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Kinetic characterization of inhibition of human thrombin with DNA aptamers by turbidimetric assay

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ABSTRACT

A sensitive turbidimetric method for detecting fibrin association was used to study the kinetics of fibrinogen hydrolysis with thrombin. The data were complemented by high-performance liquid chromatography (HPLC) measurements of the peptide products, fibrinopeptides released during hydrolysis. Atomic force microscopy (AFM) data showed that the fibril diameter is the main geometric parameter influencing the turbidity. The turbidimetric assay was validated using thrombin with the standard activity. To study thrombin inhibitors, a kinetic model that allows estimating the inhibition constants and the type of inhibition was proposed. The kinetic model was used to study the inhibitory activity of the two DNA aptamers 15-TBA (thrombin-binding aptamer) and 31-TBA, which bind to thrombin exosites. For the first time, 31-TBA was shown to possess the competitive inhibition type, whereas the shortened aptamer 15-TBA has the noncompetitive inhibition type.

Thrombin is a key enzyme of the blood coagulation system, accomplishing limited proteolysis of fibrinogen and, thus, producing fibrin associates. This process, along with platelet aggregation, provides effective blood clotting [1].

α-Thrombin is a 36-kDa serine peptidase; its specificity and catalytic activity are subtly regulated by two lysine/arginine-enriched exosites and additional loops. Thrombin exosites mediate interaction with substrates and inhibitors, among which are fibrinogen, the major substrate (exosite I), and heparin, a natural inhibition cofactor (exosite II). Thrombin is susceptible to autoproteolysis, which decreases enzymatic activity [2].

Fibrinogen, the substrate, is a 340-kDa glycoprotein consisting of three pairs of nonidentical chains (named α, β, and γ) folded into a three-domain structure 45 nm long and 5 nm wide (Fig. 1) [3]. Plasma fibrinogen has considerable heterogeneity, which results mainly from alternative splicing of α-mRNA (messenger RNA)[1] and γ-mRNA and also from degradation of C-terminal α-chain region [4].

Thrombin cleaves only 4 of 376 trypsin-sensitive peptide bonds in fibrinogen [3]. Hydrolysis leads to the formation of fibrin and four fibrinopeptides: two A (16 residues) and two B (14 residues). As a result, soluble fibrinogen converts into insoluble fibrin associates—particles with an asymmetric structure (Fig. 1) dozens of micrometers long and up to 200 nm wide [5]. Thrombin affinities to the substrate and to the final product are similar; therefore, thrombin binds to fibrin associates through exosite I and, thus, gains resistance to exosite-I-directed inhibitors [6]. This circumstance complicates modeling the reaction mathematically.

The autoproteolytic activity of thrombin and the heterogeneity of plasma-derived fibrinogen require controlling protein activities. Moreover, investigating thrombin inhibitors that block only exosites rather than catalytic center requires using a method that follows both the catalytic center activity and the exosite-binding ability. Therefore, developing a rapid and effective method for evaluating fibrinogen-hydrolyzing thrombin activity is a current need.

Because thrombin catalytic and substrate-binding sites are separated, two approaches could be used to determine the peptidase activity. The first and most used approach, hydrolysis of peptide substrates, allows estimating only the catalytic center activity. Until now, the second approach has not been applicable for routine use; it comprises tracing the hydrolysis of macromolecular substrates (i.e., fibrinogen) and allows determining the involvement of exosite I and the activity of the catalytic center.

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[1] *Abbreviations used:* mRNA, messenger RNA; HPLC, high-performance liquid chromatography; TBA, thrombin-binding aptamer; AFM, atomic force microscopy; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometer; UV, ultraviolet; NMR, nuclear magnetic resonance.

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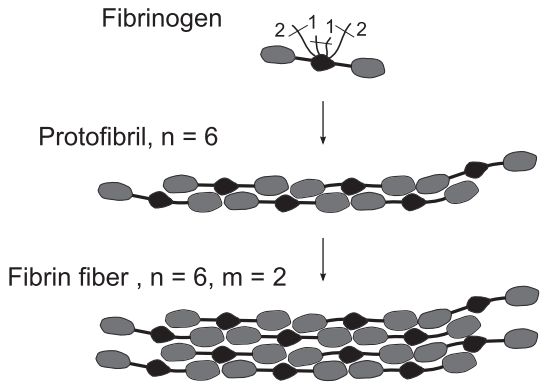

Fig.1. Scheme of fibrin association. E-domains are gray, and D-domains are black. Thrombin cleaves two fibrinopeptides A (1) and two fibrinopeptides B (2). Hydrolysis products associate in two-stranded protofibrils and their associates, fibers. Here *n* is the number of fibrin molecules in the protofibril, and *m* is the number of protofibrils in the fiber.

The first approach, using chromogenic substrates (e.g., D-Phe-Pro-Arg-*p*-nitroanilide [7], D-Phe-Pro-Phe-*p*-nitroanilide [8]), is widely applied. The approach is rather convenient for estimating both the thrombin peptidase activity and the catalytic center inhibitory effects [9].

Investigating thrombin exosite-binding ligands (i.e., fibrinogen and thrombin inhibitors) requires using the second approach, which follows both the catalytic center activity and the exosite-binding ability. The course of hydrolysis could be investigated by following the fibrinopeptide accumulation (using either high-performance liquid chromatography [HPLC] [10,11] or capillary zone electrophoresis [12]) as well as fibrin association. The latter consumes less reagent and time, but there has been no example of estimating kinetic parameters quantitatively with this approach.

Therefore, developing a rapid and efficient method for evaluating fibrinogen-hydrolyzing activity of thrombin and its inhibition on the basis of fibrin association tracing is challenging.

Materials and methods

Inorganic salts with analytical-grade purity (MP Biomedicals, USA), recombinant human α-thrombin with a specific activity of 3.6 kIU/mg (Haematologic Technologies [HTI], USA), human α-thrombin with a standard specific activity of 110 IU/ml (National Institute for Biological Standards and Control [NIBSC], UK), fibrinogen from human plasma (Calbiochem, Germany), and 31-TBA (thrombin-binding aptamer) (CACTGGTAGGTTGGTGTGGTTGGGGCCAGTG) and 15-TBA (GGTTGGTGTGGTTGG) (Synthol, Russian Federation) were used. Guanidine hydrochloride (Helicon, Russian Federation), acetonitrile, trifluoroacetic acid, and acetone with chromatographic purity (Baza No. 1 Himreactivov, Russian Federation) were used in the HPLC analysis. All reactions were carried out at 27 °C in a buffer containing 20 mM Tris–acetate (pH 7.4), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

AFM experiments

The atomic force microscopy (AFM) experiments were conducted using a Multimode AFM device with a Nanoscope IIIA Controller (Digital Instruments, USA) in tapping mode with a typical scan rate of 1 Hz. The measurements were performed in air in tapping mode using standard silicon cantilevers (NT–MDT, Russian Federation) with a guaranteed tip radius of 25 nm and sharp silicon cantilevers (NT–MDT) with a guaranteed tip radius of 10 nm.

Typically, 20 μl of 2 μM fibrinogen solution was treated with 1 μl of thrombin (final concentration 3.6 nM). The reaction mixture was then placed on freshly cleaved mica and allowed to adsorb on the surface for a certain time required in each experiment. The solution was carefully removed with filter paper, the substrate was immediately placed onto a drop of double-distilled Millipore water (this procedure was repeated twice), and the surface was then dried with air flow. This sample preparation method was used to eliminate any remaining salts and minimize artifact aggregation during drying.

HPLC separation of fibrinopeptides

Typically, 2 μM fibrinogen solution was treated with thrombin (final concentration 3.6 nM), and the reaction was stopped by adding 3 volumes of chilled acetone (–20 °C). The mixture was kept at –20 °C for 60 min and was then spun at 14 krpm (4 °C) for 10 min. The supernatant was evaporated dry, and the resultant solid was dissolved in 50 μl of 6 M guanidine hydrochloride. The whole sample volume was analyzed on a MiLiChrom A-02 chromatograph with Prontosil 120-5 C18 columns (2 × 75 mm, Econova, Russian Federation) using the Di Cera and Cantwell technique [10]. Peak areas for 214-nm absorption curves were determined with MultiChrom Spectrum software (Ampersand, USA).

Fibrinopeptide identification

The fractions collected in HPLC were analyzed using a matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometer (MS) (Bruker, Germany). Two fractions with 1537- and 1553-Da substances (calculated masses of the fibrinopeptides are 1536.5 Da for A and 1552.8 Da for B) were subsequently analyzed using a tandem MALDI–TOF/TOF MS (Ultraflex II Bruker, Germany) and identified as the respective fibrinopeptides A (ADSGEGDFLAEGGGVR) and B (GVNDNEEGFFSAR) using Biotools software (version 3, Bruker).

Turbidimetric assay for determination of thrombin-specific activity

Typically, 2 μM fibrinogen solution was placed in a UVette cuvette of a spectrophotometer (BioPhotometer, Eppendorf, Germany), and the reaction was started by adding a thrombin sample with different specific activities to a final concentration of 0.5–15 nM (total volume 70 μl). Measurements were taken relative to the same sample without thrombin at a 230-nm wavelength [13]. Thrombin with a standard specific activity was used to determine the specific activity of the uncharacterized samples.

Turbidimetric assay for determination of aptamer inhibitory activity

Aptamer solutions (200 nM) were preformed in 10 mM KCl at 95 °C for 5 min and cooled at room temperature. The turbidimetric assay was the same except for the addition of preformed aptamer solution (final concentration 2–30 nM) before adding thrombin.

Results

AFM studying of fibrin association

A series of images of fibrin associates at various reaction stages were obtained for hydrolysis of 2 μM fibrinogen with 3.6 nM thrombin (Fig. 2). For the sample of 2 μM fibrinogen without thrombin, a thin protein layer was formed on the mica (Fig. 2A). Fine fibrous structures 2 μm long appeared during the first minute of reaction (Fig. 2B). The fibril length later increased quickly, reach-