Non-enzymatic activation of prothrombin induced by interaction with fibrin β26-42 region*

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We have discovered that addition of monomeric desAB fibrin to prothrombin leads to appearance of the thrombin-like activity of prothrombin towards S2238 chromogenic substrate. DesA and desABβ(15-42), fibrin forms did not cause any activation of prothrombin. From this observation we could suggested that amino acid residues of the 15-42 fragment of BN-domain presented in desAB fibrin, cleaved in desABβ(15-42), fibrin and protected in desA fibrin, play a crucial role in the non-enzymatic activation of prothrombin. To identify the Bβ amino acid residues involved in the fibrin-prothrombin binding we used monoclonal antibodies 1-5G and 2d2a with epitopes in Bβ26-42 and Bβ12-26 fibrin fragments respectively. The thrombin-like activity in the mixture of prothrombin and desAB fibrin was monitored in the presence of each of these monoclonal antibodies. It was found that anti-Bβ12-26 antibody does not exhibit any inhibitory effect on the thrombin-like activity of the mixture. In contrast, adding of Bβ26-42 antibody into the mixture of desAB fibrin with prothrombin diminished the thrombin-like activity by 70%. Recombinant dimeric peptides Bβ(15-44) and Bβ(15-66), that mimic amino acid residues in fibrin were also tested for their ability to activate prothrombin. It was found that both peptides were able to induce non-enzymatic activation of prothrombin. The activation was more evident in the case of Bβ(15-44), peptide. From the data obtained we can conclude that desAB fibrin binds to prothrombin through the Bβ26-42 amino acid residues and the formation of such a complex caused a non-enzymatic activation of prothrombin.

Key words: prothrombin, fibrin, fibrin degradation products, staphylococcalase, thrombin

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INTRODUCTION

Prothrombin is an inactive precursor of thrombin which is activated by simultaneous cleavage of Arg271-Thr272 and Arg502-Thr503 peptide bonds by the prothrombinase complex (Wolberg et al., 2007). Interactions of thrombin and fibrinogen are well studied. Apart from binding to fibrinogen by its catalytic site, thrombin can also bind fibrinogen and fibrin through hydrophobic exosite I and heparin-binding exosite II (Mosesson et al., 1993; Hogg et al., 1996; Di Cera et al., 2003; Scheraga et al., 2004). There are high-affinity and low-affinity thrombin-binding sites on fibrinogen molecule (Pospisil et al., 2003). High-affinity sites are located at the C-terminal region of fibrinogen γ-chains (Mosesson et al., 2003) and bind to thrombin exosite II. Low-affinity sites have been localized at the N-terminal region of Aα- and Bβ-chains of fibrinogen (Binnie & Lord, 1993; Meh et al., 1996). Amino acids AαPhe10, and AβPhe10 (Kaczmarek & McDonagh, 1988; Goodwyn et al., 1992), residues Aα27-50 (Binnie & Lord, 1991) and residues Bβ15-42 (Meh et al., 1996; Mosesson et al., 2004), Bβ69-71 and Bβ76-80 (Kaczmarek & McDonagh, 1988) have been shown to be important for thrombin binding. Low-affinity binding sites of fibrinogen bind mainly to exosite I of thrombin (Lane et al., 2005).

Thrombin exosite I was shown to be exposed in a prothrombin molecule (Ni et al., 1993; van de Locht et al., 1996), though there have been no experimental data demonstrating prothrombin binding to fibrinogen or fibrin. However, prethrombin-2, a derivative formed by Arg271-Thr272 cleavage that has no active site, as well as prothrombin, has been shown to bind directly to Fibrinogen-sepharose (Kaczmarek et al., 1987). Surprisingly, thrombin-like activity induced in the mixture of prothrombin with the E-fragment of fibrin was previously reported. Such interactions provoked prothrombin activation without cleavage of the prothrombin molecule (Platonova et al., 2002).

The goal of our study was to demonstrate interaction of prothrombin with the E-region of fibrin and identify fibrin residues involved in this interaction.

MATERIALS AND METHODS

Materials. Thrombin, Lysine-Sepharose, Superdex G-75, GPRP were purchased from Sigma-Aldrich (US), chromogenic substrate S2238 (H-D-Phe-Pip-Arg-pNA) was from Chromogenix (Sweden), BrCN-activated sepharose, goat anti-rabbit secondary antibody conjugated to alkaline phosphatase, pNPP (para-Nitrophenylphosphate) (Sigma). Anti-E-fragment polyclonal antibody produced in rabbit was a kind gift from Dr O. Savchuk. Recombinant dimeric peptides Bβ(15-44), and Bβ(15-66), were generously donated by Dr S. Yakovlev, and monoclonal

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Abbreviations: DFP, Di-isopropyl fluorophosphate; FPLC, fast protein liquid chromatography; GPRP, Gly-Pro-Arg-Pro peptide; PCS, photon correlation spectroscopy; PDB, Protein Data Bank; pNA, para-Nitroaniline; pNPP, para-Nitrophenylphosphate

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antibodies: one to Bβ26-42 (1-5G) and another to Bβ12-26 (2d2a), were kindly gifted by Dr I. Kolesnikova.

Fibrinogen was purified from citrated blood plasma according to Varetska et al (1965) and plasminogen contamination was removed with Lysine-Sepharose affinity column.

Prothrombin was isolated from the human citrated blood plasma by the adsorption of prothrombin on BaSO₄, with further elution by 0.05 M Tris/HCl, 0.2 M NaCl buffer, pH 7.4, containing 0.01 benzamidine (Mann, 1976). Pure prothrombin was obtained by ion-exchange chromatography on Q-Sepharose. To avoid the spontaneous prothrombin activation, the preparation of prothrombin was inhibited by DFP and purified on PD-10 ex tempore.

E₁-fragment of fibrin was prepared by plasmin hydrolysis of stabilized fibrin. Fibrinogen (10 mg/ml) was clotted by thrombin (0.5 NIH/ml per 1 mg of fibrinogen) and stabilized at 20°C overnight in 0.05 M Tris-HCl buffer pH 7.4, 0.13 M NaCl (TBS) with 0.02 M CaCl₂. Polymeric fibrin was hydrolyzed by plasmin (0.5 NIH/ml) at 20°C for 4–5 hours. Hydrolysis was terminated by addition of 10⁻⁵ M DFP. Plasmin was removed using affinity chromatography with Lys-Sepharose. Ion-exchange chromatography on CM-sephadex was used to purify the DDE-complex. E₁-fragment was purified by size-exclusion chromatography on Sephacryl S300 in the presence of 1M KSCN (Medved et al., 1988).

DesA and desAB fibrins were prepared from plasminogen-depleted fibrinogen. Fibrin polymerization was initiated by 0.5 NIH/ml thrombin (for desAB fibrin) and 0.75 NIH/ml ancistron for desA fibrin (Solovev & Ugareva, 1993). Each mixture was incubated for 30 minutes at 37°C. Fully formed clot was removed from the incubation mixture by a glass stick, washed and re-dissolved at 37°C. Solution of pNPP (1 mg/ml in diethanolamine, pH 9.8) was added and incubated at 37°C. Optical density was measured after 30 minutes of incubation.

Dynamic light scattering was performed for individual proteins, fibrin E₁-fragment and prothrombin, and for 1:1 mixture of these proteins. Final concentration of the proteins in every probe was 0.6 mg/ml. The radius of small beads in Brownian motion in a solution was measured according to Gun’ko et al. (2003) and Lorber et al. (2012).

Thrombin-like activity in the mixture of prothrombin and different forms of monomeric fibrin was monitored by measuring hydrolytic activity towards synthetic chromogenic substrate S2238. Prothrombin (0.04 mg/ml), S2238 (0.1 mM) and GPRP (0.04 mg/ml) were mixed in TBS. Monomeric fibrin (0.1 mg/ml) dissolved in acetic acid temporo.

![Figure 1](image)

**Figure 1. Direct studies of prothrombin-E₁-fragment complex formation.**

(A) ELISA of the complex formation of fibrin E₁-fragment immobilized on tissue culture plate with prothrombin and cross-reactivity of polyclonal antibody to prothrombin with fibrin E₁-fragment immobilized on a tissue culture plate. *Significant at p<0.005, n=6, E₁, fibrin E₁-fragment.* (B) Auto-correlation function of the mixture of prothrombin and E₁-fragment of fibrin. 1, E₁-fragment particles; 2, prothrombin particles; 3, particles of complex of prothrombin and E₁-fragment. Results of a typical experiment. τ, delay time; ACF, auto-correlation function.

Thrombin polymerization was induced by 0.5 NIH/ml thrombin. Fibrin polymerization was induced by 0.5 NIH/ml thrombin. Fibrin polymerization was induced by 0.5 NIH/ml thrombin. Fibrin polymerization was induced by 0.5 NIH/ml thrombin. Fibrin polymerization was induced by 0.5 NIH/ml thrombin.
acid was added to the well. Polymerization of fibrin did not occur in the presence of GPRP in the mixture. Hydrolytic activity was continuously monitored at 405 nm. The amount of hydrolyzed substrate was calculated using a molar extinction coefficient of 10500 M⁻¹×cm⁻¹ for free pNA (Gershkovich & Kibirev, 1988).

**Protein–protein docking.** The crystal structure of human prethrombin-2 was obtained from the Brookhaven Protein Data Bank (PDB ID: 1NU9) (Friedrich et al., 2003). The prethrombin-2 subunit has been extracted from the pdb file, and staphylocoagulase has been removed from the prethrombin-staphylocoagulase complex.

The three-dimensional structure for β26-44 fragment of fibrin was predicted using web-server I-TASSER (Zhang, 2008; Roy et al., 2010; Roy et al., 2012).

Protein–protein docking of prethrombin-2 with β26-44 fragment of fibrin was performed with ZDOCK server (Pierce et al., 2014). Several amino acids in the Ile₁₆-activation pocket of prethrombin were selected as binding site residues. The complexes were visualized using the program ViewerLite v.4.2.1 (Accelrys Inc.; http://www.accelrys.com).

Statistical data analysis was performed using Microsoft Excel and “Statistica 7”. All assays were performed in series of three replicates and the data were fitted with standard errors using “Statistica 7”.

**RESULTS AND DISCUSSION**

**Prothrombin-E₁ complex formation**

The formation of E₁-fragment/prothrombin complex was confirmed independently by dynamic light scattering, sandwich ELISA and analytical size-exclusion chromatography.

The mixture of fibrin E₁-fragment and prothrombin was prepared at a 1:1 molar ratio and the complex was obtained on Superose column in 0.05 M Tris-HCl buffer with 0.13 M NaCl and 10⁻⁴ M CaCl₂ (elution speed 0.75 ml/min). Fraction of the complex eluted before elution of the individual proteins was characterized by SDS/PAGE (Savchuk et al., 2006).

Sandwich ELISA was used for a direct proof of the complex formation of prothrombin with fibrin E₁-fragment. The E₁-fragment was immobilized on a tissue culture plate; the wells were incubated with prothrombin and washed three times. Polyclonal antibody to prothrombin was used to demonstrate that prothrombin was attached to the immobilized E-fragment (Fig. 1A).

The complex of fibrin E₁-fragment with prothrombin was also studied using photon correlation spectroscopy (PCS). The mean particle diameter was measured on photon correlation spectroscopy PCS 100 (Malvern Instrument Limited, UK) with helium-neon laser LS 230. The particles with the the size of bi-molecular complex of prothrombin/E₁-fragment were detected (Fig. 1B).

Therefore, using three different methods (gel-filtration with SDS/PAGE, ELISA and PCS) we have demonstrated that fibrin E₁-fragment can directly interact with prothrombin. As we showed earlier, this interaction leads to exposure of an active thrombin-like site in prothrombin molecule. The mechanism of this activation, as well as amino acid residues involved in these interactions, remain unclear.
Mapping BβN-terminus of fibrinogen residues that interact with prothrombin

In our further studies we used monomeric fibrins instead of E₁-fibrin with aim to exclude the action of plasmin on α and β polypeptide chains N-termini of the E-region.

We prepared three forms of monomeric fibrin (Fig. 2) using the modified method by Varetskaia et al., 1965. Monomeric desAB fibrin was obtained by cleavage of fibrinopeptides A and B by thrombin. Monomeric desA fibrin was obtained by a similar procedure using ancistron — a thrombin-like enzyme from the venom of the South American recluse spider, Loxosceles reclusa. All these fibrins were composed of Bβ and Bγ chains but lacked α chains.

Figure 5. The thrombin-like activity induced in the mixture of prothrombin with monomeric desAB fibrin.

Control, without antibody; (anti-Bβ12-26) in the presence of monoclonal antibody to Bβ12-26 and (anti-Bβ26-42) in the presence of monoclonal antibody to Bβ26-42. *Significant at p<0.005, n=3.

Figure 6. Induction of amidolytic activity measured by chromogenic substrate S2238 in the mixture of prothrombin (6 μM) with peptides that mimic N-terminal fibrin Bβ-chain residues.

1. prothrombin mixed with Bβ(15-44), 2. prothrombin mixed with Bβ(15-66). The peptides were used at two concentrations: a, 3 μM; b, 1,5 μM. * Significant at p<0.005, n=3. [p], concentration of hydrolysed substrate S2238.

Figure 7. The binding mode of β26-44 fibrin fragment with prethrombin.

(A) complex of β26-44 fibrin fragment (labeled yellow) with prethrombin subunit (colored by secondary type). (B) complex of β26-44 fibrin fragment (stick display style) with amino acids of prethrombin activation pocket (line display style) located in the radius 7 Å. (C) β26-44 fibrin fragment with labeled amino acids. The atoms of amino acids are labeled red (Oxygen), blue (Nitrogen), grey (Carbon) and yellow (Sulphur) color.
of Aphi st ron b aly s that cleaves only fibrinopeptid es A (Sol ovev & Ugar ova, 19 93). Truncated form of desAB fibrin lacking both fibrinopeptid es and 15-42 residue of Bβ-chain was obtained by incubation of fibrinogen with thrombin and fibrinogen from the venom of Echis mult i tisquama tis (Chernyshenko et al., 2010; Chernyshenko et al., 2014) (Fig. 2). All proteins were characterized by SDS/PAGE (Fig. 3).

Next, we experimentally confirmed that the addition of monomeric desAB fibrin to prothrombin induced thrombin-like activity of the mixture as detected by S2238 chromogenic substrate. Other fibrin forms (desA and desABβ(15-42)2 fibrins) do not cause activation of prothrombin (Fig. 4). From these observations we could tentatively presume that 15-42 residues of BβN-domain, present in the desAB fibrin, but cleaved in desABβ(15-42)2 fibrin and protected in desA fibrin, play a crucial role in the induction of thrombin-like activity in the prothrombin-fibrin complex.

To identify amino acid residues of Bβ15-42 fragment responsible for prothrombin binding we have monitored thrombin-like activity in the mixture of prothrombin and desAB fibrin in the presence of two specific monoclonal antibodies: 1-5G antibody specific to Bβ26-42, and 2d2a antibody specific to Bβ12-26 sites on the fibrin molecule. It was found that antibody to Bβ12-26 didn’t inhibit the appearance of thrombin-like activity of the mixture. In contrast, adding Bβ26-42 antibody diminished diminished thrombin-like activity of the prothrombin-desAB fibrin mixture by two thirds (Fig. 5).

In general, antibodies can inhibit active sites of intermolecular binding sites of protein molecules when the epitope on the antigen overlaps with binding sites on substrate. Our data indicate that binding of prothrombin to desAB fibrin was mediated by fibrin residues β26-42. This region is known as the multi-functional part of the molecule and includes sites of interactions with heparin, cell receptors (VE-cadherins, VLDLP-receptors of EC), etc (Bennet, 2001; Gorlatov et al., 2002; Yakovlev et al., 2003; Lugovskoi et al., 2007; Yakovlev et al., 2009).

To study direct interaction of prothrombin with the N-terminus of fibrin β-chain we used recombinant dimeric peptides Bβ(15-44)2 and Bβ(15-66)2 that mimic corresponding residues in fibrin (Gorlatov et al., 2002). The peptides were tested for their ability to activate prothrombin using chromogenic substrate assay. It was found that each peptide was able to induce the non-enzymatic activation of prothrombin. The activation was more pronounced with the Bβ(15-44)2 peptide (Fig. 6).

**Computer modeling of prothrombin-Bβ26-44 interactions**

Previously, the non-enzymatic activation of prothrombin was reported as a result of interaction of staphylocoagulase N-terminus with the activation pocket (Ilele64, pocket) of prothrombin (DiBella & Scherga, 1996; Friedrich et al., 2006). Intermolecular interactions that cause such non-enzymatic activation were shown to occur after incorporation of N-terminal fragment of staphylocoagulase (Ilele-Tyr3) into the activation pocket of prothrombin (Khan & James, 1998; Friedrich et al., 2003). In the case of physiological activation, the activation pocket is occupied by the N-terminal peptide formed after Argβ33-Ileβ32 cleavage by the Xa factor (Bradford et al., 2010).

Thus we assumed that interaction of prothrombin with β26-44 residues could occur through the same activation site (pocket), as it was shown for physiological activation and activation of prothrombin by staphylocoagulase. Using the ZDOCK server we predicted a binding mode of the activation pocket with Bβ22-44 residues of fibrin that were shown to be sufficient for non-enzymatic activation.

Crystal structure of prethrombin-2 was virtually removed from docking calculations of the complex with staphylocoagulase. We have created a three-dimensional structure of β26-44 fibrin fragment using the I-TASSER server. ZDOCK server predicted ten best scored complexes. Binding mode of β26-44 fibrin fragment with prethrombin was similar for seven proposed complexes (Fig. 7A). The model of the complex of β26-44 fibrin fragment with the activation pocket is presented in Fig. 7B. Thus we showed that β26-44 could be incorporated into the activation pocket of a prothrombin molecule.

**CONCLUSIONS**

The appearance of thrombin-like activity in the mixture of prothrombin and the E3-fragment of fibrin was reported earlier. In the current study we showed that prothrombin can bind to the desAB fibrin and its derivatives through the Bβ26-42 fibrin residues, and the formation of such a complex can cause a non-enzymatic activation of prothrombin. Such mechanisms of prothrombin activation could lead to over-load of thrombin on fibrin matrix or in a complex with fibrin degradation products which are formed during severe pathologies.

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**Conflict of interests**

The authors declare that there are no conflicts of interest.

**REFERENCES**


