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Mechanism of thrombin inhibition by heparin cofactor II and antithrombin in the presence of the ray (*Raja radula*) skin dermatan sulfate

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ABSTRACT

Introduction: The kinetics of the thrombin inhibition by heparin cofactor II (HCII) and antithrombin (AT) have been studied as a function of the concentration of a dermatan sulfate (DS) from the skin of the ray *Raja radula*.

Materials and methods: The initial concentrations of inhibitor (I), HCII or AT, and thrombin (E) were set at equimolecular levels (3.10^{-9} M). Analysis of the experimental data obtained for DS concentrations ranging from 10^{-8} to 10^{-4} M was performed according to a previously described model in which DS binds quickly to the inhibitor and forms a complex more reactive than the free inhibitor towards thrombin.

Results: The apparent rate constant of the thrombin inhibition, k_{app}, by either HCII or AT, increased in a concentration-dependent manner for DS concentrations up to 10^{-5} M or 10^{-6} M, respectively. At higher DS concentrations, k_{app} remained unchanged for thrombin inhibition by HCII whereas a decrease in k_{app} was observed for the thrombin-AT reaction. The dissociation constant of the polysaccharide-inhibitor complex, K_{DSI}, and the rate constant of the thrombin inhibition by this complex, k, were $(7.81 \pm 0.75).10^{-7}$ M and $(2.84 \pm 0.42).10^{9}$ $M^{-1}.min^{-1}$, whereas they were $(4.93 \pm 0.31).10^{-7}$ M and $(2.47 \pm 0.28).10^{8}$ $M^{-1}.min^{-1}$, when the inhibitor was either HCII or AT, respectively.

Conclusion: DS from ray skin catalyzes the thrombin inhibition by HCII or AT primarily by forming a DS-inhibitor complex more reactive than the free inhibitor towards the protease. The affinity of DS for HCII was approximately 2-fold higher whereas the catalyzed reaction rate constant was approximately 20-fold higher when compared to AT.

Dermatan sulfate (DS) naturally occur as glycosaminoglycan (GAG) chains of proteoglycans in a variety of animal tissues [1], and differ in terms of structure and particularly mean molecular weight, sulfate content and sulfation pattern [2–4]. This heterogeneity is responsible for the various and more specialized functions of these glycosaminoglycans [5]. In particular, DS, which is a heparin-like sulfated galactosaminoglycan, has been reported to exhibit antithrombin activity through interacting with heparin cofactor II (HC II) [6,7]. Native mammalian DS exerts its anticoagulant effect through enhancing approximately 1000-fold the inactivation rate of thrombin by HC II but has no significant effect on thrombin inhibition by antithrombin (AT) [6,8]. Chemically [9] or naturally occurring [10] oversulfated dermatan sulfates enhance up to 10.000-fold the rate of the thrombin–HC II reaction, more than both native dermatan sulfate [9] and heparin [11]. It has been claimed that DS first binds to HCII [6,12] and subsequently forms with thrombin a ternary complex preceding the generation of the covalent thrombin-HCII complex (template mechanism) whereas Verhamme et al. claimed that DS first binds to thrombin and subsequently interacts with HCII [13]. In turn, other findings suggested that DS binds quickly to the inhibitor and forms a complex more reactive than the free inhibitor towards thrombin (allosteric mechanism) [11,14–16].

We have isolated from the skin of the ray *Raja radula* a DS endowed with high anticoagulant activity mediated by both HCII and AT [17]. The aim of this study was to elucidate the mechanism of catalysis of the thrombin-HCII and thrombin-AT reaction, in the presence of this ray skin DS.

Materials and methods

Materials

DS from the skin of the ray *Raja radula* skin (Mn 22 kDa, Mw 31.2 kDa, Ip = 1.41, sulfate content 29%), was extracted and purified

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by cetylpyridinium chloride and ethanol precipitation followed by anion exchange chromatography [17], human heparin cofactor II (HCII) was from HYPHEN BioMed, France, human antithrombin and human α-thrombin (3000 NIH units/mg) were purified respectively according to Bezeaud *et al.* [18]; chromogenic substrate for thrombin S2238 (H-D-Phe-Pip-Arg-pNA) was from Kabivitrum, Stockholm, Sweden; reptilase was purchased from Diagnostica Stago, Asnières, France; fibrinogen, N-(2 hydroxyethyl) piperazine-N'-(2 ethane sulfonic) acid (HEPES) and polyethylene glycol 8000 were from Sigma, USA; platelet poor plasma (PPP) was prepared from whole blood, drawn on 0.13 M sodium citrate (9:1, v/v), obtained by venipuncture of healthy volunteers.

Methods

Kinetic evaluation of the thrombin-inhibitor interaction

Kinetics of the thrombin-inhibitor reaction was studied by determining the residual enzyme activity as a function of the incubation time with the inhibitor. All components were diluted in HEPES buffer 10 mM, NaCl 150 mM and polyethylene glycol 8000 1 mg/mL, pH 7.5). The initial concentrations of enzyme (C_E) and inhibitor (C_I) were equimolar and set at 3.10^{-9} M. All assays were performed in triplicate.

Kinetics of thrombin inhibition in the absence of dermatan sulfate

25 μL HEPES buffer pH 7.5 were added to 37.5 μL inhibitor, HC II or AT, and 37.5 μL thrombin. The inhibitor and protease were set at equimolar final concentrations (fc) equal to 3.10^{-9} M. Several reaction mixtures were incubated at 37 °C for different times ranging from 0 to 40 min. Enzyme activity was then measured at each incubation time by adding 100 μL of chromogenic substrate S-2238 0.4 mM to the samples. The rate of S-2238 hydrolysis was measured. Thrombin concentration was determined using a calibration curve made with defined concentrations of thrombin.

Kinetics of thrombin inhibition in the presence of various concentrations of dermatan sulfate

25 μL DS from ray skin at concentrations ranging from 10^{-8} to 10^{-4} M were pre-incubated 1 min at 37 °C with 37.5 μL inhibitor (AT or HCII) at $C_I = 3.10^{-9}$ M. 37.5 μL thrombin at $C_E = 3.10^{-9}$ M were then added. Samples were incubated at 37 °C for various periods of time for each DS concentration. The residual enzyme activity was measured for each incubation time by adding 100 μL S-2238 0.4 mM as above.

Fibrinogen clotting activity of thrombin in the presence of various dermatan sulfate concentrations

150 μL fibrinogen 2 mg/mL were added to 50 μL HEPES buffer pH 7.5 containing or not the ray skin DS at concentrations ranging from 10^{-8} to 10^{-4} M. The mixture was pre-warmed 1 min at 37 °C. 50 μL of thrombin (5 NIH units/mL corresponding to 50 nM) were then added and the clotting time was measured for each DS concentration [11].

Fibrinogen clotting activity of Reptilase in the presence of various dermatan sulfate concentrations

100 μL platelet poor plasma (PPP) containing or not DS at concentrations ranging from 10^{-8} to 10^{-4} M were added to 100 μL Reptilase 10UB/mL (Units Batroxobine/mL). The clotting time was measured for each DS concentration.

Measurement of fibrin formation in the presence of various dermatan sulfate concentrations

50 μL DS at concentrations ranging from 10^{-8} to 10^{-4} M were added to 50 μL of thrombin at final concentrations of 1 or 3 nM. The mixture was pre-incubated 2 min at 37 °C. 100 μL fibrinogen 0.5 mg/mL were then added. The absorbance at 340 nm was recorded for 15 min. The absorbance level was proportional to fibrin formation.

Theoretical

All experimental data were analysed using a previously described and discussed model [9,11,19].

The non-catalyzed reaction is written as follows:

$$I + E \xrightarrow{k_0} \overset{\circ}{E}I \tag{1}$$

where k_0 is the second-order rate constant of free thrombin (E) inhibition by the free inhibitor (I).

The reaction rate is then written as follows:

$$\frac{-d[E]_t}{dt} = k_0.[I]_t.[E]_t \tag{2}$$

k_0 is the slope of the curve $1/[E]_t = f(t)$ obeying the second-order equation:

$$\frac{1}{[E]_t} - \frac{1}{C_E} = k_0.t \tag{3}$$

The kinetic model considers that the total protease E_T (free (E)) is inactivated by the total inhibitor I_T (free (I) and/or DS-bound I (DSI)). The total reaction is subsequently considered as a bimolecular reaction:

$$I_T + E_T \xrightarrow{k_{app}} EI \tag{4}$$

where k_{app} is the second-order rate constant of the total reaction.

The total reaction rate is then written as follows:

$$\frac{-d[E_T]_t}{dt} = k_{app}.[I_T]_t.[E_T]_t \tag{5}$$

For each DS concentration (C_{DS}), k_{app} is the slope of the curve obeying the second-order equation:

$$\frac{1}{[E_T]_t} - \frac{1}{C_E} = k_{app}.t \tag{6}$$

$[E_T]_t$ is the residual enzyme concentration at the end of the reaction time t.

This model also considers that the polysaccharide (DS) binds quickly to the inhibitor. The complex formed, DSI, rapidly reacts with the free protease in a second step which is rate-limiting. This leads to the formation of an inactive inhibitor-thrombin complex and release of the free polysaccharide according to:

$$DS + I \overset{K_{DSI}}{\longleftrightarrow} DSI + E \xrightarrow{k} DS + EI \tag{7}$$

where K_{DSI} is the dissociation constant of DSI and k the second-order rate constant of the free thrombin inhibition by DSI, eventually followed by the binding of a second polysaccharide molecule, at high DS concentrations, and inhibition of the free enzyme by the complex DS-DSI according to:

$$DS + DSI \overset{K_{DSI}}{\longleftrightarrow} DS - DSI + E \xrightarrow{k'} EI + 2DS \tag{8}$$

where k' is the bimolecular rate constant of the free enzyme inhibition by the complex DS-DSI.

At low polysaccharide concentrations, the total reaction is considered to be reduced to the sum of reactions 1 and 7. In this case, the equation of the total reaction rate was expressed by:

$$\frac{-d[E_T]_t}{dt} = k_0.[I]_t.[E]_t + k.[DSI]_t.[E]_t \tag{9}$$

DS concentration is considered constant and equal to C_{DS}. The development of Eq. (9) and the identification of its terms with those