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Note

Thrombin inhibition by antithrombin in the presence of oversulfated dermatan sulfates

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Abstract—DSS_1 and DSS_2 are two oversulfated dermatan sulfate derivatives with sulfur contents of 7.8% and 11.5%, respectively. DSS_1 and DSS_2 both enhanced the rate at which antithrombin (AT) inactivates thrombin according to a concentration dependent manner. The analysis of the experimental data, using our previously described kinetic model [*Biomaterials* **1997**, *18*, 203] (i) suggested that both DSS_1 and DSS_2 catalyzed the thrombin–AT reaction according to a mechanism in which the oversulfated derivative quickly formed with AT a complex, which was more reactive towards thrombin than the free inhibitor and (ii) allowed us to determine the dissociation constants of the polysaccharide–inhibitor complexes, which were $(1.15 \pm 0.74) \times 10^{-7}$ and $(7.17 \pm 0.65) \times 10^{-9}$ M, and the catalyzed reaction rate constants, which were $(2.29 \pm 0.15) \times 10^8$ and $(8.71 \pm 0.08) \times 10^8$ M^{-1} min^{-1}, for DSS_1 and DSS_2, respectively. These data suggested that the oversulfation confers an affinity for AT to dermatan sulfate and that the higher the sulfur content the higher the affinity for AT. They also suggested that the reactivities of the polysaccharide–AT complexes formed towards the protease increased with the sulfur content.

Keywords: Heparin; Dermatan sulfate; Oversulfation; Antithrombin; Thrombin; Anticoagulant mechanism

Antithrombin (AT), a plasma derived single chain glycoprotein, is a serine-protease inhibitor (serpin), sharing about 30% homology with other serpins such as heparin cofactor II (HC II). AT exerts its inhibitory action by forming an inactive, extremely stable, equimolar complex between AT and the proteases such as thrombin. The interaction of AT with these serine-proteases is considerably enhanced by heparin,[1] a highly sulfated glycosaminoglycan with a widespread clinical use as an anticoagulant drug.[2,3] However, because bleeding[4] and heparin-induced thrombocytopenia[5] represent major side effects of heparin, it is likely that alternative drugs are being sought.[3,6]

Dermatan sulfate, a heparin-like sulfated galactosaminoglycan, exerts its anticoagulant effect through potentiating HC II inhibitory activity towards thrombin[7] but has no significant effect on thrombin inhibition by AT.[8] Chemically oversulfated dermatan sulfates enhance the rate of the thrombin–HC II reaction more than both the native dermatan sulfate[8–10] and heparin[11,12] whereas they have been reported to have no effect on the rate at which AT inactivates thrombin.[10,13] We found however that the oversulfation confers to dermatan sulfate a new acquired ability to enhance the rate of the thrombin–AT reaction.[8]

We present in this study kinetic data specifying the mechanism of the catalysis of the thrombin–AT reaction by two oversulfated dermatan sulfates.

Abbreviations: AT, antithrombin; HC II, heparin cofactor II; DS, dermatan sulfate.

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The inhibitor (AT) and the enzyme (E) were both set at equimolar initial concentrations ($C_{AT} = C_E = 10^{-8}$ M). The residual thrombin $[E_T]_t$ was measured for various incubation times t and for each initial polysaccharide concentration (C_{PS}) ranging from 10^{-10} to 5.10^{-5} M. The reciprocal of the residual enzyme was then plotted versus the incubation time for each C_{PS} (Fig. 1a and b). The curves $1/[E_T]_t = f(t)$ were linear and indicated that the total reaction was second order at any C_{PS}, when either DSS_1 or DSS_2 was used. We postulated thereafter that the data obtained here fit a kinetic model assuming a total reaction wherein the total protease (free (E) and/or polysaccharide-bound (PSE)) was inactivated by the total inhibitor (free (AT) and/or polysaccharide-bound (PSAT)). The slopes of these $1/[E_T]_t = f(t)$ curves were the experimental values of the total reaction rate constants k_{app}, which were subsequently plotted versus DSS_1 and DSS_2 concentrations, respectively (Fig. 2).

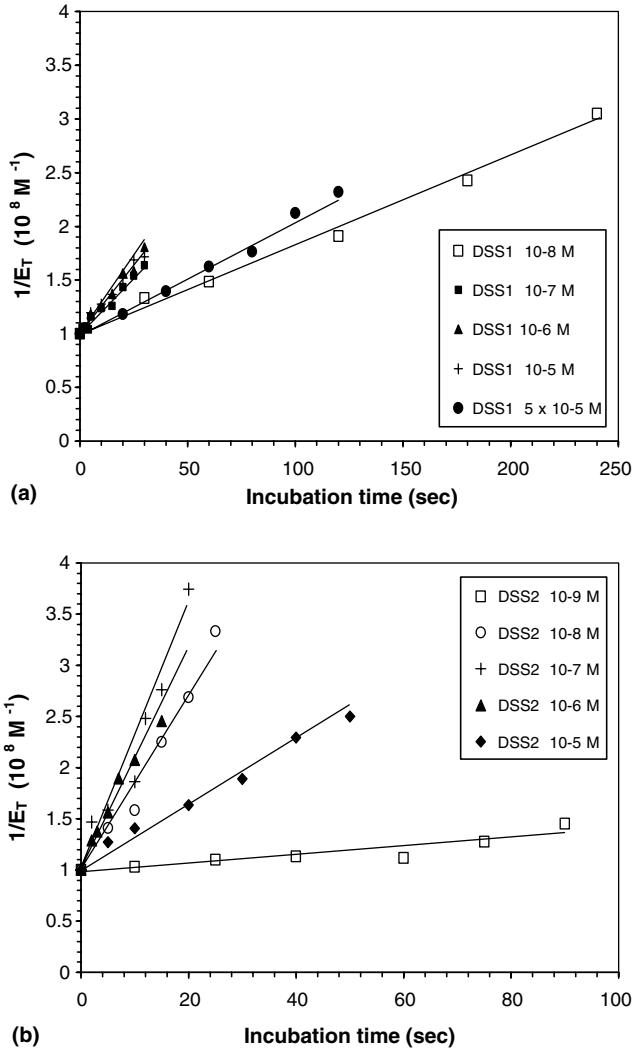

Figure 1. Thrombin inactivation by antithrombin in the presence of various DSS_1 (a) and DSS_2 (b) concentrations. The reciprocal of the residual enzyme was plotted versus the incubation time.

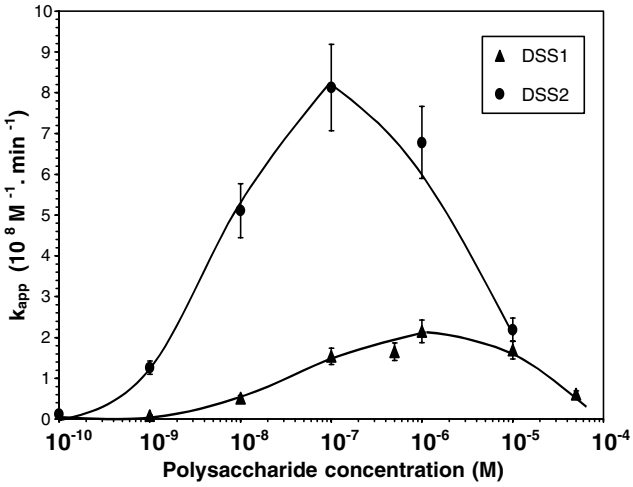

Figure 2. Thrombin antithrombin reaction rate as a function of DSS_1 (▲) and DSS_2 (●) concentrations. The total reaction rate constant (k_{app}) was plotted versus the polysaccharide concentration.

When DSS_1 was used, the graph obtained showed a significant increase in k_{app} as C_{PS} was raised up to 10^{-6} M, reaching the maximal value of $(2.15 \pm 0.24) \times 10^8$ M^{-1} min^{-1}; at higher concentrations, the reaction rate diminished significantly. In turn, when DSS_2 was used, at concentrations up to 10^{-7} M, the increase in k_{app}, up to $(8.15 \pm 0.24) \times 10^8$ M^{-1} min^{-1}, was also followed by a significant decrease.

At optimal concentrations, the acceleration of the reaction in the presence of DSS_1 or DSS_2 was 108-fold and 408-fold, respectively, by comparison with the non-catalyzed reaction (2×10^6 M^{-1} min^{-1}). Heparin was previously found to accelerate the thrombin–AT reaction 450-fold, under the same experimental conditions.[11]

We observed previously, at concentrations up to 10^{-6} M that neither DSS_1 nor DSS_2 prolonged the fibrinogen clotting time. Therefore, we assumed that neither DSS_1 nor DSS_2 could bind to thrombin, in this concentration range, and considered that fibrinogen is a heavy macromolecular substrate, which should be displaced from thrombin upon polysaccharide binding to the enzyme.[12] Taking this into account, we assumed that the formation of a polysaccharide–AT complex, which was more reactive than the free inhibitor towards thrombin was involved in the mechanism of the reaction catalysis, as already established when the inhibitor was HC II.[12] Thereafter, the higher C_{PS} the more polysaccharide–AT (PSAT) complex formed, until saturation of the inhibitor. This was illustrated by the sharp increase in k_{app} observed in the increasing part of the bell-shaped curve $k_{app} = f(C_{PS})$ for both DSS_1 and DSS_2 (Fig. 2).

The polysaccharide affinity for AT, K_{PSAT}, and the bimolecular rate constant, k, of the free thrombin

Table 1. (a, b) K_{PSAT} and K_{PSE} represent the dissociation constants of the polysaccharide–AT and polysaccharide–thrombin complexes, respectively

	K_{PSAT} (M)	k ($M^{-1}\,min^{-1}$)
(*a*)		
DSS1	$(1.15 \pm 0.74) \times 10^{-7}$	$(2.29 \pm 0.15) \times 10^{8}$
DSS2	$(7.17 \pm 0.65) \times 10^{-9}$	$(8.71 \pm 0.08) \times 10^{8}$
Heparin[a]	$(3.7 \pm 0.8) \times 10^{-7}$	$(1.3 \pm 0.1) \times 10^{9}$
(*b*)		
	K_{PSE} (M)	k' ($M^{-1}\,min^{-1}$)
DSS_1	$(8.26 \pm 0.64) \times 10^{-6}$	$(1.60 \pm 0.53) \times 10^{7}$
DSS_2	$(2.03 \pm 0.45) \times 10^{-6}$	$(1.46 \pm 0.65) \times 10^{8}$
Heparin[a]	$(4.0 \pm 0.4) \times 10^{-6}$	$(2.0 \pm 0.4) \times 10^{8}$

k and k' represent the bimolecular rate constants of the polysaccharide-bound AT with free thrombin and polysaccharide-bound thrombin, respectively.

Mean values of K_{PSAT}, k, K_{PSE} and k' are calculated from all the k_{app} values obtained at the corresponding C_{PS} values, ranging from 10^{-9} to 10^{-4} M.

[a] Data for heparin are from a previous study.[11]

inactivation by the polysaccharide-bound inhibitor were computed by means of Eq. 8 from all the k_{app} experimental values obtained for C_{PS} up to 10^{-6} M for DSS_1 and 10^{-7} M for DSS_2 (Table 1a). K_{PSAT} and k were $(1.15 \pm 0.74) \times 10^{-7}$ M and $(2.29 \pm 0.15) \times 10^{8}\,M^{-1}\,min^{-1}$ for DSS_1 whereas they were $(7.17 \pm 0.65) \times 10^{-9}$ M and $(8.71 \pm 0.08) \times 10^{8}\,M^{-1}\,min^{-1}$ for DSS_2.

At concentrations higher than 10^{-6} M for DSS_1, or 10^{-7} M for DSS_2, the decrease in k_{app} was attributed to a polysaccharide–thrombin complex the formation of which was no longer negligible, particularly for C_{PS} over 10^{-6} M. Indeed, at such high concentrations, both DSS_1 and DSS_2 prolonged the fibrinogen clotting time,[12] strongly suggesting that both polysaccharides bound to the protease and that the formed complex would be inactivated at a slower rate than the free protease by the polysaccharide–AT complex. PSE formation accounted here for the decrease of the total reaction rate for such concentrations. The polysaccharide affinity for thrombin, K_{PSE}, and the bimolecular rate constant, k', of the polysaccharide-bound thrombin inactivation by the polysaccharide-bound inhibitor were computed by means of Eq. 10 from all the k_{app} experimental values obtained for C_{PS} from 10^{-6} M to higher concentrations for DSS_1 and from 10^{-7} M to higher concentrations for DSS_2; K_{PSE} and k' were $(8.26 \pm 0.64) \times 10^{-6}$ M and $(1.60 \pm 0.53) \times 10^{7}\,M^{-1}\,min^{-1}$ for DSS_1 whereas they were $(2.03 \pm 0.45) \times 10^{-6}$ M and $(1.46 \pm 0.65) \times 10^{8}\,M^{-1}\,min^{-1}$ for DSS_2 (Table 1b).

The data obtained from the study of the oversulfated DS accelerated thrombin–AT reaction were analyzed according to the previously established model for the heparin-catalyzed thrombin–AT reaction.[11]

The theoretical and experimental data were found to be in good agreement with the kinetic model in which the polysaccharide bound quickly to the inhibitor. The formed complex thereafter reacted much more rapidly than the free inhibitor with the protease. As observed for heparin, the oversulfated dermatans, used at high concentrations, caused a decrease in the reaction rate. Indeed, as mentioned above, data from fibrinogen clotting assays[12] strongly suggested the formation of polysaccharide-bound thrombin whose inactivation occurred at a slower rate than the free protease.

DSS_2 had a stronger affinity ($K_{PSAT} = 7.17 \times 10^{-9}$ M) than DSS_1 ($K_{PSAT} = 1.15 \times 10^{-7}$ M) for AT. This might be attributed to the higher sulfur content in DSS_2. However, DSS_1, though less sulfated, exhibited an almost equal or even higher affinity than heparin ($K_{PSAT} = 3.7 \times 10^{-7}$ M) for AT. In turn DSS_2, almost as sulfated as heparin, had an affinity higher than those of both DSS_1 and heparin for the inhibitor (Table 1a).

Altogether, these data suggested that the oversulfation may have brought in a specific sulfation pattern of the dermatan sulfate polysaccharide chains enabling them to interact with AT. Indeed, specific sulfate groups distribution along the polysaccharide chain rather than the whole polysaccharide negative charge is needed for GAG–protein interactions.[14]

At optimal concentrations, the reaction rate of the DSS_1 catalyzed thrombin–AT reaction was $2.15 \times 10^{8}\,M^{-1}\,min^{-1}$ whereas it was $8.15 \times 10^{8}\,M^{-1}\,min^{-1}$ for DSS_2. The first reaction rate was weaker whereas the second was almost similar in comparison with the reaction rate obtained previously for the heparin catalyzed reaction ($9.1 \times 10^{8}\,M^{-1}\,min^{-1}$).[11] Indeed, the computed values of the bimolecular rate constants of the inactivation of free thrombin by the polysaccharide-bound AT $-2.29 \times 10^{8}\,M^{-1}\,min^{-1}$ and $8.71 \times 10^{8}\,M^{-1}\,min^{-1}$ for DSS_1 and DSS_2, respectively, in comparison with $1.3 \times 10^{9}\,M^{-1}\,min^{-1}$ for heparin–AT (Table 1a) indicated first that the reactivity of the oversulfated dermatan-bound AT increased with the sulfur content and suggested that the extent of the binding of the GAG to the inhibitor might be independent from the ability of the polysaccharide-bound AT to react with the protease as the inhibitor bound to DSS_1 or DSS_2 had a weaker reactivity towards thrombin in comparison with heparin-bound AT. More precisely, the level of the reactivity of the polysaccharide–inhibitor complex towards thrombin might not be proportional to the extent of the affinity of the polysaccharide for the inhibitor. This has to be further investigated. In other respects, heparin contains an AT-high-affinity pentasaccharide sequence bearing a unique 3-O-sulfate group of a glucosamine residue,[15] which has proven to be of great importance for antithrombin activation and the anticoagulant action of heparin[16–18] and may explain the differences observed above between the reactivities of the different polysaccharide-bound AT complexes towards thrombin.

The analysis of our data also indicated that the chemical oversulfation of DS conferred likewise a newly acquired affinity for thrombin knowing that the native DS has no affinity for the protease. In turn, fibrinogen clotting experiments indicated that DSS_1 and DSS_2, at concentrations higher than 10^{-6} M and 10^{-7} M, respectively, bound to thrombin.[12] The polysaccharide affinities (K_{PSE}) for thrombin were 8.26×10^{-6} M for DSS_1 and 1.15×10^{-6} M for DSS_2 (Table 1b). Fibrinogen clotting experiments also indicated that heparin, at high concentrations, bound to thrombin; the heparin affinity for the protease, previously calculated according to the same kinetic model was $K_{PSE} = 4 \times 10^{-6}$ M[11] (Table 1b). DSS_1 affinity was thus lower than both heparin and DSS_2 affinities for the protease. This observation could be explained by the higher sulfation of heparin and DSS_2 polysaccharide chains in comparison with DSS_1, if assumed that the oversulfated dermatans bound to thrombin the way heparin did. Indeed, binding of heparin to thrombin seems to be uniquely due to a polyelectrolyte effect where the interaction of the protease with heparin is entirely ionic-based, without specific hydrophobic or hydrogen bonding interactions.[14]

At high polysaccharide concentrations, the decrease in the catalyzed reaction rate was attributed to the formation of a polysaccharide–thrombin complex, where inactivation by the polysaccharide–inhibitor complex occurred at a slower rate in comparison with the free protease, as indicated by the computed bimolecular rate constants k', which were 1.6×10^7 M^{-1} min^{-1} for DSS_1 and 1.46×10^8 M^{-1} min^{-1} for DSS_2. k' was previously found to be 2×10^8 M^{-1} min^{-1} when heparin was used[11] (Table 1b). The reactivities of the polysaccharide–AT complexes towards the heparin-bound thrombin were in this case statistically similar for heparin and DSS_2 whereas that of DSS_1–AT was weaker. The difference between heparin–AT and DSS_2–AT reactivities towards the protease bound to the polysaccharide was smaller in comparison with the free protease inactivation.

Taken together, our data indicated that (i) the oversulfation conferred to dermatan sulfate a newly acquired affinity for AT, possibly by bringing in a specific sulfation pattern, (ii) the oversulfated dermatans DSS_1 and DSS_2, as already shown for heparin or when the inhibitor was heparin cofactor II, preferentially bound to AT in the first step of the reaction, inducing a much more rapid reaction of the polysaccharide–inhibitor complex than that of the free inhibitor with free thrombin in the second step of the reaction, (iii) the polysaccharide-bound AT reactivity towards thrombin might be independent from the extent of the polysaccharide affinity for AT; this was suggested by the lower reactivity of the DSS_1 or DSS_2-bound AT in comparison with the heparin-bound AT towards thrombin, although DSS_1 and DSS_2 affinities were found to be, respectively, almost similar and higher than that of heparin for AT.

1. Experimental

1.1. Proteins and polysaccharides

Purified human thrombin (3300 u NIH/mg) was from Sigma, St. Louis, USA, purified human antithrombin from Kabivitrum, Stockholm, Sweden and chromozym-TH from Diagnostica Stago, Asnières, France. Unfractionated dermatan sulfate (intestinal bovine mucus, 6% sulfur/disaccharide) and its two oversulfated derivatives DSS_1 and DSS_2 (7.8% and 11.5% sulfur/disaccharide, respectively), were kindly provided by Mr. Mardiguian, Pharmuka, Gennevilliers, France.

1.2. Assays of biologic activity

Thrombin was assayed by its amidolytic activity on a chromogenic substrate and the initial rate of amidolysis was measured at 405 nm, as previously described.[11]

1.3. Measurement of the inhibitor concentration and determination of equimolar inhibitor and thrombin concentrations

The inhibitor stock solution concentration was evaluated by incubating AT with an excess of thrombin at saturating levels of heparin. The amount of AT was then proportional to the part of the thrombin inhibited.[11]

The molar equivalence between thrombin and AT was established by neutralizing a fixed amount of thrombin with increasing levels of inhibitor, in the presence of saturating amounts of heparin. The decline in enzyme activity was a linear function of the inhibitor concentration, and extrapolation to 100% inactivation was used to compute the level of AT equimolar to thrombin.[11]

1.4. Kinetics of the thrombin inhibition in the presence or absence of polysaccharide

The kinetics of the thrombin–AT interaction was studied by determining the residual enzyme activity as a function of the incubation time of the protease with the inhibitor in the absence or in the presence of various polysaccharide concentrations.[11]

1.5. Theoretical

All experimental data were analyzed using a previously described and discussed kinetic model[11,19] in which the polysaccharide (PS) binds quickly to the inhibitor (AT). The formed complex, PSAT, reacts rapidly with the free protease, in a second step, which is rate limiting. This leads to the formation of an inactive inhibitor–thrombin complex and the release of the free polysaccharide according to

$$PS + AT \overset{K_{PSAT}}{\leftrightarrows} PSAT + E \overset{k}{\rightarrow} PS + E^{\circ}AT \quad (1)$$

where K_{PSAT} is the dissociation constant of PSAT and k the second-order rate constant of the free thrombin inhibition by PSAT, eventually followed by

$$PS + E \overset{K_{PS,E}}{\leftrightarrows} PSE + PSAT \overset{k'}{\rightarrow} PS + E^{\circ}AT \quad (2)$$

where K_{PSE} is the dissociation constant of PSE and k' the second-order rate constant of the polysaccharide-bound thrombin inhibition by PSAT.

The non-catalyzed reaction is written as follows:

$$AT + E \overset{k_0}{\rightarrow} E^{\circ}AT \quad (3)$$

Where k_0 is the second-order rate constant of free thrombin inhibition by free AT.

The kinetic model considers that the total protease E_T (free (E) and/or polysaccharide-bound (PSE)) is inactivated by the total inhibitor AT_T (free (AT) and/or polysaccharide-bound (PSAT)). The total reaction is subsequently considered as a bimolecular reaction:

$$AT_T + E_T \overset{k_{app}}{\rightarrow} E^{\circ}AT \quad (4)$$

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

The total reaction rate is then written as follows:

$$\frac{-d[E_T]_t}{dt} = k_{app} \cdot [AT_T]_t \cdot [E_T]_t \quad (5)$$

For each PS concentration (C_{PS}), k_{app} is the slope of the curve $1/[E_T]_t = f(t)$ obeying the second-order equation:

$$\frac{1}{[E_T]_t} - \frac{1}{C_E} = k_{app} \cdot t \quad (6)$$

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

At low polysaccharide concentrations, the total reaction is considered to be reduced to the sum of reactions 3 and 1. In this case, the equation of the total reaction rate was expressed by

$$\frac{-d[E_T]_t}{dt} = k_0 \cdot [AT]_t \cdot [E]_t + k \cdot [PSAT]_t \cdot [E]_t \quad (7)$$

PS concentration is considered constant and equal to C_{PS}. The development of Eq. 7 and the identification of its terms with those of the total reaction 5 gave the expression of the experimentally measured k_{app} as a function of K_{PSAT}, k_0, k and C_{PS}:

$$k_{app} = \frac{k_0 \cdot K_{PSAT} + k \cdot C_{PS}}{K_{PSAT} + C_{PS}} \quad (8)$$

At high polysaccharide concentrations, the total reaction was considered to be reduced to the sum of reactions 1 and 2, whose rate constants were k and k', respectively. The total reaction rate decreased as PSE formed until E saturation. Taking the above considerations into account, the equation of the total reaction rate was here expressed by

$$\frac{-d[E_T]_t}{dt} = k \cdot [PSAT]_t \cdot [E]_t + k' \cdot [PSAT]_t \cdot [PSE]_t \quad (9)$$

The development of Eq. 9 and the identification of its terms with those of the total reaction 5 gave the expression of the experimentally measured k_{app} as a function of K_{PSE}, k, k' and C_{PS}:

$$k_{app} = \frac{k.K_{PS,E} + k'}{CK_{PS,E} + C_{PS}} \quad (10)$$

1.6. Statistical analysis

The analysis of variance was performed and standard deviations were determined for all k_{app}, k_{PSAT}, k_{PSE}, k and k' values.

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