Mechanism of the anticoagulant action of heparin

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Summary

The anticoagulant effect of heparin, a sulfated glycosaminoglycan produced by mast cells, requires the participation of the plasma protease inhibitor antithrombin, also called heparin cofactor. Antithrombin inhibits coagulation proteases by forming equimolar, stable complexes with the enzymes. The formation of these complexes involves the attack by the enzyme of a specific Arg-Ser bond in the carboxy-terminal region of the inhibitor. The complexes so formed are not dissociated by denaturing solvents, which indicates that a covalent bond may contribute to their stability. This bond may be an acyl bond between the active-site serine of the enzyme and the arginine of the cleaved reactive bond of the inhibitor. However, the native complexes dissociate slowly at near-neutral pH into free enzyme and a modified inhibitor, cleaved at the reactive bond. So, antithrombin apparently functions as a pseudo-substrate that traps the enzyme in a kinetically stable complex.

The reactions between antithrombin and coagulation proteases are slow in the absence of heparin. However, optimal amounts of heparin accelerate these reactions up to 2 000-fold, thereby efficiently preventing the formation of fibrin in blood. The accelerating effect, and thus the anticoagulant activity, is shown by only about one-third of the molecules in all heparin preparations, while the remaining molecules are almost inactive. The highly active molecules bind tightly to antithrombin, i.e. with a binding constant of slightly below 10^8 M^{-1} at physiological ionic strength, while the relatively inactive molecules bind about a thousand-fold more weakly. The binding of the high-affinity heparin to antithrombin is accompanied by a conformational change in the inhibitor that is detectable by spectroscopic and kinetic methods. This conformational change follows an initial, weak binding of heparin to antithrombin and causes the tight interaction between polysaccharide and inhibitor that is prerequisite to heparin anticoagulant activity. It has also been postulated that the conformational change leads to a more favourable exposure of the reactive site of antithrombin, thereby allowing the rapid interaction with the proteases.

Heparin also binds to the coagulation proteases. Recent studies indicate that this binding is weaker and less specific that the binding to antithrombin. Nevertheless, for some enzymes, thrombin, Factor IX_a and Factor XI_a, an interaction between heparin and the protease, in addition to that between the polysaccharide and antithrombin, apparently is involved in the accelerated inhibition of the enzymes. The effect of this interaction may be to approximate enzyme with inhibitor in an appropriate manner. However, the bulk of the evidence available indicates that binding of heparin to the protease alone cannot be responsible for the accelerating effect of the polysaccharide on the antithrombin-protease reaction.

Heparin acts as a catalyst in the antithrombin-protease reaction, i.e. it accelerates the reaction in non-stoichiometric amounts and is not consumed during the reaction. This ability can be explained by heparin being released from the antithrombin-protease complex for renewed binding to antithrombin, once the complex

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has been formed. Such a decreased affinity of heparin for the antithrombin complex, compared to the affinity for antithrombin alone, has been demonstrated.

The structure of the antithrombin-binding region in heparin has been investigated following the isolation of oligosaccharides with high affinity for antithrombin. The smallest such oligosaccharide, an octasaccharide, obtained after partial random depolymerization of heparin with nitrous acid, was found to contain a unique glucosamine-3-O-sulfate group, which could not be detected in other portions of the high affinity heparin molecule and which was absent in heparin with low affinity for antithrombin. The actual antithrombin-binding region within this octasaccharide molecule has been identified as a pentasaccharide sequence with the predominant structure: →N-acetyl-D-glucosamine(6-O-SO_3)→D-glucuronic acid→D-glucosamine(N-SO_3; 3,6-di-O-SO_3)→L-iduronic acid(2-O-SO_3)→D-glucosamine(N-SO_3; 6-O-SO_3)→. In addition to the 3-O-sulfate group, both N-sulfate groups as well as the 6-O-sulfate group on the N-acetylated glucosamine unit appear to be essential for the interaction with antithrombin. The remarkably constant structure of this sequence, as compared to other regions of the heparin molecule, suggests a strictly regulated mechanism of biosynthesis.

The ability of heparin to potentiate the inhibiton of blood coagulation by antithrombin generally decreases with decreasing molecular weight of the polysaccharide. However, individual coagulation enzymes differ markedly with regard to this molecular-weight dependence. Oligosaccharides in the extreme low-molecular weight range, i.e. octa- to dodecasaccharides, with high affinity for antithrombin have high anti-Factor X_a-activity but are virtually unable to potentiate the inhibition of thrombin. Furthermore, such oligosaccharides are ineffective in preventing experimentally induced venous thrombosis in rabbits. Slightly larger oligosaccharides, containing 16 to 18 monosaccharide residues, show significant anti-thrombin as well as antithrombotic activities, yet have little effect on overall blood coagulation. These findings indicate that the affinity of a heparin fragment for antithrombin is not in itself a measure of the ability to prevent venous thrombo-genesis, and that the anti-Factor X_a activity of heparin is only a partial expression of its therapeutic potential as an antithrombotic agent.

The biological role of the interaction between heparin and antithrombin is unclear. In addition to a possible function in the regulation of hemostasis, endogenous heparin may serve as a regulator of extravascular serine proteinases. Mouse peritoneal macrophages have been found to synthesize all the enzymes that constitute the extrinsic pathway of coagulation. Moreover, tissue thromboplastin is produced by these cells in response to a functional interaction with activated T-lymphocytes. The inhibition of this extravascular coagulation system by heparin, released from mast cells, may be potentially important in modulating inflammatory reactions.

Introduction

Heparin is a sulfated glycosaminoglycan that is widely distributed in mammalian tissues (1–6), and also has been reported to occur in certain invertebrates (7, 8). In the tissues it has been found, to date, only inside mast cells, where it is stored in basophilic granules (9–12). The heparin of these granules may be released from the mast cell during certain pathological conditions, such as anaphylactic shock (13). However, the normal occurrence of heparin extracellularly, e.g. in blood, has not been conclusively demonstrated (14). While the biological function of endogenous heparin remains obscure, the polysaccharide has pronounced effects in a number of biological systems that may or may not be related to its function. The most prominent and longest known of these is its anticoagulant activity in vitro (1, 2). This effect has led to the widespread use of heparin for prophylaxis and therapy of thrombosis in vivo.

The mechanism of the anticoagulant action of heparin has long been illusive. It was suggested over fifty years ago that heparin requires a cofactor in plasma for its action (15), and this was confirmed by later studies (16, 17). A close relation between the heparin cofactor and the plasma protease inhibitor antithrombin* was apparent in several subsequent investigations (19–21). However, it was not

* In a previously used nomenclature of possible physiological mechanisms for thrombin inhibition (18), the factor in plasma responsible for the progressive inactivation of the enzyme was termed antithrombin III. The effect was later shown to be exerted by a specific inhibitor, which inherited the designation antithrombin III. However, the roman numeral has ceased to fulfil a purpose and will not be used in this review.

until antithrombin was isolated in a pure form that the identity of the two was convincingly demonstrated (22). The purified protein thus showed both progressive antithrombin activity (i.e. a slow inhibition of thrombin in the absence of heparin) and heparin cofactor activity (i.e. a rapid inhibition of thrombin in the presence of heparin). This finding led to the general agreement that the major effect of heparin on blood coagulation requires the participation of antithrombin. Based on more detailed studies it was later suggested that heparin exerts its effects by binding to and changing the conformation of antithrombin, thereby greatly accelerating the normal slow rate with which the inhibitor inactivates thrombin (23). Although this hypothesis subsequently has been challenged and modified, it remains the foundation of our present understanding of how heparin affects blood coagulation.

The emphasis of this review will be on recent developments regarding the interactions of heparin with antithrombin and with coagulation proteases and the effect of these interactions on the inactivation of the proteases by the inhibitor. Other possible effects of heparin in blood coagulation and the binding of heparin to non-coagulation proteins will not be dealt with. The reader is referred to previous review articles by Rosenberg (24) and Barrowcliffe et al. (25) for thorough discussions of earlier work in the field. Also, a recent monograph on heparin (26) gives a wealth of information on most chemical and biological aspects of the polysaccharide.

The interacting molecular species – a presentation

This review concerns the interaction between three kinds of molecules: coagulation proteases; the protease inhibitor, antithrombin; and the polysaccharide, heparin. Before details of the concerted action of these molecules are presented, some background information relating to their basic structural and functional properties will be provided. In the case of heparin, for which the relationship between structure and function is highly complex, this information will include a brief description of the biosynthesis of the polysaccharide and its regulation.

Coagulation proteases

The clotting of blood can be effected by two partly different routes (Fig. 1). Contact of blood in vivo with subendothelium exposed by vascular damage (or with a foreign surface in vitro) initiates the intrinsic pathway, while release of tissue factor, or thromboplastin, from cells in the damaged tissue activates the extrinsic pathway. Either initiating event leads to a sequential activation of a number of serine proteases by limited proteolysis of inactive proenzymes present in plasma. Several of these activation reactions occur on the thrombocyte surface and require calcium ions and specific cofactors. The two pathways involve different enzymes in their early stages but converge at the level of Factor X_a. The final enzyme, thrombin, cleaves fibrinogen, resulting in formation of a fibrin gel. A detailed account of the various coagulation proteases lies outside the scope of this presentation; the reader is referred to a recent review by Jackson and Nemerson (27).

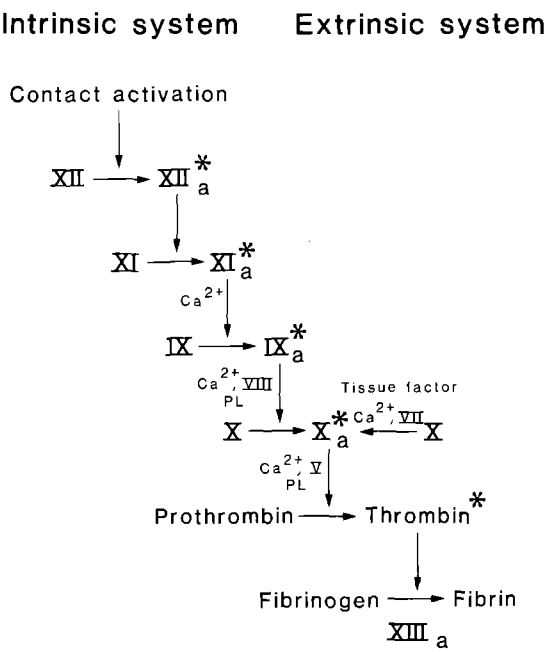

Fig. 1. An overview of the coagulation mechanism. The coagulation factors are represented by Roman numerals; a subscript denotes a proteolytically activated factor, and an asterisk indicates enzymes inactivated by antithrombin. PL, phospholipid.

Antithrombin

A prominent feature of the regulation of blood coagulation is the inactivation of activated coagulation factors by protease inhibitors in plasma. The most important of these is the specific coagulation inhibitor antithrombin (28–30), although certain general protease inhibitors, such as α_1-protease inhibitor and α_2-macroglobulin, may also inhibit coagulation enzymes, primarily thrombin (29). In addition, recent findings suggest the occurrence in plasma of a second specific inhibitor of coagulation proteases; while the action of this inhibitor resembles antithrombin in some respects, it requires higher heparin concentrations to become activated (31) (E. Holmer, personal communication). Antithrombin inactivates not only thrombin, as the name implies, but all the activated factors of the intrinsic coagulation system, i.e. also Factors XII_a, XI_a, IX_a, and X_a (22, 23, 32–36), and also some non-coagulation enzymes, such as plasmin and trypsin (37, 38). In contrast, Factor VII_a of the extrinsic system is unaffected by antithrombin (39). The inactivation of all the susceptible coagulation proteases is slow when heparin is absent but is greatly accelerated in the presence of the polysaccharide. Although detailed information on the role of antithrombin in the regulation of blood coagulation is lacking, evidence indicates that one of the physiological functions of the inhibitor may be to act as a scavenger of activated coagulation enzymes that have escaped into the circulation from their normal site of action and therefore are potentially harmful. Antithrombin thus does not inactivate Factor X_a, neither in the presence nor in the absence of heparin, when the protease is bound to the thrombocyte surface, as it is during normal blood clotting (40, 41).

Antithrombin can easily be isolated in a homogeneous form by a procedure based on affinity chromatography on heparin-agarose (42). The antithrombins of several species are highly similar; the inhibitor is a slightly assymmetric, single-chain glycoprotein with a molecular weight of about 55 000 (42–46). The concentration of antithrombin in human plasma is 125–200 mg/l (25, 47, 48). The sequence of human antithrombin is almost completely known; the protein has about 430 amino acid recidues, an amino-terminal histidine, a carboxy-terminal lysine, three intra-chain disulfide loops and four carbohydrate attachment sites (49). The structure of the identical carbohydrate units has been elucidated (50, 51). The sequence of antithrombin is homologous to those of α_1-protease inhibitor and ovalbumin (49, 52, 53). However, the biological significance of the homology with the latter protein is not yet obvious.

Heparin

The structure of heparin, summarized in Fig. 2, is that of a sulfated glycosaminoglycan consisting of alternating hexuronic acid (D-glucuronic or L-iduronic) and D-glucosamine residues (for reviews, see refs. 54–56). The polymer is heavily sulfated, carrying an N-sulfate group at C-2 of the glucosamine units as well as O-sulfate groups in various positions. However, the sulfation pattern is variable, as is the distribution of hexuronic acid units, resulting in marked structural microheterogeneity. This variability is at least partly non-random, in consequence of the regulatory mechanisms operating during heparin biosynthesis. The simplest way to provide some insight into the structural rules that apply to heparin-like polysaccharides is to briefly review the process of heparin biosynthesis (for more detailed, recent accounts, see refs. 56–60).

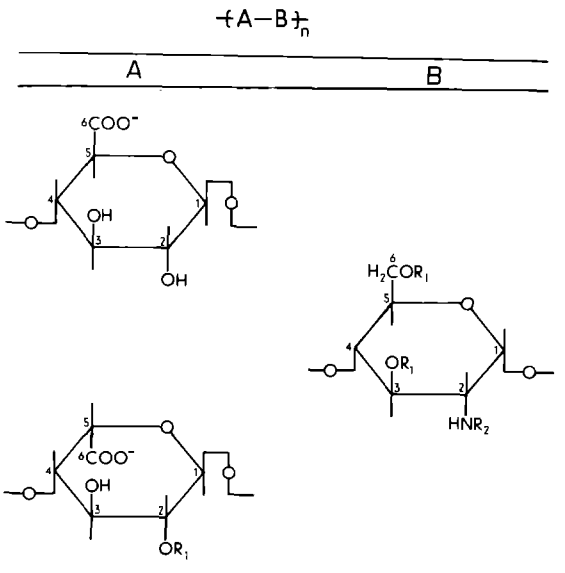

Fig. 2. Structure of heparin. The polysaccharide chain is composed of alternating A- and B-units, where A is D-glucuronic acid (top) or its C5-epimer, L-iduronic acid, and B is D-glucosamine. $R_1 = -H$ or $-SO_3^-$; $R_2 = -SO_3^-$ or $-COCH_3$.

Heparin is synthesized as a proteoglycan composed of several polysaccharide chains linked to a peptide core of alternating serine and glycine residues (61). The molecular weights of the individual polysaccharide chains range from 60 000 to 100 000 and thus by far exceed that of commercially available heparin (5 000 to 25 000). The initial polymerization product has a simple structure, the polysaccharide chains consisting of glucuronic acid and N-acetylglucosamine units in alternating sequence. The resulting nonsulfated proteoglycan is subsequently carried through a series of polymer-modification reactions which take place in a rapid and ordered, sequential manner leading to the formation of a number of distinct intermediate species. These reactions, mentioned in the order of occurrence during the biosynthetic process, include deacetylation of N-acetylglucosamine residues, N-sulfation of the resulting free amino groups, conversion of D-glucuronic acid into L-iduronic acid units by epimerization at C-5, O-sulfation at C-2 of iduronic acid residues, and finally O-sulfation at C-6 of glucosamine units (Fig. 3; 3-O-sulfation of glucosamine residues has not been studied).

The reaction sequence illustrated in Fig. 3 implies that a polymer composed of exclusively trisulfated disaccharide units is formed. However, as pointed out above, heparin has a heterogeneous structure. This heterogeneity is due to incomplete polymer Modification; only a fraction of the total disaccharide units is involved in each reaction. Studies of the substrate specificities of the corresponding enzymes indicated that the selection of target units is, at least in part, a nonrandom process. The introduction of N-sulfate groups thus has a directory effect on the subsequent polymer-modification reactions, since both uronosyl C-5-epimerization (and consequently 2-O-sulfation of iduronic acid) and 6-O-sulfation of glucosamine units occur, preferentially or exclusively, in the N-sulfated regions of the polysaccharide. The initial modification reaction, deacetylation of N-acetylglucosamine residues, is prerequisite to N-sulfation and, therefore, has a key role in the regulation of the overall modification process. In the final product, the N-sulfated regions are abundant in iduronic acid and O-sulfate groups, whereas the N-acetylated regions remain essentially unmodified (Fig. 4). The mechanism regulating the extent and distribution of N-deacetylation is at present unknown.

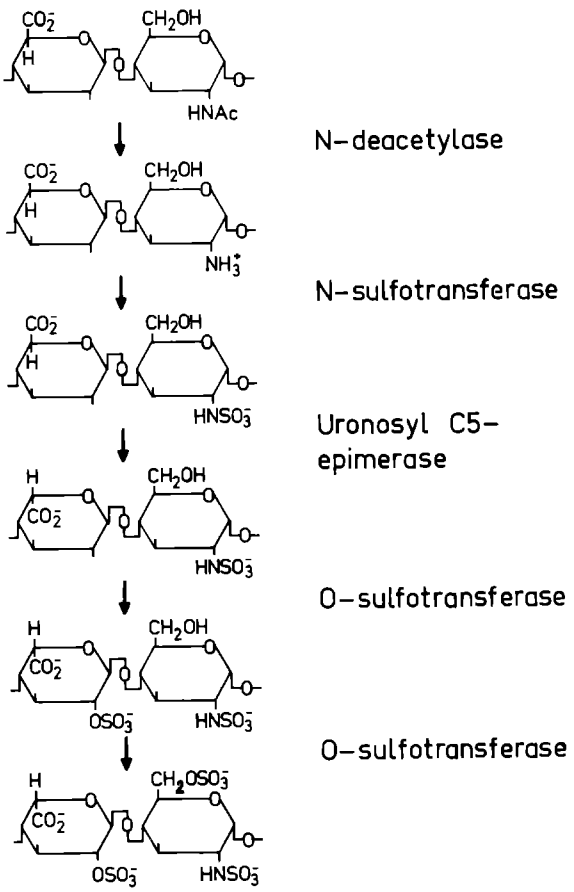

Fig. 3. Sequence of polymer-modification reactions involved in the biosynthesis of heparin. The 3-O-sulfation of D-glucosamine residues has not been included (see the text).

As mentioned above, the polysaccharide chains in heparin proteoglycans are larger than the molecules recovered in conventional heparin preparations. Various tissues have been shown to contain endoglycosidases capable of depolymerising the proteoglycans to fragments similar in molecular size to commercially available heparin (62–66). One of these enzymes, an endo-β-D-glucuronidase (64), thus degrades the newly synthesized proteoglycan, in the mast cell, to products that are stored in the basophilic granules of the cell (67).

Inhibition of coagulation proteases by antithrombin in the absence of heparin

Antithrombin inhibits serine proteases by forming equimolar, stable complexes with the enzymes

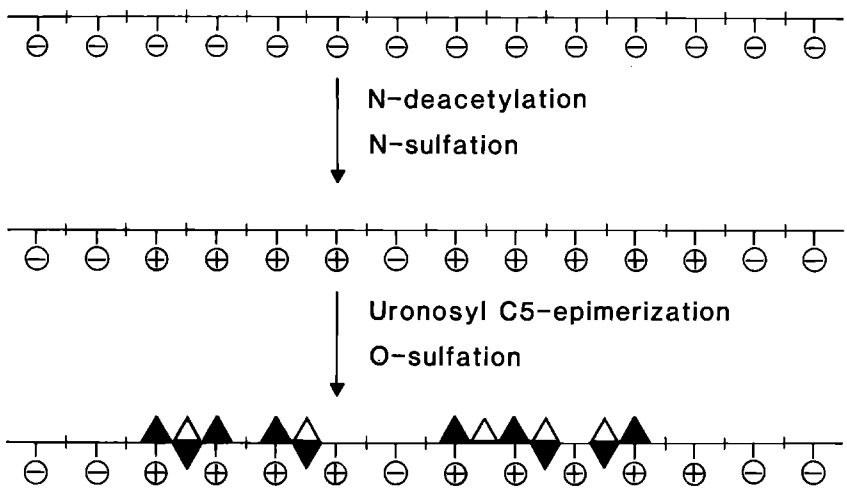

Fig. 4. Schematic representation of the polymer-modification process concluding the biosynthesis of a heparin-like polysaccharide. The process is incomplete in the sense that every disaccharide unit is not involved in each modification reaction. The scheme displays the directory effect of incorporated N-sulfate groups on subsequent modification reactions; L-iduronic acid units and O-sulfate groups will be preferentially located in the N-sulfated regions of the polysaccharide chain. The upper sequence illustrates the initial polymerization product, consisting of alternating D-glucuronic acid and N-acetyl-D-glucosamine units. The symbols are: —|—, D-glucuronic acid; ⊖, N-acetylated D-glucosamine; ⊕, N-sulfated D-glucosamine; △, L-iduronic acid; ◆, 2-O-sulfated L-iduronic acid; ▲, 6-O-sulfated D-glucosamine. 3-O-sulfated D-glucosamine units (see the text) have not been included in the structure. Due to the large number of different structural permutations included, the proportion of trisulfated disaccharide units in the final product is too low for a typical heparin.

(23, 33, 34–38). The kinetics of association of bovine antithrombin with two coagulation proteases, Factor X_a and thrombin, have been studied; both reactions are characterized by second-order kinetics, with rate constants at pH 7.5, 37 °C of 3.9×10^3 and 1.4×10^4 M^{-1} s^{-1}, respectively (39, 68). The two reactions have been suggested to lead to the formation of initial, reversible enzyme-inhibitor complexes with association constants at pH 7.5, 37 °C of 2×10^9 M^{-1} and 8×10^9 M^{-1}, respectively. The inhibition of the non-coagulation enzyme trypsin also follows second-order kinetics with a rate constant of about 2×10^5 M^{-1} s^{-1} at pH 7.4, 25 °C (69, 70). This reaction is thus appreciably faster than the reaction of antithrombin with either of the coagulation enzymes. However, heparin only insignificantly accelerates the antithrombin-trypsin reaction.

Circular dichroism analyses have suggested that the binding of antithrombin to thrombin is accompanied by conformational changes of either the enzyme or the inhibitor moiety of the complex, or of both (71). This may be a general aspect of the interaction of antithrombin with all its target enzymes.

The antithrombin-protease complexes are stable in 1% sodium dodecyl sulfate or 6 M guanidine hydrochloride (23, 35, 72) but can be dissociated by nucleophiles or at extreme pH values (72–76). This indicates that a covalent bond may contribute to the stabilization of the complexes. The products of dissociation of the complexes between the inhibitor and Factor IX_a, Factor X_a and thrombin have been analysed. The dissociation produces intact protease, but no intact antithrombin; instead the form of the inhibitor that is released from these complexes is proteolytically modifed (73–77). The modified forms of antithrombin isolated from the three complexes are all cleaved at a single, identical site, near the carboxy-terminus of the chain. The cleavage is between Arg-385 and Ser-386 in human antithrombin, the sequence of which is known (Fig. 5); an identical site at a homologous position is cleaved in the bovine protein (76–78). Available evidence indicates that this bond is the active-site bond of antithrombin, i.e. the functional site for interaction with proteases. A major part of this evidence is that three enzymes with different specificities give rise to modified inhibitors cleaved at the same site, i.e. non-specific cleavage is unlikely. Moreover, the amino-acid sequence around the cleavage site in

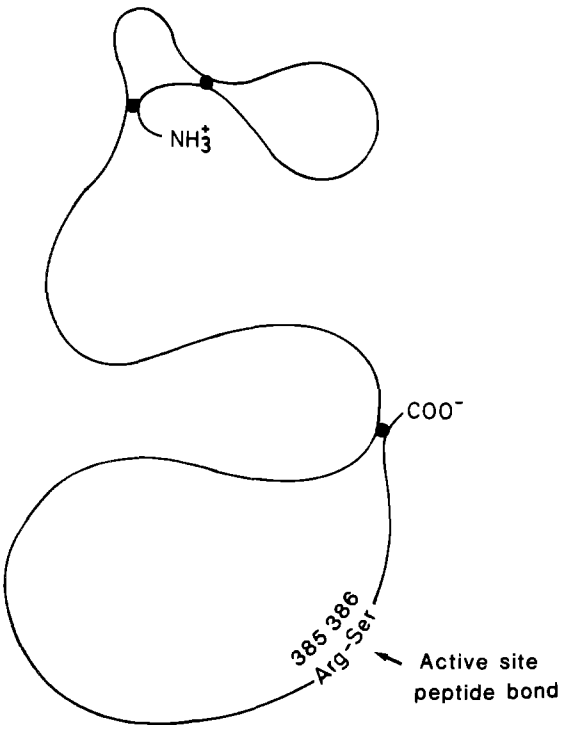

Fig. 5. The active-site bond of antithrombin. The length of the solid line is proportional to the number of amino acids in the different segments of the polypeptide chain of the inhibitor. Filled circles denote disulfide bonds.

antithrombin is homologous to that around the proposed active-site bond in α_1-proteinase inhibitor (52). Also, the participation of an arginine at the active site is in agreement with chemical modification studies (23) and with the demostration that an arginine can be released by carboxypeptidase B digestion of antithrombin-thrombin complex that has been dissociated with methoxyamine hydrochloride (75).

In spite of their stability in denaturing solvents, the complexes of antithrombin with proteases dissociate spontaneously, albeit slowly, in their native state, as inferred from studies of the antithrombin-thrombin complex. The first-order rate constant for the dissociation of this complex was determined to be $1.4 \times 10^{-6}\,s^{-1}$ at pH 7.5, 37 °C from the rate of appearance of active thrombin from the complex (68). Thus, the half life of the complex is about 5.7 days. The dissociation rate increases markedly at pH values higher than 8.5 (79). Besides active thrombin, the dissociation produces the same modified form of antithrombin as that which can be released from the complex by nucleophilic agents (80). Although it has been suggested that intact, active antithrombin dissociates from the complex at neutral pH (79), this has not been verified (80) (Danielsson, Å. and Björk, I., unpublished results). These findings show that the complex is not thermodynamically, but only kinetically stable and slowly decomposes to free enzyme and proteolytically modified inhibitor.

The most likely interpretation of all these observations is that antithrombin acts essentially as a substrate for the proteases it inhibits, but as a substrate with certain unique properties. Thus, it has an unusually high affinity for the enzyme in the initial enzyme-substrate complex (a low K_M) and, what is more important, an extremely low turnover rate (a low k_{cat}), i.e. it is cleaved only very slowly. The reaction most likely proceeds via the same type of intermediates that occur in normal proteolysis by serine proteases (81, 82), but one of the reaction steps is extremely slow, due to the specific conformation around the active site of the inhibitor. This rate-limiting step has not yet been conclusively identified, but some circumstantial evidence indicates that it may be the deacylation step (75, 76, 83). The covalent bond that aids in stabilization of the antithrombin-protease complexes thus may be an acyl bond between the arginine of the cleaved active-site bond of the inhibitor and the reactive serine of the protease, as exemplified for the antithrombin-thrombin complex in Fig. 6. A possible reason for the hydrolysis of this bond being slow could be that the interaction between antithrombin and pro-

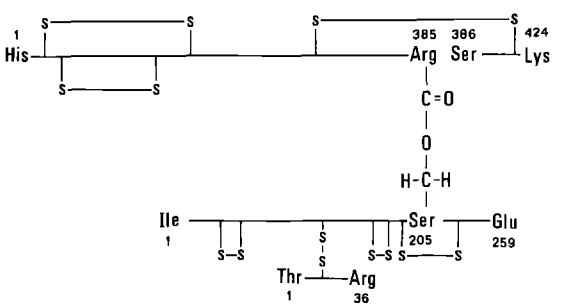

Fig. 6. The covalent acyl bond proposed to stabilize the complex between antithrombin (top) and thrombin (bottom). The amino- and carboxyterminal amino acids of the different polypeptide chains are indicated, as are the active serine of thrombin (in position 205) and all disulfide bonds.

tease is so tight, even after formation of the first proteolytic product (i.e. the small fragment of the modified inhibitor) by scission of the active-site bond of antithrombin, that water is efficiently excluded. A hydrophobic hexapeptide amino-terminal to the active-site bond of antithrombin has been suggested to contribute to the non-covalent stabilization of the complex between the inhibitor and thrombin by interacting with a complementary hydrophobic site of the enzyme (77).

A recently demonstrated feature of the reactions between antithrombin and thrombin or Factor X_a is that a non-complexed modified inhibitor, identical to the modified inhibitor that can be released from the antithrombin-protease complexes, is formed concurrent with these complexes (76, 84, 85). The mechanism which results in the production of this free modified antithrombin, cleaved at the active site, has not been fully elucidated. However, since the modified protein appears rapidly, it must be formed by a reaction that competes with the formation of the stable complexes and not by dissociation of these complexes. Markedly higher amounts of the modified antithrombin are produced when the antithrombin-protease reaction occurs in the presence of heparin, specifically the form of the polysaccharide that has high affinity for antithrombin (76, 85). This indicates that the binding of heparin to antithrombin increases the rate of the reaction by which the modified inhibitor is formed more than it increases the rate of complex formation. The modified antithrombin has no protease-inhibiting activity and a drastically reduced affinity for heparin (84, 85). The latter aspect suggests that the conformations of the active site and the heparin-binding site of antithrombin are intimately linked.

Binding of heparin to antithrombin and the effect of this binding on the conformation and activity of the inhibitor

Unfractionated heparin

Early studies of the interaction between heparin and purified antithrombin were done with commercial heparin, which was not fractionated further, except in some cases with regard to molecular weight. Rosenberg and coworkers in a series of papers showed that heparin greatly increases the rate of formation of the complexes between antithrombin and all the activated coagulation factors of the intrinsic system, without altering the stoichiometry and dissociability of these complexes (23, 33, 34, 36). Heparin was shown to bind to antithrombin, and it was postulated that this binding causes a conformational change of antithrombin which results in a more favourable exposure of the reactive site of the inhibitor, thereby allowing a rapid interaction with the proteases (23). Villanueva and Danishefsky (86) and Einarsson and Andersson (87) subsequently presented spectroscopic evidence consistent with a conformational change of antithrombin on heparin binding. However, small-angle X-ray scattering studies (45) showed that this binding does not appreciably change the size and shape of the inhibitor. Attempts to quantitatively study the binding to antithrombin of heparin not fractionated with regard to its affinity for the inhibitor (see below) have given results that are difficult to interpret due to the heterogeneity of the ligand (87–90).

Several studies with unfractionated heparin have established the concept that heparin acts as a catalyst in the reaction between antithrombin and thrombin (and presumably also other coagulation proteases), i.e. that it accelerates the reaction in non-stoichiometric amounts and is not consumed during the reaction (91–94). This ability can be explained by heparin being released from the antithrombin-thrombin complex for renewed binding to antithrombin, once the complex has been formed (Fig. 7). A decreased affinity of heparin for the antithrombin-thrombin complex, compared to the affinity for antithrombin alone, was, in fact, qualitatively demonstrated by Carlström et al. (95) and Andersson et al. (96). Jordan et al. (97) later presented quantitative evidence indicating that heparin binds to the antithrombin-thrombin complex about 100-fold more weakly than to antithrombin.

Heparin fractionated with respect to its affinity for antithrombin

A major breakthrough in the elucidation of heparin anticoagulant action was the demonstration by three different groups that commercial heparin can be fractionated into two distinct fractions differing markedly in their affinity for antithrombin

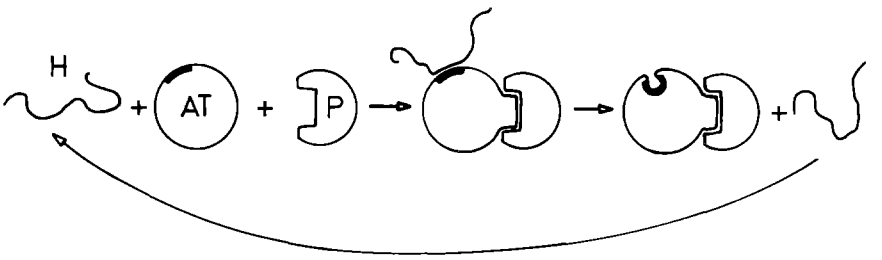

Fig. 7. A schematic illustration of the function of heparin as a catalyst in the antithrombin-protease reaction. After formation of the inactive complex, the affinity of the antithrombin moiety of the complex for heparin is decreased. H, heparin; AT, antithrombin; P, protease.

(98–100). This separation can be accomplished by sucrose density gradient centrifugation or gel chromatography of mixtures of heparin and antithrombin (97, 98) or, more conveniently, by affinity chromatography of heparin on matrix-linked antithrombin (Fig. 8), which allows separation of large amounts of material (99, 100). The three procedures separate all heparin preparations examined so far into a fraction with low affinity and a fraction with high affinity for antithrombin. The low affinity fraction comprises about two-thirds of the parent heparin preparation and has low anticoagulant activity, typically less than 20 B.P. units/mg, while the high affinity fraction comprises about one-third of the starting material and has high anticoagulant activity, typically about 300 B.P. units/mg. The high affinity heparin fraction accounts for about 90% of the anticoagulant activity of the unfractionated polysaccharide. The structural basis for the different affinities of the two fractions will be discussed in a later section.

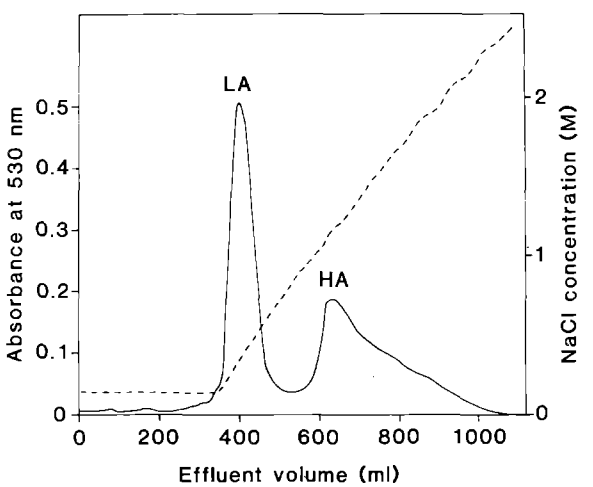

Fig. 8. Affinity chromatography of commercial heparin on a column of matrix-linked antithrombin. An amount of 50 mg of polysaccharide was separated on a 75-ml column. Elution was done with a linear salt gradient (.....) from 0.05 M to 3 M NaCl in 50 mM Tris-HCl, pH 7.4, and the effluent was analysed for uronic acid by the carbazole reaction (———). LA, low affinity heparin; HA, high affinity heparin.

Studies of the binding of separatd low affinity and high affinity heparin fractions to antithrombin proved to be highly informative. An initial report (87) indicated that a high-affinity fraction with a molecular weight of about 11 000 bound to antithrombin with a binding constant of about 2×10^6 M^{-1}. More comprehensive studies were done with preparations of the two heparin fractions that had molecular weights of about 15 000 (101–103). Both these fractions changed the absorption, near-ultraviolet circular dichroism and fluorescence emission spectra of antithrombin in a manner suggesting local perturbations of aromatic amino acids of the protein on heparin binding. However, the high affinity fraction caused much larger spectral changes than the low affinity fraction, indicating different modes of binding of the two fractions to the inhibitor. These findings are compatible with the concept that the highly active, high affinity fraction induces a conformational change of antithrombin that is related to the activation of the inhibitor. It cannot be completely excluded, however, that the spectral changes are due only to perturbations of surface chromophores without a conformational change. Quantitative studies showed that the low affinity and high affinity heparin fractions competed for the same binding site on antithrombin. They both bound to this site in an equimolar ratio to the inhibitor but with affinities differing by about a

thousand fold. The binding constant of the high affinity fraction at physiological ionic strength and pH thus was found to be about 8×10^7 M^{-1}, while that of the low affinity fraction was about 5×10^4 M^{-1}.

These findings were essentially confirmed by Jordan et al. (97) for low affinity and high affinity fractions with lower molecular weights, i.e. about 6 000, although somewhat lower binding constants (1×10^4 M^{-1} and 1×10^7 M^{-1}) were reported. These authors also studied the kinetics of the antithrombin-thrombin reaction in the presence of the high-affinity fraction and found that optimal amounts of this fraction increased the rate of thrombin inhibition about 2 000-fold. Heparin concentrations higher than those required to saturate the inhibitor decreased the rate below the maximum, possibly due to the formation of heparin-thrombin complexes.

The quantitative studies of the binding of high affinity heparin to antithrombin have been extended to fractions with molecular weights other than those originally studied. Rosenberg et al. (104) reported the isolation of a high affinity heparin species with a molecular weight of 20 000 that had two binding regions for antithrombin. In a more detailed study, Danielsson and Björk (105) investigated a number of high affinity fractions with molecular weights from 6 000 to 35 000. Most high affinity heparin species were found to bind to antithrombin with a stoichiometry of 1:1, regardless of molecular weight. Moreover, the minimum affinity for high affinity heparins of different molecular weights was shown to be about the same, viz. slightly below 10^8 M^{-1} at physiological pH and ionic strength. However, as the molecular weight increased, the high affinity fractions showed an increasing proportion of chains with two binding sites for antithrombin, confirming the results of Rosenberg et al. (104), and also with higher affinities. The highest binding constant demonstrated was about 3×10^8 M^{-1} at physiological ionic strength. In a recent paper (106), a highly active heparin fraction with a molecular weight of 35 000, apparently of the high affinity type but isolated by gel chromatography only, was shown to bind to antithrombin with a 1:1 stoichiometry and with a binding constant of about 7×10^7 M^{-1}.

The interaction between high-affinity heparin and antithrombin is highly dependent on ionic strength and pH (97, 107). The binding constant at an ionic strength of 0.5 thus is about 100-fold lower than under physiological conditions (107). The dependence of the binding constant on the activity of NaCl has been interpreted to indicate that maximally 5 to 6 charged groups on each molecule are directly involved in the interaction (107). The affinity of high-affinity heparin for antithrombin increases about 25-fold from pH 5.5 to 8.5 (97, 107). Outside these pH-values the binding constant decreases rapidly; the decrease in the acid region is due to an irreversible conformational change of the inhibitor (107).

Only a limited amount of information is available on the nature and localization of the heparin binding site in antithrombin. The ionic strength dependence of the binding shows that the interaction is mainly, or exclusively, electrostatic (97, 107). Chemical modification studies have demonstrated that some or all of the interacting charges on antithrombin are contributed by lysine residues (23). In addition, the spectroscopic changes observed in antithrombin on heparin binding indicate that one or more tryptophan residues are affected by the binding of the polysaccharide (86, 87, 97, 101, 102). This conclusion has now been amply verified by chemical modification studies (108–110). The modification of one specific tryptophan residue in antithrombin was shown to markedly decrease heparin binding, and heparin to some extent protected this residue from being modified. The tryptophan residue of importance thus probably is at or near the heparin binding site. However, it cannot be fully excluded that it is located elsewhere and only is affected by the heparin-induced conformational change of the protein. It is also known that heparin binding to antithrombin requires that the carboxy-terminal disulfide bridge of the protein is intact (111, 112).

As discussed previously, the spectroscopic changes observed on binding of high-affinity heparin are consistent with a conformational change of antithrombin, but do not prove conclusively that such a change occurs. However, recent work by Olson and Shore (113) and Olson et al (114) has provided more definite evidence for a change of the conformation of the protein on heparin binding. It was shown that the enhanced fluorescence of the antithrombin-heparin complex arises from an altered environment of buried tryptophan residues

and not merely from a perturbation of surface tryptophans without a conformational change. Moreover, in stopped-flow kinetic studies, the observed pseudo first order rate constant for the binding of high affinity heparin to antithrombin was found to vary hyperbolically with heparin concentration, approaching a limiting rate with a rate constant of about 440 s^{-1}. This indicates that heparin binding is a two-step process, involving an initial binding, followed by a conformational change. Evidence was presented consistent with a mechanism in which the conformational change in antithrombin is induced by the binding of heparin, rather than heparin perturbing an equilibrium between two conformational states of the protein. The observation of single pseudo first order binding rates over a large heparin concentration range with no detectable lags is compatible with the initial binding step being in a rapid equilibrium, for which an association constant of 2.3×10^4 M^{-1} at pH 7.4, 25 °C and an ionic strength of 0.15 was calculated. Comparison with the directly determined binding constant of 1.4×10^7 M^{-1}, and consideration of the errors involved in the analyses, suggests that a major function of the conformational change is to increase the affinity of heparin for antithrombin at least 300-fold and thus to cause the tight interaction required for heparin anticoagulant activity.

Isolation and characterization of the antithrombin-binding region in heparin

The findings that only a fraction of the molecules in heparin preparations have high affinity for antithrombin, and, furthermore, that only the high affinity molecules show significant anticoagulant activity, would seem to provide important clues to the relationship between polysaccharide structure and function. However, a direct structural comparison of the intact polysaccharide molecules failed to provide any conclusive information, showing merely a slight excess of N-acetylated glucosamine, glucuronic acid and nonsulfated iduronic acid in the high affinity relative to the low affinity species (115, 116). Continued attempts to define the structure responsible for the anticoagulant activity of heparin followed two somewhat different lines.

Rosenberg and coworkers searched for structural differences between heparin with high and with low affinity for antithrombin, respectively, by comparing the products obtained on exhaustive deaminative cleavage with nitrous acid (115, 117). A tetrasaccharide, iduronosyl → N-acetyl-glucosaminyl-(6-O-sulfate) → glucuronosyl → anhydromannose-(6-O-sulfate) (where the anhydromannose corresponds to an N-sulfated glucosamine unit in the intact polysaccharide chain), was obtained in significant quantity from the high affinity fraction. Since this tetrasaccharide was practically absent from the deamination products of the low affinity fraction, it was considered to represent the critical site responsible for anticoagulant activity.

In a different approach, Lindahl et al. subjected heparin to partial depolymerization, either by digestion with a bacterial heparinase (118) or by deamination with nitrous acid (116), followed by affinity chromatography of the products on immobilized antithrombin. High-affinity oligosaccharides obtained by the deamination technique were shown to contain as an integral component the tetrasaccharide sequence implicated by Rosenberg et al. The smallest such oligosaccharide obtained was an octasaccharide, in which the specific tetrasaccharide sequence occupied one-half of the molecule, with the nonsulfated iduronic acid unit at the nonreducing terminus (unit *1* in Fig. 9) (119). This finding suggested that the tetrasaccharide segment forms part of, but does not constitute the entire, antithrombin-binding region. High affinity oligosaccharides of similar molecular weight have also been isolated following partial enzymic depolymerization of heparin (120, 121), and, in low yield, from 50 kg(!) of a commercial heparin preparation, by fractionation procedures not involving depolymerization of the polysaccharide (122).

Three of the sugar residues of the tetrasaccharide sequence described above, viz. nonsulfated iduronic acid, N-acetylated glucosamine, and glucuronic acid, are known to be minor constituents of heparin. However, these components are unique neither to the antithrombin-binding region, nor to heparin molecules with high affinity for antithrombin. Furthermore, they are abundant in heparan sulfate, a polysaccharide structurally related to heparin but essentially devoid of anticoagulant activity. These considerations raised the question as to the occurrence in the antithrombin-binding sequence of a unique component, previously not recognized in heparin. A clue to the existence and nature of such a

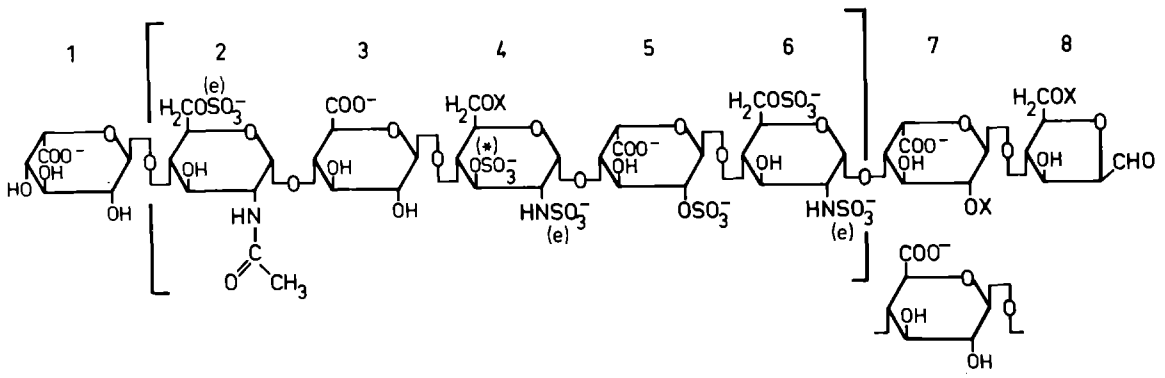

Fig. 9. Structure of the antithrombin-binding octasaccharide isolated after partial deaminative cleavage of heparin with nitrous acid. The 2,5-anhydro-D-mannose unit *8* corrresponds to a N-sulfated D-glucosamine residue in the intact polysaccharide. The actual binding region is represented by the pentasaccharide sequence (units *2–6*) within brackets. Structural variants are indicated by -X(-H or SO_3^-) or by the sugar residue (unit *7*) drawn below the main structure. The 3-O-sulfate group of unit *4* (marked by an asterisk) is unique to the antithrombin-binding region of the heparin molecule. In addition, each one of the sulfate groups indicated by (e) is essential to the high affinity binding of antithrombin. For further information, see the text.

component emerged through the demonstration by Leder of an enzyme in human urine capable of desulfating a 3-O-sulfated glucosaminide (123). 3-Desulfation occurred only provided that the glucosaminide was also N-sulfated, thus strongly suggesting that the natural substrate for the enzyme is a heparin-like polysaccharide. The occurrence of a 3-O-sulfate group in the antithrombin-binding sequence could indeed be demonstrated, by incubating the pentasaccharide corresponding to units *4–8* (isolated after cleavage of the octasaccharide by periodate-alkali treatment) with the 3-sulfatase (124). A sulfate group was released from a fraction of the pentasaccharide molecules, presumably from the nonreducing-terminal glucosamine residue corresponding to unit *4* of the intact octasaccharide sequence (Fig. 9). In addition, this glucosamine unit was found to be predominantly 6-O-sulfated, and the occurrence of a 3-O-sulfate group could therefore be verified by the isolation of a 3,6-di-O-sulfated anhydromannitol monosaccharide derivative. This compound was not obtained from heparin with low affinity for antithrombin, suggesting that the 3-O-sulfate group is indeed a unique component of the antithrombin-binding region. These conclusions have been confirmed by ^{13}C-nuclear-magnetic-resonance spectroscopy (121, 125).

Recent studies on the antithrombin-binding heparin octasaccharide have focused on attempts to evaluate the functional role of the various components of this molecule. While the detailed mode of interaction between the octasaccharide and antithrombin is unknown, some groups of functional significance, presumably involved in the binding to the protein, may be discerned; the information available at present has been included in Fig. 9. Selective, partial N-desulfation of the octasaccharide, followed by affinity chromatography, thus showed that the N-sulfate groups of units *4* and *6* are both required for high affinity binding (126). Furthermore, due to its unique distribution in the heparin molecule, the 3-O-sulfate group of unit *4* is assumed (but not yet demonstrated) to be essential for the interaction. Removal of the N-acetyl group from unit *2* by hydrazinolysis did not affect the affinity of the octasaccharide for antithrombin, suggesting that this substituent is not directly involved in the interaction (127). However, removal of the entire disaccharide corresponding to units *1* and *2*, by selective deamination of the N-deacetylated glucosamine residue, resulted in virtually complete loss of binding affinity. The critical component within this disaccharide segment was identified by stepwise degradation of the intact octasaccharide with exoenzymes (U. Lindahl, G. Bäckström and L. Thunberg, unpublished results). The heptasaccharide corresponding to units *2–8*, produced by the action of α-L-iduronidase, retained high affinity for antithrombin, showing, somewhat unexpectedly, that the nonsulfated iduronic acid unit *1* is not essential for binding. However, the product obtained on digesting this heptasaccharide

with N-acetylglucosamine 6-sulfatase lacked affinity for antithrombin, and it is therefore concluded that the 6-O-sulfate group of unit *2* is required for the interaction.

Additional information was obtained by identifying structural variants in the high-affinity octasaccharide (Fig. 9), the rationale being that only components invariably present in the molecule could be essential for binding of antithrombin. The lack of a 6-O-sulfate group at unit *4* in a fraction of the molecules thus argues against this particular substituent being required for the binding (124). Similar variability was displayed by the 6-O-sulfate group at the terminal unit *8*. Furthermore, in some octasaccharide species the 2-O-sulfated iduronic acid unit *7* was replaced by either nonsulfated iduronic acid or glucuronic acid (127). The extensive variability within the reducing-terminal disaccharide segment (units *7* and *8*) strongly suggests that this portion of the octasaccharide molecule falls outside the actual antithrombin-binding sequence. This conclusion is not contradicted by the observed lack of high-affinity hexasaccharide following partial depolymerization of heparin with nitrous acid (119), since formation of a hexasaccharide by deaminative cleavage of the glucosaminidic linkage between units *6* and *7* would entail loss not only of the nonessential units *7* and *8*, but also of the functionally important N-sulfate group of unit *6*. Indeed, Casu et al. recently showed that cleavage of this glucosaminidic bond by a bacterial heparinase, with retention of the N-sulfate group, was compatible with the formation of oligosaccharides having high affinity for antithrombin (121). These findings, in conjunction with the functional properties of the nonreducing-terminal disaccharide unit (see above), indicate that the antithrombin-binding site in heparin is represented by the pentasaccharide sequence extending from unit *2* to unit *6* of the structure shown in Fig. 9. The evaluation of the functions of the various groups in this sequence is still incomplete and should be extended to include the carboxyl groups of units *3* and *5*, as well as the O-sulfate groups of units *5* and *6*. The two latter sulfate groups appear to be invariably present in the binding sequence (119, 127). However, while structural variability for a given component may be regarded as evidence against a critical role in antithrombin binding, the inverse is not necessarily true for constant components (as shown, for example, by the nonsulfated iduronic acid unit *1*). It is recalled (see above) that the number of charged groups in the heparin molecule that are directly involved in binding to antithrombin has been estimated to at most 5 or 6 (107). The critical groups so far identified (Fig. 9) would already account for four such interaction sites.

Binding of heparin to coagulation proteases

Heparin also binds to proteases of the coagulation system, besides to antithrombin. These interactions have been more difficult to investigate, because, unlike the interaction with antithrombin, they give rise to only minimal changes of the spectroscopic properties of the proteins (71). Most studies have been done with thrombin. This enzyme has been found to bind to matrix-linked unfractionated heparin (128–131), from which it elutes at an ionic strength of about 0.5, i.e. at a lower ionic strength than antithrombin (42). A further difference to the antithrombin-heparin interaction is that all heparin molecules bind to thrombin with similar, although not identical, affinity, as shown qualitatively by several techniques (132–134). Specifically, heparin fractions with low and with high affinity for antithrombin bind similarly to thrombin. Nevertheless, it is possible to fractionate commercial heparin preparations on matrix-linked thrombin into a continuum of species differing somewhat in anticoagulant activity (135, 136). However, this separation shares several characteristics with the fractionation of heparin achieved by simple ion-exchange chromatography. Notably, the differences in anticoagulant activity are due primarily to different contents of heparin with low and with high affinity for antithrombin in the various fractions (136). This fractionation thus does not have a functional significance similar to that shown by affinity chromatography on immobilized antithrombin.

Early quantitative studies (137), done with the proflavine dye binding technique and with unfractionated heparin, suggested that 3–4 heparin molecules may bind to each thrombin molecule with an average binding constant of about $10^7\ M^{-1}$. Further estimates for the affinity of heparin for thrombin of about $6 \times 10^8\ M^{-1}$ (138) and about $10^9\ M^{-1}$ (139) have been obtained from kinetic analyses of the

effect of heparin on the hydrolysis of synthetic tripeptide thrombin substrates. However, these values are highly uncertain, as conflicting results have been reported regarding the effect of heparin on the thrombin-catalyzed cleavage of such substrates (132, 134, 138, 140–142). This discrepancy may be due partly to heparin reacting directly with the substrate (132). A similar value, about 1.5×10^8 M^{-1}, was derived from the inhibiting effect of active-site blocked thrombin on the antithrombin-thrombin reaction in the presence of heparin, but the analyses involved certain doubtful assumptions (143). The most reliable quantitative data for the interaction between heparin and coagulation enzymes to date have been obtained by Jordan et al. (144). These authors used fluorescence depolarization to study the binding to several proteases of fluorescently labelled heparin (M_r ~6 000) with high affinity for antithrombin. The analyses suggested that the heparin fraction used bound to thrombin with a stoichiometry of two heparin molecules per molecule of thrombin and with a binding constant for both sites of about 1.2×10^6 M^{-1}. In contrast, the binding to Factors X_a and IX_a occurred with a 1:1 stoichiometry and with binding constants of about 1.1×10^5 and 3.9×10^6 M^{-1}, respectively.

There are no data so far suggesting a conformational change of thrombin or any other coagulation enzyme on binding of heparin. Moreover, there is no evidence to indicate the existence of a specific protease-binding sequence in heparin. Thus, the binding of heparin to coagulation proteases may be due merely to a relatively non-specific electrostatic interaction.

Relative importance of the interactions of heparin with antithrombin and with coagulation proteases for heparin anticoagulant activity

The ability of heparin to bind both to antithrombin and to coagulation enzymes has given rise to three different hypotheses regarding the relative contributions of these interactions to the heparin-induced increased rate of inhibition of the enzymes by antithrombin (Fig. 10). The classical theory, already mentioned, suggests that heparin binds only to antithrombin, thereby inducing a conformational change in the inhibitor that makes the latter react more rapidly with its target proteases (23). Another theory, proposed originally for the antithrombin-thrombin reaction, is that heparin in an analogous fashion binds to and changes the conformation of the protease, so that this becomes more susceptible to inhibition by antithrombin (129, 145–147). A third hypothesis proposes that heparin accelerates the antithrombin-protease reac-

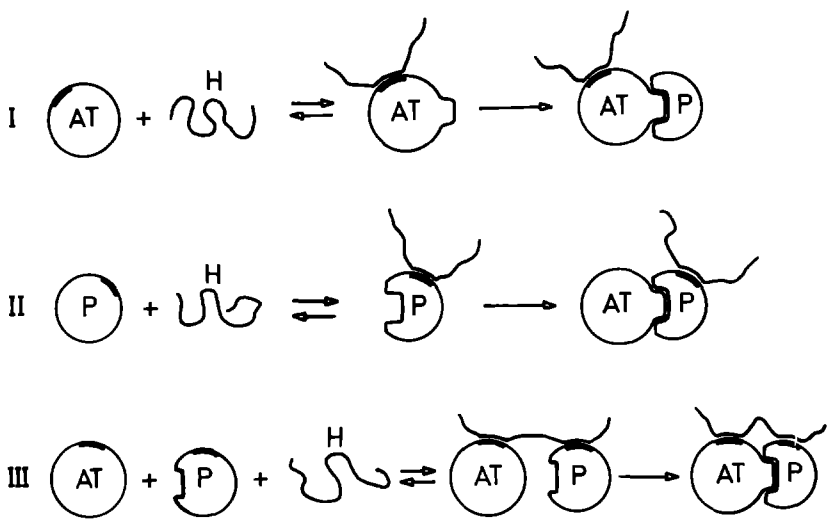

Fig. 10. Three hypotheses for the mechanism of the rate-enhancing effect of heparin on the reactions between antithrombin and proteases. I. Heparin binds only to antithrombin. II. Heparin binds only to the protease. III. Heparin binds to both antithrombin and protease. AT, antithrombin; P, protease; H, heparin.

tion by binding both reactants and bringing them together in a manner that allows them to react rapidly (133, 148–150). These three theories have been discussed extensively in recent years. A wealth of evidence, most of which has already been presented, suggests that the binding of heparin to antithrombin is essential for the accelerating effect of the polysaccharide. A major point is thus that there is a striking correlation between the anticoagulant activity of the low-affinity and high-affinity heparin fractions and the affinity of these fractions for antithrombin, while no corresponding correlation exists between affinity of heparin species for thrombin and activity. Moreover, the binding of heparin to antithrombin is tight, requires a specific monosaccharide sequence in the polysaccharide and induces a conformational change of the inhibitor, while the interaction between heparin and the proteases is weaker, less specific and apparently does not affect the conformation of the enzymes. There is also direct evidence from rapid kinetics studies that is inconsistent with a binding of heparin only to thrombin being responsible for the accelerating effect of heparin on thrombin inhibition (151). Premixing antithrombin with heparin thus accelerated the antithrombin-thrombin reaction markedly in stopped-flow experiments, while premixing thrombin with heparin was without effect. Other, more recent, kinetic experiments have also indicated that the major effect of heparin on the inhibition of proteases by antithrombin is exerted via the inhibitor (152). In these analyses, an excellent correlation was demonstrated between the initial velocities of inactivation of several proteases by antithrombin at varying heparin concentrations and the concentrations of the heparin-antithrombin complex. In contrast, no such correlation was noted between the inhibition rates and the concentrations of the heparin-protease complexes. The hypothesis that heparin accelerates the antithrombin-protease reaction by binding only to the protease therefore is extremely unlikely.

Although most evidence thus indicates that the binding of heparin to antithrombin is necessary for heparin action, data have accumulated suggesting that this binding alone may not be sufficient to fully increase the rate of inactivation of several of the coagulation proteases. Early indications that the binding of heparin to thrombin may be involved in the accelerated inhibition of this enzyme came from chemical modification studies (149, 153–155). Modification of arginine or lysine residues in thrombin thus was found to decrease the rate of inhibition of the enzyme by antithrombin in the presence of heparin but to have no effect on the rate in the absence of the polysaccharide. Pomerantz and Owen (149) also showed that antithrombin and thrombin can bind to the same heparin molecule, suggesting the possibility that such binding may be of importance for heparin action. Analyses of the molecular-weight dependence of the activity of high affinity heparin (presented in a separate section below) have provided additional evidence that the interaction of the polysaccharide with thrombin, and also with Factors IX_a and XI_a, is involved in increasing the rate of inactivation of these enzymes. This hypothesis has also received support from a comprehensive investigation of the kinetics of inhibition of several hemostatic proteases by antithrombin in the presence of high-affinity heparin (152). These studies indicated that the heparin-induced acceleration of the reactions between antithrombin and thrombin or Factor IX_a is to a limited extent dependent on heparin-enzyme interaction, while acceleration of the inhibition of Factor X_a or plasmin does not appear to involve such interactions. From analyses of the activity and binding to antithrombin of heparin oligosaccharides with increasing lengths, Oosta et al. (156) have suggested the existence of an enzyme-binding domain in heparin adjacent to the antithrombin-binding site (see also ref. 148). Interaction between this domain and the enzyme apparently is required for heparin-catalyzed inhibition of thrombin by anti-thrombin, but not for the corresponding inhibition of Factor X_a (156, 157).

All the observations presented in this section lead to the conclusion that the effect of heparin on the reactions between antithrombin and coagulation proteases is mediated primarily by interaction with the inhibitor. However, for some enzymes, thrombin, Factor IX_a and Factor XI_a, an additional interaction with the protease is involved in the reaction mechanism. The effect of this interaction may be to directly position the enzyme beside the inhibitor in a manner appropriate for optimal rate of the reaction. The interaction may also accelerate the antithrombin-protease reaction by allowing one-dimensional diffusion of the randomly and non-specifically bound protease along the heparin chain, so that rapid contact between the protease and the

antithrombin firmly bound to its specific site of the polysaccharide is established.

Relationship between the molecular weight, the anticoagulant activity and the antithrombotic activity of heparin

When polydisperse heparin preparations are fractionated according to molecular weight, the anticoagulant activities of the fractions generally increase with increasing degree of polymerization of the heparin (see ref. 148). This phenomenon was partly explained by Laurent and collaborators, who postulated on statistical grounds that the probability of finding a specific oligosaccharide sequence of defined length, such as the antithrombin-binding region, in a heparin molecule should be a function of the degree of polymerization of the polysaccharide (148). Heparin fractions of different molecular weights conformed to the predicted probability relationship, as judged by the proportion of molecules capable of binding to antithrombin. However, it was also recognized that this probability could not be the only determining factor, since the anticoagulant activities of heparin molecules selected by their high affinity for antithrombin (and thus containing at least one high-affinity binding site) were still dependent on molecular weight (148, 158). Even more complex relationships were suggested by the finding of Andersson et al. that this dependency varied with the method used to determine anticoagulant activity (100). The decrease in activity with decreasing molecular weight of the heparin, recorded in multiple-role clotting assays such as the APTT method, was thus not observed, or was markedly less pronounced (158), in measurements of Factor X_a inhibition. The implications of this observation were examined in more systematic studies (159) on the effects of heparin fractions, with high affinity for antithrombin and different molecular weights, on the antithrombin-mediated inhibition of individual coagulation enzymes. Two groups of enzymes were discerned, one, consisting of thrombin, Factor IX_a and Factor XI_a, that showed increasing inhibition with increasing molecular weight of the heparin, and another, consisting of Factor X_a, Factor XII_a and kallikrein, which was markedly less influenced by variations in the molecular weight of the polysaccharide. When such studies were extended to high affinity oligosaccharides, isolated after partial depolymerization of heparin by chemical or enzymatic methods, striking differences between different coagulation enzymes were observed (121, 156, 157). Oligosaccharides in the extreme low-molecular weight region, i.e. octa- to dodecasaccharides, were thus essentially unable to potentiate the inhibition of thrombin (however, see ref. 120), but showed Factor X_a-inhibiting activities as high, or higher, as that of intact high affinity heparin. With increasing molecular size the oligosaccharides were found to gain thrombin-inhibitory activity, which first appeared in a tetradecasaccharide fraction (156). However, a high affinity heparin fraction with a molecular weight of 5 600 (on the average 18 sugar residues per molecule) still showed only 45% the specific thrombin-inhibitory activity of the corresponding full-sized molecules (mol.wt. 10 000 to 25 000) (see Tables 1 and 2 in ref. 148). These results were interpreted in terms of the hypothesis, discussed in a previous section, that certain coagulation enzymes, such as thrombin, are inactivated by antithrombin with maximal efficiency only when the enzyme and the inhibitor are both simultaneously bound to the same heparin molecule. With such enzymes, only heparin molecules sufficiently large to accommodate both proteins show maximal activity. The inactivation of other enzymes, such as Factor X_a, is less dependent on actual binding of the proteinase molecule to the heparin chain. In the extreme case, represented by the octasaccharide shown in Fig. 9, the carbohydrate is almost completely occupied by the antithrombin molecule and is therefore unable to provide any attachment site for proteinases. This inability is of no major consequence to the inhibition of Factor X_a, whereas the inhibition of thrombin will be severly impeded.

The capacity of a heparin preparation to prevent thrombus formation has generally been associated with its overall anticoagulant activity, i.e. its ability to prolong clotting time in multiple-role clotting assays. However, parallel with the development of more specific coagulation tests this relationship has been qualified; it has thus been suggested that the antithrombotic property of heparin reflects its ability to potentiate the inhibition of Factor X_a (160–162) or other enzymes acting earlier in the coagulation cascade (159, 163). The availability of heparin preparations or derivatives with selective

anticoagulant activity has recently enabled a deeper insight into the mechanism of antithrombotic action. Heparin preparations of low molecular weight, having high anti-Factor X_a activity but moderately decreased overall anticoagulant activity, as compared to unfractionated heparin, efficiently prevented experimental serum-induced thrombosis in rabbits (164) (see also ref. 165), in accord with the postulated key role of Factor X_a. It was therefore unexpected to find that a heparin decasaccharide (mol. wt. ~3 000) with high affinity for antithrombin (differing from the structure shown in Fig. 9 by an additional disaccharide unit), and a specific anti-Factor X_a activity of 1 000 to 1 300 units/mg, was much less efficient than standard heparin in preventing experimental thrombosis (166). The effectiveness of heparin as an antithrombotic drug apparently does not depend solely on its capacity to activate antithrombin and inhibit Factor X_a. In contrast to the decasaccharide, a high affinity oligosaccharide fraction containing molecules composed of 16 or 18 sugar units showed antithrombotic activity approaching that of normal heparin, yet had considerably lower overall anticoagulant activity (D. P. Thomas, R. E. Merton, T. W. Barrowcliffe, L. Thunberg and U. Lindahl, unpublished results). Similar results were obtained by Holmer et al. (167), with oligosaccharides not fractionated with regard to their affinity for antithrombin. The antithrombotic effect of heparin thus appears to depend on the presence of an oligosaccharide sequence involving about 10 sugar residues, in addition to the region required to bind and activate antithrombin. The functional significance of this additional segment is unknown. It may reflect the need for inactivation of some coagulation enzyme other than Factor X_a by a mechanism involving a more extended saccharide sequence (see above). However, other possibilities are equally plausible. The antithrombotic action may be primarily aimed at events occurring at the cell surface of the vascular endothelium. Endothelial cells in culture have been shown to possess external binding sites for heparin-like polysaccharides (168). If binding to endothelial cells is indeed prerequisite to the antithrombotic effect of heparin, it would in all probability involve a portion of the polysaccharide molecule separate from the antithrombin-binding region. Oligosaccharides lacking such a component would be unable to simultaneously interact with both antithrombin and the endothelial cell surface.

Finally, a clinical aspect of anticoagulation may be briefly considered in relation to the heparin oligosaccharides. Treatment (or prevention) of thromboembolic disease with heparin is often hampered by complications due to bleeding, aggregation of thrombocytes, activation of lipases, skeletal demineralization, and difficulties in adjusting dosage to individual needs. Most of these problems can probably be ascribed to relatively nonspecific polyelectrolyte effects of the heparin molecule (55), or to specific, but generalized, effects on blood coagulation. The properties of the oligosaccharides found to be effective against experimental thrombosis offer novel prospects for an antithrombotic regime which lacks most of the drawbacks of conventional heparin therapy.

Biosynthesis of heparin with high affinity for antithrombin

The structure-function characteristics of the antithrombin-binding sequence add a new dimension to the problem of regulation in heparin biosynthesis. In contrast to the extensive microheterogeneity shown by the whole heparin molecule, the antithrombin-binding region has a remarkably constant, yet complex, structure. This region is thus composed of three non-repetitive disaccharide units (including the iduronic acid unit *1;* see Fig. 9) in fixed sequence, with at least four sulfate groups in constant positions (see p. 24–26). Moreover, one of the sulfate groups appears to be unique to this particular region. Which are the regulatory mechanisms required to achieve this degree of biosynthetic fidelity, so far unprecedented in glycosaminoglycans?

As outlined in a previous section, the polymer-modification reactions that conclude the formation of heparin take place in a strictly stepwise manner. Within the individual polysaccharide chain (or even the entire proteoglycan molecule) N-sulfation is thus completed before O-sulfation is initiated (169). Similar temporal separation apparently applies to the different O-sulfation reactions, 2-O-sulfation preceding the incorporation of 6-O-sulfate groups (58). Since the product of each reaction becomes the substrate in the subsequent reaction, the location of, for instance, a particular sulfate group will

be determined by the recognition properties of the corresponding sulfotransferase, as expressed in relation to previously introduced structural elements. The incorporation of 3-O-sulfate groups into heparin, yet to be demonstrated in a biosynthetic system, probably occurs late in the polymer-modification process. Since this unique substituent occupies a fixed position in relation to at least three other sulfate groups (one 6-O-sulfate group and two N-sulfate groups; see Fig. 9) the latter groups may be prerequisite to substrate recognition by the 3-O-sulfotransferase. Additional invariant structural elements of the antithrombin-binding sequence could similarly serve as biosynthetic signals, with specific regulatory (directory) functions in selected polymer-modification reactions. By providing such a signal a given sugar residue or substituent may prove essential to the formation of a functional binding site without being directly involved in the interaction with antithrombin.

An additional problem in this context is that of initiation: at which stage of polymer modification is a particular region of the heparin-precursor polysaccharide committed to conversion into a high affinity binding site? This question assumes particular importance if, as suggested by Horner and Young (66), the antithrombin-binding regions are not randomly distributed, but rather are concentrated to a limited fraction of the polysaccharide chains within a heparin proteoglycan molecule. These high affinity chains appear to have multiple binding sites for antithrombin. Without any information on the nature of the initiation mechanism, all that can be stated at present is that the critical modification reactions apparently take place in a nonrandom manner. Such order might conceivably be due to, for instance, the spatial distribution of the corresponding enzymes in the membranes of the endoplasmic reticulum of the cell. However, the organization of the biosynthetic apparatus is poorly understood and other more or less speculative possibilities appear equally plausible.

The problem of regulation may be extended to include the post-biosynthetic modification process. Is is recalled (see p. 165) that the heparin stored in mast-cell granules is generally a degradation product of the newly synthesized proteoglycan, formed by limited cleavage of the constituent polysaccharide chains by one or more endoglycosidases. The majority of the resulting high affinity heparin molecules contain a single antithrombin-binding region (105), which suggests that the sites of endoglycosidase attack are located between these regions. The substrate specificity of an endo-β-D-glucuronidase responsible for such post-biosynthetic modification (64, 67) was recently investigated in relation to the structure of the antithrombin-binding sequence in heparin. The high affinity octasaccharide described above (Fig. 9) was found to be resistant to the enzyme, as postulated, in spite of the presence of a β-glucuronidic linkage between units *3* and *4* (170). In contrast, incubation of the same octasaccharide with a β-glucuronidase derived from blood platelets, apparently designed to inactivate heparin, resulted in cleavage of the same linkage. The biosynthetic and post-biosynthetic events leading to the formation of biologically active heparin molecules seem to be under control of an intricate network of regulatory mechanisms, many aspect of which remain to be elucidated.

Biological significance of the interaction between heparin and antithrombin

Heparin interacts with a large number of macromolecules, mostly proteins, forming complexes of varying stability. Such interactions often affect the biological properties of the proteins, for instance, the activity of an enzyme, and have therefore been interpreted as expressions of the biological function of heparin (55). However, in most instances these interactions are nonspecific, reflecting only the marked polyelectrolyte character of the polysaccharide, and there is generally no evidence to indicate that they actually occur in vivo. The binding between heparin and antithrombin is exceptional, in that a defined region of the heparin molecule seems to be specifically tailored for this particular interaction. The specificity of the interaction strongly suggests, albeit on teleological grounds, that the binding of antithrombin reflects a true biological function of endogenous heparin. Because of the striking effects of the interaction on blood coagulation, this function has been tacitly assumed to involve the regulation of hemostasis. On the other hand, while such a function remains a realistic possibility, current information regarding the distribution and availability of heparin in the tissues point to alternative roles (as does indeed the

abundance of heparin with low affinity for antithrombin). The mast cells, in which heparin is synthesized and stored, thus are located outside the vascular system. Release of heparin from these cells occurs in special situations, not necessarily associated with deranged blood coagulation, such as allergic reactions or other inflammatory conditions.

A novel aspect of the functional role of heparin emerged with the recent finding that coagulation enzymes are produced and secreted by macrophages (171–173). Also these cells, which occur in association with mast cells in the tissues, have been ascribed various functions in inflammatory reactions. Mouse peritoneal macrophages were found to produce not only the proteinase precursors (Factor VII, Factor X and prothrombin) required to induce the deposition of fibrin by the extrinsic coagulation mechanism (Fig. 1), but also the auxiliary proteins (tissue thromboplastin and Factor V) that are prerequisite to activation of the enzymes. The production of thromboplastin, i.e. the trigger of the extrinsic pathway, depends on an interaction between the macrophages and activated T-lymphocytes (173–175), suggesting that the coagulation mechanism of the macrophage may be activated in response to an inflammatory process. The biological role of this coagulation system is unknown, but may relate to numerous observations of extra-vascular fibrin deposition in various inflammatory conditions. The system is susceptible to inhibition by heparin in the presence of antithrombin, as shown in recent experiments with Factor X_a produced by mouse peritoneal macrophages (176). As expected, heparin with high affinity for antithrombin greatly potentiated the inhibition of Factor X_a, whereas low affinity heparin was much less effective. A function for heparin as a modulator of the inflammatory response may thus be envisaged. Speculative as this proposal may seem at present, it should nevertheless provide a novel and potentially usefull approach to further studies on the biological role of this polysaccharide.

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