytic removal of the fragment 1 domain. In the absence of phospholipids, R155A-meizothrombin converted itself into thrombin des-1—13 by a combination of intramolecular (k = 0.8 × 10^4 s^-1) and intermolecular autocatalysis (k = 0.2 × 10^2 M^-1 s^-1). Intramolecular autocatalytic conversion of R155A-meizothrombin into thrombin was not affected by the presence of phospholipids (k = 0.8 × 10^-4 s^-1), whereas intermolecular autocatalysis was accelerated 25-fold (k = 5.6 × 10^3 M^-1 s^-1) by phospholipid vesicles. Since factor Xa/Va-catalyzed conversion of meizothrombin into thrombin occurs with k = 5.5 × 10^8 M^-1 s^-1, we conclude that in reaction systems containing purified proteins autocatalysis of meizothrombin hardly contributes to thrombin formation during factor Xa-catalyzed prothrombin activation.

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5 The numbering used corresponds to the cDNA sequence coding for the human prothrombin (cf. Degen & Davie, 1987). Human prothrombin consists of 579 residues whereas bovine prothrombin contains 582 residues. Consequently, the numbering of amino acids of human prothrombin differs by three residues from that of the bovine molecule.
F1 (Doyle & Mann, 1990; Esmon et al., 1974). Autoproteolysis at Arg^{284} in human prothrombin will give rise to a form of thrombin (thrombin des-1—13), with a 13 amino acid shorter A-chain, which has the same catalytic properties as α-thrombin (Downing et al., 1975; Lanchantin et al., 1973). When this cleavage occurs in meizothrombin, fragment 1.2.3 and thrombin des-1—13 are liberated (Doyle & Mann, 1990; Rabiet et al., 1986). Other thrombin cleavage sites within fragment 1 have been reported at Arg^{24} (Walz et al., 1996) and at Arg^{54} (Board & Shaw, 1983). The latter cleavage was recently reported as Arg^{55}—Asp^{56} by Stevens et al. (1996) in a recombinant stable meizothrombin with the cleavage site mutations R155A, R271A, and R284A (in the human cDNA numbering1 this is, however, Arg^{52}—Asp^{53}). In thrombin, a number of additional bonds can be cleaved by autocatalysis at the carboxyl-terminus of the molecule (B-chain), resulting in forms of thrombin (β- and γ-thrombin) with reduced procoagulant activity (Boissel et al., 1984).

In plasma or whole blood only low amounts of fragment 1 (Rabiet et al., 1986; Aronson et al., 1977) or meizothrombin (Tans et al., 1991) accumulate. This suggests the presence of additional mechanisms which may protect prothrombin from feedback proteolysis and alter the peptide bond cleavage pattern during plasma prothrombin activation. To allow evaluation of feedback reactions during prothrombin activation, we determined in a model system the rate constants of autocatalytic cleavages in prothrombin and R155A-prothrombin, a recombinant prothrombin in which Arg155 is replaced by Ala and which, due to the amino acid substitution, cannot be converted into prethrombin 1 or meizothrombin des-F1. This study was undertaken in order to elucidate the possible physiological significance of feedback and/or autocatalytic reactions during in vivo prothrombin activation.

**EXPERIMENTAL PROCEDURES**

**Materials.** Heps, Tris, EDTA, bovine serum albumin, and ovalbumin were purchased from Sigma Chemical Co. DOPC and DOPS were obtained from Avanti Polar Lipids, Pelham, AL. Small unilamellar phospholipid vesicles were prepared as described before (Rosing et al., 1980). The chromogenic substrate S2238 and the reversible thrombin inhibitor I2581 were supplied by Chromogenix, Mölndal, Sweden. Ecarin, the prothrombin activator from *Echis carinatus* venom, was purchased from Pentapharm, Switzerland. Heparin (USP activity 175 units/mg) was purchased from Organon. PPACK was obtained from Calbiochem, and p-NPGB was from Nutritional Biochemicals. Micro BCA protein assay kits were from Pierce, Rockford. Immobilon-P membranes were obtained from Millipore, Bedford, MA. Antibodies were supplied by Kordia, The Netherlands, and Sigma Chemical Co. Rabbit antihuman F1.2 was a kind gift of Dr. T. Lindhout, Maastricht University, The Netherlands.

Materials used for protein purification were purchased from Pharmacia.

**Proteins.** The human coagulation factors used in this study were purified from fresh frozen plasma. Human prothrombin was purified according to DiScipio et al. (1977). Human thrombin was prepared from prothrombin activation mixtures by the method of Fletcher and Nesestuen (1982). Recombinant prothrombin (R155A-prothrombin) was obtained and purified as described before (Tans et al., 1994). Human antithrombin III was isolated according to Thaler and Schmer (1975). Prethrombin 1 and fragment 1 were purified from a reaction mixture of human prothrombin with thrombin in 50 mM Tris (pH 7.9) and 175 mM NaCl. After inhibition of thrombin with PPACK, the reaction mixture was applied to a column (XK 5/10) with Q Sepharose fast flow. Prethrombin 1 did not adhere to the resin, and fragment 1 and noncleaved prothrombin were eluted with a gradient of 250 to 600 mM NaCl. Fragment 1 was separated from prothrombin by gel permeation chromatography on a Superdex 200 HiLoad (16/60) column in 25 mM Heps (pH 7.7) and 175 mM NaCl. R155A-meizothrombin, meizothrombin, and meizothrombin des-fragment 1 were obtained from reaction mixtures in which R155A-prothrombin or prothrombin were incubated with purified prothrombin activators from *Echis carinatus* or *Echis coloratus* (Tans et al., 1994). Prothrombin, R155A-prothrombin, antithrombin III, prethrombin 1, fragment 1, and thrombin were stored at −80 °C. Protein preparations were homogeneous and >95% pure as judged by SDS—PAGE according to Laemmli (1970).

**Protein Concentrations.** Protein concentrations were determined with the micro BCA protein assay (Smith et al., 1985). Molar thrombin concentrations were determined by active site titration with p-NPGB (Chase & Shaw, 1969). Prothrombin concentrations were determined after complete activation of prothrombin with *Echis carinatus* venom and quantitation of thrombin with p-NPGB. Meizothrombin and R155A-meizothrombin concentrations were determined with S2238, considering that the amidolytic activity per mole is the same as that of thrombin (Rosing et al., 1986; Doyle & Mann, 1990).

**Measurement of Prothrombin Activation.** Amidolytic activity (thrombin, meizothrombin, and/or meizothrombin des-F1) generated during prothrombin activation was determined by transferring samples from reaction mixtures to cuvettes containing a final volume of 1 mL of 50 mM Tris·HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg/mL ovalbumin, and 235 μM S2238 at 37 °C, as described earlier (Rosing et al., 1986). Quantitation of meizothrombin plus meizothrombin des-F1 was performed in appropriate dilutions by measurement of the activity toward S2238, that remained after 1 min incubation with 5 nM AT III in the presence of 30 μg/mL heparin, as described before (Rosing et al., 1986). This assay does not discriminate between meizothrombin and meizothrombin des-F1, since under the assay conditions they exhibit the same activity toward S2238 and are equally inhibited by AT-III plus heparin.

**Gel Electrophoretic and Immunoblot Analysis.** Products of prothrombin proteolysis were analyzed after SDS—PAGE on 13% slabs gels according to Laemmli (1970) and identified after staining with Coomassie Brilliant Blue R-250 or after electrophoretic transfer of proteins from the gel to Immo-
bilon-P membranes and visualization with a polyclonal rabbit-antibody directed against the human F1.2 and goat anti-rabbit IgG conjugated with alkaline phosphatase as described by Blake et al. (1984). The immunoblots were scanned on a Pharmacia Image Master DTS scanner and the amounts of proteins were quantified using the “Image Master 1D” program as percentage of staining intensity of the same protein bands in the sample taken at time zero. The relation between the staining intensity and the amount of protein on the gel was determined on separate gels that contained varying known amounts of prothrombin, prethrombin 1, or fragment 1 per lane.

Amino-Terminal Sequence Analysis. Prothrombin (5 μM) was incubated at 37 °C for 2 h with 2 μM thrombin in the absence of Ca2+ in 25 mM Hepes (pH 7.7) and 175 mM NaCl. The reaction products were separated by SDS–PAGE on 13% reduced gels, transferred to Immobilon-P membranes in 50 mM Tris-borate (pH 8.4) and 20% methanol, and stained with diluted Coomassie Brilliant Blue R-250. Protein bands were cut from the blots and subjected to automated amino-terminal sequencing on a Procise 494 Sequencer from Applied Biosystems, Foster City, CA (Protein Laboratory, IMB-Jena Germany).

Kinetic Analysis of Time Courses of Proteolysis. Time courses of prothrombin, prethrombin1, meizothrombin, or fragment 1 proteolysis were followed by SDS–PAGE, immunoblotting, and quantitation of the intensity of the protein bands on the blots as described above. The fraction of substrate remaining at each time interval (S/S0) was plotted vs time and the pseudo-first-order rate constants (k) were obtained by fitting the time courses of prothrombin, prethrombin 1, meizothrombin, or fragment 1 disappearance to a single exponential (S0 = S0e−kt, in which S0 = the intensity of the protein band at T = 0 and S = the intensity of the protein band at T = t) using non-linear least squares regression analysis. Second-order rate constants were obtained by dividing the pseudo-first-order rate constant by the enzyme concentration.

RESULTS

Peptide Bonds in Human Prothrombin Susceptible to Feedback Proteolysis by Thrombin. The time course of product generation during feedback reactions of thrombin on prothrombin was followed by SDS–PAGE and visualization of protein bands by staining with Coomassie Blue (Figure 1). In the absence of Ca2+ ions, considerable amounts of prethrombin 1 and fragment 1 were formed in the early phase of the incubation. Their concentration decreased after prolonged incubation with formation of products with lower molecular weights (designated band 1, 2, and 3, cf. Figure 1). The appearance of these bands is indicative for thrombin-catalyzed cleavages in prethrombin 1 and fragment 1.

Amino-terminal sequence analysis of the protein bands 1, 2, and 3 formed during incubation of prothrombin with thrombin in the absence of Ca2+ yielded the following sequences: band 1, Thr-Phe-Gly-Ser-Gly-Glu-Ala-Asp; band 2, Ser-Glu-Gly-Ser-Ser-Val-Asn-Leu; and band 3, Thr-Pro-Arg-Asp-Lys-Leu-Ala-Ala and Asp-Lys-Leu-Ala-Ala-Thr-Val-Asn-Leu-Glu. Comparison with the primary amino acid sequence of human prothrombin (Degen & Davie, 1987) indicates that these products result from thrombin-catalyzed cleavages at Arg284—Thr285 in prethrombin 1 giving rise to prethrombin 2 des-1-13 (band 1) and fragment 2.3 (band 2) and at Arg51—Thr52 and Arg54—Asp55 in the fragment 1 region resulting in the formation of fragments with apparent Mr ≈ 20 kDa (band 3) and 7 kDa (not visible on the gel) derived from fragment 1.

Effect of Ca2+ Ions on Proteolysis of Human Prothrombin, Prethrombin 1, and Fragment 1 by Thrombin. Earlier investigations on the removal of fragment 1 from bovine prothrombin by thrombin and on the cleavage at Arg54—Asp55 in recombinant meizothrombin demonstrated inhibition by Ca2+ (Silverberg, 1979; Stevens et al., 1996). To evaluate the effect of Ca2+ ions on the thrombin-catalyzed cleavages at Arg155—Ser156, Arg284—Thr285, and Arg51—Thr52/Arg54—Asp55, the disappearance of the substrates for these peptide...
slowed down about 25-fold, whereas no significant changes
human prothrombin to prethrombin 1 and fragment 1 was
fragment 1 was completely inhibited and proteolysis of
were observed (Table 1).

Variation of the concentrations of reactants showed that the
first-order rate constants obtained from the exponential fits.
of second-order rate constants (Table 1) from the pseudo-
substrate (0
rates of peptide bond cleavages were first-order in both
Figure 2: Time courses of prothrombin, prethrombin 1, and
fragment 1 proteolysis by thrombin. Proteolysis of 0.5 μM
prothrombin (B), prethrombin 1 (●), or fragment 1 (▲) was
followed at 37 °C in reaction mixtures containing 25 mM Hepes
(pH 7.7), 175 mM NaCl, and 0.4 μM thrombin without CaCl2 (panel
A) or 0.8 μM thrombin in the presence of 2.5 mM CaCl2 (panel B).
After various time intervals aliquots from the reaction mixture
were diluted in SDS—electrophoresis buffer (with 5% β-mercaptoethanol)
and 10 μL of these samples was subjected to SDS—PAGE on 13% slab gels, immunoblotting and quantification of the
staining intensity of protein bands as described in the Experimental
Procedures. The solid lines represent exponential curves obtained
by fitting the data to the equation given in the Experimental
Procedures.

Figure 2A shows the time courses of thrombin-catalyzed cleavages in prothrombin, prethrombin 1, and fragment 1 in
the absence of Ca2+ ions. The time courses could be fitted
with a single exponential equation and yielded pseudo-first-order
rate constants for thrombin-catalyzed cleavages at Arg155—Ser156, Arg245—Thr285, Arg51—Thr52, and Arg54—Asp55.
Variation of the concentrations of reactants showed that the
rates of peptide bond cleavages were first-order in both
substrate (0—2 μM prothrombin, prethrombin 1, or fragment 1) and enzyme (0—2 μM thrombin). This allows calculation of
second-order rate constants (Table 1) from the pseudo-
first-order rate constants obtained from the exponential fits.

In the presence of Ca2+ ions (Figure 2B), proteolysis in
fragment 1 was completely inhibited and proteolysis of
human prothrombin to prethrombin 1 and fragment 1 was
slowed down about 25-fold, whereas no significant changes of the rate of cleavage at Arg284—Thr285 in prethrombin 1
were observed (Table 1).

Table 1: Rate Constants of Proteolytic Cleavages in Human Prothrombin

<table>
<thead>
<tr>
<th>substrate</th>
<th>enzyme</th>
<th>peptide bond</th>
<th>products</th>
<th>no Ca2+</th>
<th>2.5 mM Ca2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>thrombin</td>
<td>Arg155—Ser156</td>
<td>PT1, F1</td>
<td>2 × 10^4</td>
<td>0.08 × 10^4</td>
</tr>
<tr>
<td>PT1</td>
<td>thrombin</td>
<td>Arg245—Thr285</td>
<td>PT2 des-1—13, F2.3</td>
<td>0.02 × 10^4</td>
<td>0.02 × 10^4</td>
</tr>
<tr>
<td>F1</td>
<td>thrombin</td>
<td>Arg51—Thr52</td>
<td>7 kDa, 20 kDa fragments</td>
<td>0.4 × 10^4</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arg54—Asp55</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Rate constants of thrombin-catalyzed cleavage of human prothrombin, prethrombin 1, and fragment 1 were obtained by fitting the time courses presented in Figure 2 to a single exponential equation. Further experimental details are given in the Experimental Procedures. PT = prothrombin, PT1 = prethrombin 1, F1 = fragment 1.

autocatalytic conversion of meizothrombin. The observation that thrombin can cleave the Arg284—Thr285 bond in
prothrombin/prethrombin 1 encouraged us to test whether
meizothrombin can also cleave this peptide bond in itself
and thus contribute to thrombin formation (thrombin des-1—13) via autocatalysis. Meizothrombin is a reaction
intermediate that is formed in the early phase of factor Xa-
catalyzed prothrombin activation and that is subsequently
converted into thrombin (Rosing et al., 1986; Krishnaswamy
et al., 1986). However, human meizothrombin is rapidly
converted into meizothrombin des-F1 by autocatalytic feed-
back cleavage (Doyle & Mann, 1990). Since meizothrombin
des-F1 lacks the gla-containing phospholipid-binding domain
this reaction precludes studies of the autocatalytic cleavage at Arg284—Thr285 in intact meizothrombin, especially in the
presence of phospholipids.

Therefore, we first determined the rate constant for conversion of meizothrombin into meizothrombin des-F1 (Figure 3). Time courses of meizothrombin disappearance in the absence and presence of phospholipids were fitted to a single exponential equation and it appeared that phospholipids did not influence the rate of conversion of meizothrombin into meizothrombin des-F1 (Figure 3, Table 2). Since the half-life of meizothrombin was independent of the concentration (0.1—1 μM meizothrombin, data not shown), we conclude that the removal of fragment 1 from meizothrombin is a first-order reaction in which each meizothrombin molecule cleaves off its own fragment 1.
Table 2: Rate Constants of Peptide Bond Cleavages in Meizothrombin*

<table>
<thead>
<tr>
<th>substrate</th>
<th>enzyme</th>
<th>cleavage site</th>
<th>product</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT</td>
<td>MT</td>
<td>Arg25–Ser156</td>
<td>MT des-F1, F1</td>
</tr>
<tr>
<td>MT des-F1</td>
<td>MT des-F1</td>
<td>Arg254–Thr285</td>
<td>T des-1–13, F2.3</td>
</tr>
<tr>
<td>R155A-MT</td>
<td>FXa/Va</td>
<td>Arg271–Thr272</td>
<td>T, F1.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>0 PL</th>
<th>+ 25 μM PL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_1$ (s$^{-1}$)</td>
<td>$k_2$ (M$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>MT des-F1</td>
<td>207 × 10$^{-4}$</td>
<td>0.7 × 10$^{-4}$</td>
</tr>
<tr>
<td>R155A-MT</td>
<td>245 × 10$^{-4}$</td>
<td>0.4 × 10$^{-4}$</td>
</tr>
</tbody>
</table>

* Rate constants of intramolecular ($k_1$) and intermolecular ($k_2$) autocatalytic cleavage in meizothrombin and R155A-meizothrombin were obtained from plots of $k_{obs}$ as function of the meizothrombin concentration (Figure 5). Second-order rate constants of factor Xa/Va-catalyzed conversion of R155A-meizothrombin into thrombin were obtained from initial rates of thrombin formation. MT = meizothrombin, T = thrombin, F1 = fragment 1, F1.2 = fragment 1.2, F1.2.3 = fragment 1.2.3, F2.3 = fragment 2.3, nd = not determined.
not due to meizothrombin itself but is caused by a contaminating proteolytic enzyme present in the reaction mixture, e.g., in the ecarin used for preparation of meizothrombin or in ovalbumin, the carrier protein routinely used in our reaction mixtures. Since addition of excess ecarin or ovalbumin did not affect the time courses of autocatalytic thrombin formation, we exclude this possibility and we conclude that this results of a mixed intramolecular and intermolecular autocatalysis with rate constants \( k_1 \) and \( k_2 \), respectively. The values for \( k_1 \) and \( k_2 \) calculated from Figure 5 and summarized in Table 2 show that the rate constants for intramolecular autocatalysis \( (k_1, \text{intersection at } [MT] = 0) \) hardly differed for meizothrombin des-F1 or R155A-meizothrombin and were not affected by the presence of phospholipids. In addition, the rate constants for intermolecular autocatalysis \( (k_2, \text{slope of the line}) \) are similar for meizothrombin des-F1 (with and without phospholipids) and R155A-meizothrombin (without phospholipids). Phospholipids only affected intermolecular autocatalysis of R155A-meizothrombin the rate constant of which was enhanced some 25-fold (Table 2).

**DISCUSSION**

Conversion of prothrombin into thrombin by factor Xa is accompanied by generation of several intermediates and products due to feedback reactions catalyzed by thrombin and meizothrombin. The data presented in this paper provide insight in the potential significance of autocatalytic reactions during the activation of human prothrombin.

In agreement with earlier studies (Walz et al., 1977; Board & Shaw, 1983; Stevens et al., 1996), thrombin cleavages in human prothrombin were identified at Arg\(^{51}\)-Thr\(^{52}\)/Arg\(^{54}\)-Asp\(^{55}\), Arg\(^{155}\)-Ser\(^{56}\), and Arg\(^{284}\)-Thr\(^{285}\). The rate of cleavage at these peptide bonds is greatly affected by the reaction conditions. In the absence of Ca\(^{2+}\) ions, thrombin relatively efficiently proteolyzes peptide bonds within the fragment 1.2 region (Arg\(^{51}\)-Thr\(^{52}\)/Arg\(^{54}\)-Asp\(^{55}\) and Arg\(^{155}\)-Ser\(^{56}\)). The presence of calcium ions inhibits the removal of fragment 1 some 25-fold and prevents cleavage at Arg\(^{51}\)-Thr\(^{52}\)/Arg\(^{54}\)-Asp\(^{55}\) in fragment 1. Stevens et al. (1996) observed a similar inhibition for cleavage at Arg\(^{54}\)-Asp\(^{55}\) which is presumably due to sequestering of the peptide bond inside the fragment 1 region upon the binding of calcium to the gla-domain (Stevens et al. 1996). This conformational change, possibly in combination with a reduction of the negative charge of the gla-domain after binding Ca\(^{2+}\) ions, may also be responsible for the 25-fold inhibition of the rate of cleavage at Arg\(^{155}\) (Silverberg, 1979; Bjork & Stenflo, 1973; Nelsestuen & Lim, 1977). The fact that prothrombin is significantly protected from proteolysis by the presence of Ca\(^{2+}\) likely explains why low amounts of fragment 1 are detectable during prothrombin activation in plasma (Boissel et al., 1985; Silverberg, 1979).

It has been reported that during prothrombin activation in plasma fragment 1.2.3 is formed (Rabiet et al., 1986), and it was hypothesized that this may be indicative for a contribution of thrombin/meizothrombin to thrombin formation by cleavage at Arg\(^{284}\)-Thr\(^{285}\). However, comparison of the rates of peptide bond cleavage at Arg\(^{51}\)-Thr\(^{52}\) by factor Xa-Va \( [k = 5.5 \times 10^3 \text{ M}^{-1} \text{s}^{-1}, \text{cf. Mann et al. (1990) and Table 2}] \) and cleavage at Arg\(^{284}\)-Thr\(^{285}\) by thrombin \( (k = 0.2 \times 10^3 \text{ M}^{-1} \text{s}^{-1}) \) shows that on a kinetic basis thrombin itself will hardly contribute to its own formation when factor Xa is present.

It is possible, however, that meizothrombin can autocatalytically cleave the Arg\(^{284}\)-Thr\(^{285}\) at a faster rate. Meizothrombin has an exposed active site and contains a gla-domain that enables its binding to phospholipids which may result in enhancement of its catalytic activity (Tans et al., 1990). Studies on autocatalytic cleavages in and by intact meizothrombin are, however, hampered by the fact that the gla-containing fragment 1 domain is readily cleaved off by autocatalysis (Doyle & Mann, 1990). In this paper, we show that the Arg\(^{155}\)-Ser\(^{56}\) bond in meizothrombin is indeed cleaved at a high rate. In the presence of Ca\(^{2+}\) ions, the rate of cleavage in 1 \( \mu \text{M} \) meizothrombin is approximately 25-times higher than thrombin-catalyzed cleavage of the same bond in prothrombin. In contrast to the latter reaction the autocatalytic removal of the fragment 1 domain from meizothrombin yields a constant half-life at all meizothrombin concentrations \( (t_{1/2} = \sim 30 \text{ s}) \), which is indicative of a first-order reaction in which each meizothrombin molecule proteolyses itself via intramolecular autocatalysis [cf. Sanny et al. (1975) for intramolecular activation of pepsinogen]. Stevens et al. (1996) also reported an intramolecular mechanism of autocatalysis for cleavage at Arg\(^{54}\). Considering the high rate of intramolecular autocatalysis, meizothrombin is apparently folded in such a way that the fragment 1 region is readily available for entry into the active site and cleavage at Arg\(^{155}\).

The rapid removal of fragment 1 from meizothrombin precludes studies on autocatalytic cleavage of the Arg\(^{284}\)-Thr\(^{285}\) bond in intact meizothrombin. This cleavage was, therefore, quantitated in meizothrombin des-F1 and in a recombinant derivative (R155A-meizothrombin) that is resistant to removal of fragment 1. In the absence of phospholipids, the Arg\(^{284}\)-Thr\(^{285}\) bond was cleaved in meizothrombin des-F1 and R155A-meizothrombin at similar rates. Phospholipids did not affect autocatalytic thrombin formation with meizothrombin des-F1 as substrate and considerably enhanced this reaction in the case of R155A-meizothrombin (Figure 4).

The dependency of the observed rate constant as a function of the meizothrombin concentration (Figure 5) indicates that cleavage of the Arg\(^{284}\)-Thr\(^{285}\) bond results from a combination of an intramolecular first-order reaction (rate constant independent of meizothrombin concentration) and an intermolecular second-order reaction (pseudo-first-order rate constant linearly dependent on the meizothrombin concentration). The rate constant for intramolecular autocatalysis appeared to be similar for both forms of meizothrombin \( [k_1 = (0.8–0.9) \times 10^{-4} \text{ s}^{-1}] \) and was not affected by the presence of phospholipids (Table 2).

The fact that intramolecular autocatalytic cleavage of Arg\(^{284}\)-Thr\(^{285}\) contributes to the conversion of meizothrombin into thrombin conversion (cf. Figure 5 and Table 2) came somewhat as a surprise. Recently, van de Locht et al. (1996) reported that residues 284–320, which form the truncated A-chain of α-thrombin, are located to the back of the molecule, away from the active site. On the basis of this and on crystallographic data available for the complex of fragment 2 with thrombin (Arni et al., 1993), they proposed a hypothetical conformation which would position residues
Autocatalysis at Arg 284 (lipid surface [cf. Mann et al. (1990)]. This is not surprising since intermolecular autocatalysis is a bimolecular reaction which, due to coordinated binding of substrate and enzyme and enhanced enzyme-substrate complex formation, will proceed at higher rates at the phospholipid surface [cf. Mann et al. (1990)].

A comparison of the rate constant for meizothrombin autocatalysis at Arg$_{284}$ ($k_2 = 5.6 \times 10^3$ M$^{-1}$ s$^{-1}$) and factor Xa/Va-catalyzed cleavage at Arg$_{271}$ ($k = 5.5 \times 10^8$ M$^{-1}$ s$^{-1}$) indicates that on a theoretical basis autoproteolysis in meizothrombin complex has catalyzed 10$^5$ turnovers, i.e., when the concentration of meizothrombin is 10$^3$ times higher than that of the factor Xa/Va complex. Moreover, meizothrombin-catalyzed cleavage at Arg$_{284}$ was not stimulated by factor Va [alone or in combination with phospholipid vesicles (data not shown)]. Our observation that the fragment 1 domain is rapidly removed from meizothrombin ($t_{1/2} \approx 30$ s) further reduces the contribution of meizothrombin to thrombin formation since the rate of Arg$_{284}$-Thr$_{285}$ cleavage in meizothrombin des-F1 is even 15 times slower than that in intact meizothrombin (Table 2).

The data obtained in model systems cannot explain the formation of fragment 1.2.3 during prothrombin activation in plasma (Rabiet et al., 1986), which indicates that cleavage at Arg$_{284}$-Thr$_{285}$ contributes to plasma thrombin formation. It is possible that as yet unknown plasma factors may influence the actual rate of peptide bond cleavages in prothrombin. However, further conclusions regarding the contribution of cleavage at Arg$_{284}$-Thr$_{285}$ to thrombin formation have to await kinetic analysis of plasma prothrombin activation.

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