Anti-thrombin activities of heparin

Effect of saccharide chain length on thrombin inhibition by heparin cofactor II and by antithrombin

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The interactions of two proteinase inhibitors, heparin cofactor II and antithrombin, with thrombin are potentiated by heparin. Using two methods, we have studied the potentiating effects of a series of heparin (poly)saccharides with high affinity for antithrombin and mean M_r ranging from approx. 1700 to 18800. First, catalytic amounts of heparin (poly)saccharide were added to purified systems containing thrombin and either heparin cofactor II or antithrombin. Residual thrombin activity was determined with a chromogenic substrate. It was found that only the higher- M_r polysaccharides (M_r greater than 8000) efficiently catalysed thrombin inhibition by heparin cofactor II, there being a progressive catalytic effect with increasing M_r of the polysaccharide. Weak accelerating effects were noted with low- M_r saccharides (M_r less than 8000). This contrasted with the well-characterized interaction of heparin with antithrombin and thrombin, where heparin oligosaccharides of M_r less than 5400 had absolutely no ability to accelerate the reaction, while (poly)saccharides of M_r exceeding 5400 showed rapidly increasing catalytic activity with increasing M_r . Secondly, these and other heparin preparations were added in a wide concentration range to plasma with which ^{125}I -labelled thrombin was then incubated for 30 s. Inhibited thrombin was determined from the distribution of labelled thrombin amongst inhibitor–thrombin complexes, predominantly antithrombin–thrombin and heparin cofactor II–thrombin complexes. In this situation, where the inhibitors competed for thrombin and for the (poly)saccharides, it was found that, provided the latter were of high affinity for antithrombin and exceeded a M_r of 5400, thrombin inhibition in plasma was mediated largely through antithrombin. Polysaccharides of M_r exceeding 8000 that were of low affinity for antithrombin accelerated thrombin inhibition in plasma through their interaction with heparin cofactor II. High concentrations of saccharides of M_r 1700–5400 exhibited a size-dependent acceleration of thrombin inhibition, not through their interaction with antithrombin, but through their interaction with heparin cofactor II.

INTRODUCTION

Heparin is a structurally heterogeneous glycosaminoglycan whose chains are comprised of alternating 1 → 4 linked and variously sulphated residues of uronic acid (L-iduronic and D-glucuronic acids) and glucosamine. Heparin acts as an anticoagulant primarily by accelerating the inhibitory action of the proteinase inhibitor antithrombin against proteinases of the blood coagulation system (Damus *et al.*, 1973; Bjork & Lindahl, 1982). It has been claimed that all of the proteinases generated in the intrinsic pathway of coagulation can be inhibited by antithrombin and heparin (Damus *et al.*, 1973). The mechanism of such inhibition may vary. Thus, Jordan *et al.* (1980) and Holmer *et al.* (1981) identified two classes of proteinase that could be distinguished on the basis of the size dependency of their heparin-accelerated inhibition. Inhibition of Factor Xa, Factor XIIa and kallikrein by antithrombin could be catalysed by heparin regardless of the polysaccharide size, while the inhibition of thrombin, Factor IXa and Factor XIa by antithrombin was size-dependent and could only be appreciably accelerated by heparin of relatively high M_r . These

observations can be explained in terms of a model of heparin containing different functional domains. A unique pentasaccharide sequence of heparin is required for its binding to antithrombin (Lindahl *et al.*, 1980; Casu *et al.*, 1981; Thunberg *et al.*, 1982; Choay *et al.*, 1983; Atha *et al.*, 1984, 1985). Heparin oligosaccharide fragments containing the pentasaccharide are apparently able to accelerate inhibition of Factor Xa-like enzymes. However, this unique pentasaccharide sequence alone is unable to catalyse inhibition of thrombin (and related enzymes) by antithrombin. Rather, it appears that additional, less strictly defined, sequences are required to accommodate both antithrombin and thrombin simultaneously on the polysaccharide chain, before inhibition can take place (Pomerantz & Owen, 1978; Holmer *et al.*, 1979). This type of proposed interaction has sometimes been termed a template reaction (Griffith, 1983). The minimum chain length required for acceleration of thrombin inhibition by antithrombin has been shown to be 18 monosaccharide units (Lane *et al.*, 1984; Danielsson *et al.*, 1986).

A second heparin-dependent proteinase inhibitor was identified in plasma by Tollefsen and coworkers

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(Tollefsen & Blank, 1981; Tollefsen *et al.*, 1982) and is variously known as heparin cofactor II, dermatan sulphate cofactor (Abildgaard & Larsen, 1984) and human leuserpin 2 (Ragg, 1986). Unlike antithrombin, the only coagulation proteinase that this second factor inhibits is thrombin (Parker & Tollefsen, 1985). Heparin cofactor II inhibitory activity towards thrombin can also be accelerated by dermatan sulphate (Tollefsen *et al.*, 1982; Griffith & Marbet, 1983). Dermatan sulphate is a less sulphated glycosaminoglycan than heparin, and contains uronic acid residues (L-iduronic and D-glucuronic acids) that alternate with galactosamine, rather than glucosamine, residues. The size-dependency of the accelerating effect of dermatan sulphate on thrombin inhibition by heparin cofactor II has been studied by Tollefsen *et al.* (1986). They found that fragments containing a minimum of 12–14 sugar residues are required for acceleration. There is contradictory information regarding the effects of heparin of different sizes on this reaction (Linhardt *et al.*, 1986; Scully *et al.*, 1987; Petitou *et al.*, 1988; Sie *et al.*, 1988; Maimone & Tollefsen, 1988). This may be caused in part by the heterogeneity of the heparin preparations that have been used. In the present study, we have compared the heparin-induced acceleration of thrombin inhibition by heparin cofactor II and by antithrombin, with regard to dependency on the molecular size of the polysaccharide. We have used heparin oligosaccharides and fractions that have been well characterized in terms of their interaction with proteins of the coagulation system (Denton *et al.*, 1983; Lane *et al.*, 1984, 1986, 1987a).

MATERIALS AND METHODS

Materials

Heparin (M_r approx. 14000) isolated from porcine intestinal mucosa was separated into fractions with high or low affinity for antithrombin by chromatography upon antithrombin immobilized to Sepharose, as described (Höök *et al.*, 1976). High-affinity fractions of reduced polydispersity and different M_r values (Fractions 1, 3 and 6; M_r values approx. 18800, 12400 and 7800 respectively) were prepared by gel chromatography of commercial heparin followed by affinity chromatography on antithrombin–Sepharose (Denton *et al.*, 1983; Lane *et al.*, 1984). Heparin oligosaccharides were prepared by partial depolymerization of heparin with nitrous acid, followed by affinity chromatography on antithrombin–Sepharose and finally, repeated gel chromatography (Lane *et al.*, 1984). All even-numbered components containing 8–18 monosaccharide units (M_r 2400 to 5400) were obtained in homogeneous form; in addition, two fractions comprised predominantly of 20 and approx. 24 units (M_r values approx. 6000 and 7200 respectively) were isolated. A synthetic pentasaccharide containing the essential antithrombin-binding sequence of heparin (Casu *et al.*, 1981; Choay *et al.*, 1983) was also used (this was kindly supplied by Dr. Jean Choay, Institute Choay, Paris, France). The 4th International Heparin Standard was obtained from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts., U.K. The anticoagulant potencies of all of these saccharide preparations in purified systems containing antithrombin and enzymes have been reported previously (Lane *et al.*, 1984, 1986, 1987a).

Chromogenic substrates for the determination of

Factor Xa (S2222) or thrombin (S2238) were purchased from Flow Laboratories Ltd., Rickmansworth, Herts., U.K. Human antithrombin was either purchased from KabiVitrum, Uxbridge, Middx., U.K. or prepared from fresh human plasma as described (Lane *et al.*, 1987b). The latter protein contained no detectable contaminating proteins, and migrated as a single band on SDS/polyacrylamide-gel electrophoresis with an approx. M_r of 60000. No differences could be distinguished between these two preparations as regards their performance in assays and procedures used in this study. Bovine Factor Xa was purchased from Diagen, Thame, Oxon., U.K. and human thrombin (specific activity exceeding 2500 units/mg) was prepared as described (Fenton *et al.*, 1977). Labelled thrombin was prepared as described by Fernandez *et al.* (1987), but Iodogen (Pierce Warriner) (Fraker & Speck, 1978) was used instead of chloramine-T. Some inactivation of thrombin during iodination could not be avoided. This could be demonstrated by a small (< 10%) drop in specific activity (assessed by its decreased ability to clot normal human plasma) and by failure of inhibitors to form complexes with more than 80% ^{125}I -thrombin when added to plasma containing high concentrations of heparin (see also Fernandez *et al.*, 1987). Inexplicably, some preparations of ^{125}I -thrombin had minimal decreases in specific activity, yet failed to interact effectively with inhibitors in heparinized plasma. The differing response with inhibitors of different labelled thrombin preparations made quantitative comparison of results carried out on different occasions difficult (see below).

Human heparin cofactor II was a generous gift from Dr. Pierre Sie, Centre de Transfusion Sanguine, Toulouse, France. This preparation migrated as a single band on SDS/polyacrylamide gel electrophoresis with an M_r of approx. 70000, and was free of antithrombin as determined by immunological assays (Sie *et al.*, 1985, 1988; Petitou *et al.*, 1988).

Methods

The abilities of heparin and related saccharides to catalyse the inhibition of thrombin or Factor Xa by antithrombin were determined using purified reagents and the appropriate substrate, as described in detail elsewhere (Lane *et al.*, 1986, 1987a). The abilities of these saccharides to catalyse thrombin inhibition by heparin cofactor II were determined in a similar manner. Briefly, thrombin (30 nM) and heparin cofactor II (260 nM) were incubated for 30 s in 50 mM-Tris/7.5 mM-EDTA/175 mM-NaCl buffer, pH 8.4, in the presence of catalytic concentrations of saccharide (0.5–7.0 $\mu\text{g}/\text{ml}$). Residual enzyme activity was determined with the chromogenic substrate S2238 (0.5 mM). For each saccharide preparation, at least three dilutions were made into the range of the assay. Response in the assay (change in absorbance caused by hydrolysis of the substrate) was then plotted against the logarithm of saccharide concentration. Potencies were determined from displacement of the linear curves from that obtained using the 4th International Heparin Standard (this type of assay is known as a parallel line assay). The interassay coefficient of variation of this type of assay is less than 10% (Ireland *et al.*, 1984).

The ability of heparin to accelerate thrombin inhibition by antithrombin and heparin cofactor II in plasma was determined by adding ^{125}I -labelled human thrombin to a

pool of normal (defibrinated) human plasma ($n = 6$) and, after 30 s, analysing the amount of inhibitor-thrombin complexes formed by SDS/polyacrylamide-gel electrophoresis followed by autoradiography. The method used (Tew *et al.*, 1988) was similar to that originally described by Tollefsen *et al.* (1982) and was adapted from that of Fernandez *et al.* (1987). In brief, citrated plasma was defibrinated with Arvin (Berk Pharmaceuticals, Shalford, Surrey) and then reacted for 30 s with an equal volume of 1 unit of ^{125}I -labelled thrombin/ml. The reaction of labelled thrombin with plasma was terminated with an excess of boiling SDS-reducing agent solution, and electrophoresis was performed on small 10–15% polyacrylamide gels (Phast-System, Pharmacia). Following fixing, staining and drying, autoradiography was performed with overnight contact of the gel and film (Kodak X Omat) at -20°C .

Each saccharide preparation was studied repeatedly ($n = 2-4$) in at least four different concentrations in plasma, using different batches of ^{125}I -thrombin. A separate gel was used on each occasion for each saccharide. Autoradiography was performed and size- and dose-dependent effects on thrombin inhibition (see Results section) could be clearly seen.

In an attempt to quantify the clearly visible changing patterns of thrombin inhibition that were observed, the gels were then sliced into strips corresponding to the positions of thrombin and the main inhibitor-thrombin complexes. Because of their close proximity on these small gels, no attempt was made to resolve, discriminate and quantify antithrombin-thrombin and heparin cofactor II-thrombin complex bands. The broad band corresponding to the region where both complexes migrated on the gels was sliced. The sliced gels were then counted in a gamma counter and the thrombin contained in the inhibitor-thrombin complex band expressed as a percentage of total thrombin.

As might be expected from its description, this procedure results in more variability than the chromogenic substrate assays described above. To examine this formally, on separate occasions ($n = 8$) increasing concentrations (0–12 $\mu\text{g}/\text{ml}$) of the 4th International Heparin Standard were added to plasma samples from normal individuals. The plasma was treated with ^{125}I -thrombin, autoradiography and gel slicing were performed, and the percentage of ^{125}I -thrombin which was inhibited was determined. An interassay coefficient of variation of up to 34% was calculated. A large part of this variability was thought to be contributed by variability in activity of the ^{125}I -thrombin. Because of this relatively high inter-assay variability, seen also when separate attempts were made to quantify the effects of other saccharides, the same preparation of labelled thrombin and normal pooled plasma were used in the depicted Results.

RESULTS

The catalytic effects of the different heparin oligosaccharides and fractions upon the heparin cofactor II and thrombin reactions were first studied in purified systems, containing only these proteins. The chromogenic substrate S2238 was used to determine the residual thrombin activity and the potencies of the saccharides were compared to that of the 4th International Heparin Standard. The low M_r oligosaccharides had very small, yet measurable, accelerating activity (Fig. 1a). As the size

Fig. 1. Catalytic activities of high affinity (for antithrombin) heparin oligosaccharides and fractions studied in purified systems

High-affinity heparin oligosaccharides (of size 8 to approx. 24 monosaccharide units) and fractions (1, 3 and 6; see the Materials and methods section) were added (0.5–7.0 $\mu\text{g}/\text{ml}$) to incubation mixtures containing purified human heparin cofactor II and human thrombin (a), human antithrombin and human thrombin (b) and human antithrombin and bovine Factor Xa (c). Residual enzyme activities were determined with appropriate chromogenic substrates. The activities of the heparin preparations were compared with that of the 4th International Heparin Standard. For details of the conditions of the heparin cofactor II/thrombin/heparin reactions, see the Materials and methods section. Those of the antithrombin/enzyme/heparin reactions are contained elsewhere (see Lane *et al.*, 1986, 1987a). The catalytic activities of oligosaccharides in the heparin cofactor II-thrombin incubation experiments (a) were not zero, as was the case in the antithrombin-thrombin experiments (b) (see also the Results section). Under the former circumstance, the actual values for the catalytic activities ranged from 0.01 to 0.18 unit/nmol as the size of the oligosaccharides increased from 12 to 24 monosaccharide units.

of the heparin increased, there was a gradual and progressive rise in catalytic activity, until maximal activity was observed with the highest- M_r fraction. This clearly contrasts with the changing activities of the same oligosaccharides studied in purified systems containing antithrombin and thrombin (fig. 1b), and antithrombin and Factor Xa (Fig. 1c) (the latter results are included for completeness here). In the incubations of antithrombin and thrombin, absolutely no activity of oligosaccharides was observed until an M_r of 5400 (cor-

Fig. 2. Heparin-accelerated inhibition of ^{125}I -labelled thrombin by plasma containing the 4th International Standard (IS) and low-affinity (LA) heparin

^{125}I -labelled thrombin (1 unit/ml) was added to an equal volume of defibrinated plasma and incubated for 30 s; the reaction was terminated with boiling SDS solution. SDS/polyacrylamide-gel electrophoresis was performed using 10–15% gradient gels. After staining, fixing and drying, autoradiography was performed. The defibrinated plasma contained increasing amounts of the International Heparin Standard (a), or low affinity heparin (b). Abbreviations: T, thrombin; AT-T, antithrombin–thrombin complex; HCII-T, heparin cofactor II–thrombin complex.

responding to a heparin chain of 18 monosaccharide units) was reached. In the antithrombin and Factor Xa incubation mixtures, all of the oligosaccharides and fractions had high catalytic activities, although there was an overall 5-fold increase with increasing size of saccharide.

The above experiments were carried out using proteins purified from plasma and with catalytic concentrations of saccharides. Additional experiments were carried out in normal human plasma to explore the consequences of competition between the two proteinase inhibitors for thrombin and for heparin saccharides added in a wide concentration range. In order that the accelerating effect of the added saccharides upon thrombin inhibition by antithrombin and heparin cofactor II could be distinguished, an alternative to the chromogenic substrate method was employed. Labelled thrombin was added directly to the undiluted (defibrinated) plasma and incubated for 30 s before the reaction was inhibited with buffer containing SDS. SDS/polyacrylamide-gel electrophoresis was then carried out to separate unreacted

thrombin from its complexes with antithrombin and heparin cofactor II. Fig. 2 shows typical autoradiographs obtained following addition of increasing concentrations of the 4th International Heparin Standard (Fig. 2a), and heparin with low affinity for antithrombin (Fig. 2b). In the absence of any polysaccharide, approx. 30% of the thrombin was taken into complex with its inhibitors (Fig. 2a). The great majority of this inhibitor–thrombin complex comprised antithrombin–thrombin complex under the conditions employed. However, other unidentified thrombin complexes are also visible as trace bands on these autoradiographs. As increasing amounts of the International Heparin Standard were added to plasma, almost complete inhibition of thrombin (determined by the extent of formation of inhibitor–thrombin complexes) occurred when the heparin concentration exceeded 3 µg/ml. Again, most inhibition of thrombin appeared to result primarily from antithrombin–thrombin complex formation. Possibly some heparin cofactor II–thrombin complex occurred, but this could not be clearly demonstrated. Addition of increasing concentrations of low-affinity heparin to plasma also resulted in some thrombin inhibition, as confirmed by slicing the gels after autoradiography had been performed and counting the radioactivity corresponding to the thrombin and inhibitor–thrombin bands (Fig. 3). However, in this situation, the major inhibitor involved appeared to be heparin cofactor II, and much higher concentrations of polysaccharide were required to produce appreciable inhibition of thrombin (Figs. 2b and 3).

Similar experiments were then carried out with the heparin oligosaccharides and fractions having high affinity for antithrombin. Addition of oligosaccharides composed of 8–18 monosaccharide units at low concentrations (less than or equal to 1 µg/ml) had negligible effect upon inhibition of thrombin, judged by formation of inhibitor–thrombin complexes (Fig. 4). The amounts of antithrombin–thrombin complex were approximately similar to those generated in the absence of added saccharides (results not shown), and no significant heparin cofactor II–thrombin complex was detected. However, as the concentration of these oligosaccharides in plasma was increased up to 50 µg/ml, appreciable formation of heparin cofactor II–thrombin was observed. The accelerated inhibition of thrombin thus appeared to be due primarily to potentiation of heparin cofactor II–thrombin complex formation rather than potentiation of antithrombin–thrombin complex formation. Furthermore, as the length of the polysaccharide chain increased from 8 to 18 monosaccharide units, the ability of the oligosaccharides to accelerate thrombin inhibition increased. This is again shown in quantitative terms in Fig. 3, which also illustrates a weak accelerating effect upon inhibitor–thrombin complex formation by the synthetic antithrombin-binding pentasaccharide. The effect of the pentasaccharide was also due to potentiation of heparin cofactor II–thrombin complex formation (results not shown).

The larger high-affinity saccharides (oligosaccharides composed of 20 and 24 monosaccharide units and fractions HA6 and HA1) were more able to potentiate thrombin inhibition than the smaller oligosaccharides and the low-affinity heparin (Figs. 3 and 5), and did so at lower concentrations than these other preparations. The higher M_r saccharides catalysed thrombin inhibition primarily through antithrombin–thrombin complex for-

Fig. 3. Anti-thrombin activities of heparin oligosaccharides and fractions

Defibrinated plasma containing heparin, its oligosaccharides or fractions, was reacted with labelled thrombin, as described in the legend to Fig. 2. SDS/PAGE was performed. Dried polyacrylamide gels were aligned with their autoradiographs (see, for example, Fig. 2) and sliced corresponding to the positions of ^{125}I -thrombin and the combined heparin cofactor II-thrombin and antithrombin-thrombin complexes (no attempt was made to slice these latter two bands individually as they migrated closely on these small gels). The amount of thrombin inhibited was then calculated from the ratio of free thrombin to that contained in inhibitor-thrombin complexes. Normal plasma in the absence of added heparin inhibited 30% of added thrombin, while addition of large amounts (50 $\mu\text{g}/\text{ml}$) of the 4th International Standard resulted in 85% inhibition of thrombin. For the purposes of this illustration the former was designated 0% and the latter designated 100% thrombin inhibition, and all other results were calculated in relation to these. Abbreviations: IS, 4th International Heparin Standard; LA, low-affinity heparin; 5 to 24, high-affinity heparin oligosaccharides with the specified number of monosaccharide units; HA1 and HA6, high-affinity heparin fractions of M_r 18800 and 7800 respectively. The molar concentrations of oligosaccharides and heparin fractions at 50 $\mu\text{g}/\text{ml}$ are as follows: 5, 33 μM ; 8, 21 μM ; 10, 17 μM ; 12, 14 μM ; 18, 9.2 μM ; 20, 8.3 μM ; 24, 6.9 μM , HA1, 2.7 μM ; HA6, 6.3 μM ; LA, 3.7 μM ; IS, 3.3 μM . Each data point represents the results of a single determination, using the same preparation of ^{125}I -thrombin for all the experiments. Similar results have been obtained with different preparations of labelled thrombin,

Fig. 4. Heparin-accelerated inhibition of ^{125}I -labelled thrombin by plasma containing high-affinity heparin oligosaccharides of size 8 to 18 monosaccharide units

For further details, see the Materials and methods section, and legend to Fig. 2. Abbreviations: T, thrombin; AT-T, antithrombin-thrombin complex; HCII-T, heparin cofactor II-thrombin complex; OS, oligosaccharide.

Fig. 5. Heparin-accelerated inhibition of ^{125}I -labelled thrombin by plasma containing high-affinity heparin oligosaccharide of size 20 and 24 monosaccharide units and high-affinity fractions HA6 and HA1

For further details see the Materials and methods section, and the legend to Fig. 2. Abbreviations: T, thrombin; AT-T, antithrombin-thrombin complex; HCII-T, heparin cofactor II-thrombin complex; OS, oligosaccharide; HA1 and HA6, high-affinity heparin fractions of M_r 18800 and 7800 respectively.

mation. Even at very high concentrations (50 $\mu\text{g}/\text{ml}$), there was little evidence of heparin cofactor II-thrombin complex bands with these saccharide preparations, although it cannot be excluded that some thrombin inhibition may have occurred through heparin cofactor II.

but for the reasons outlined in the Materials and methods section, individual results would not necessarily be superimposable with those illustrated.

DISCUSSION

The results of the present study demonstrate that the size-dependency of the heparin-accelerated heparin cofactor II–thrombin and antithrombin–thrombin reactions differ in two distinct ways. First, a larger heparin chain is required for efficient acceleration (in the following referred to as catalytic effect) of the heparin cofactor II–thrombin reaction. Secondly, oligosaccharides of small chain length (M_r less than 5400) with high affinity for antithrombin, which can accelerate antithrombin–factor Xa complex formation, are unable to catalyse antithrombin–thrombin complex formation, but do have weak accelerating effects upon the heparin cofactor II–thrombin complex formation. This weak interaction produces appreciable accelerated inhibition of thrombin when the saccharide concentration is increased above that usually encountered with heparin ($> 10 \mu\text{g/ml}$).

These results relating to the catalytic effects of the higher M_r fractions are in general agreement with work of Griffith & Marbet (1983) and Scully *et al.* (1987). The latter group studied the effects of less well-characterized heparin with narrow M_r range, prepared by gel filtration, on the inhibitor–thrombin reactions. They measured the increase in pseudo-first-order rate constant of inhibition of thrombin by heparin cofactor II and concluded that (high- M_r) heparin could act as a template, accommodating both inhibitor and proteinase, to accelerate inhibition. The template or ternary complex model of thrombin inactivation by heparin cofactor II, proposed by Griffith (1983), has received recent additional support from Yamagishi *et al.* (1987). They found that chemical (pyridoxal 5'-phosphate) modification of either heparin cofactor II or thrombin resulted in loss of the heparin-binding properties of these proteins, along with their ability to respond to heparin by accelerated complex formation. Scully *et al.* (1987) found greater peak stimulation at lower heparin concentrations with higher- M_r than with lower- M_r heparin. Like Scully *et al.* (1987), we have been unable to identify a critical heparin chain length required for this type of reaction to occur. Appreciable catalytic effects were not observed, however, in the heparin cofactor II–thrombin reaction unless the M_r of heparin exceeded 8000. Similar findings have recently been reported by Sie *et al.* (1988).

A weak accelerating effect upon thrombin inhibition by heparin cofactor II was clearly indicated in the present study with the small oligosaccharides (of size less than 18 monosaccharide units), and this progressively increased with increasing M_r of the saccharide chain. This weak accelerating effect was just detectable under the conditions of the purified system experiments but was not so apparent when low concentrations of oligosaccharides were added to plasma. This discrepancy was attributed to the better precision of the substrate assay and conceivably, to an inhibitory influence of non-specific interactions of other proteins in the ^{125}I -thrombin assay. The weak accelerating effect was more clearly revealed when higher concentrations of oligosaccharides were added to plasma and SDS/polyacrylamide-gel electrophoresis and autoradiography performed. This mode of heparin cofactor II acceleration is probably not due to a template mechanism, as it seems unlikely that, for example, an octasaccharide would be able to simultaneously accommodate thrombin and heparin cofactor II. It should be

Fig. 6. Mechanisms of heparin-accelerated thrombin inhibition by heparin cofactor II

(a) Weak interaction of low- M_r (1700–8000) heparin, (b) template mechanism ($M_r > 8000$). Abbreviations: HCII, heparin cofactor II; Ila, thrombin.

recalled that the minimum size of oligosaccharide that can potentiate thrombin inhibition by antithrombin is 18 monosaccharide units (Lane *et al.*, 1984; Danielsson *et al.*, 1986) and that heparin cofactor II has an M_r exceeding that of antithrombin (Tollefsen & Blank, 1981; Tollefsen *et al.*, 1982). The size of a heparin chain required for the formation of a ternary complex with heparin cofactor II and thrombin might be expected, therefore, to exceed that required for antithrombin–thrombin and heparin. Two alternative explanations for the weak accelerating effect of the small oligosaccharide on the heparin cofactor II–thrombin reaction can be proposed. The oligosaccharide could bind either to the enzyme or to the inhibitor. The three possible alternative mechanisms of accelerated thrombin inhibition by heparin cofactor II are summarized in Fig. 6.

Results of the present study are compatible with the view that there is no specific region of the heparin chain that is responsible for its interactions with heparin cofactor II (Scully *et al.*, 1987; Petitou *et al.*, 1988). The interaction appears to be mainly determined by charge density (Sie *et al.*, 1988) and the length of the saccharide chain. A contrasting study was presented by Linhardt *et al.* (1986). They fragmented heparin with heparinase and identified a unique hexasaccharide fragment with potent ability (half of that of unfractionated heparin) to accelerate heparin cofactor II–thrombin complex formation. This hexasaccharide apparently contained part of the antithrombin-binding sequence of heparin. A feature of this hexasaccharide fragment was its ready ability to catalyse heparin cofactor II–thrombin complex formation, but it had low ability to catalyse the antithrombin–thrombin and antithrombin–Factor Xa reactions. Such behaviour has not been observed with oligosaccharides of similar size (this study; Petitou *et al.*, 1988; Maimone & Tollefsen, 1988). A weak size-dependent accelerating effect upon the heparin cofactor II–thrombin reaction was observed with very small heparin oligosaccharides in the present study, in partial agreement with Linhardt *et al.* (1986). Sie *et al.* (1988) have also reported very weak accelerating effects of small oligosaccharides. Petitou *et al.* (1988), though, could not

detect acceleration of the heparin cofactor II-thrombin reaction by very high concentration of a hexasaccharide fragment. It should be noted, however, that they did not use the minimally diluted plasma and ^{125}I -labelled thrombin method employed here.

The results of the present study are relevant to the continuing debate regarding the mechanisms by which heparin and other related saccharides inhibit thrombin and prevent thrombosis. It is increasingly being claimed that acceleration of thrombin inhibition is the major mechanism involved in this latter property (Ofosu *et al.*, 1985, 1987; Béguin *et al.*, 1988). Thus, Fernandez *et al.* (1987) recently compared the antithrombotic and anticoagulant properties of a variety of glycosaminoglycans in an experimental model in the rabbit. Comparable and effective inhibition of thrombosis occurred when the concentration of glycosaminoglycans produced a 2-fold acceleration of inhibition of thrombin, measured *ex vivo*. Some of the glycosaminoglycan preparations they studied, such as dermatan sulphate, apparently produce their inhibitory effects upon thrombin solely by potentiating the action of heparin cofactor II. The situation with heparin and related compounds (low- M_r heparin, heparan sulphate) is less clear. It is known that these compounds can catalyse thrombin inhibition through the action of antithrombin, through heparin cofactor II, or through both inhibitors. However, it has not always been clear which property of the saccharides determines their interactions with these inhibitors. The results of the present study provide a basis for an understanding of the selectivity of the heparin polysaccharides (in their interaction with these inhibitors). Firstly, it has to be recognized that the most important determinant of inhibitor selectivity is the presence of the antithrombin-binding pentasaccharide in the saccharide sequence. This enables the saccharide to bind to antithrombin (and, consequently, to catalyse the inhibition of Factor Xa). This binding reaction will occur regardless of the size of the (poly)saccharide chain provided that it contains the antithrombin-binding pentasaccharide sequence. The second determinant is the polysaccharide chain size. When this exceeds 5400 and the antithrombin-binding sequence is present, the polysaccharide will be of sufficient size to act catalytically to inhibit thrombin through antithrombin. If, on the other hand, the polysaccharide content of the specific pentasaccharide sequence is low but the polysaccharide chain length exceeds 8000, the inhibition of thrombin by heparin cofactor II will be promoted. If the size of the saccharide with or without the antithrombin-binding site is less than 18 monosaccharide units, then acceleration of thrombin inhibition will be possible only at high concentrations of saccharide and will occur through heparin cofactor II.

This work was supported by Grant No. 2309 from the Swedish Medical Research Council.

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Received 22 November 1988/21 March 1989; accepted 4 April 1989