THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 261, No. 33, Issue of November 25, pp. 15467-15473, 1986
Printed in U.S.A.

Role of Ternary Complexes, in Which Heparin Binds Both Antithrombin and Proteinase, in the Acceleration of the Reactions between Antithrombin and Thrombin or Factor Xa*

(Received for publication, July 15, 1986)

Åke Danielsson, Elke Raub, Ulf Lindahl, and Ingemar Björk

From the Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, The Biomedical Center, Box 575, S-751 23 Uppsala, Sweden

Oligosaccharides (10–20 monosaccharide units) with high affinity for antithrombin, as well as larger high-affinity heparin fractions (having relative molecular masses between 6,000 and 21,500), all markedly accelerated the inhibition of Factor Xa by antithrombin. Moreover, all high-affinity oligosaccharides and heparins enhanced, to a similar extent, the amount of free proteolytically modified antithrombin cleaved at the reactive bond by Factor Xa. In contrast, a minimum high-affinity heparin size of ~18 monosaccharide units was required to significantly accelerate the inactivation of thrombin by antithrombin and to enhance the production of modified antithrombin by this enzyme. All high-affinity fractions studied had similar affinities for antithrombin, as determined by fluorescence titrations. In competition experiments, binary complexes of antithrombin with octadecasaccharide or larger high-affinity heparins, but not with smaller oligosaccharides, displaced inactivated ^{125}I-thrombin from matrix-linked low-affinity heparin. Moreover, similar binary complexes with ^{3}H-labeled octadecasaccharide or larger chains, but not with smaller oligosaccharides, were capable of binding to matrix-linked inactivated thrombin. These results indicate that simultaneous binding of antithrombin and thrombin to high-affinity heparin is a prerequisite to the acceleration of the antithrombin-thrombin reaction and that the minimum heparin sequence capable of binding both proteins comprises ~18 monosaccharide units. Similar complex formation apparently is not required for the acceleration of the antithrombin-Factor Xa reaction.

Heparin, a sulfated glycosaminoglycan, dramatically increases the rate at which the plasma proteinase inhibitor antithrombin forms inactive equimolar complexes with serine proteinases of the coagulation system (Rosenberg and Damus, 1973). The polysaccharide also markedly promotes the concurrent production of a modified form of antithrombin, cleaved at the reactive bond, in the reactions with thrombin and Factor Xa (Jörnvall *et al.*, 1979; Marciniak, 1981; Björk *et al.*, 1982; Björk and Fish, 1982). Only about one-third of commercial preparations of heparin is active in these respects and binds with high affinity to antithrombin (high-affinity heparin). The remaining fraction (low-affinity heparin) is inactive and binds only weakly to the inhibitor (for a review, see Björk and Lindahl, 1982). High-affinity heparin contains a specific pentasaccharide sequence which constitutes the binding site for antithrombin (Thunberg *et al.*, 1982; Lindahl *et al.*, 1984; Petitou, 1984).

Several mechanisms have been suggested for the accelerating effect of heparin on the inhibition of coagulation proteinases by antithrombin (see Björk and Lindahl, 1982). The available data indicate that the effect is primarily dependent on the tight heparin-antithrombin interaction (Rosenberg and Damus, 1973; Björk and Lindahl, 1982). This interaction, which induces a conformational change in antithrombin (Villanueva and Danishefsky, 1977; Nordenman and Björk, 1978a; Olson *et al.*, 1981), appears to be sufficient for the acceleration of the inactivation of some coagulation enzymes, such as Factor Xa. A chemically synthesized high-affinity pentasaccharide, containing only the antithrombin-binding sequence of heparin, thus accelerates the inhibition of Factor Xa by antithrombin to nearly the same extent as full-length heparin (Choay *et al.*, 1983). However, an interaction also between heparin and the target enzyme has been suggested to be essential for the acceleration of the inhibition of thrombin and certain other proteinases (Laurent *et al.*, 1978; Pomeranz and Owen, 1978; Holmer *et al.*, 1979). In support of this proposal, short high-affinity heparin fragments have little effect on the antithrombin-thrombin reaction, while larger high-affinity chains show increasing activity (Holmer *et al.*, 1980; Oosta *et al.*, 1981; Lane *et al.*, 1984). Moreover, simultaneous binding of antithrombin and thrombin to the same molecule of full-length high-affinity heparin was indicated by affinity chromatography (Pomeranz and Owen, 1978), and the importance of such complexes for the acceleration of the inactivation of thrombin has been suggested by kinetic analyses (Griffith, 1982; Pletcher and Nelsestuen, 1983; Nesheim, 1983; Hoylaerts *et al.*, 1984).

In this work, we have studied the ability of well-defined high-affinity heparin oligosaccharides, differing in length by only one disaccharide unit, to accelerate the reactions between antithrombin and thrombin or Factor Xa and to promote the formation of the modified form of antithrombin in these reactions. The results are consistent with ternary complexes, in which antithrombin and proteinase bind to the same heparin molecule (AT·H·proteinase complexes[1,2]), being in-

* This work was supported by Grants 2309 and 4212 from the Swedish Medical Research Council, Grant 600/83 from the Swedish Council for Forestry and Agricultural Research, and by grants from KabiVitrum AB, Stockholm, the National Board for Technical Development, and the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This notation will be used to indicate simultaneous binding of antithrombin (AT) and proteinase to the same heparin (H) chain. Such ternary complex formation presumably precedes the reaction between the bound antithrombin and proteinase at their reactive

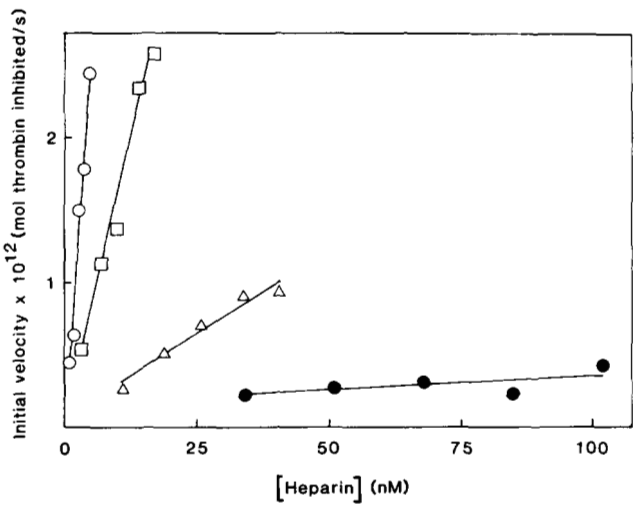

FIG. 1. **Initial rates of inactivation of thrombin by antithrombin in the presence of increasing concentrations of high-affinity heparin or heparin oligosaccharides of different lengths.** The antithrombin and thrombin concentrations were 194 and 20 nM, respectively, in all analyses, and the reaction volume was 2.3 ml. ●——●, hexadecasaccharide; △——△, octadecasaccharide; □——□, eicosasaccharide; ○——○, heparin, molecular weight ~8400.

volved in the mechanism of both these effects in the reaction with thrombin, but playing only a minor role in the Factor Xa reaction. Moreover, the analyses indicate that such complexes with thrombin may form only with oligosaccharides at least 18 monosaccharide units long. In support of this conclusion, ternary complexes between heparin, antithrombin, and inactive thrombin were demonstrated by two different methods essentially only with oligosaccharides of this size and longer. The amount of such complexes that could be formed increased with the size of the oligosaccharide in parallel with the accelerating ability and the ability to increase the formation of modified antithrombin.

MATERIALS AND METHODS[3]

RESULTS

Kinetics of the Reactions between Antithrombin and Thrombin or Factor Xa in the Presence of Different Size Heparins—Initial rates of inactivation of 20 nM thrombin in the presence of a constant 10-fold higher concentration of antithrombin were obtained at an ionic strength of 0.15 as a function of heparin concentration for a number of high-affinity heparin fractions with different molecular weights. The conditions of the experiments were such that all heparin fractions essentially bound stoichiometrically to antithrombin. At the heparin concentrations studied, plots of the observed initial rates of thrombin inactivation *versus* heparin concentration were linear (Fig. 1). The apparent accelerating ability of heparin on the antithrombin-thrombin reaction was defined from the slope of these plots as a specific heparin activity (mol of thrombin inhibited/s × mol of heparin) and was plotted as a function of heparin molecular weight (Fig. 2). It is evident that heparin oligosaccharides shorter than 18 monosaccharide units minimally affect the rate of the antithrombin-thrombin reaction, while the accelerating ability increases markedly with molecular weight for longer heparin chains. Since all heparin fractions essentially were saturated with antithrombin, this increase most likely is due to an increased binding of thrombin. The heparin fraction with the highest molecular weight showed the largest effect, *i.e.* about a 600-fold increase in accelerating ability over that shown by the hexadecasaccharide.

The observed initial rates of Factor Xa inactivation, obtained in a similar manner as those of thrombin inactivation, also increased linearly with heparin concentration for all fractions of the polysaccharide at the concentrations analyzed (data not shown). A plot of the accelerating ability, defined as described previously, *versus* heparin molecular weight (Fig. 2) showed that heparin fractions below a molecular weight of about 6000 accelerated the antithrombin-Factor Xa reaction to about the same extent. This accelerating effect was considerably higher than that observed for the inactivation of thrombin. At molecular weights higher than about 6000, the ability of heparin to accelerate the inactivation of Factor Xa increased notably. However, less than a 10-fold increase in accelerating ability occurred over the entire molecular weight range.

Production of Modified Antithrombin—The ability of high-affinity heparins of different molecular weights to increase the production of modified antithrombin by thrombin or Factor Xa was studied with a constant molar heparin to antithrombin ratio of 0.2 (Björk and Fish, 1982). A 4-fold increase of this ratio was used in selected control experiments, which gave identical results. Time course analyses monitored by gel electrophoresis showed that the incubation times used were sufficient for virtual completion of all reactions, *i.e.* all free antithrombin was consumed. Little modified antithrombin was produced by thrombin in the absence of heparin and also in the presence of high-affinity heparin oligosaccharides containing ≤18 monosaccharides (Fig. 3). However, a sharp increase in the amount of modified inhibitor formed occurred

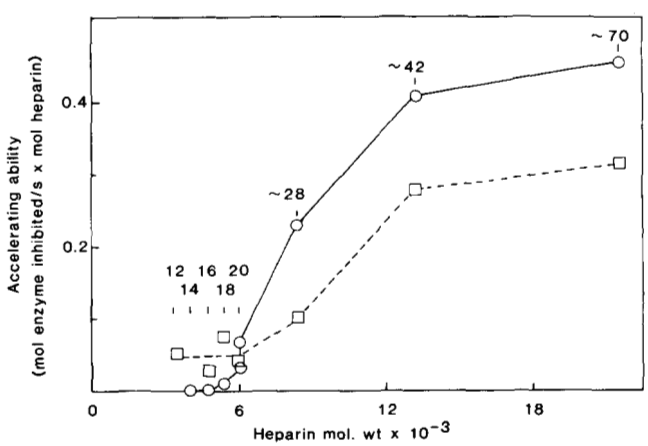

FIG. 2. **The molecular weight dependence of the accelerating ability of high-affinity heparin and heparin oligosaccharides on the antithrombin-thrombin (○——○) and antithrombin-Factor Xa (□– – –□) reactions.** The accelerating ability was calculated from the plots of Fig. 1 and additional plots not shown, as described under "Results." The number of monosaccharide units is given for each heparin fraction.

sites. This type of complex thus differs from a ternary H·AT·proteinase complex (apparently involved in the inhibition of Factor Xa), in which heparin binds only to antithrombin in an intermediate ternary complex on the inactivation pathway.

[2] The abbreviations used are: AT, antithrombin; H, heparin; MS-thrombin, methanesulfonyl fluoride-inactivated thrombin.

[3] "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2386, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

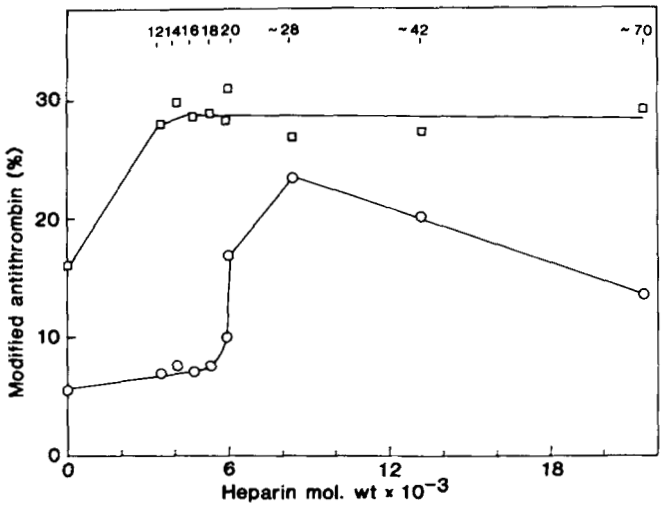

FIG. 3. **Production of modified antithrombin by thrombin or Factor Xa in the presence of high-affinity heparin or heparin oligosaccharides of different lengths.** Modified antithrombin was analyzed by gel electrophoresis as described under "Materials and Methods." The amount of modified antithrombin formed is given in percent of the total amount of antithrombin added to the reaction mixture, the remainder occurring largely in complex with the proteinase. All points represent averages of duplicate experiments. O——O, reaction with thrombin; □——□, reaction with Factor Xa. The number of monosaccharide units is given for each heparin fraction.

TABLE I

Binding constants for the binding of high-affinity heparin oligosaccharides or undegraded high-affinity heparin to antithrombin at I = 0.3

Number of monosaccharide units	Molecular weight	Binding constant
		$\times 10^{-7}\ M^{-1}$
Oligosaccharides		
10	2,860	2.0[a]
12	3,480	2.9[a]
14	4,090	5.0
16	4,710	6.0
18	5,320	6.5
20	5,940	6.5
Undegraded heparin		
~20	6,000	0.8[b]
~28	8,400	1.0[b]
~44	13,200	0.8[b]
~70	21,500	0.6[b]

[a] Published earlier by Lindahl *et al.* (1984).
[b] Published earlier by Danielsson and Björk (1981).

with longer chains, *i.e.* in the same molecular weight range as the increase in accelerating ability. The decreased production of modified antithrombin in the presence of still longer polysaccharide chains has also been noted by Marciniak (1982). Factor Xa generated more of the modified antithrombin than did thrombin in the absence of heparin (Fig. 3), and the proportion of this component was further increased in the presence of heparin. However, in contrast to the results with thrombin, small oligosaccharides also increased the formation of modified antithrombin by Factor Xa to about the same extent as long heparin chains.

Binding Constants—The affinities between the high-affinity oligosaccharides or larger heparin chains used in this work and antithrombin were measured by fluorescence titrations at an ionic strength of 0.3 (Table I). A small increase in affinity from deca- to tetradecasaccharide was apparent, while the affinity of the larger oligosaccharides was essentially identical. Notably, all fractions of undegraded heparin had lower binding constants than the oligosaccharides. Possibly, the degradation and isolation procedure selected oligosaccharides with somewhat higher affinities than that of the major population of undegraded molecules.

Formation of Ternary AT·H·Proteinase Complexes with Inactive Thrombin—The ability of a single high-affinity heparin chain to bind both antithrombin and thrombin in a ternary AT·H·proteinase complex was investigated as a function of the size of the heparin by two different procedures (Fig. 4). Inactive thrombin, which has the same affinity for heparin as the active enzyme (Nordenman and Björk, 1978b) was used to avoid direct binding to antithrombin; methanesulfonyl fluoride was chosen as inactivating reagent because of its small size (Sonder and Fenton, 1984). Affinity chromatography of inactive thrombin on antithrombin-Sepharose with or without heparin, a method previously used to demonstrate ternary complex formation with full-length heparin (Pomeranz and Owen, 1978), was found to be unsuitable. Attachment of antithrombin to the matrix in the absence of heparin thus led to a complete loss of heparin-binding ability. Moreover, attempts to include heparin (even only a small excess of extensively *N*-acetylated high-affinity heparin) in the coupling reaction to protect the heparin binding site (Höök *et al.*, 1976) always led to the binding of a small amount of polysaccharide to the gel, which thus also bound thrombin in control experiments. Two other procedures, therefore, were developed.

Distribution of Inactive Thrombin between Heparin-Antithrombin Complexes and Matrix-linked Low-affinity Heparin—In this method, the high-affinity heparin sample was mixed with antithrombin, radiolabeled MS-thrombin, and low-affinity heparin-agarose, and the amount of inactive thrombin not bound to the immobilized low-affinity heparin at equilibrium was analyzed. The rationale of the experiment was that only complexes between antithrombin and a high-affinity heparin chain sufficiently long to also accommodate a thrombin molecule would be able to bind the inactive thrombin and thus prevent it from binding to the matrix-linked low-affinity heparin (Fig. 4*A*). In contrast, complexes of antithrombin with shorter high-affinity chains would show no such effect. The conditions of the experiments were chosen

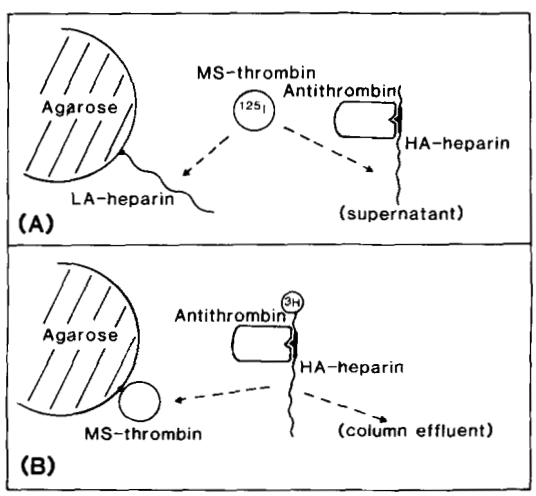

FIG. 4. **Outline of experimental design for the study of ternary complex formation between antithrombin, heparin, and inactivated thrombin by competition experiments (*A*) and affinity chromatography (*B*).** For experimental details, see "Materials and Methods." *LA-heparin*, low-affinity heparin; *HA-heparin*, high-affinity heparin.

such that >95% of the high-affinity heparin was bound to antithrombin, as calculated from the binding constants (Table I; Nordenman *et al.*, 1978; Danielsson and Björk, 1981; Lindahl *et al.*, 1984). The expected maximal concentration of free high-affinity heparin could only minimally have affected the analyses, as shown by a control experiment in which this concentration (0.2 μM) was used in the absence of antithrombin. The concentrations of the reactants were also chosen such that most of the inactive thrombin was bound to the immobilized low-affinity heparin in the absence of any added heparin in solution. Low-affinity heparin-agarose, which binds thrombin as tightly as matrix-linked high-affinity heparin (Nordenman and Björk, 1978b) but has only weak affinity for antithrombin (Höök *et al.*, 1976; Nordenman and Björk, 1978a), was used to minimize the binding of antithrombin to the gel.

Complexes between antithrombin and high-affinity heparin oligosaccharides containing less than 18 monosaccharide units were unable to compete with the matrix-linked low-affinity heparin for binding of inactive thrombin (Fig. 5). In contrast, complexes between antithrombin and larger heparin chains did compete, the effect increasing with increasing size of the high-affinity heparin. This behavior indicates that appreciable amounts of ternary AT·H·proteinase complexes are formed only if the heparin chain is at least 18 monosaccharide residues long. The alternative possibility, that binding of inactive thrombin to the longer high-affinity heparin chains dramatically decreases the heparin-antithrombin affinity so that antithrombin is displaced and no ternary complexes are formed, is highly unlikely for several reasons. Thus, no evidence for such an effect has been observed in previous studies, in which ternary complex formation was analyzed with active thrombin and full-length heparin by enzymatic methods (Griffith, 1982; Pletcher and Nelsestuen, 1983; Nesheim, 1983). Moreover, the high-affinity oligosaccharides and larger heparin chains were found to associate quantitatively with antithrombin in gel chromatography on Sephadex G-100 and also when the two species were mixed with a 10-fold excess of inactive thrombin before the experiment (results not shown). No ternary complexes were observed under the non-equilibrium conditions of these analyses, presumably because of insufficient affinity between inactive thrombin and heparin.

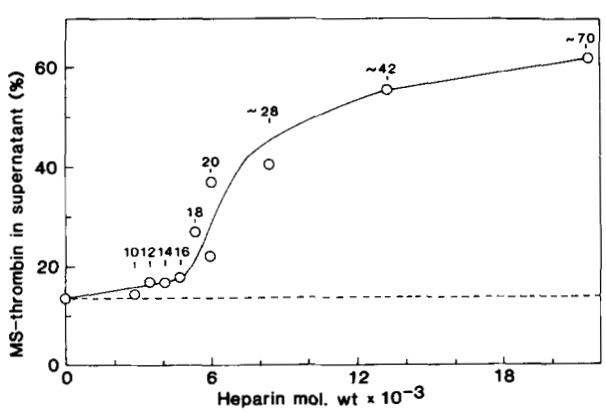

FIG. 5. **Displacement of ^{125}I-labeled MS-thrombin from low-affinity heparin-agarose by complexes between antithrombin and high-affinity heparin or heparin oligosaccharides of different lengths.** The *dashed line* indicates the equilibrium level of free MS-thrombin, *i.e.* the proportion of MS-thrombin which was not bound to low-affinity heparin-agarose in a control experiment where high-affinity heparin was omitted. Incubations were performed and analyzed as detailed under "Materials and Methods." Each point represents the average of duplicate experiments. The number of monosaccharide units is given for each heparin fraction.

Affinity Chromatography of Heparin-Antithrombin Complexes on Matrix-linked Inactive Thrombin—In this procedure, radiolabeled high-affinity heparin fractions were applied to a column of agarose-bound MS-thrombin in the presence of antithrombin, and the column was eluted with a salt gradient, also in the presence of antithrombin. As in the previous experiment, the antithrombin concentration was such that all heparin species were essentially saturated with the protein throughout the analyses. The proportion of heparin bound to the column in the presence of antithrombin thus must reflect the amount of ternary AT·H·proteinase complex formed with the different heparin fractions (see Fig. 4*B*); a displacement of antithrombin from the heparin chain by the immobilized inactive thrombin is highly unlikely for the reasons discussed earlier. Control experiments showed that all heparin fractions bound quantitatively to the column in the absence of antithrombin and that the interaction between antithrombin and the matrix-linked inactive thrombin was negligible. In the presence of antithrombin, only small amounts of the short oligosaccharides bound to the column, while increasing proportions of the polysaccharide were bound as the length of the chain increased over 18 monosaccharide residues (Figs. 6 and 7). The bound fraction eluted at the same ionic strength as in the absence of antithrombin. Appreciable amounts of ternary AT·H·proteinase complex thus were detected by this method only with heparin chains longer than 18 monosaccharide units, in good agreement with the preceding analyses.

DISCUSSION

Early proposals regarding the occurrence of ternary AT·H·proteinase complexes in which heparin binds both antithrombin and thrombin (Laurent *et al.*, 1978; Pomerantz and Owen, 1978; Machovich and Aranyi, 1978; Holmer *et al.*, 1979) and the implications of such interactions for the mechanism of the heparin effect were based on limited experimental evidence. These proposals were supported by subsequent kinetic analyses with full-length heparin (Jordan *et al.*, 1980; Griffith, 1982; Pletcher and Nelsestuen, 1983; Nesheim, 1983; Petersen and Jörgensen, 1983) or with heparin covalently linked to antithrombin (Hoylaerts *et al.*, 1984), which were consistent with AT·H·proteinase complexes increasing the rate of inactivation of thrombin by approximating the enzyme

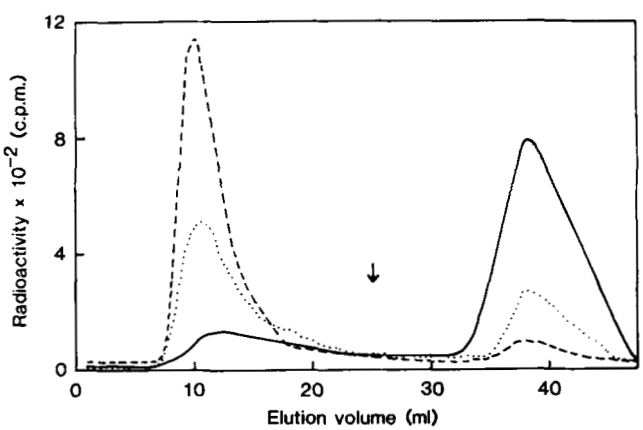

FIG. 6. **Affinity chromatography on immobilized MS-thrombin of complexes between antithrombin and ^{3}H-labeled high-affinity heparins of different lengths.** The experimental details are described under "Materials and Methods." The *arrow* marks the start of the NaCl gradient. The labeled heparin fractions contained 10 (– – –), 20 (· · · ·), and ~28 (——) monosaccharide units.

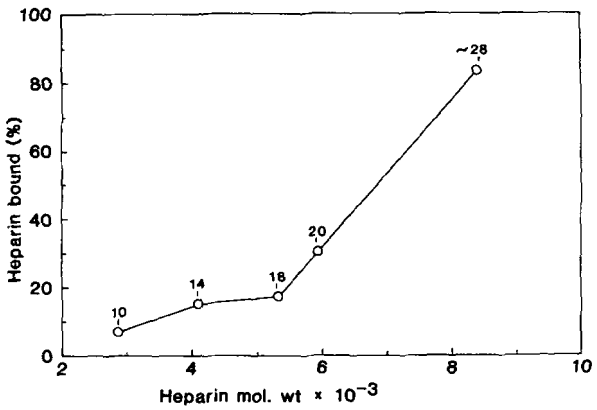

FIG. 7. **Binding of complexes between antithrombin and ^{3}H-labeled high-affinity heparin or heparin oligosaccharides of different lengths to immobilized MS-thrombin.** The vertical axis shows the proportion of labeled polysaccharide which was bound to and eluted from a column of matrix-linked inactive thrombin in the presence of antithrombin. Three such experiments are shown in Fig. 6. For further experimental detail, see "Materials and Methods." The number of monosaccharide units is given for each heparin fraction.

to the inhibitor. Heparin has also been shown to markedly increase the stability of the initial antithrombin-thrombin complex, possibly by ternary complex formation (Olson and Shore, 1982). In contrast, kinetic analyses have suggested that AT·H·proteinase complexes are only of minor importance for the acceleration of the reaction between antithrombin and Factor Xa (Jordan *et al.*, 1980; Griffith, 1983; Pletcher and Nelsestuen, 1983).

The present study more directly implicates AT·H·proteinase complexes in the heparin-accelerated inhibition of thrombin by antithrombin. MS-thrombin, with similar heparin affinity as active thrombin, was shown to bind to binary complexes of heparin and antithrombin only when the heparin was at least 18 monosaccharide units long, whereas all free heparin species bound similarly to the inactive enzyme. Moreover, the ability of heparins of increasing size to form a ternary complex with antithrombin and thrombin strictly paralleled the ability of the fractions to accelerate the inactivation of the enzyme by the inhibitor. Although the ternary AT·H·proteinase complexes of necessity could be demonstrated only with inactive thrombin, this parallel increase strongly suggests that such complexes are involved in the effects of the polysaccharide on the antithrombin-thrombin reaction. This conclusion does not exclude the possibility that interactions of antithrombin and thrombin at their reactive sites in the ternary complex may somewhat influence complex formation. The size dependence of the accelerating ability is similar to that of the anticoagulant activity (a more approximate measure of the accelerating ability), determined in a pure system with thrombin as the target enzyme by Lane *et al.* (1984), but differs somewhat from the results of Oosta *et al.* (1981). These authors thus also found significant activity in hexadeca- and tetradecasaccharide fractions, possibly due to impurities of larger fragments. All oligosaccharides tested, from dodecasaccharide upward, showed appreciable ability to accelerate the inactivation of Factor Xa by antithrombin, in agreement with previous findings (Holmer *et al.*, 1980, 1981; Oosta *et al.*, 1981; Choay *et al.*, 1983; Lane *et al.*, 1984) and with the suggestion that AT·H·proteinase complexes are of minor importance in this reaction. The moderate increase in the ability to accelerate the inactivation of Factor Xa observed in the present study for heparin chains longer than about 30 monosaccharide units does not necessarily contradict this suggestion, since the increase may at least partly reflect the increasing occurrence of two antithrombin-binding sites in these long polysaccharide chains (Rosenberg *et al.*, 1979; Jordan *et al.*, 1982).

The binding constants for the interaction between antithrombin and high-affinity heparin were found to be largely independent of the molecular size of the saccharide. This observation suggests that the antithrombin-heparin interaction is essentially restricted to the specific pentasaccharide binding region and that no additional interactions contributing appreciable binding energy thus occur with longer heparin chains (see also Lindahl *et al.*, 1984). It therefore appears likely that the minimal additional sequence of about 12 monosaccharide units required to obtain a significant acceleration of the antithrombin-thrombin reaction serves mainly to bind the thrombin molecule by nonspecific electrostatic interaction. The enzyme may also be captured by more extended saccharide sequences and then be transferred by unidimensional diffusion toward the antithrombin bound at the pentasaccharide segment (see Björk and Lindahl, 1982; Hoylaerts *et al.*, 1984). The efficiency of this process would be promoted by the increasing length of the polysaccharide chain, thus partly explaining the sharp molecular weight dependence of the ability of heparin to accelerate the inactivation of thrombin.[4] The subsequent reaction between enzyme and inhibitor presumably is further facilitated by the conformational change induced in antithrombin by binding to the pentasaccharide segment (Villanueva and Danishefsky, 1977; Nordenman and Björk, 1978a; Olsen *et al.*, 1981; Lindahl *et al.*, 1984).

The effects of heparin on the production of a modified form of antithrombin, cleaved at the reactive bond, followed essentially the same dependence on the size of the polysaccharide as the effects on proteinase inactivation. This behavior is consistent with ternary AT·H·proteinase complexes being similarly involved in the mechanisms of the two phenomena, *i.e.* playing a major role in the thrombin reactions but being of considerably less importance in the reactions with Factor Xa. Modified antithrombin presumably is formed by a reaction pathway which branches off from that leading to formation of the stable antithrombin-proteinase complex and in which antithrombin serves as a normal substrate for the proteinase (Björk and Fish, 1982; Olson, 1985). In the antithrombin-thrombin reaction, a heparin chain sufficiently long to bind both the inhibitor and the enzyme may remain bound in this ternary complex past the intermediate stage of the reaction at which the two pathways diverge. In this way, the heparin molecule may accelerate the formation of both the stable antithrombin-thrombin complex and modified antithrombin. However, the rate of the reaction branch leading to the modified inhibitor presumably is preferentially increased, and thus a higher proportion of antithrombin is distributed along this pathway. Both effects most likely depend on activation of the antithrombin molecule by the heparin-induced conformational change, in addition to binding of thrombin to the heparin chain. In the Factor Xa reaction, however, presumably neither the acceleration of inhibitor-enzyme complex formation nor the increase in the production of modified antithrombin requires that AT·H·proteinase complexes are formed; instead both effects can be ascribed

[4] In addition it should be noted that the antithrombin-binding region presumably is randomly located in the high-affinity oligosaccharides obtained by deaminative cleavage (Lindahl *et al.*, 1984). For the octadecasaccharides, thus only the fraction of the molecules in which this region is maximally shifted toward one end should be able to bind both antithrombin and thrombin. The proportion of molecules with this ability should increase with increasing molecular size of the oligosaccharides.

only to the conformational change in antithrombin induced by the bound heparin.

REFERENCES

Björk, I., and Fish, W. W. (1982) *J. Biol. Chem.* **257,** 9487–9493

Björk, I., and Lindahl, U. (1982) *Mol. Cell. Biochem.* **48,** 161–182

Björk, I., Jackson, C. M., Jörnvall, H., Lavine, K. K., Nordling, K., and Salsgiver, W. J. (1982) *J. Biol. Chem.* **257,** 2406–2411

Carlström, A.-S., Lieden, K., and Björk, I. (1977) *Thromb. Res.* **11,** 785–797

Chase, T., and Shaw, E. (1970) *Methods Enzymol.* **19,** 20–27

Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B., and Gatti, G. (1983) *Biochem. Biophys. Res. Commun.* **116,** 492–499

Danielsson, Å., and Björk, I. (1981) *Biochem. J.* **193,** 427–433

Fish, W. W., Orre, K., and Björk, I. (1979) *FEBS Lett.* **98,** 103–106

Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11,** 4882–4891

Griffith, M. J. (1982) *J. Biol. Chem.* **257,** 7360–7365

Griffith, M. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 5460–5464

Holmer, E., Söderström, G., and Andersson, L.-O. (1979) *Eur. J. Biochem.* **93,** 1–5

Holmer, E., Lindahl, U., Bäckström, G., Thunberg, L., Sandberg, H., Söderström, G., and Andersson, L.-O. (1980) *Thromb. Res.* **18,** 861–869

Holmer, E., Kurachi, K., and Söderström, G. (1981) *Biochem. J.* **193,** 395–400

Höök, M., Björk, I., Hopwood, J., and Lindahl, U. (1976) *FEBS Lett.* **66,** 90–93

Höök, M., Riesenfeld, J., and Lindahl, U. (1982) *Anal. Biochem.* **119,** 236–245

Hoylaerts, M., Owen, W. G., and Collen, D. (1984) *J. Biol. Chem.* **259,** 5670–5677

Iverius, P.-H. (1971) *Biochem. J.* **124,** 677–683

Jackson, C. M., Johnson, T. F., and Hanahan, D. J. (1968) *Biochemistry* **7,** 4492–4505

Jesty, J., and Esnouf, M. P. (1973) *Biochem. J.* **131,** 791–799

Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) *J. Biol. Chem.* **255,** 10081–10090

Jordan, R. E., Favreau, L. V., Braswell, E. H., and Rosenberg, R. D. (1982) *J. Biol. Chem.* **257,** 400–406

Jörnvall, H., Fish, W. W., and Björk, I. (1979) *FEBS Lett.* **106,** 358–362

Kisiel, W., Hermodson, M. A., and Davie, E. W. (1976) *Biochemistry* **7,** 4506–4517

Lane, D. A., Denton, J., Flynn, A. M., Thunberg, L., and Lindahl, U. (1984) *Biochem. J.* **218,** 725–732

Laurent, T. C., Tengblad, A., Thunberg, L., Höök, M., and Lindahl, U. (1978) *Biochem. J.* **175,** 691–701

Lindahl, U., Thunberg, L., Bäckström, G., Riesenfeld, J., Nordling, K., and Björk, I. (1984) *J. Biol. Chem.* **259,** 12368–12376

Lundblad, R. L., Uhteg, L. C., Vogel, C. N., Kingdon, H. S., and Mann, K. G. (1975) *Biochem. Biophys. Res. Commun.* **66,** 482–489

Machovich, R., and Aranyi, P. (1978) *Biochem. J.* **173,** 869–875

Marciniak, E. (1981) *Br. J. Haematol.* **48,** 325–336

Marciniak, E. (1982) *Thromb. Res.* **28,** 411–421

Miller-Andersson, M., Borg, H., and Andersson, L.-O. (1974) *Thromb. Res.* **5,** 439–452

Nesheim, M. E. (1983) *J. Biol. Chem.* **258,** 14708–14717

Nordenman, B., and Björk, I. (1978a) *Biochemistry* **17,** 3339–3344

Nordenman, B., and Björk, I. (1978b) *Thromb. Res.* **12,** 755–765

Nordenman, B., Nyström, C., and Björk, I. (1977) *Eur. J. Biochem.* **78,** 195–203

Nordenman, B., Danielsson, Å., and Björk, I. (1978) *Eur. J. Biochem.* **90,** 1–6

Olson, S. T. (1985) *J. Biol. Chem.* **260,** 10153–10160

Olson, S. T., and Shore, J. D. (1982) *J. Biol. Chem.* **257,** 14891–14895

Olson, S. T., Srinivasan, K. R., Björk, I., and Shore, J. D. (1981) *J. Biol. Chem.* **256,** 11073–11079

Oosta, G. M., Gardner, W. T., Beeler, D. L., and Rosenberg, R. D. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 829–833

Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 594–605

Peterson, L. C., and Jörgensen, M. (1983) *Biochem. J.* **211,** 91–97

Petitou, M. (1984) *Nouv. Rev. Fr. Hematol.* **26,** 221–226

Pletcher, C. H., and Nelsestuen, G. L. (1983) *J. Biol. Chem.* **258,** 1086–1091

Pomeranz, M. W., and Owen, W. G. (1978) *Biochim. Biophys. Acta* **535,** 66–77

Rosenberg, R. D., and Damus, P. S. (1973) *J. Biol. Chem.* **248,** 6490–6505

Rosenberg, R. D., Jordan, R. E., Favreau, L. V., and Lam, L. H. (1979) *Biochem. Biophys. Res. Commun.* **86,** 1319–1324

Sonder, S. A., and Fenton, J. W., II (1984) *Biochemistry* **23,** 1818–1823

Thunberg, L., Bäckström, G., and Lindahl, U. (1982) *Carbohydr. Res.* **100,** 393–410

Villanueva, G. B., and Danishefsky, I. (1977) *Biochem. Biophys. Res. Commun.* **74,** 803–809

Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412

SUPPLEMENTARY MATERIAL TO

Role of Ternary Complexes, in which Heparin binds both Antithrombin and Proteinase, in the Acceleration of the Reactions between Antithrombin and Thrombin or Factor Xa

Åke Danielsson, Elke Raub, Ulf Lindahl and Ingemar Björk

MATERIALS AND METHODS

Bovine antithrombin was isolated by affinity chromatography on heparin-agarose (Miller-Andersson et al., 1974). Bovine α-thrombin was prepared from prethrombin 1 as described earlier (Owen et al., 1974; Lundblad et al., 1975; Carlström et al., 1977); the purified enzyme was 90% active by active-site titration (Chase and Shaw, 1970) and the specific activity was 2900 NIH units/mg. Bovine Factor X was a generous gift from Dr Johan Stenflo, Malmö Allmänna Sjukhus, Sweden. Factor Xa was prepared from Factor X by reaction with the activating enzyme from Russell's viper venom (Kisiel et al., 1976; Fujikawa et al., 1972; Jesty and Esnouf, 1973).

Thrombin was inactivated with methanesulfonyl fluoride as described by Sonder and Fenton (1984). The methanesulfonyl fluoride-treated enzyme (MS-thrombin; 2.5 mg) was applied to a heparin-Sepharose column (1 x 5 cm) in 0.05 M Tris-HCl buffer, 0.1 M NaCl, pH 7.4, and the column was eluted with a NaCl gradient. The peak which eluted at the same salt concentration as active thrombin, i.e. at 0.42 M NaCl (Nordenman and Björk, 1978b), constituted about 70% of the material and was used for further analyses. An aliquot was radiolabelled with $Na[^{125}I]$ with the use of the Enzymobead reagent (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The labelled thrombin had a specific radioactivity of 2.6×10^5 dpm/μg and eluted as unlabelled thrombin on rechromatography on heparin-Sepharose. It was mixed with an appropriate amount of unlabelled MS-thrombin before most experiments.

MS-Thrombin was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in the presence of low-affinity heparin (N-acetylated to eliminate any free amino groups; Höök et al., 1976), essentially as described earlier for the attachment of active thrombin to Sepharose (Nordenman and Björk, 1978b). The estimated MS-thrombin content of the gel was 0.8 mg/ml.

High-affinity heparin fractions with reduced polydispersity and different average molecular weights were prepared from porcine mucosal heparin (stage 14, Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A.) by gel chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and affinity chromatography on antithrombin-Sepharose, as described by Danielsson and Björk (1981). The average molecular weights of the fractions were determined by sedimentation equilibrium ultracentrifugation (Danielsson and Björk, 1981). A high-affinity heparin fraction with an average molecular weight of 8400 was radiolabelled by reduction with $Na[^3H]BH_4$ (Thunberg et al., 1982), with a resulting specific radioactivity of ~6000 dpm/μg.

Heparin fragments with high affinity for antithrombin were prepared by partial depolymerization of porcine mucosal heparin with nitrous acid (20 min reaction time at ~10°C), followed by chromatography on antithrombin-Sepharose (Thunberg et al., 1982). Fragments of defined lengths were then obtained by repeated chromatography on a calibrated Sephadex G-50 column (3 x 250 cm). All even-numbered oligosaccharides containing from 8 to 20 monosaccharide units gave separate peaks in this procedure (Lane et al., 1984). The molecular weights of the oligosaccharides were calculated as described previously (Lindahl et al., 1984).

For preparation of radiolabelled oligosaccharides, heparin was first N-deacetylated by hydrazinolysis at 100°C for 1 h and was then re-N-^{3}H-acetylated by treatment with [^{3}H]acetic anhydride (Höök et al., 1982). Labelled polysaccharide (10 mg) was depolymerized with nitrous acid, and oligosaccharides with high affinity for antithrombin were isolated and further fractionated by gel chromatography as described above. The specific radioactivity of the products was estimated to ~10^6 dpm/nmol of oligosaccharide.

Low-affinity heparin, obtained by chromatography on antithrombin-Sepharose (Laurent et al., 1978), was coupled to cyanogen bromide-activated Sepharose 4B by the method developed by Iverius (1971). The resulting heparin content was about 2.2 mg/ml gel.

The kinetics of the antithrombin-thrombin reaction in the presence of heparin were studied at 25°C in 0.05 M Tris-HCl buffer, 0.1 M NaCl, 1% poly(ethylene glycol), pH 7.4. Antithrombin (final concentration 194 nM) and heparin or heparin fragments of increasing concentrations were mixed in a cuvette, and thrombin (final concentration 20 nM) was added after 2 min. The total reaction volume was 2.3 ml. Residual thrombin activity was measured after an appropriate reaction time by addition of the chromogenic thrombin substrate D-phenylalanyl-L-pipecolyl-L-arginine p-nitroanilide (S-2238, KabiVitrum, Stockholm, Sweden) to a final concentration of 0.16 mM. The substrate solution contained polybrene (final concentration 0.8 mg/ml) in order to neutralize the heparin and thus to essentially quench further inhibition of thrombin. The increase in absorbance at 405 nm was recorded for >60 s. Five different antithrombin-thrombin reaction times, covering 0 to ~50% of the reaction, were evaluated for each heparin concentration. The initial rate of thrombin inactivation, expressed as mol thrombin inhibited/s, was obtained from the initial slopes of plots of thrombin activity vs. time in conjunction with the specific activity of thrombin determined separately.

The kinetics of the antithrombin-Factor Xa reaction in the presence of heparin were studied in a similar manner as the antithrombin-thrombin reaction. Antithrombin (final concentration 240 nM) was incubated for 2 min with heparin or heparin fragments of increasing concentrations, and Factor Xa was then added to a final concentration of 24 nM. After an appropriate reaction time, a portion of the solution was transferred to a cuvette containing the chromogenic Factor Xa substrate N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine p-nitroanilide (S-2222, KabiVitrum) and polybrene at final concentrations of 0.8 mM and 0.12 mg/ml, respectively. The increase in absorbance at 405 nm was then recorded. Other conditions were as in the analyses of the inactivation of thrombin.

The production of modified antithrombin by thrombin or Factor Xa in the presence of heparin was monitored by dodecyl sulphate/polyacrylamide gel electrophoresis under reducing conditions. Antithrombin (final concentration 15 μM) was incubated with different high-affinity heparin fractions or fragments (final concentration 3 μM) in 0.02 M sodium phosphate buffer, 0.11 M NaCl, pH 7.0, for 5 min at 25°C. Thrombin or Factor Xa was then added to a final concentration of 15 μM. After reaction times of 5 min for the antithrombin-thrombin reaction or 10 min for the reaction with Factor Xa, the samples were made 1% in sodium dodecyl sulphate and 1.3 M in 2-mercaptoethanol and were kept on a boiling water bath for 60 s. Gels were stained with Coomassie brilliant blue R 250 after electrophoresis (Weber and Osborn, 1969) and were scanned at 570 nm. The absorbance given by the large fragment of modified antithrombin was determined in per cent of the total absorbance of all bands on the gel, and the fraction of antithrombin converted to the modified form was calculated from this value (Björk and Fish, 1982).

Titrations of antithrombin with high-affinity heparin fractions for determination of binding constants were monitored by fluorescence in an SLM 4800S spectrofluorimeter (SLM Instruments Inc., Urbana, IL, U.S.A.). The procedure followed that of Nordenman et al., (1978), except that the analyses were made by successive additions of heparin to the same solution of antithrombin (100-200 nM) in 0.05 M Tris-HCl buffer, 0.26 M NaCl, pH 7.4 (I=0.3). The sample was stirred continuously during measurements. Binding constants were calculated as described earlier (Nordenman and Björk, 1978a).

The formation of ternary complexes, in which heparin binds both antithrombin and inactive thrombin (AT-H-P complexes), was studied by competition experiments, based on the ability of the antithrombin-heparin complex to prevent ^{125}I-labelled MS-thrombin from binding to matrix-linked low-affinity heparin. The experimental design is outlined in Fig. 5A. The radiolabelled, inactive enzyme (0.85 μM) was incubated with low-affinity heparin-Sepharose (in an amount corresponding to 3 μg of low affinity heparin), antithrombin (5.2 μM) and heparin fractions or fragments (3.8 μM) of different lengths. The amount of radioactivity in each incubation was ~5000 c.p.m. and the total volume was 0.3 ml. The buffer was 0.05 M Tris-HCl, 0.1 M NaCl, 1% poly(ethylene glycol), pH 7.4. The samples were rotated end-over-end at 22±2°C for 3 h. The tubes were then centrifuged, and the amount of radioactivity in the supernatant, proportional to the amount of ternary complex formed, was determined.

The formation of ternary AT-H-P complexes with inactive thrombin was also studied by affinity chromatography of heparin-antithrombin complexes on immobilized MS-thrombin in the manner outlined in Fig. 5B. A 200 μl sample, containing antithrombin (100 μM) and ^{3}H-labelled heparin or heparin oligosaccharides (25 μM, 10000 c.p.m.), was applied to a column (1.6 x 1.5 cm) of MS-thrombin-Sepharose at 4°C. The column was previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 3.5 μM antithrombin. Unbound material was eluted with ~25 ml of this buffer, and a gradient (10 + 10 ml) to 0.6 M NaCl in the antithrombin-containing buffer was then started. The flow rate was 5 ml/h, and 1-ml fractions were collected. A volume of 800 μl from each fraction was analysed by liquid scintillation counting. The proportion of bound radioactive material, reflecting the formation of ternary complexes, was estimated from the areas under the retarded and unretarded peaks. In control experiments, antithrombin was omitted from both sample and buffer.

Protein concentrations were measured spectrophotometrically at 280 nm. The specific absorption coefficients used for antithrombin, thrombin and Factor Xa were 0.67 (Nordenman et al., 1977), 1.75 (Fish et al., 1979) and 1.22 (Jackson et al., 1968) liter g^{-1} cm^{-1}, respectively.