Effect of oligodeoxynucleotide thrombin aptamer on thrombin inhibition by heparin cofactor II and antithrombin

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Abstract 'Thrombin aptamers' are based on the 15-nucleotide consensus sequence of d(GGTTGGTGTGGTTGG) that binds specifically to thrombin's anion-binding exosite-I. The effect of aptamer–thrombin interactions during inhibition by the serine protease inhibitor (serpin) heparin cofactor II (HCII) and antithrombin (AT) has not been described. Thrombin inhibition by HCII without glycosaminoglycan was decreased ∼two-fold by the aptamer. In contrast, the aptamer dramatically reduced thrombin inhibition by >200-fold and 30-fold for HCII–heparin and HCII–dermatan sulfate, respectively. The aptamer had essentially no effect on thrombin inhibition by AT with or without heparin. These results add to our understanding of thrombin aptamer activity for potential clinical application, and they further demonstrate the importance of thrombin exosite-I during inhibition by HCII–glycosaminoglycans. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Aptamer; Serpin; Thrombin; Heparin cofactor II; Antithrombin

1. Introduction

α-Thrombin is a trypsin-like serine protease involved in a multitude of physiological processes [1,2]. Thrombin is the last protease in the clotting cascade functioning to cleave soluble fibrinogen to insoluble fibrin that forms the fibrin gel either of a physiologic plug or a pathologic thrombus [1–4]. In addition, thrombin will potentiate the procoagulant process by activating Factors V, VIII, XI and XIII. Conversely, following its binding to thrombomodulin, thrombin aids the anticoagulant process by activating protein C [5,6]. Thrombin is also involved with other activities including inflammation and wound healing [7–9].

Thrombin and other coagulation proteases are regulated in vivo by a series of mechanisms including the protein C pathway already mentioned, tissue factor pathway inhibitor complex formation, and also a collection of plasma proteins including antithrombin (AT) and heparin cofactor II (HCII)

[10–12]. AT and HCII are members of the serine protease inhibitor (serpin) superfamily [13,14], and these inhibitors form 1:1 complexes with thrombin that render thrombin proteolytically inactive. Once formed the complexes are cleared via the low-density lipoprotein receptor-related protein [15]. The rates of protease inhibition by these serpins are significantly increased in the presence of glycosaminoglycans such as heparin/heparan sulfate for both AT and HCII and dermatan sulfate for HCII.

A new class of thrombin inhibitors has recently been described [16–19]. These inhibitors are based on sequence-specific single-stranded DNA oligonucleotides, termed 'thrombin aptamers'. Bock and coworkers selected a pool of single-stranded DNA oligonucleotides that were found to inhibit thrombin's ability to clot fibrinogen at nM concentrations [16–18]. The 15-nucleotide consensus sequence d(GGTTGG-TGTGGTTGG) found in nearly all samples of thrombin aptamer was found to display significant AT activity. The thrombin aptamer works by binding to thrombin's anion-binding exosite-I, one of thrombin's two highly basic regions that form secondary binding sites on the surface of the molecule [20,21]. This basic region of thrombin contributes to the macromolecular substrate specificity of thrombin with substances like fibrinogen, hirudin, and HCII [22,23]. Thrombin aptamers represent a new type of inhibitor with significant antithrombotic and anticoagulant properties, and due to their chemistry they may prove useful in various biomedical applications [16–19,24].

The goal of the project was to evaluate the influence of the DNA aptamer–thrombin interaction during inhibition by the serpins HCII and AT. The interactions were measured in both the presence and absence of the glycosaminoglycans heparin and dermatan sulfate. Based on these experiments, we have further shown the importance of thrombin exosite-I in regulating the HCII–thrombin inhibition reaction in the presence of glycosaminoglycans.

2. Materials and methods

2.1. Proteins and reagents
Plasma derived human α-thrombin, γ_T-thrombin, HCII and AT were prepared and purified to homogeneity as described previously [25–27]. The thrombin aptamers were provided by Dr. Craig S. Gibbs (Gilead Sciences, Foster City, CA, USA) and were: d(GGTTGGTG-TGGTTGG) aptamer; and d(GGTGGTGGTTGTGGT) scrambled aptamer. Unbleached crude heparin was obtained from Diosynth (Oss, The Netherlands). Dermatan sulfate (Calbiochem) was treated with nitrous acid to remove contaminating heparin/heparan sulfate as detailed [28].

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Abbreviations: serpin, serine protease inhibitor; HCII, heparin cofactor II; AT, antithrombin; BSA, bovine serum albumin

2.2. Serpin/thrombin activity

Prior to performing the serpin–thrombin assays, it was necessary to choose a buffer with the appropriate metal ions to promote aptamer–thrombin interactions [16,17]. A fibrinogen assay was used with the aptamer (or scrambled aptamer) at 20 nM and thrombin at 7 nM (pre-incubated for 60 s), and bovine fibrinogen (2.0 mg/ml) was then added to the buffer/aptamer–thrombin solution. Of the buffers examined, we found that HNPK (20 mM HEPES, 145 mM NaCl, 5 mM KCl, 0.1% polyethylene glycol 8000, 0.02% NaN_3 at pH 7.4) promoted the best activity with the aptamer (data not included).

Inhibition assays were performed using HNPK/BSA (bovine serum albumin)-coated microtiter plates as described previously [29–32]. In the absence of glycosaminoglycan, 1 nM thrombin was incubated with 500 nM serpin, and either 1 μM aptamer or scrambled aptamer. In the presence of fixed amounts of glycosaminoglycan, 1 nM thrombin (α- or γ_T-) was incubated with 10 nM serpin, various amounts or aptamer or scrambled aptamer (from 0.0001 to 1 μM), and glycosaminoglycan (10 μg/ml heparin for HCII; 1 or 5 μg/ml heparin for ATIII; or 20 μg/ml dermatan sulfate for HCII). Assays with HCII– or AT–thrombin in the presence of varying amounts of heparin (0.10–100 μg/ml for AT and 1–1000 μg/ml for HCII) were performed with 10 nM serpin, 1 nM thrombin, and 5 nM aptamer. Inhibition reactions and residual thrombin activity were determined using both continuous and discontinuous assays, and rate constants were calculated as described previously [29–32].

2.3. Molecular modeling

The HCII–thrombin complex was constructed essentially as described previously using the Homology module of the Insight II molecular modeling package (Version 2.3.0, BIOSYM TECHNOLOGIES, Inc., San Diego, CA, USA) [33,34]. The X-ray crystal structure of α-thrombin complexed with the thrombin aptamer and D-Phe-Pro-Arg-chloromethyl ketone ([35]; Brookhaven entry 1HAO) was obtained from the Brookhaven Protein Data Bank. The coordinates of the backbone atoms in the thrombin molecule were aligned in Insight II. Connolly solvent accessible surfaces for HCII, thrombin, aptamer, and D-Phe-Pro-Arg-chloromethyl ketone were created in Insight II using a probe radius of 1.4 Å.

3. Results

3.1. Influence of thrombin aptamer on HCII/AT–thrombin inhibition in the absence of glycosaminoglycans

Without glycosaminoglycan present, the aptamer slightly reduces the thrombin inhibition rate by HCII to $1.52 \pm 0.07 \times 10^4$ M^{-1} min^{-1}, compared to $2.51 \pm 0.01 \times 10^4$ M^{-1} min^{-1} with the scrambled aptamer and $2.60 \pm 0.07 \times 10^4$ M^{-1} min^{-1} in the absence of aptamer. The interaction of the aptamer with thrombin showed no effect in AT inhibition of thrombin activity with an inhibition rate in the presence of aptamer of $2.76 \pm 0.38 \times 10^5$ $M^{-1}min^{-1}$, in comparison to $2.62 \pm 0.33 \times 10^5$ M^{-1} min^{-1} with the scrambled aptamer and $2.48 \pm 0.22 \times 10^5$ M^{-1} min^{-1} in the absence of aptamer.

3.2. Influence of thrombin aptamer on HCII/AT–thrombin inhibition in the presence of glycosaminoglycans

In contrast to the data above, HCII inhibition in the presence of heparin and dermatan sulfate was dramatically altered by aptamer–thrombin interactions. In the presence of 1 μM aptamer, there was a greater than 96% attenuation of the glycosaminoglycan-enhanced inhibition reaction (Table 1). A significant loss of thrombin inhibitory activity by HCII was found when using various aptamer amounts from 0.001 to 0.1 μM in the presence of either heparin and dermatan sulfate, with an aptamer $I_{50\%}$ of ∼ 5 nM for both glycosaminoglycans (Fig. 1). There was an effect of the scrambled aptamer sequence in the presence of dermatan sulfate but not with heparin, possibly attributed to dermatan sulfate being more dependent on thrombin exosite-I during inhibition by HCII (Fig. 1). The thrombin aptamer substantially reduced the rate of thrombin inhibition over a wide range of heparin concentrations with a maximal inhibition of 12×10^7 M^{-1} min^{-1} in the presence of aptamer compared to 41×10^7 M^{-1} min^{-1} in its absence (Fig. 2). These results suggest that thrombin aptamer–thrombin interactions alter the HCII–glycosaminoglycan inhibition reaction for thrombin.

The thrombin aptamer only had a minor effect on the AT–heparin inhibition of thrombin, and there was no effect of the scrambled aptamer to alter the inhibition reaction (Table 1 and Fig. 1). The small influence for the aptamer on AT–heparin was mimicked when γ_T-thrombin, a derivative lacking anion-binding exosite-I, was used in place of α-thrombin (Table 1). Varying the concentration of heparin with a fixed amount of thrombin aptamer showed an ∼ 1.4-fold loss of activity for AT–heparin (Fig. 2). These results indicate that

Table 1
Influence of thrombin aptamer on serpin–glycosaminoglycan thrombin inhibition[a]

Serpin–protease	Conditions	k_2 (M^{-1} min^{-1}) $\times 10^7$	Ratio (serpin–aptamer/serpin alone)
HCII/α-thrombin	*Heparin*		
	HCII alone	19.6 ± 3.5	1.0
	HCII+aptamer	< 0.10	< 0.001
	HCII+scrambled	20.9	1.1
HCII–α-thrombin	*Dermatan sulfate*		
	HCII alone	13.8 ± 1.0	1.0
	HCII+aptamer	$0.37 \pm .31$	0.03
	HCII+scrambled	7.13 ± 1.6	0.52
AT–α-thrombin	*Heparin*		
	AT alone	133 ± 15	1.0
	AT+aptamer	82.0 ± 5.6	0.62
	AT+scrambled	133 ± 13	1.0
AT–γ_T-thrombin	*Heparin*		
	AT Alone	71.5 ± 3.8	1.0
	AT+Aptamer	58.1 ± 4.2	0.81
	AT+Scrambled	71.9 ± 1.9	1.0

[a]Serpin/thrombin assay was performed at room temperature in HNPK with 10 nM serpin, 1 nM (α- or γ_T-)thrombin, 1 μM aptamer or 1μM scrambled sequence and 10 μg/ml (or 667 nM) heparin with HCII, 5 μg/ml (or 334 nM) heparin with ATIII, and 20 μg/ml (or 444 nM) dermatan sulfate with HCII. The assays were done three separate times in triplicate (except for the scrambled aptamer with HCII–heparin, which was done two separate times).
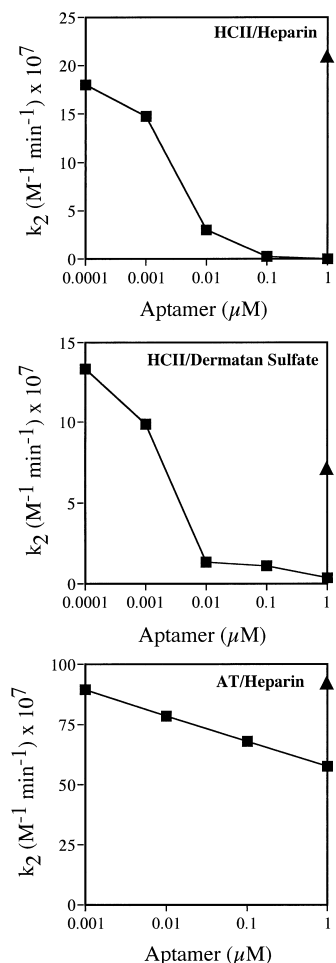
Fig. 1. HCII and AT inhibition of thrombin in the presence of thrombin aptamer and glycosaminoglycans. Reaction conditions were 10 nM serpin, 1 nM thrombin, 1 μg/ml (or 67 nM) heparin with AT, 10 μg/ml (or 667 nM) heparin with HCII, or 20 μg/ml (or 444 nM) dermatan sulfate with HCII, and 0.001–1 μM aptamer (■) or 1 μM scrambled aptamer (▲). Top is HCII/heparin; middle is HCII/dermatan sulfate and bottom is AT/heparin.

the thrombin aptamer has little influence on AT–heparin thrombin inhibition, which is consistent with a less important role for exosite-I during the AT inhibition reaction.

4. Discussion

The goals of anticoagulant therapy in cardiovascular disease are to inhibit fibrin deposition and platelet aggregation, which prevent ongoing ischemic events and to prolong the patients life [36–39]. There are many established antithrombotic agents like aspirin, coumarin and unfractionated heparin. While these agents have well-established biological actions there are some limitations to their effectiveness; thus, a search for new and novel antithrombotics has been underway for the past 15 years [36–39]. The thrombin aptamer described by Bock and co-workers [16–18] is a potent antithrombotic, both in vitro and in vivo, and it possesses some potential for clinical application [24]. Aptamers have now been prepared to both exosite-I [16–18] and exosite-II of thrombin [19]. The specificity of thrombin aptamers provides a unique

biomedical/biochemical tool to probe thrombin's interactions with macromolecular substrates.

We evaluated the effect of the exosite-I-specific thrombin aptamer on serpin-catalyzed thrombin inhibition reactions, comparing HCII to AT. The aptamer had virtually no influence on thrombin inhibition by AT in either the absence or presence of glycosaminoglycans. The results verified that AT has no major interaction with exosite-I of thrombin with or without heparin ([30,40] and references cited therein). The inhibitory activity of HCII in the absence of glycosaminoglycan was slightly affected by the thrombin aptamer, with the k_2 value decreased $\sim$ two-fold, and is comparable to decreases with γ_T-thrombin and the dysthrombin variant Quick I ($Arg^{67} \rightarrow Cys$) with HCII [27,41]. These results are consistent with the thrombin aptamer decreasing the positive electrostatic potential of exosite-I, which could slightly reduce the association rate constant between HCII and thrombin.

A large decrease in the rate of thrombin inhibition was found for HCII–glycosaminoglycans in the presence of the thrombin aptamer. These results agree with the evidence that exosite-I is critical for HCII inhibition especially catalyzed by glycosaminoglycans ([30] and references cited therein). HCII contains an N-terminal acidic region (residues 56–75) similar in sequence to the C-terminal tail of hirudin [42]. The HCII acidic domain is required for inhibition of thrombin in the presence of glycosaminoglycan by interacting with thrombin's exosite-I, and it is also postulated to bind to the glycosaminoglycan-binding site in the absence of glycosaminoglycans [32,43,44]. We recently found that Arg^{67} and Arg^{73} in exosite-I of thrombin have a major role in complex formation with HCII in the presence of glycosaminoglycan (Fig. 3,
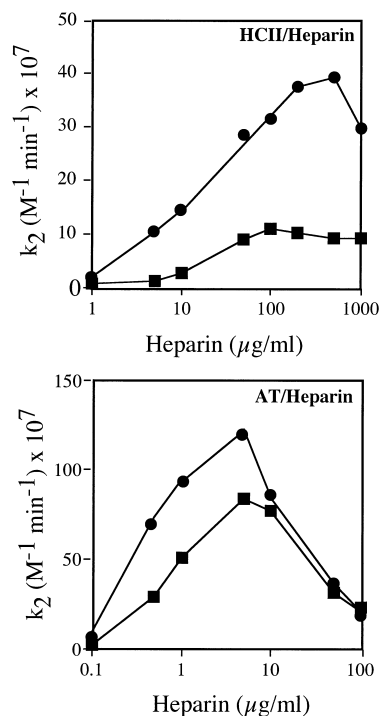

Fig. 2. HCII and AT inhibition of thrombin in the presence of thrombin aptamer and heparin. Final concentrations were 10 nM serpin, 1 nM thrombin, without (●) or with 5 nM aptamer (■), and heparin from 0.1 to 1000 μg/ml (or 6.7–66 667 nM). Top is HCII; bottom is AT.
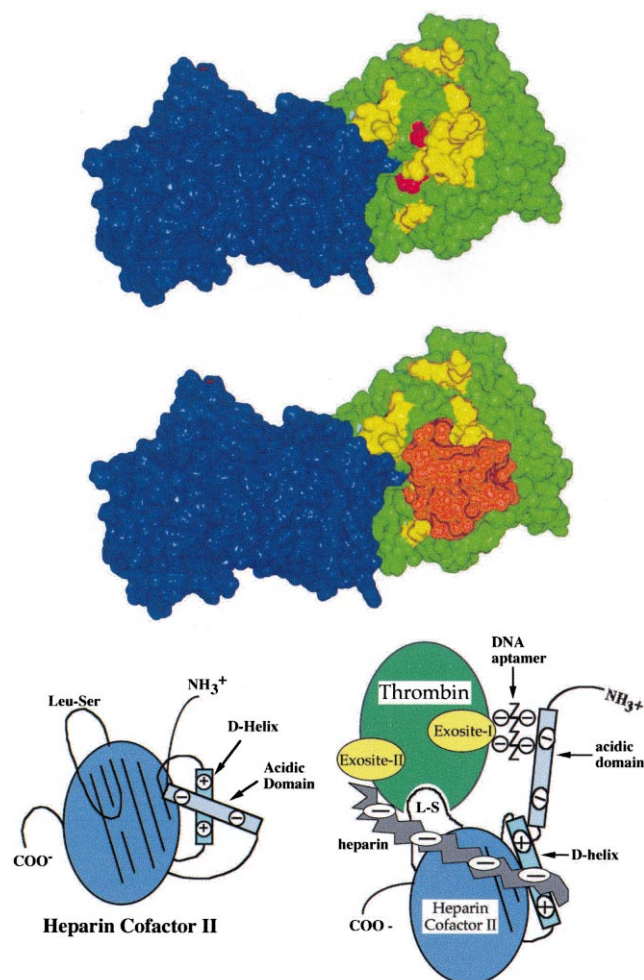
Fig. 3. Molecular models of HCII complexed with thrombin and thrombin–thrombin aptamer. Top: Critical thrombin exosite-I residues for HCII–glycosaminoglycan inhibition, Arg[67] and Arg[73] are highlighted in red, while the other exosite-I residues are in yellow [31]. Thrombin–HCII complex. The first residue (Gly[100]) of the HCII model is shown in red and the acidic domain is not shown because it can not be displayed using homology modeling. Middle: Thrombin–aptamer–HCII complex. This depicts the structure of thrombin–thrombin aptamer (aptamer is shown in orange) bound to exosite-1 of thrombin. The key residues in exosite-I for thrombin–HCII interactions (Arg[67] and Arg[73]) are masked by the thrombin aptamer. Bottom: Schematic of postulated mechanism of HCII–thrombin inhibition with heparin and the thrombin aptamer: left side is HCII alone showing the intramolecular interaction of acidic domain with the D-helix; and right side is HCII–heparin-thrombin with the aptamer blocking access to exosite-I.

top panel) [31]. In contrast, there was only a minimal effect of the other basic residues in exosite-I on HCII–glycosaminoglycan thrombin inhibition (Fig. 3, top panel) [31]. Interestingly, the thrombin aptamer interacts with both Arg[67] and Arg[73] of thrombin (Fig. 3, middle and bottom panels). Consistent with our past mutagenesis study, aptamer binding to Arg[67] and Arg[73] would greatly contribute to the loss of inhibitory activity by HCII in the presence of glycosaminoglycans (Fig. 3, bottom panel). We conclude that the interaction of the complementary electrostatic field of thrombin exosite-I for the HCII acidic domain would be greatly diminished following thrombin aptamer binding to thrombin.

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