Effect of a pentosan polysulphate upon thrombin and factor Xa inactivation by antithrombin III

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The kinetics of inhibition of human and bovine α -thrombin and human factor Xa by antithrombin III were examined under pseudo-first-order conditions as a function of the concentration of pentosan polysulphate [a fully sulphated (β 1-4)-linked D-xylopyranose with a single laterally positioned 4-O-methyl- α -D-glucuronic acid]. Double-reciprocal plots of the observed first-order rate constant against concentration of pentosan polysulphate gave straight lines, intercepts on the axes giving values for maximum increase in second-order rate constant (by calculation) and apparent dissociation constant. These values were: for human α -thrombin $1.52 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $3.6 \mu\text{M}$ respectively, for bovine α -thrombin $6.56 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $0.16 \mu\text{M}$ and for factor Xa $6.86 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $20 \mu\text{M}$. In the presence of pentosan polysulphate the dissociation constant for the initial complex of antithrombin III and thrombin was shown to be reduced from approx. $2 \times 10^{-3} \text{ M}$ to $61 \times 10^{-6} \text{ M}$ without apparent change in the limiting rate constant of 750 min^{-1} . An oligosaccharide (primarily 8–10 saccharide units) prepared from heparin and with high affinity for antithrombin III but low potency in the thrombin–antithrombin III interaction did not diminish the rate of interaction catalysed by pentosan polysulphate. The catalysis was shown to be due to a weak electrostatic interaction, since it was completely reversed by concentrations of NaCl greater than 0.3 M . It is concluded that the mechanism is independent of the heparin high-affinity binding site on antithrombin III and is probably due to binding of the high-charge-density polysaccharide to the proteinase. It is calculated that the acceleration in rate achieved, although lower than that of heparin, approaches that required to be of physiological significance and may be of importance in the anticoagulation role of antithrombin III at sites of high charge density which may occur *in vivo*.

Heparin functions as an anticoagulant primarily by accelerating the velocity at which antithrombin III neutralizes serine proteinases of the haemostatic mechanism (Damus *et al.*, 1973). A number of phenomena are important in this catalysis, but a unique feature is an alteration in the conformation of the antithrombin III upon binding to a specific domain on the heparin molecule, which increases the reactivity of the inhibitor as a pseudosubstrate (Lindahl *et al.*, 1980, 1982; Casu *et al.*, 1981; Bjork & Lindahl, 1982).

A number of other sulphated polysaccharides

have been identified as having the property of prolonging the clotting time of whole blood or plasma, and these have been grouped under the heading of heparinoids (Pulver, 1961). One of such is a pentosan polysulphate prepared by sulphation of carbohydrate from the bark of beech trees, *Fagus sylvatica*. The structure of the parent polysaccharide has been elucidated as chains of (β 1-4)-linked D-xylopyranose with laterally positioned 4-O-methyl- α -D-glucuronic acid at C-2 of xylose (Aspinall, 1959). All available hydroxy groups are occupied upon sulphation (Raveux *et al.*, 1966). The molecular mass has been estimated by ultracentrifugation to be 3000 Da with a narrow distribution (no portion > 5000 Da or < 1500 Da).

The properties of the pentosan polysulphate as

Abbreviation used: SDS, sodium dodecyl sulphate.

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an anticoagulant show it to be of low potency as compared with heparin (Soria *et al.*, 1980), about 10 times less effective than heparin on a weight-to-weight basis (Scully *et al.*, 1983). The mechanism of its anticoagulant effect was shown to be partly independent of antithrombin III (Soria *et al.*, 1980), but a number of workers have shown it to accelerate the rate of interaction between antithrombin III and thrombin, and between antithrombin III and coagulation factor Xa (Czapek *et al.*, 1980; Fischer *et al.*, 1982).

In this present paper we have investigated this property and have obtained evidence which, together with our previous findings (Scully & Kakkar, 1984), suggests that the pentosan polysulphate brings about this catalysis by a mechanism which does not involve binding to the high-affinity site on antithrombin III, and may be due to binding to the proteinase.

Materials and methods

Bovine α -thrombin, human antithrombin III and human factor Xa were prepared and characterized as previously described (Scully & Kakkar, 1984; Ellis *et al.*, 1982). Human α -thrombin was provided generously by Dr. J. Fenton, New York State Department of Health, U.S.A. Pentosan polysulphate was obtained from Clin Midy, Paris, France, as a solution of 50 mg/ml. An oligosaccharide fraction prepared from heparin was provided generously by Dr. D. A. Lane, Charing Cross Medical School, London, U.K. (Denton *et al.*, 1983). Chondroitin sulphates A, B and C, and ovalbumin, were obtained from Sigma. Triethanolamine and poly(ethylene glycol) 6000 were obtained from BDH.

H-D-Phe-Pip-Arg-*p*-nitroanilide 2HCl (S2238), and Bz-Ile-Glu(OR)-Gly-Arg-*p*-nitroanilide [R=H (50%) or R=CH₃ (50%)] (S2222) were obtained from Flow Laboratories.

Kinetics of the reaction between thrombin or factor Xa in the presence of pentosan polysulphate

This was carried out by mixing the enzyme into a solution containing both inhibitor and substrate as described previously by Griffith (1982a). The disappearance of enzyme from a mixture of inhibitor and enzyme in conditions in which the concentration of antithrombin $\gg$ enzyme follows first order kinetics such that:

$$k' = k'' [\text{antithrombin III}]$$

k'' being the second-order rate constant for interaction between antithrombin III and enzyme. In the presence of substrate there is competitive inhibition of the interaction according to the

concentration of substrate and its K_m with the enzyme such that:

$$k'_{\text{obs.}} = \frac{k'' [\text{antithrombin III}]}{1 + [S]/K_m}$$

First-order rate constants were calculated from semilogarithmic plots of V_1 against time where:

$$V_1 = \frac{V}{V_0}$$

V being the rate of substrate hydrolysis at time t and V_0 the rate of hydrolysis at time zero (measured in the absence of antithrombin III). The linear inter-relationship between $1/k'_{\text{obs.}}$ and the substrate concentration has been shown previously (Griffith, 1982a; Petersen & Jorgensen, 1983).

In practice 200 μ l of antithrombin III/substrate mixture was incubated in plastic cuvettes at 37°C for 2 min. An aliquot of enzyme (50 μ l, also pre-warmed) was added at $t=0$. All reactions, unless stated, were carried out in 0.1M-triethanolamine/HCl (pH 7.8)/0.15M-NaCl containing 0.1% poly(ethylene glycol) 6000 (Griffith *et al.*, 1980) and the change in absorbance was followed at timed intervals for up to 120s. These determinations were made in an Abbott Biochromatic Analyser 100 with a 380/450 nm filter. Determinations were performed in triplicate.

The K_m for the various enzymes and substrates was measured under the conditions of assay from Lineweaver-Burk plots and was determined to be the following: S2238 and human α -thrombin, K_m 6 μ M; S2238 and bovine α -thrombin, 4 μ M; S2222 and human factor Xa, 0.8 mM. Pentosan polysulphate at a concentration of 20 μ M had no significant effect on the K_m for these substrates under the conditions used throughout the work.

Results

Effect of pentosan polysulphate on antithrombin III/proteinase interaction

In Fig. 1 is shown the increase in rate of interaction between antithrombin III and human α -thrombin according to the concentration of pentosan polysulphate. Linear semilogarithmic plots were obtained and $k'_{\text{obs.}}$ calculated; a plot of $1/k'_{\text{obs.}}$ against $1/[\text{pentosan polysulphate}]$ was linear (Fig. 2a), from which a maximum $k'_{\text{obs.}}$ of 5.5 min^{-1} under these conditions was calculated and an apparent dissociation constant for pentosan polysulphate of 3.6 μ M. In Fig. 3 is shown the effect of the pentosan polysulphate upon the reaction rate as observed on SDS/polyacrylamide gels. As high concentrations of inhibitor and enzyme were necessary for visualization on the gels the reaction

Fig. 1. Effect of increasing concentrations of pentosan polysulphate on the interaction between antithrombin III and human α -thrombin

Thrombin and antithrombin III at final concentrations of 10 nM and 10 μ M respectively were mixed with substrate S2238 (final concentration 0.16 mM) and increasing concentrations of pentosan polysulphate $\circ$, 0; Δ , 0.312 μ M; $\times$, 0.625 μ M; $\bullet$, 1.25 μ M; $\square$, 2.5 μ M; $\circ$, 5 μ M; $\blacktriangle$, 10 and 20 μ M). The rate of nitroaniline release, expressed as V_1 (see the Materials and methods section) is plotted against time of incubation.

was carried out at 25°C and in the presence of substrate. The results show clearly the acceleration of the formation of the antithrombin III–thrombin complex in the presence of pentosan polysulphate (gels a, b and c) as compared with in its absence (gels d, e and f).

The effect of pentosan polysulphate on the antithrombin III–factor Xa interaction was also determined and k'_{obs} was calculated. A plot of $1/k'_{\text{obs}}$ versus $1/[\text{pentosan polysulphate}]$ (Fig. 2b) was linear, intercepts on the ordinate abscissa giving values for maximum k'_{obs} of 2.85 min^{-1} and K_d of 20 μ M. In Fig. 2(c) is shown the effect of the heparinoid on the interaction between bovine α -thrombin and antithrombin III. Maximum k'_{obs} of 1.6 min^{-1} and K_d of 0.16 μ M were calculated.

Fig. 2. Effect of pentosan polysulphate on the reaction between antithrombin III and proteinases

The reciprocal of k'_{obs} is plotted as a function of the reciprocal of the concentration of pentosan polysulphate for enhancement of the rate of interaction between antithrombin III and (a) human α -thrombin, (b) human factor Xa and (c) bovine α -thrombin. The final concentration of other reactants was: in (a) antithrombin III 10 μ M, thrombin 10 nM, S2238 0.16 μ M; in (b) antithrombin III 0.5 μ M, factor Xa 43 nM, S2222 0.26 μ M; and in (c) antithrombin III 10 μ M, thrombin 2 nM, S2238 0.16 mM.

Effect of pentosan polysulphate on the dissociation constant of the antithrombin III–thrombin complex

The rate of reaction between antithrombin III and human α -thrombin was measured in the presence of a constant concentration of pentosan polysulphate but varying the concentration of antithrombin III. When $1/k'_{\text{obs}}$ is plotted against $1/[\text{antithrombin III}]$ according to the method of Kitz & Wilson (1962), a maximum k'_{obs} of

Fig. 3. Effect of pentosan polysulphate on the rate of interaction between antithrombin III and human thrombin determined by SDS/polyacrylamide-gel electrophoresis

Human thrombin and antithrombin III at final concentrations of 7 and 14 μM respectively were mixed in the presence of S2238 (final concentration 0.16 mM) with pentosan polysulphate (final concentration 10 μM) at 25°C. Samples (50 μl) were removed at 5, 10 and 20 s and added to 50 μl of 0.1 M-Tris/phosphate (pH 7.0) containing 0.1% SDS at 100°C. After 2 min heating the samples were cooled to room temperature and run on 7% SDS/polyacrylamide gels according to the method of Weber & Osborn (1969). Gels (a), (b) and (c) show the changes in electrophoretic mobility in the reaction mixture containing pentosan polysulphate and gels (d), (e) and (f) that in its absence. Gels (a) and (d), 5 s sampling; (b) and (e), 10 s sampling; (c) and (f), 20 s sampling. Abbreviations used: T, thrombin; A, antithrombin III; C, antithrombin III- α -thrombin complex.

28.5 min^{-1} and K_d of 66 μM were found (Fig. 4b). In the absence of pentosan polysulphate and at concentrations of 100–500 μM -antithrombin III, the inverse plot of rate constant against inhibitor extrapolated through the origin indicating a K_d of >1 mM (Fig. 4a).

Effect of antithrombin III-binding oligosaccharide derived from heparin upon the acceleration of the antithrombin III-thrombin interaction by pentosan polysulphate

An oligosaccharide containing 8–10 monosaccharide units obtained by HNO_2 depolymerization of heparin, gel chromatography and separation of molecules with high affinity for antithrombin III has been shown previously to have the property of exhibiting very low potency in the antithrombin III-thrombin interaction but high potency in the antithrombin III-factor Xa interaction (Thunberg *et al.*, 1980; Oosta *et al.*, 1981; Denton *et al.*, 1983). Values for specific activity have been found of 800 units/mg and 1600 units/ μmol measured by factor Xa neutralization and 10 units/mg and 0 units/ μmol by thrombin neutralization (Denton *et al.*, 1983; Lane *et al.*,

1984). Its effect on the antithrombin III-thrombin interaction is shown in Fig. 5; a 3-fold increase in rate is observed, a plateau occurring at concentrations above 0.6 μM (binding characteristics similar to those previously reported for octa- and decasaccharides; Oosta *et al.*, 1981). When oligosaccharide was added, at this latter, near-saturating, concentration to a pentosan polysulphate/antithrombin III/thrombin mixture it caused no reduction in the observed rate (Table 1).

Effect of increasing ionic strength upon the pentosan polysulphate-induced acceleration of the thrombin-antithrombin III interaction

With increasing ionic strength an increase in k'_{obs} was observed, reaching a plateau at concentrations of NaCl between 0.0375 and 0.11 mM. At concentrations of NaCl beyond this the potentiation was reduced and the reaction rate was the same as the antithrombin III-thrombin control at 0.3 M-NaCl (Fig. 6). At all concentrations examined, NaCl had no significant effect on the interaction of antithrombin III with thrombin when alone, as others have shown previously (Griffith *et al.*, 1980).

Fig. 4. Effect of concentration of antithrombin III on the rate of interaction with human α -thrombin in the absence and presence of pentosan polysulphate

The reciprocal of k_{obs} is plotted as a function of the reciprocal of the concentration of antithrombin III in the absence (a) and presence (b) of $20 \mu M$ -pentosan polysulphate (Kitz & Wilson, 1962). The final concentration of other reactants was: thrombin $10 nM$ and S2238 $0.16 mM$ in (a) and (b) together with pentosan polysulphate ($20 \mu M$) in (b).

Fig. 5. Effect of increasing concentrations of an antithrombin III-binding oligosaccharide derived from heparin (primarily 8–10 saccharide units) on the interaction between antithrombin III and human α -thrombin

Thrombin and antithrombin III at final concentrations of $5 nM$ and $10 \mu M$ respectively were mixed with S2238 (final concentration $0.16 mM$) in the presence of increasing concentrations of oligosaccharide. The rate of nitroaniline release, expressed as V_1 (see the Materials and methods section) was plotted against time. The k_{obs} was calculated from the slope of these plots.

Table 1. Effect of an antithrombin III binding oligosaccharide (primarily 8–10 saccharide units) derived from heparin on the rate of interaction between antithrombin III and human α -thrombin in the presence of pentosan polysulphate

Thrombin and antithrombin III at final concentrations of $5 nM$ and $10 \mu M$ respectively were mixed with S2238 (final concentration $0.16 mM$) in the presence of pentosan polysulphate alone (final concentration $1.6 \mu M$), heparin oligosaccharide alone (final concentration $0.625 \mu M$) or pentosan polysulphate and heparin oligosaccharide together and the k'_{obs} was calculated from plots measuring the release of nitroaniline (measured as V_1 ; see the Materials and methods section).

Mixture inclusion	k'_{obs} (min^{-1})
Oligosaccharide ($0.625 \mu M$)	0.37
Pentosan polysulphate ($1.6 \mu M$)	1.6
Pentosan polysulphate ($1.6 \mu M$) + oligosaccharide ($0.625 \mu M$)	1.6

Fig. 6. Effect of increasing concentrations of NaCl on the rate of interaction between antithrombin III and human α -thrombin in the presence of pentosan polysulphate

Thrombin and antithrombin III at final concentrations of $5 nM$ and $10 \mu M$ respectively were mixed with S2238 (final concentration $0.16 mM$) in the presence of pentosan polysulphate (final concentration $1.6 \mu M$). The reaction was carried out in $0.1 M$ -triethanolamine/HCl (pH 7.8) containing 0.1% poly(ethylene glycol) 6000 increasing concentrations of NaCl. The rate of nitroaniline release expressed as V_1 (see the Materials and methods section) was plotted against time and k_{obs} was calculated from these plots.

Discussion

The results presented here show clearly a potentiation of the rate of interaction between antithrombin III and human and bovine α -thrombin and human factor Xa (Table 2). The mechanism of inhibition of proteinases (P) by anti-

Table 2. Effect of pentosan polysulphate on the interaction between antithrombin III and various proteinases

Proteinase	$k''(\text{M}^{-1} \cdot \text{min}^{-1})$			K_d (μM)
	– Pentosan polysulphate	+ Pentosan polysulphate	Increase (fold)	
Human α -thrombin	$3.78 \times 10^5*$	1.52×10^7 ($\pm 0.114 \times 10^7$)	40	3.6
Human factor Xa	$2.64 \times 10^4\dagger$	8.66×10^6 ($\pm 1.14 \times 10^6$)	260	20
Bovine α -thrombin	6.24×10^5	65.6×10^5 ($\pm 8.26 \times 10^5$)	10	0.16

* From Downing *et al.* (1978).† From Ellis *et al.* (1983).

thrombin III (AT) may be considered as a two-step reaction scheme analogous to that of other serine proteinase inhibitors.

The reaction is assumed to involve an initial binding interaction or Michaelis complex, P-AT, before irreversible formation of the stable P-AT* complex. Heparin has been shown previously to increase the affinity of the Michaelis complex with little or no effect on the irreversible step (Olson & Shore, 1982). In this present study we have demonstrated that a 1:1 molar complex between antithrombin III and thrombin is formed (Fig. 3) and that the effect of pentosan polysulphate is to increase the affinity of the Michaelis complex. The rate constant of 750 min^{-1} in the presence of pentosan polysulphate can be compared with that found by Griffith (1982b) of 785 min^{-1} in the presence of heparin. Assuming the limiting rate constant to be the same in the absence of heparin, a dissociation constant of approx. $2 \times 10^{-3} \text{ M}$ can be calculated (Fig. 4a) corresponding to an increased affinity of 30-fold in the presence of pentosan polysulphate.

To provide a preliminary explanation of the mechanism by which the pentosan polysulphate may be causing an increase in affinity of the antithrombin III-proteinase complex we have considered mechanisms by which heparin is thought to exert its effect on the inhibition of coagulation proteinases. There are three mechanisms for which evidence has been provided over the past years. Firstly, binding of heparin to antithrombin III causes a conformational change increasing its receptivity to interaction (Rosenberg & Damus, 1973; Li *et al.*, 1976; Villanueva & Danishefsky, 1977; Nordenman *et al.*, 1978); secondly, binding of heparin to the proteinase increases its receptivity (Machovich, 1975; Machovich *et al.*, 1975; Smith, 1977; Hatton & Regoeczi, 1977) and thirdly, binding to antithrombin III and proteinase

(in the case of thrombin and factor IXa) causes increased reactivity (Laurent *et al.*, 1978; Pomerantz & Owen, 1978; Machovich & Aranyi, 1978; Jordan *et al.*, 1980b; Holmer *et al.*, 1981; Griffith, 1982b).

From the evidence we consider that the second mechanism may explain the potentiation caused by the pentosan polysulphate. Firstly, we have shown previously that pentosan polysulphate has no effect on the binding of heparin to antithrombin III but can interfere with the binding of heparin to thrombin (Scully & Kakkar, 1984). Data from others show that pentosan polysulphate binds to thrombin but not to antithrombin III [indicated by alterations in electrophoretic mobility (Fischer *et al.*, 1982)]. Secondly, a 100-fold variation in apparent dissociation constants is observed according to the proteinase involved (K_d for factor Xa $20 \mu\text{M}$, for bovine thrombin $0.16 \mu\text{M}$), suggesting that the effect is related to the binding to the enzyme rather than to a common site on the antithrombin III. Thirdly, preliminary binding to the high-affinity site of an oligosaccharide derived from heparin [prepared by elution from antithrombin III-Sepharose at 0.9 M-NaCl (Denton *et al.*, 1983)] has no significant effect on the acceleration of rate by pentosan polysulphate (Table 1).

When the potentiation is measured in the presence of increasing concentrations of NaCl a decrease is observed at greater than 0.11 M . This shows the potentiation to arise from a weak electrostatic binding as has been shown to be the property of heparin binding to thrombin (Petersen & Jorgensen, 1983). Although heparin binding to thrombin is necessary for the acceleration of the thrombin-antithrombin III reaction [K_d for heparin-thrombin $8 \times 10^{-7} \text{ M}$ (Jordan *et al.*, 1980a,b) or $3.5 \times 10^{-8} \text{ M}$ (Griffith, 1982c)] this does not appear to be the case for the antithrombin III-factor Xa reaction [K_d for heparin-factor Xa $8.73 \times 10^{-6} \text{ M}$ (Jordan *et al.*, 1980a,b)]. It is interesting to note that the potentiation of the factor Xa-antithrombin III interaction by pentosan poly-

sulphate does occur, but at higher concentrations than are required for thrombin. It appears therefore that potentiation of the antithrombin III-proteinase reaction by pentosan polysulphate may be due to binding to the proteinase, although increased reactivity due to binding at a site on the antithrombin III remote from the heparin binding site cannot, as yet, be excluded. When tested at 18 µg/ml neither chondroitin sulphate A nor B nor C were observed to accelerate the antithrombin III-thrombin interaction (experimental conditions as in Table 1). This property of pentosan polysulphate may be related to its high charge density, (approx. 4 sulphate groups/disaccharide unit versus 2.5 for heparin and 1.0 for chondroitin sulphate). Properties of acceleration of interaction by heparin which are independent of the antithrombin III-binding site but dependent upon charge density have recently been described for heparin cofactor II (Hurst *et al.*, 1983). In that paper it was shown, also, that although the catalytic effect of heparin on the antithrombin III-thrombin interaction was primarily dependent on the antithrombin III binding sequence, increase in charge density also increased potency.

Although the acceleration by pentosan polysulphate is much less than that of heparin [antithrombin III-thrombin interaction: heparin, 7000-fold calculated from Griffith (1982*b*), pentosan polysulphate, 40-fold] the lower rates of acceleration may be significant *in vivo*. In a recent review Travis & Salvesen (1983) state that to be effective physiologically the rates of interaction of inhibitor and target enzyme in plasma should be such as to achieve a t_1 for enzyme activity of less than 100 ms. As example, the t_1 of plasma due to interaction with fast α -antiplasmin in plasma at 25°C is 29 ms (Wiman & Collen, 1978). By using the maximum second-order rate constant obtained herein, t_1 in plasma (ignoring contribution by other plasma proteinase inhibitors) may be calculated from the formula of Bieth (1980):

$$t_1 \simeq \frac{1}{k'' [\text{antithrombin III}]}$$

Assuming a plasma concentration of antithrombin III of 4.4 µM (Kueppers & Black, 1974) a value is obtained from thrombin of 909 ms and for factor Xa of 1980 ms. The possibility that antithrombin III may function as an inhibitor, physiologically, by a mechanism dependent upon binding of the proteinases to high-charge-density sites is in need of investigation.

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