Differentiation between proteolytic activation and autocalytic conversion of human prothrombin. Activation of recombinant human prothrombin and recombinant D419N-prothrombin by snake venoms from Echis carinatus and Oxyuranus scutellatus

Bernhard E. Fischer¹, Uwe Schlukat, Artur Mitterer, Leopold Grillberger, Manfred Reiter, Wolfgang Mundt, Friedrich Dorner and Johann Eibl

IMMUNO AG, Biomedical Research Center, Uferstrasse 15, A-2304 Orth/Donau, Austria

¹To whom correspondence should be addressed

Recombinant human prothrombin (r-prothrombin) and recombinant mutant prothrombin with active site Asp419 substituted by Asn (D419N-prothrombin) were expressed in recombinant CHO cells, isolated and purified from the fermentation supernatant. The r-Prothrombin and D419N-prothrombin were digested by both Echis carinatus venom and Oxyuranus scutellatus venom. Prior to, during and after activation, generation of thrombin activity and the proteolytic degradation of the prothrombin polypeptide chain were analysed. Owing to the recombinant preparation and inactivity of D419N-prothrombin and its activation products, the proteolytic action of E. carinatus and O. scutellatus venom could be studied without addition of thrombin inhibitor, without interference from autocalytic digestion of prothrombin and in the absence of any other blood coagulation protease. The comparison between the activation of r-prothrombin and D419N-prothrombin by snake venom permitted differentiation between proteolytic activation and autocalytic conversion of prothrombin. Incubation of D419N-prothrombin with E. carinatus venom resulted in the generation of stable D419N-meizothrombin by hydrolysis of the peptide bond Arg320—Ile321. By contrast, O. scutellatus venom exhibited activity towards peptide bonds Arg320—Ile321 and Arg271—Thr272 and lower activity towards peptide bond Arg155—Ser156, thus converting D419-prothrombin into D419N-thrombin and also liberating Fragment-1, Fragment-2 and Fragment-1/2 activation peptide. Activation of r-prothrombin by E. carinatus and O. scutellatus venom demonstrated the autocalytic potential of prothrombin-derived molecules and indicated that meizothrombin hydrolysed the cleavage between Fragment-2 and thrombin A-chain in the meizothrombin molecule, but not in prothrombin, preferentially at position Arg284—Thr285. By contrast, both meizothrombin and thrombin exhibited no detectable activity towards peptide bond Arg320—Ile321 between thrombin A- and B-chain, although this site exhibits the optimum sequence for thrombin cleavage.

Keywords: activation/human prothrombin/mutant protein/recombinant expression/snake venom

Introduction

Human prothrombin is the single polypeptide chain precursor of the blood clotting proteinase thrombin. Prothrombin contains 579 amino acid residues and is composed of several distinct domains: Fragment-1 (residues 1—155), Fragment-2 (residues 156—271), thrombin A-chain (residues 272—320) and thrombin B-chain (residues 321—579). Thrombin (coagulation factor IIa) is the activation product of prothrombin (coagulation factor II) that initiates the final stages of blood coagulation. It is a serine protease that cleaves the fibrinogen molecule into fibrin monomers and activates coagulation factor XIII, which then catalyses fibrin crosslinking reactions. Thrombin also controls other reactions in the blood clotting system by a feedback mechanism and is a highly potent stimulant of platelet release (Lundblad et al., 1977). In vivo thrombin is liberated when prothrombin is activated by Factor Xa, Factor V and calcium ions (Jackson and Nemerson, 1980) by hydrolysis of the Arg271—Thr272 and Arg320—Ile321 peptide bonds, yielding thrombin composed of thrombin A-chain and B-chain. Detailed in vitro analysis of prothrombin by the prothrombinase complex and Factor Xa alone revealed the prothrombin activation pathway (Stenn and Blout, 1972; Heldebrant et al., 1973a, b; Esmon and Jackson, 1974; Esmon et al., 1974a-c; Owen et al., 1974; Downing et al., 1975; Jackson et al., 1975; Magnusson et al., 1975; Taswell et al., 1975; Morita et al., 1976; Krishnaswamy et al., 1986, 1987; Rosing et al., 1986; Rosing and Tans, 1988; Jackson, 1994). Figure 1 shows a schematic diagram of human prothrombin and summarizes the prothrombin activation pathway. The catalytic centre of thrombin is contained in the B-chain and comprises the amino acids His363, Asp419 and Ser525.

However, prothrombin can also become activated through effects of exogenous sources, such as snake venoms (Denson et al., 1971; Girolami et al., 1975; Morita et al., 1975; Novoa et al., 1980; Walker et al., 1980; Briet et al., 1982; Rhee et al., 1982; Speijer et al., 1986; Yukelson et al., 1991; Rosing and Tans, 1992; Tans and Rosing, 1993; Marsh, 1994). Snake venoms contain a rich variety of factors affecting the haemostatic mechanism by action of coagulant enzymes and anticoagulant proteins (Marsh, 1994). Snake venoms also affect platelets either by inducing or inhibiting platelet aggregation and cause haemorrhage via proteolysis of the blood vessel wall. Snake venoms exhibit potential as well as therapeutic and diagnostic applications. Thus, detailed information on the structure and function of snake venoms is essential.

The study of effects of activators on human coagulation factors, such as prothrombin, are limited owing to several complications. Even highly purified human prothrombin preparations may contain traces of other coagulation proteases, such as thrombin, Factor X and Factor Xa. Prothrombin-derived molecules which result from activator action, such as meizothrombin, may exhibit a notable proteolytic activity. Thrombin itself exhibits a strong autocalytic potential. In the past, much effort was necessary to develop experimental conditions and to produce results which were able to differentiate between prothrombin activation by proteases and autocalytic processing (for a review, see Jackson, 1994). Even in the presence of inhibitors the stability of transient prothrombin activation products was low (Doyle and Mann, 1990). Other
Purified venom proteases were stabilized by addition of albumin. Polyclonal rabbit anti-human prothrombin immunoglobulin (Assera factor II) was obtained from Diagnostica Stago (Asnières, France). Peroxidase-conjugated goat anti-rabbit IgG was purchased from Bio-Rad (Hercules, CA, USA).

Construction of expression vectors

The β-galactosidase NcoI fragment of plasmid pSVb (MacGregor and Caskey, 1989) was removed. The resulting plasmid pSV was digested with HindIII and XbaI, filled in with Klenow and religated to yield pSVD. The chemically synthesized multiple cloning site 5′-CCATGG ACAAGTTTATCGGCCCAGATCGTACTGACTG A-3′ was inserted via compatible cohesive ends into the unique XhoI site to yield pSV-ATG+2. The human prothrombin cDNA fragment from pTKemc-PT2 was cloned as a partial NcoI, complete SmaI fragment into analogously digested pSV-ATG+2, yielding expression vector pSV-FII for recombinant human prothrombin.

Expression of proteinase fragments

To retain as much as possible the native structure and functionality of prothrombin and thrombin but to eliminate thrombin activity, for this study (i) recombinant mutant prothrombin with active site Asp419 substituted by Asn (D419N-prothrombin) and (ii) recombinant human prothrombin (r-prothrombin) were expressed, purified and characterized. D419N-prothrombin and its activation products exhibited no proteolytic activity. Owing to recombinant preparation, any contamination with other plasma proteases of the blood clotting system was excluded. D419N-prothrombin and r-prothrombin were used as model substrates for functional characterization of prothrombin activators isolated from Echis carinatus venom (Briet et al., 1982; Rhee et al., 1982) and Oxyuranus scutellatus venom (Speijer et al., 1986) to test whether recombinant and inactive coagulation factors are useful tools to study the action of snake venoms and to differentiate between proteolytic activation and autocatalytic conversion of prothrombin.

Materials and methods

**Materials**

Prothrombin-activating proteases from *E. carinatus* venom and *O. scutellatus* venom were obtained from Pentapharm (Basle, Switzerland) and Sigma (St Louis, MO, USA), respectively, and were additionally purified by gel filtration as described previously (Owen and Jackson, 1973; Morita et al., 1976).
Activation of r-prothrombin and D419N-prothrombin

Activation of r-prothrombin and D419N-prothrombin by E. carinatus and O. scutellatus venoms was performed in 20 mmol/l Tris–HCl buffer, pH 7.4, 150 mmol/l NaCl, 10 mmol/l CaCl₂ at a prothrombin:protease ratio of 50:1 at room temperature for up to 22 h. The prothrombin concentration during activation was 200 μg/ml (2.8 μM). During incubation the thrombin activity was determined. Protein samples were separated by SDS–PAGE, blotted onto a nitrocellulose membrane and prothrombin-derived peptides were stained immunoenzymatically. Immunoenzymatically stained blots were further analysed by densitometry. Activation end-products were subjected to N-terminal amino acid sequence analysis. N-Terminal amino acid sequence analysis of peptides generated during activation was performed directly from blots on to a PVDF membrane using standard Edman chemistry in an Applied Biosystems Model 477A PepSequencer.

Electrophoretic analysis

Electrophoretic analysis of protein was performed under reducing and non-reducing conditions by SDS–PAGE in 12% acrylamide slab gels using the buffer system described by Laemmli (1970).

Immunoblotting

Polypeptides resolved by electrophoresis were transferred onto nitrocellulose membranes in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol buffer (Towbin et al., 1979). Visualization of prothrombin-related peptides was carried out by immunoenzymatic staining. As primary antibody, polyclonal rabbit anti-human prothrombin immunoglobulin was used in a dilution of 1:1000. As secondary antibody, peroxidase-conjugated affinity-purified goat anti-rabbit IgG was used in a dilution of 1:1000. Pre-stained molecular weight markers, separated by SDS–PAGE and blotted onto nitrocellulose membranes, were used for the estimation of the molecular weights of peptides.

Determination of prothrombin activity

The clotting activity of prothrombin was determined by a one-step prothrombin time test as described in detail previously (Fischer et al., 1995) using a prothrombin standard to prepare the calibration curve.

Determination of thrombin activity

Thrombin activity was determined photometrically by the hydrolysis of the synthetic thrombin-specific chromogenic substrate AcOH–HD–CHG–Ala–Arg–pNA at 37°C in 50 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl, 0.1% PEG 6000 and a final substrate concentration of 200 μM. The release of p-nitroaniline that resulted from the hydrolysis of the peptidyl p-nitroanilide substrate was followed by measuring the increase in absorbance at 410 nm. A thrombin standard was used to prepare the calibration curve. Using assay times of 10 min the detection limit of thrombin was 2.5 ng/ml.

Results

Activation of r-prothrombin and D419N-prothrombin by E. carinatus venom protease

Activation of r-prothrombin and D419N-prothrombin by E. carinatus venom is shown in Figure 3. Digestion of D419N-prothrombin resulted in two peptides, labelled 55K and 32K (Figure 3A). N-terminal amino acid sequence analysis of the final activation product resulted in the detection of the two sequences (i) Ala–Asn–Thr–Phe–Leu–Glu–Glu–Val and (ii) Ile–Val–Glu–Gly–Ser–Asp–Ala–Glu, indicating that generated D419N-meizothrombin was composed of the Fragment-1/
Fragment-2/A-chain polypeptide (F1/F2/A-chain, 55K peptide) and of the thrombin B-chain polypeptide (32K peptide). Thus, exclusively peptide bond Arg320-Ile321 of D419N-prothrombin localized between thrombin A- and B-chain was hydrolysed. Owing to conversion of active site Asp419 to Asn, no thrombin activity was detected at any time during activation. Electrophoretic analysis under non-reducing conditions of protein samples prior to, during and after incubation of D419N-prothrombin with E.carinatus venom protease resulted in the constant detection of a peptide with a molecular weight of 72 000, thus F1/F2/A-chain polypeptide and thrombin B-chain were still connected by the Cys293-Cys439 disulphide bond.

By contrast, activation of r-prothrombin by E.carinatus venom resulted in a very different activation pattern (Figure 3B). First, r-prothrombin is converted into r-meizothrombin, composed of thrombin B-chain (32K peptide) and F1/F2/A-chain polypeptide (55K peptide). The appearance of a 52K peptide, identified by N-terminal amino acid sequence analysis as F1/F2-activation peptide, apparently resulted from autocatalytic action of generated r-meizothrombin. The F1/F2/A-chain polypeptide is then further degraded into Fragment-1 (27K peptide), Fragment-2 (16K peptide) and thrombin A-chain by proteolytic action of prothrombin-derived activation products. The appearance of a 19K peptide, identified as F2/A-chain polypeptide, indicated the temporary accumulation of a polypeptide resulting from the hydrolysis at position Arg320-Ile321 by E.carinatus venom and autocatalytic hydrolysis at position Arg155-Ser156. There was no detectable accumulation of prothrombin-2, indicating that E.carinatus venom-catalysed hydrolysis of the Arg320-Ile321 bond preceded cleavage of the peptide bond between Fragment-2 and thrombin A-chain. N-terminal amino acid sequence analysis of the final activation products identified Fragment-1, Fragment-2, thrombin B-chain and a shortened thrombin A-chain starting at Thr285. Thus, the peptide bond Arg284-Thr295 had been cleaved autocatalytically. Electrophoresis under non-reducing conditions of the final activation product resulted in a polypeptide with an apparent molecular weight of 36 000, indicating that thrombin A- and B-chains were still connected by the intermolecular disulphide bond to form an active r-thrombin molecule. In contrast to D419N-prothrombin activation, activity measurement during r-prothrombin activation resulted in the detection of proteolytic activity (Figure 4A). Maximum thrombin activity was found after 40 min of incubation.

Activation of r-prothrombin and D419N-prothrombin by O.scutellatus venom

Figure 5 shows the activation of D419N-prothrombin and r-prothrombin by O.scutellatus venom. Early activation products of D419N-prothrombin by O.scutellatus venom are peptides with molecular weights of 55 000, 52 000, 36 000 and 32 000, labelled 55K, 52K, 36K and 32K peptides, respectively (Figure 5A). Owing to Asp419 → Asn modification, no thrombin activity was generated. The main final degradation products are the 52K and 32K peptides. Amino acid sequence analysis revealed that the 52K peptide corresponded to the F1/F2-activation peptide with the N-terminal sequence Ala-Asp-Thr-Phe-Leu-Gla-Gla-Val, while the 32K peptide represented the thrombin B-chain with the N-terminal sequence Ile-Val-Glu-Gly-Ser-Asp-Ala. The 55K peptide was identified as F1/F2/A-chain polypeptide. Additionally, N-terminal sequence analysis of the final activation product resulted in the detection of the sequence Thr-Ala-Thr-Ser-Glu-Tyr-Gln, corresponding to the thrombin A-chain. Thus, peptide bonds between thrombin A- and B-chain (Arg320-
Ile321) and between Fragment-2 and thrombin A-chain (Arg271-Thr272) have been cleaved by *O. scutellatus* venom with similar activities. This suggested that the temporarily appearance of a 36K peptide corresponds to prothrombin-2, composed of the linear sequence of thrombin A- and B-chain. This was confirmed by sequence analysis of the 36K peptide and electrophoretic analysis under non-reducing conditions (not shown). Prolonged proteolysis resulted in the appearance of a 27K and a 16K peptide, identified as Fragment-2 and Fragment-1, respectively, unveiling a third cleavage site between Arg155 and Ser156. Electrophoretic analysis of the activation products under non-reducing conditions resulted in the detection of polypeptides with molecular weights of 52 000, 36 000, 27 000 and 16 000 (not shown), confirming the generation of the F1/F2-activation peptide, of thrombin (composed of A- and B-chain), of Fragment-2 and of Fragment-1 as final products, respectively.

By contrast, activation of r-prothrombin by *O. scutellatus* venom (Figure 5B) resulted in no substantial accumulation of the 52K peptide, but rapid formation of 55K, 36K, 32K, 27K and 16K peptides, identified as F1/F2/A-chain polypeptide, thrombin A-B-chain polypeptide, thrombin B-chain, Fragment-1 and Fragment-2, respectively. Thus, early generation of active thrombin molecules was instantly followed by autocatalytic hydrolysis of the peptide bond Arg155-Ser156, liberating Fragment-1 (27K peptide) and Fragment-2 (16K peptide). This was confirmed by amino acid sequence analysis of activation products and by electrophoresis under non-reducing conditions (not shown). Since activations of D419N-prothrombin and r-prothrombin by *O. scutellatus* venom were performed at the same time and under identical experimental conditions, it appeared that generated thrombin molecules inactivated *O. scutellatus* venom, thus slowing the cleavage between thrombin A- and B-chain. Although *O. scutellatus* venom exhibited sufficient proteolytic activity towards peptide bond Arg320-Ile321 in D419N-prothrombin (Figure 5A), about 25% of the final activation product of r-prothrombin was composed of prethrombin-2 (36K peptide). However, amino acid sequence analysis of prethrombin-2 resulted in the sequence Thr-Phe-Gly-Ser-Gly-Glu-Ala, indicating that peptide bond Arg284-Thr285 is preferentially cleaved in meizothrombin. Temporary accumulation of F2/A-chain polypeptide indicates that autocatalytic cleavage of the Arg155-Ser156 bond occurs faster than hydrolysis between Fragment-2 and thrombin A-chain.

Discussion

The physiologically important activator of prothrombin is the complex consisting of factor Xa, factor Va, phospholipids and Ca$^{2+}$ ions (prothrombinase complex). Activations of prothrombin by the prothrombinase complex and by factor Xa alone have been studied in detail previously (for a review, see Jackson, 1994). Two pathways for prothrombin activation have been predicted: activation can occur via prethrombin-2 and Fragment-1/Fragment-2, or via meizothrombin (Stenn and Blout, 1972; Heldebrant et al., 1973a,b; Esmon and Jackson, 1974; Esmon et al., 1974a-c; Owen et al., 1974; Downing et al., 1975; Jackson et al., 1975; Magnusson et al., 1975; Taswell et al., 1975; Morita et al., 1986; Rosing et al., 1986; Rosing and Tans, 1988; Jackson, 1994). However, prothrombin can also become activated through effects of exogenous sources, such as snake venoms (Denson et al., 1971; Girolami et al., 1975; Morita et al., 1975; Novoa et al., 1980; Walker et al., 1980; Briet et al., 1982; Rue et al., 1982; Speijer et al., 1986; Yukelson et al., 1991; Rosing and Tans, 1992; Tans and Rosing, 1993; Marsh, 1994). Judging from its mode of action in prothrombin activation, *E. carinatus* venom appears capable of cleaving the Gly155-Ser156 and Arg320-Ile321 bonds (Morita et al., 1975; Briet et al., 1982; Tans and Rosing, 1993) in prothrombin. *Oxyuronus scutellatus* venom appears capable of cleaving the Arg271-Thr272 and Arg320-Ile321 bonds (Speijer et al., 1986; Tans and Rosing, 1993).

However, even highly purified human prothrombin preparations may contain traces of other coagulation proteases, such as thrombin and Factor Xa. Prothrombin-derived molecules, such as meizothrombin, may exhibit a notable proteolytic activity. Thrombin itself exhibits a strong autocatalytic potential.

In this study, the substitution of Asp419 by Asn in human prothrombin by mutagenesis and recombinant expression yielded D419N-prothrombin exhibiting no coagulant activity. Owing to recombinant preparation of prothrombin, other coagulation factors and plasma proteases were excluded. Proteolytic digestion of D419N-prothrombin by *E. carinatus* venom resulted the detection of only a single cleavage site between thrombin A-chain and B-chain. Inactive but structural stable D419N-meizothrombin was formed. Thus, previously reported *E. carinatus* venom cleavage site at prothrombin Gly158-Ser159 (Briet et al., 1982) apparently resulted from additional protease contamination. Activation of r-prothrombin by *E. carinatus* venom confirmed previous results (Krishnaswamy et al., 1986; Rosing et al., 1986; Rosing and Tans, 1988) that meizothrombin exhibits proteolytic activity for cleavage of the peptide bonds between Fragment-2 and A-chain and between Fragment-1 and Fragment-2. This study showed, that at the start of r-prothrombin activation by *E. carinatus* venom, generated r-meizothrombin and r-prothrombin existed simultaneously in solution. However, no generation of prethrombin-2 was observed. Thus, our results indicate for the first time that meizothrombin cleaves a peptide bond between Fragment-2 and A-chain exclusively in meizothrombin and not in prothrombin. Analysis of r-prothrombin activation by *E. carinatus* venom under neither reducing nor non-reducing conditions resulted in the detection of the entire thrombin A-chain starting at position Thr272; however, a shortened thrombin A-chain starting at Thr285 was found. Apparently the peptide bond Arg284-Thr285 is preferentially cleaved in meizothrombin. Temporary accumulation of F2/A-chain polypeptide indicates that autocatalytic cleavage of the Arg155-Ser156 bond occurs faster than hydrolysis between Fragment-2 and thrombin A-chain.

Incubation of D419N-prothrombin with *O. scutellatus* venom confirmed (Speijer et al., 1986; Tans and Rosing, 1993) that this enzyme catalyses the hydrolysis of prothrombin Arg320-Ile321 and Arg271-Thr272 peptide bonds at similar rates, yielding simultaneous formation of meizothrombin, prothrombin-2 and thrombin. However, continuation of digestion resulted in the additional detection of activity towards the Arg155-Ser156 peptide bond. Activation of r-prothrombin by *O. scutellatus* venom confirmed that activated prothrombin-

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derived molecules catalyse the hydrolysis of Arg155–Ser156 bonds, thus preventing accumulation of F1/F2-activation peptide. Interestingly, our results indicated for the first time that O.scutellata venom protease is inactivated by thrombin and/or meizothrombin, resulting in the accumulation of prethrombin-2. Although the first 13 amino acids are released from thrombin A-chain of prethrombin-2 by an autotryptic mechanism, prethrombin-2 was not autotryptically converted into thrombin.

Previous studies of the activation pathway of prethrombin and meizothrombin included at least the addition of Factor Xa to human or bovine prethrombin solution (Stenn and Blout, 1972; Heldebrant et al., 1973; Esmon et al., 1974a,b; Owen et al., 1974; Jackson et al., 1975; Magnusson et al., 1975; Morita et al., 1976; Krishnaswamy et al., 1986, 1987; Rosing et al., 1986; Rosing and Tans, 1988). The results of this study obtained in the absence of other coagulation factors show that meizothrombin itself has sufficient capacity for its own conversion into thrombin. By contrast, although the site between thrombin A- and B-chain exhibits an amino acid sequence favoured by thrombin (Chang, 1985), apparently the Arg320–Ile321 peptide bond in prethrombin-2 is not recognized by activated prothrombin-derived molecules. Thus, hydrolysis of the Arg320–Ile321 bond by Factor Xa appears to be the most important step in prothrombin activation.

In summary, the results of this study show that using recombinant and inactive prothrombins, the proteolytic actions of two different snake venoms were easily analysed. The combination of activation experiments of recombinant active and inactive prothrombin proved to be effective in obtaining detailed information about proteolytic properties of the coagulation factor itself and in differentiating clearly between protease action and autotryptic function. Thus, the methods described in this paper offer several benefits over previous strategies for the functional characterization of coagulation factors and their activators.

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References