Effect of a heparan sulphate with high affinity for antithrombin III upon inactivation of thrombin and coagulation Factor Xa

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The kinetics of inhibition of human α -thrombin and coagulation Factor Xa by antithrombin III were examined under pseudo-first-order reaction conditions as a function of the concentration of heparan sulphate with high affinity for antithrombin III. The maximum observed second-order rate constant was, for the antithrombin III–thrombin reaction, $1.2 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ compared with $2.4 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ in the presence of high-affinity heparin. However, the maximum rate was catalysed by much higher concentrations of heparan sulphate ($1.3 \mu\text{M}$) than of heparin ($0.025 \mu\text{M}$). Differences were also observed in the maximal acceleration of the antithrombin III–Factor Xa interaction: $1.2 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ at $0.2 \mu\text{M}$ -heparan sulphate compared with $2.2 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ at $0.04 \mu\text{M}$ -heparin. The differences in properties of heparan sulphate and heparin were analysed by using the random bi-reactant model of heparin action [Griffith (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5460–5464]. It was observed that the apparent binding affinity for thrombin was higher for heparan sulphate (180 nm) than for heparin (14 nm). The rate constant for transformation of the antithrombin III–Factor Xa complex into irreversible product differed between heparan sulphate (96 min^{-1}) and heparin (429 min^{-1}). These properties of the high-affinity heparan sulphate may be of importance in consideration of a putative role in the control of intravascular haemostasis.

INTRODUCTION

Antithrombin III, a serine-proteinase inhibitor, interacts with all of the proteinases of the intrinsic pathway of coagulation, more especially with thrombin and coagulation Factor Xa [1,2]. This property would appear to be of physiological importance, since hereditary deficiency of the protein is associated with recurrent thrombotic disease [3]. However, at plasma concentrations of the inhibitor (approx. $3 \mu\text{M}$) the rate of interaction with thrombin or Factor Xa is below that necessary to achieve physiological control of enzyme activity [4–7].

Heparin is a well-established antithrombotic drug, the mechanism of action of which is to accelerate, catalytically, the rate of inhibition of coagulation proteases by antithrombin III (up to four orders of magnitude higher than the basal rate) [8–10]. Heparin is located almost exclusively in mast cells and is not considered to be available for the control of coagulation. Instead, heparan sulphate molecules present on the vascular wall have been proposed to fulfil this function [11]. The heparin-like property that has been used to identify these catalytic heparan sulphate molecules is the ability to bind tightly to antithrombin III. Heparin itself contains 30–40% of molecules with this property and also with high anticoagulant potency [12–14]. Heparan sulphate molecules with this property have been isolated from aortic tissue [15] and from endothelial cell cultures [16], but no investigation by kinetic methods has hitherto been made of the properties of this class of molecules to accelerate antithrombin III–proteinase interactions. This is the subject of the present study.

MATERIALS AND METHODS

Materials

Human coagulation Factor Xa and antithrombin III were prepared as described previously [5,17]. Human α -thrombin was obtained from Dr. J. Fenton, Albany, NY, U.S.A., and had a specific activity of 2500 units/mg. Chromogenic substrate for thrombin (S2238, D-Phe-Pro-Arg-*p*-nitroanilide hydrochloride) and Factor Xa (S2765, *N*-benzyloxycarbonyl-D-Arg-Gly-Arg-*p*-nitroanilide) were obtained from KabiVitrum, Uxbridge, Middx., U.K.

Heparan sulphate fractionation

Heparan sulphate isolated from pig intestinal mucosa was obtained from Mediolanum Farmaceutici, Milan, Italy. The heparan sulphate was isolated by extraction with urea and NaCl and subsequent fractional precipitation with acetone. The purity of this preparation is given by the manufacturer as specific anticoagulant activity 5 units/mg (measured as the influence on activated partial thromboplastin time); it had a sulphate/carboxylate ratio 1.68 (measured by conductimetric titration [18]), and a single band was obtained upon cellulose acetate electrophoresis in the presence of barium acetate, pH 5.8 [19].

In order to separate heparan sulphate with high affinity for antithrombin III, the starting material (500 mg) was passed over a column (30 ml) of concanavalin A–Sephacrose (Sigma Chemical Co., Poole, Dorset, U.K.) to which antithrombin III (80 mg) had been bound previously and equilibrated with 0.05 M-NaCl/1 mM- CaCl_2 /1 mM- MnCl_2 /1 mM- MgCl_2 [20]. Unbound heparan sul-

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phate was removed by washing, before elution with a linear gradient generated by mixing the buffer (100 ml) with buffer containing 1.5 M-NaCl (100 ml). Fractions (3.5 ml) were collected at a flow rate of 42 ml/h and analysed for heparan sulphate by metachromatic assay with Azure A [12]. Pooled material was dialysed extensively against water before being freeze-dried. The high-affinity fraction was then subjected to chromatography on DE-52 DEAE-cellulose (Whatman): 1 mg was adsorbed on a 6 cm × 0.8 cm column of DE-52 DEAE-cellulose equilibrated with 50 mM-LiCl/50 mM-sodium acetate buffer, pH 4.0. After the column had been washed, elution was effected by a linear gradient generated by mixing the buffer (60 ml) with buffer containing 1.5 M-LiCl (60 ml) [21]. Fractions (3.5 ml) were collected at a flow rate of 9 ml/h and analysed for uronic acid by the method of Bitter & Muir [22]. After extensive dialysis against water, pooled material was concentrated by freeze-drying. High-affinity heparin (average M_r 16500) was prepared from commercial heparin (Leo Pharmaceuticals, Aylesbury, Bucks., U.K.) as described previously [17].

Binding affinity for antithrombin III

This was determined by observing the increase in intrinsic fluorescence of antithrombin III upon binding of heparin [23,24]. Antithrombin III dissolved in 0.1 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.4, was titrated with increasing concentrations of heparan sulphate or heparin (added as small portions). Separate mixtures were prepared at each concentration, and the fluorescence emission was measured in a Perkin-Elmer LS-5B luminescence spectrophotometer at 342 nm (excitation at 280 nm). The fluorescence increase was calculated as $(F - F_0)/F_0$, where F is the fluorescence intensity of the sample containing heparan sulphate or heparin and F_0 that of the control solution.

Binding constants were estimated from titration data by manual fitting of data to the equation [25]:

$$F = \frac{F_{\max}}{2A} = \{A + H + K - [(A + H + K)^2 - 4HA]^{1/2}\}$$

where A is the concentration of antithrombin III, H is the concentration of heparin or heparan sulphate and K represents the dissociation constant for binding of either to antithrombin III.

Measurement of M_r

This was carried out by size-exclusion chromatography as described previously [26] and with standard heparin fractions as described elsewhere [27]. Heparan sulphate was applied to a TSK-G-2000 SW column (HPLC Technology, Macclesfield, Cheshire, U.K.) in a 100 μ l fixed-volume loop. The column was eluted at 0.4 ml/min with 1.5 M-NaCl, pH 3.5, and peaks were detected at 232 nm. Retention times of peaks were recorded by a Spectraphysics SP 4290 integrator. The void and total volumes were measured with dilute solutions of Blue Dextran and *p*-aminobenzoic acid.

Kinetic measurements

Antithrombin III-thrombin interaction. This was carried out as described previously [28]. A mixture of inhibitor

and chromogenic substrate in buffer (for details see the legend to Fig. 3) was pre-warmed to 37 °C in plastic optical cuvettes in an Abbott bichromatic analyser (with 380/450 nm filter) before addition of enzyme at time zero. Under the conditions employed, namely [antithrombin III (ATIII)] $\gg$ [enzyme], the rate:

$$k_1 = k_2[\text{ATIII}] \quad (1)$$

k_2 being the second-order rate constant for interaction between the inhibitor and enzyme. The reaction is followed by inclusion of substrate, so there is competitive inhibition of the interaction according to the concentration of the substrate and its K_m with the enzyme, such that:

$$k_{1(\text{obs.})} = \frac{k_2[\text{ATIII}]}{1 + \frac{[S]}{K_m}} \quad (2)$$

First-order rate constants were calculated from the slope of V_1 against time, where:

$$V_1 = \frac{V}{V_0} \quad (3)$$

V is the rate of substrate hydrolysis at time t and V_0 is the rate of hydrolysis at time zero (measured in the absence of inhibitor).

Antithrombin III-Factor Xa interaction. This was measured also under pseudo-first-order conditions, but in the absence of chromogenic substrate. Factor Xa was mixed with antithrombin III in optical cuvettes in the presence of heparan sulphate (final volume 120 μ l; for details see the legend to Fig. 4). At timed intervals (5 and 10 s) after addition the reaction was quenched by addition of 70 μ l of buffer containing Polybrene (14 mg/ml) and substrate S2765 (final concentration 0.18 mM). Residual Factor Xa was measured in an Abbott bichromatic analyser and k_2 was calculated (eqn. 1).

Analysis of the properties of heparan sulphate according to the random bi-reactant model proposed by Griffith

According to this model the catalysis of an antithrombin III-proteinase reaction by heparin is considered to be analogous to a random-order bi-reactant enzyme-catalysed reaction [29,30]. The system is described by the following equilibria:

Proteinase (P) and antithrombin III (I) bind randomly to heparin (H). K_p and K_a are the heparin-proteinase and heparin-antithrombin III dissociation constants respectively and k is the apparent first-order rate constant for the rate-limiting step in product formation. K_c is the dissociation constant for heparin and the product of the reaction.

Assuming rapid equilibrium, the initial reaction velocity (v_i) is described by the following rate equation:

$$v_i = \frac{k \cdot [H] \cdot \frac{[I][P]}{K_a K_p}}{\left(1 + \frac{[I]}{K_a}\right) \cdot \left(1 + \frac{[P]}{K_p}\right)} \quad (5)$$

Values for K_a and k were determined by measuring the rate of interaction with various concentrations of antithrombin III in the presence of a fixed concentration of proteinase and a low concentration of heparan sulphate or heparin. Values for K_p and k were determined similarly but with various concentrations of proteinase in the presence of a fixed concentration of inhibitor (for details see legends to Figs. 5 and 6).

RESULTS

Fractionation of heparan sulphate

When chromatographed over antithrombin III immobilized upon concanavalin A-Sepharose, the bulk of the heparan sulphate was not bound to the column (Fig. 1a). Upon chromatography of a similar amount of heparin, three large pools corresponding to heparin with

no affinity, low affinity and high affinity were identified. The amount of heparin in each pool, as a percentage of the total, was 44 % with no affinity, 33 % with low affinity and 23 % with high affinity. Pools of similar fractions from chromatography of heparan sulphate were characterized according to the following percentages: 97.4 % with no affinity (fractions 10–20), 2.0 % with low affinity (fractions 40–50) and 0.6 % with high affinity (fractions 60–80). The high-affinity fraction was further purified by chromatography on DEAE-cellulose. It eluted as a peak at 0.8 M-LiCl as opposed to the rather shallow peak at 1.2 M-LiCl observed when heparin was chromatographed (Fig. 1b). The difference in elution properties between heparan sulphate and heparin is related to the higher degree of sulphation of heparin [31].

Average M_r values of the high-affinity and starting material were determined as 14000 with a range 3000–35000. The sulphate/carboxylate ratio of the high-affinity heparan sulphate was determined by the method of Wessler [32], with heparin, heparan sulphate and chondroitin sulphate A as references, and found to be similar to that of the starting material (1.68). Upon HNO_3 degradation both high-affinity heparan sulphate and starting material were degraded to low- M_r oligosaccharides as determined by polyacrylamide-gel

Fig. 1. Chromatographic properties of heparan sulphate and heparin

(a) Fractionation on antithrombin III bound to concanavalin A-Sepharose 4B: ■, NaCl gradient; ●, heparan sulphate; ▲, heparin. (b) Fractionation on DE-52 DEAE-cellulose: ■, LiCl gradient; ●, heparan sulphate with high affinity for antithrombin III; ▲, heparin. For details see the Materials and methods section.

Fig. 2. Titration of antithrombin III with heparan sulphate or heparin monitored by fluorescence

Heparan sulphate or heparin was added at increasing concentration to separate portions of antithrombin III (final concentration 125 nM) dissolved in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.1 M NaCl. Increase in fluorescence emission at 342 nm (excitation at 280 nm) is plotted against the ratio of mol of heparan sulphate or heparin per mol of antithrombin III. ●, Heparan sulphate; □, heparin.

electrophoresis [26]. The anticoagulant potency was determined in human plasma by influence on thrombin time and also upon the inhibition of clotting by Factor Xa as described previously [33]. As measured in comparison with a standard commercial heparin, the specific activity with respect to thrombin time was determined for the high-affinity and unfractionated heparan sulphate as 19 units/mg and 5 units/mg respectively, and to inhibition of Factor Xa as 75 units/mg and 15 units/mg respectively.

Binding affinity for antithrombin III

This was examined by the increase in the intrinsic fluorescence intensity of antithrombin III as described previously for heparin (Fig. 2). Compared with heparin, heparan sulphate caused somewhat less of an increase in fluorescence (50% and 39% respectively). The binding constants were determined for heparan sulphate and heparin as $7.7 \times 10^7 \text{ M}^{-1}$ and $5.2 \times 10^7 \text{ M}^{-1}$ respectively [24].

Antithrombin III–thrombin interaction

The influence of an increasing concentration of high-affinity heparan sulphate upon the rate of interaction between antithrombin III and thrombin is shown on a double-logarithmic plot of k_2 against concentration in Fig. 3. A progressive increase in rate constant was observed, reaching a maximum of $1.2 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ at $1.3 \mu\text{M}$. The profile differed from that of high-affinity heparin, which reached a peak of $2.4 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ at $0.025 \mu\text{M}$ and was much more potent at the lower concentrations tested than was heparan sulphate (nearly two orders of magnitude faster at 1.2 nM).

Antithrombin III–Factor Xa interaction

The influence of an increasing concentration of high-affinity heparan sulphate upon the rate of interaction between antithrombin III and Factor Xa is shown in Fig. 4. A maximum rate constant of $1.2 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ was observed at $0.2 \mu\text{M}$ -heparan sulphate. The ascending

Fig. 3. Effect of various concentrations of heparan sulphate and heparin on the interaction between antithrombin III and thrombin

The second-order rate constants for the inhibition of thrombin are plotted against the concentration of heparan sulphate or heparin on a double-logarithmic scale. The second-order rate constant was calculated from the rate of inhibition of 1 nM-thrombin by 10 nM-antithrombin III in 0.1 M-triethanolamine/HCl buffer, pH 7.8, containing 0.1 M-NaCl and 0.1% (w/v) poly(ethylene glycol) 6000 at 37 °C in the presence of chromogenic substrate S2238 at a concentration of $0.2 \mu\text{M}$. ●, Heparan sulphate; ■, heparin.

Fig. 4. Effect of various concentrations of heparan sulphate and heparin on the interaction between antithrombin III and Factor Xa

The second-order rate constants for the inhibition of Factor Xa are plotted against the concentration of heparan sulphate or heparin on a double-logarithmic scale. The second-order rate constant was calculated from the rate of inhibition of 1 nM-Factor Xa by 10 nM-antithrombin III in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl, 5 mM-CaCl₂ and 0.1% (w/v) poly(ethylene glycol) 6000 at 37 °C. ■, Heparan sulphate; ●, heparin.

part of the profile observed with an increasing concentration of high-affinity heparin ran parallel to that of heparan sulphate and reached a maximum of $2.2 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ at $0.04 \mu\text{M}$.

Fig. 5. Kinetic plot for the catalysis of the antithrombin III-thrombin interaction by heparan sulphate or heparin

The initial rate of thrombin inhibition by antithrombin III was determined in the presence of 1.0 nM-heparan sulphate (●) or 0.33 nM-heparin (■). (a) Initial antithrombin III concentration was 500 nM for heparan sulphate and 125 nM for heparin. (b) Initial thrombin concentration was 5 nM for heparan sulphate and for heparin. Antithrombin III and thrombin dissolved in 0.1 M-triethanolamine/HCl buffer, pH 7.8, containing 0.1 M-NaCl and 0.1 % (w/v) poly(ethylene glycol) 6000 were mixed in the presence of heparan sulphate or heparin (final volume of reaction mixtures 0.2 ml) in plastic optical cuvettes in an Abbott bichromatic analyser at 37 °C. At timed intervals the reaction was quenched by addition of 0.3 ml of buffer containing Polybrene (0.5 mg/ml) and chromogenic substrate S2238 (0.5 mM), and residual thrombin was then measured. The data are plotted as the ratio of substrate concentration $[S]$ to velocity (v) against substrate concentration [34].

Fig. 6. Kinetic plot for the catalysis of the antithrombin III-Factor Xa interaction by heparan sulphate or heparin

The initial rate of Factor Xa inhibition by antithrombin III was determined in the presence of 2.0 nM-heparan sulphate (●) or 1.0 nM-heparin (■). (a) Initial antithrombin III concentration was 330 nM. (b) Initial Factor Xa concentration was 10 nM. Antithrombin III and Factor Xa dissolved in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl, 5 mM-CaCl₂ and 0.1 % (w/v) poly(ethylene glycol) 6000 were mixed in the presence of heparan sulphate or heparin (final volume of reaction mixtures 0.03 ml) in plastic optical cuvettes in an Abbott bichromatic analyser at 37 °C. At timed intervals the reaction was quenched by addition of 0.17 ml of buffer containing Polybrene (0.5 mg/ml) and chromogenic substrate S2765 (0.4 mM), and residual Factor Xa was then measured. The data are plotted as the ratio of substrate concentration $[S]$ to velocity (v) against substrate concentration [34].

Analysis of properties according to the random bi-reactant model

When analysed by this model [30], the data obtained for the antithrombin III-thrombin (Fig. 5) and antithrombin III-Factor Xa (Fig. 6) interactions permitted calculation of values for K_a , K_p and k (Table 1). The values given for k are calculated from the maximum reaction velocity observed when the inhibitor concentration is kept constant as $V_{\max} = k[H]/(1 + K_a/[antithrombin\ III])$ and when the proteinase concentration is kept constant as $V_{\max} = k[H]/(1 + K_p/[proteinase])$ [29]. The values obtained under each set of conditions were for the antithrombin III-thrombin interaction in the presence of heparan sulphate 330 min⁻¹ and 312 min⁻¹ respectively, and in the presence of heparin 276 min⁻¹ and 300 min⁻¹ respectively. For the antithrombin III-Factor Xa interaction in the presence of heparan sulphate the values were 72 min⁻¹ and 120 min⁻¹ re-

Table 1. Kinetic parameters for the heparin- or heparan sulphate-catalysed antithrombin III-thrombin and antithrombin III-Factor Xa interactions

	K_a (nM)	K_p (nM)	k (min ⁻¹)
Thrombin			
Heparin	90	14	288
Heparan sulphate	100	180	321
Factor Xa			
Heparin	25	220	429
Heparan sulphate	25	240	96

spectively, and in the presence of heparin 480 min⁻¹ and 378 min⁻¹ respectively. In Table 1 the value for k is given as the average of the two estimates in each instance.

DISCUSSION

Heparan sulphate and heparin are chemically related and belong to the sulphated glycosaminoglycans, characterized as alternating co-polymers of hexosamine (in this case glucosamine) and alduronic acid (glucuronic acid or iduronic acid) [35]. They differ from each other, principally, in iduronic acid content and in the degree of sulphation [31]. A percentage (30–40%) of heparin molecules has a potent anticoagulant property, which is mediated by a specific pentasaccharide sequence containing as a unique component a 3-*O*-sulphated glucosamine residue [36,37]. Heparin molecules possessing this structure bind with high affinity to antithrombin III and catalyse the reaction between the inhibitor and serine proteinases of the coagulation cascade. The binding causes conformational change in the antithrombin III molecule, observed as changes in intrinsic fluorescence [23,24] and u.v.-absorption spectra [38].

In mammals heparin is localized almost exclusively to the mast cells, where it is probably involved in the inflammatory response [39,40]. On the vascular endothelium a variety of sulphated glycosaminoglycans is present as proteoglycans, forming a glycocalyx lining the vessel lumen. On isolation of heparan sulphate from arterial tissue, Marcum & Rosenberg [15] observed that a small but finite percentage of the molecules had the property of binding to antithrombin III and, moreover, comprised most of the heparin-like activity of the vascular material. It was proposed, therefore, that these latter molecules bound antithrombin III at the endothelial cell surface [41] and acted to control coagulation [11]. Although present at quite low concentrations relative to the bulk of the glycosaminoglycans (<0.3%), this high-affinity material (and possessing the 3-*O*-sulphated glucosamine residue) has since been isolated from human endothelial cell cultures [16], human aorta [21], tumour cell cultures [42,43] and pig intestinal mucosa [21].

The anticoagulant potency of high-affinity heparan sulphate has been investigated previously by clotting and heparin assay methods and found to be considerably less than that of heparin [21,44,45]. The potency is dependent on the sulphate content [44] and M_r , and, since there is considerable heterogeneity in these respects between heparan sulphate derived from different sources, our findings must be considered in relationship to the properties of the preparation that we have studied, i.e. in the middle range of degree of sulphation and M_r [46]. The potency of the starting material and high-affinity fraction, as measured in plasma, were found to be similar to those reported previously at this degree of sulphation [44].

In the present study we have investigated the ability of high-affinity heparan sulphate to influence the kinetic rate of the interaction between the inhibitor and thrombin or Factor Xa. In general, it was observed that this ability was somewhat less than that of heparin. At lower concentrations (approx. 1 nM) of heparin or heparan sulphate this was particularly marked in the antithrombin III–thrombin interaction, for which the second-order rate constant was nearly two orders of magnitude lower for heparan sulphate than for heparin. Furthermore, heparin caused a maximal increase in rate that was higher than that seen with heparan sulphate and at a lower concentration (Fig. 3). In the potentiation of the antithrombin III–Factor Xa interaction less difference was observed between heparan sulphate and heparin.

However, a 5-fold difference in rate was observed and maximum acceleration occurred at higher concentrations of heparan sulphate (0.2 μ M) than of heparin (0.04 μ M).

The reasons for the different properties of heparin and heparan sulphate were examined by using the random bi-reactant molecular model for heparin action. The principal mechanisms by which heparin influences the rate of interaction between antithrombin III and thrombin has been shown to be to increase the affinity of the initial (reversible) complex between inhibitor and proteinase [47]. According to this mechanism, heparin acts as a template binding both inhibitor and proteinase. When heparin is present at limiting concentration the kinetics of interaction between antithrombin III and thrombin or Factor Xa is described by an equation similar to that characterizing an enzyme with two substrates, the heparin being equivalent to the enzyme [17,29,30,48–52]. This approach has proved useful in quantifying the key features that are important in the catalysis by heparin. For example, we have shown previously that the inhibition of the heparin-catalysed antithrombin III–thrombin interaction by pentosan polysulphate is due to an 18-fold increase in apparent K_p for thrombin [17]. It was, moreover, possible to use the data to predict the experimentally observed degree of inhibition. By measurement of these parameters in this present study we conclude that the lower activity of heparan sulphate (relative to heparin) in potentiating the antithrombin III–thrombin interaction is due to an apparent 13-fold weaker binding of thrombin (heparan sulphate K_p 180 nM, heparin K_p 14 nM).

Various values have been reported for the binding affinity of thrombin to heparin, as determined by the acceleration of the antithrombin III–thrombin interaction at physiological ionic strength, from less than 2 nM [51] up to 2 μ M [49]. Measurement of the binding of heparin to thrombin directly gives values of 10^{-7} – 10^{-6} M [53–55]. The disparity between the values obtained kinetically has been considered recently as arising from experimental conditions in which the increased affinity of thrombin for the antithrombin III–heparin complex is measured also [56]. Our experiments observe an affinity of thrombin for heparin at the higher level reported by Griffith [30] and Pletcher & Nelsestuen [51], and the affinity of thrombin for heparan sulphate that we have determined must be evaluated with regard to the above considerations. However, our findings do show a difference between the apparent affinities for thrombin of heparan sulphate and heparin measured under similar conditions, and this predicts the observed dissimilarity in the activity–concentration profile of each (Fig. 3). Moreover, it is notable that the apparent affinity (180 nM) is similar to that observed for unfractionated heparan sulphate (300 nM) [57] with the use of another experimental approach [17,56].

In the case of the antithrombin III–Factor Xa interaction the difference between heparan sulphate and heparin was observed as being due to a lower rate (k) for the formation of product (heparan sulphate 96 min⁻¹, heparin 429 min⁻¹). From the mechanistic argument the values for k should be similar (as they were for the antithrombin III–thrombin interaction), since heparin/heparan sulphate is presumed to be affecting only the binding of antithrombin III to Factor Xa. However, differences have been reported for values of k from one heparin preparation to another by other workers

[29,30,49]. It may be that the differences observed here are related to additional antithrombin III-binding properties of heparin not found in heparan sulphate and that influence this step [58,59]. We have previously reported that the maximal accelerations of the antithrombin III–Factor Xa reaction by heparin of various M_r values differ by 35-fold [60]. These previous observations are not entirely explicable by differences in binding of Factor Xa, and may again partly arise from an influence on this step, as we propose here.

The values for these parameters (Table 1) were substituted into the appropriate equation [see eqn. (5) in the Materials and methods section] and used to calculate the rate of interaction that should be observed under the conditions of experiment in Figs. 3 and 4 and at a selected single concentration of heparin. With regard to the antithrombin III–thrombin interaction in the presence of 2 nM-heparin we calculate a second-order rate constant of $3.8 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ and observed $9.0 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$. In the presence of 2 nM-heparan sulphate we calculate a rate of $3.0 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ and observed $2.6 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$. For the antithrombin III–Factor Xa interaction in the presence of 2 nM-heparin we predict a rate of $1.2 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ and observed a rate of $3.0 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$. In the presence of 2 nM-heparan sulphate we predict $2.4 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ and observe $6.0 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$. The values for K_p , K_a and k that we have calculated for heparan sulphate and heparin can therefore be used to predict the rate of interaction catalysed by each and the values calculated correspond reasonably well to those observed experimentally.

In conclusion, although the tightness of binding of antithrombin III to high-affinity heparan sulphate is similar to that of heparin, it exhibits lower potency as a catalyst of antithrombin III–proteinase interactions than heparin of an equivalent M_r (heparin 15000, heparan sulphate 14000), and its properties in this respect are more akin to those of low- M_r heparin [21,27,60–62]. In accelerating the antithrombin III–thrombin interaction, the lower potency is attributable to differences in binding of thrombin rather than antithrombin III (as is the case for low- M_r heparin). The binding affinity for thrombin is similar to that observed with low-affinity heparan sulphate [57] and suggests that there would be competition for thrombin between these two forms within the vasculature. The lower potency with respect to the antithrombin III–Factor Xa interaction arises from difference in the rate constant, k , and not in the binding of antithrombin III or Factor Xa [60].

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