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Glycosaminoglycans: Synthetic fragments and their interaction with serine protease inhibitors

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ABSTRACT

Glycosaminoglycans such as e.g. heparin, heparan sulphate and dermatan sulphate display a broad variety of biological activities. Unique, well-defined domains in some glycosaminoglycans have been characterized that are responsible for the biological activity. For instance, a unique pentasaccharide domain in heparin could be identified which binds and activates the serine protease inhibitor (serpin) anti-thrombin III (AT III). The structure-activity relationships of various synthetic counter-parts of the heparin pentasaccharide fragment reveal the highly specific nature of the pentasaccharide mediated activation of AT III. With the aid of molecular modelling and the availability of crystal structures of serpins and their target proteases, the activation process of AT III by heparin becomes understood at the molecular level. Some attention will also be paid to well-defined domains in heparan sulphate and dermatan sulphate.

INTRODUCTION

Glycosaminoglycans comprise a family of complex anionic polysaccharides, including glucosaminoglycans (heparin, heparan-sulphate), galactosaminoglycans (chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate), hyaluronic acid, and keratan sulphate.
The chemically related polysaccharides heparin and heparan sulphate have been identified in numerous living organisms. (1,2) Heparin is the most well-known glycosaminoglycan because of its widespread use in anticoagulant therapy.
Thus, since 1936, heparin has been used in clinics for the prevention and treatment of thrombosis. Its main antithrombotic activity is explained by its ability to potentiate the activity of the serine protease inhibitor antithrombin III (AT-III), which inactivates a number of serine proteases in the coagulation cascade. Later, it was established that dermatan sulphate also displays antithrombotic activity. Its mode of action, however, is ascribed to the activation of a different serine protease inhibitor called heparin cofactor II (HC II).

An unique pentasaccharide domain in heparin
By the end of the 1970's heparin fragments (obtained by chemical or enzymatic degradation) had been isolated by affinity chromatography on immobilized AT-III and the high-affinity fractions had been analyzed. From these studies it was deduced that a unique pentasaccharide fragment, that occurs in about one-third of the heparin polysaccharide chains, constitutes the minimal binding domain for AT-III. The pentasaccharide fragment was synthesized a couple of years later to confirm the earlier proposal. The finding that a unique domain of heparin is associated with its biological activity contradicted the old dogma that the action of heparin is mainly due to its polyanionic character (the synthetic counterparts are compounds **1a** and **1b** in Fig. 1) (3).

The pentasaccharide fragment (**1a, b**) elicits a very selective antithrombotic mode of action, in that it catalyzes only the AT-III mediated inhibition of coagulation factor Xa but not that of thrombin (4).

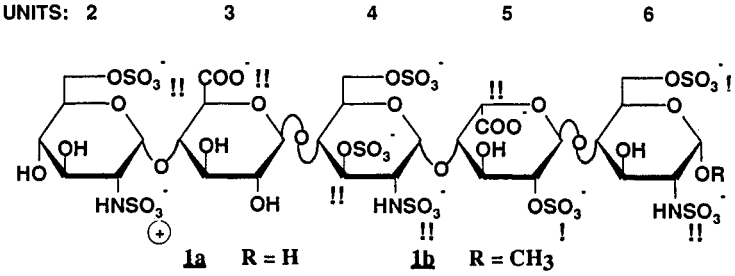

Figure 1. Unique AT III binding domain of heparin.

Other unique domains in glycosaminoglycans

There is good evidence that other well-defined domains exist in glycosaminoglycans. For instance heparan sulphate of the blood vessel wall reveals domains closely resembling the AT III binding domain of heparin (5) while oversulphated domains in dermatan sulphate are involved in the activation of HC II (vide infra). Presently the role of unique domains of glycosaminoglycans in cell adhesion and cell growth processes becomes also recognized.

A representative example is the recent elucidation of well defined domains in heparan sulphate that may interact either with bFGF, its receptor or with both (6).

STRUCTURE-ACTIVITY RELATIONSHIPS OF THE UNIQUE PENTASACCHARIDE DOMAIN OF HEPARIN

We felt that the interaction of the unique pentasaccharide domain of heparin with AT-III should be highly specific in nature since other polyanions (e.g. low-affinity heparin, synthetic polyanions, and other glycosaminoglycan fragments) at similar concentrations cannot substitute for the interaction of pentasaccharide with AT-III. The specificity of the interaction of the sulphated pentasaccharide with the protein was confirmed when several heparin pentasaccharide analogues were synthesized and tested for inhibition of blood coagulation factor Xa at both N.V. Organon and Sanofi (7). For instance, it has been elucidated which of the charged groups play an important role in the activation of AT-III (see Fig. 1). Some groups are strictly required for the activation of AT-III (denoted by !! in compound **1b**) in that the removal of one of these functions leads to at least 90% or complete loss of the anti-factor Xa (αXa) activity. Other groups (denoted ! in compound **1a**) contribute significantly during the AT-III activation process, since removal of one of these groups is accompanied by a serious decrease (70-80%) of αXa activity. It was found that essential sulphate groups cannot be replaced by phosphate groups (8). Analogues in which only one chiral center has been inverted (e.g. that of iduronic acid) were found to be inactive (7), while the presence of a rigid carbohydrate moiety (i.e. the glucuronic acid unit) may be as important as the presence of the essential charged groups (9).

Taking into account these structure-activity relationships and by contemplating molecular modelling (10) data we postulated a simplified heparin-AT-III interaction model (see Fig. 2) (11). On the basis of this model we introduced an extra sulphate group at position 3 of unit 6 of the naturally occurring fragment to give analogue **2** in Fig. 2 (11). This extra-sulphated analogue displays higher affinity towards AT-III and, an enhanced AT-III mediated αXa activity (1250 U/mg for **2** vs. 700 U/mg for **1a**).

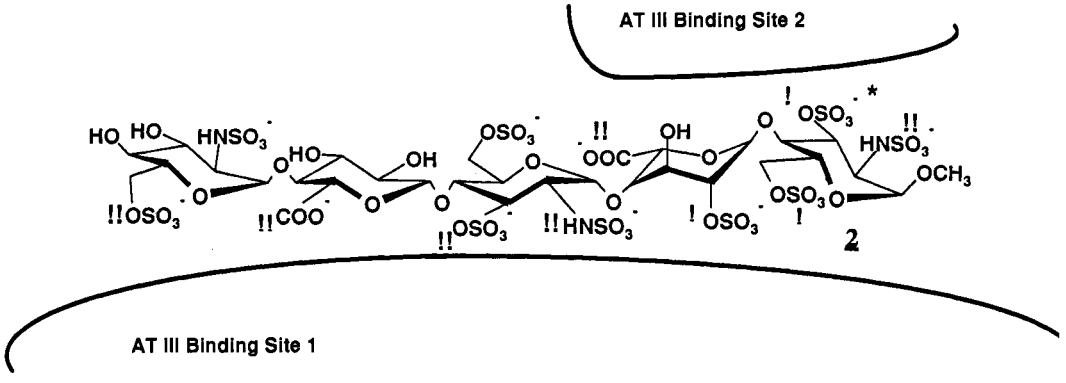

Figure 2. Model of the interaction of pentasaccharide with AT III.

"NON-GLYCOSAMINO" GLYCAN ANALOGUES OF THE HEPARIN PENTASACCHARIDE

When the essential, negatively charged groups of the heparin fragment were designated and compounds with high activity were obtained we turned our attention to a new simplified series in which all hydroxyl groups are methylated and in which all the N-sulphate groups are replaced by O-sulphate groups (e.g. pentasaccharide **3** in Fig. 3 (12)). Such compounds are called "non-glycosamino" glycan analogues. This approach was inspired by the observations that an analogue with an O-instead of N-sulphate group at the reducing end (13) and several analogues with O-methyl instead of hydroxyl groups were still biologically active (14). Actually the latter modification increased the biological activity (Compare αXa activity of **2** (1250 U/mg) with **3** (1320 U/mg) (12)). Apparently, neither the hydroxyl groups nor the $HNSO_3^-$ groups of the pentasaccharide are involved as hydrogen bond donors in the interaction process with AT-III. The latter observation is unique: in all carbohydrate-protein interactions studied so far at least a few hydrogen bridge donating "key-polar" hydroxyl groups could be identified (15). It should be noted that the synthesis of alkylated analogues is much easier than that of heparin-like fragments, which is a distinct advantage in economically feasible drug development.

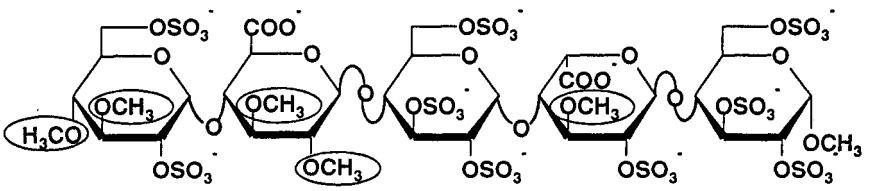

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Figure 3. A "non-glycosamino" glycan analogue of the heparin pentasaccharide.

Having the simplified derivative **3** at our disposal, we wondered whether even shorter synthetic routes could be devised towards this compound or closely related analogues. With this in mind we designed (16) analogue **4** (see Fig. 4), which has a semi-alternating sequence, that is, because of the 2-sulphate group at unit 3, the disaccharide fragments "3, 4" and "5, 6" have the same substitution pattern. The only remaining difference between the disaccharide fragments "3, 4" and "5, 6" of compound **4** is the opposite configuration at C5 of the uronic acid moieties.

Hence, a shorter synthetic route to **4** could be developed in which the disaccharide fragment "3, 4" is obtained from its counterpart "5, 6" by epimerization.

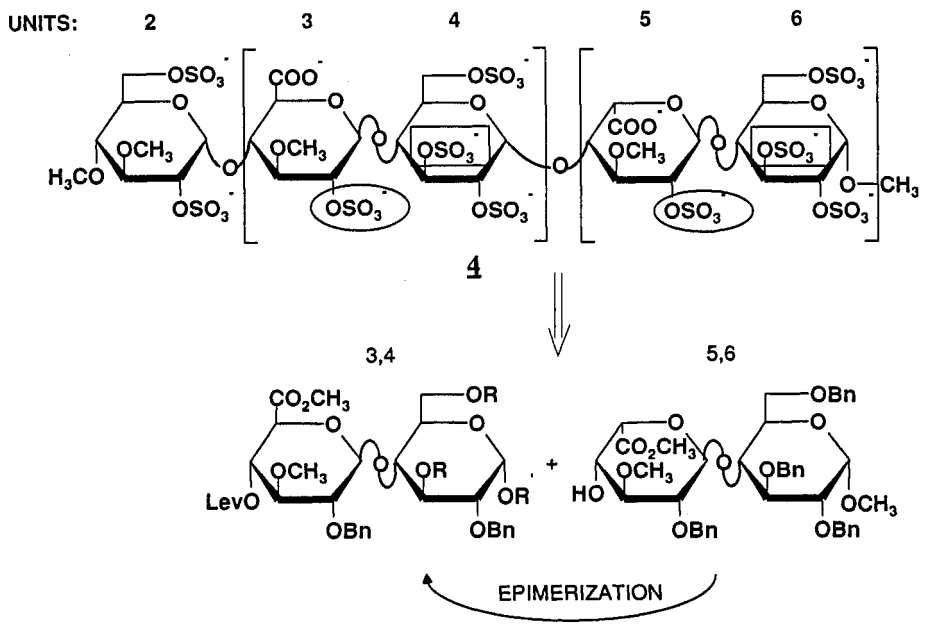

Figure 4. Retro synthetic strategy for compounds with semi-alternating sequence.

Analogue **4** displays an αXa activity of 1150 U mg^{-1} implying that the presence of a 2-sulphate instead of 2-O-methyl group at glucuronic acid (unit 3) reduces the activity slightly.
Following a similar synthetic route as described for pentasaccharide **4**, we prepared several analogues methylated at the 2-O and 3-O positions of both uronic acid moieties (i.e. units 3 and 5). At first sight it was expected that such analogues would loose at least half of their biological activity as was observed for counterparts in the series of heparin fragments lacking the 2-O-sulphate groups of iduronic acid. However, quite unexpectedly, the methylated analogue **5** (Fig. 5) turned out to be highly potent, displaying 1600 αXa U/mg (17).

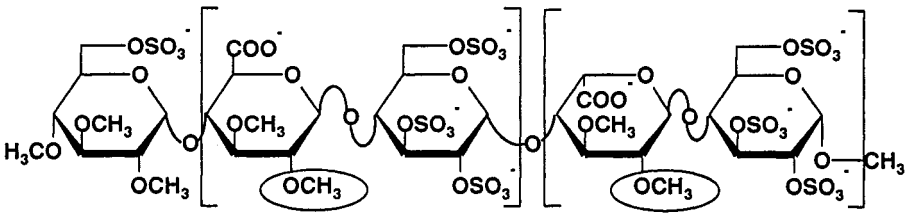

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Figure 5. A highly active per-O-methylated analogue.

The methyl groups at these positions may possibly interact even better with AT-III than the corresponding sulphates, implying that the loss of electrostatic interactions may be compensated by favorable hydrophobic or van der Waals interactions. Alternative explanations for this phenomenon may be found in a reduced free energy of dehydration of permethylated analogues with respect to nonalkylated derivatives, or by assuming a decreased dielectric constant in the pentasaccharide binding region of AT-III.

OTHER "NON-GLYCOSAMINO" GLYCAN ANALOGUES OF "UNIQUE DOMAINS"

i) Heparan Sulphate
It was also interesting to make alkylated pentasaccharides (and longer heparin-like molecules) with a "full" alternating sequence that resembles heparan sulphate sequences. In this respect it is noteworthy that an analogue of compound **2** in which ß-D-glucuronic acid has been replaced by α-L-iduronic acid-2-O-sulphate (i.e. **6** in Fig. 6) still elicts 115 αXa U/mg (7). The "non-glycosamino" glycan analogue, containing 2-O-methylated instead of 2-O-sulphated iduronic acid moieties was synthesized and showed 290 αXa U/mg. The activity of the latter two analogues suggests that heparan-sulphate (HS) from the glomerular basement membrane containing special alternating sequences (18) of the type [IdoA (2-O-SO_3-) α 1 → 4 GlcNSO_3 (3-O-SO_3^-) α1 → 4] may also activate AT-III.

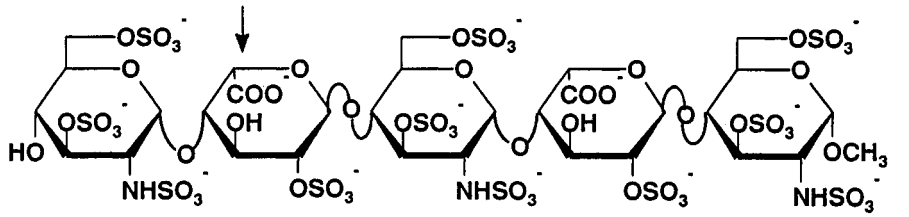

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Figure 6. A highly symmetric pentasaccharide fragment resembling heparan sulphate.

ii)Dermatan Sulphate "Non-glycosamino" glycan analogue
As it is known that dermatan sulphate (DS) catalyzes the inhibition of thrombin by heparin cofactor II (HC II, a member of the Serpin family that is homologous to AT III), it has been assumed that DS also contains a unique domain responsible for HC II binding and activation. Indeed, it was found recently that a heterogenous extra sulphated domain of DS containing a cluster of GalNAc (4-O-SO_3^-) → Ido (2-O-SO_3^-) disaccharide moieties is involved in the HC II activation process (19). For instance a hexasaccharide fragment comprising the latter disaccharide repeating unit elicits HC II affinity and

activation. This finding stimulated us to synthesize a number of "non-glycosamino" glycan analogs displaying the same sulphation pattern, such as the synthetic hexasaccharide 7 in Fig. 7 (20).
Indeed analogue 7 displayed HC II activating activity.

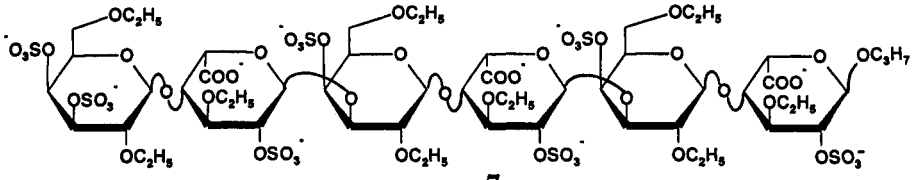

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Figure 7. A "non-glycosamino" glycan dermatan sulphate fragment.

THE COMPLEX OF GLYCOSAMINOGLYCANS WITH THEIR TARGETS

Another aspect in our research towards glycosaminoglycans is to study the interaction of the carbohydrate with its protein target at the molecular level.

AT-III pentasaccharide interaction

More detailed information about the interaction of the pentasaccharide with AT-III has been obtained after construction of a three-dimensional molecular model of the complex (21). First a molecular model of AT-III was built based on the crystal structure of cleaved α_1-antitrypsin (a member of the serpin superfamily to which AT-III also belongs). Then the positively charged amino acid residues of AT-III that have been identified to be essential in the interaction process (Lys 125, Arg 129, Arg 132, Lys 133, Lys 136, Arg 46, Arg 47) were studied. It appeared that these amino acids display an asymmetric assembly of interaction points complementary to the essential charged groups of the pentasaccharide and therefore the relative orientation of AT-III and pentasaccharide toward each other could be established unambiguously. The model was optimized by molecular dynamic calculations in which water was included explicitly.
Recently, we performed a similar study now using the crystal structure (22) of intact AT-III (see Figs. 8 and 9). Although the two interaction schemes resemble each other closely some binding characteristics differ significantly.

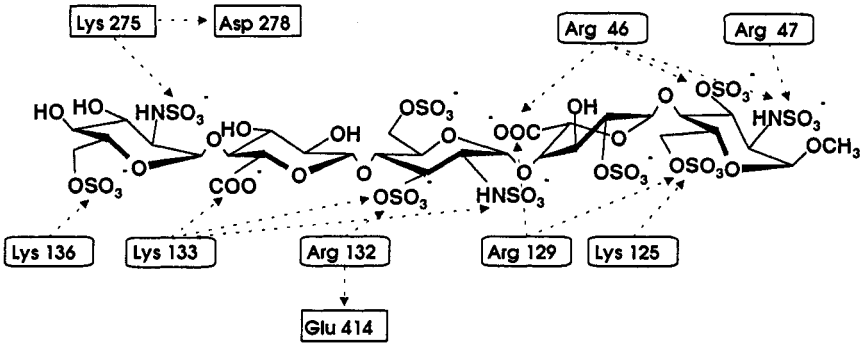

Figure 8. Schematic overview of interaction of compound 2 with AT III.

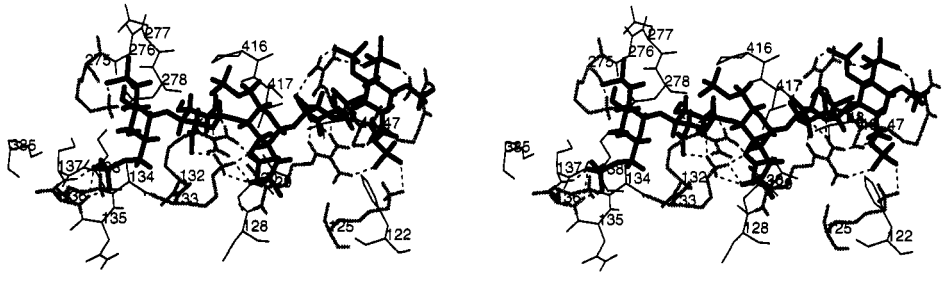

Figure 9. Stereoview of part of the complex between compound 2 and AT III.

Furthermore it was observed that within a few picoseconds of molecular dynamics (in the presence of water molecules) the $^1C_4$ (starting) conformation of L-iduronic acid was converted into the $^2S_0$ skew boat conformation. The latter conformational change is well documented for α-L-iduronic acid moieties in heparin-like molecules (10). The fact that the conformational change is simulated by a molecular dynamics simulation indicates that the force field parameters (Quanta/CHARMm 3.3.1; MSI) for these systems seem reasonable. Probably, the conformational flexibility of the iduronic acid moiety is important for this highly specific mode of interaction.

The heparin-mediated AT-III activation process at the molecular level

It is interesting to note that in the crystal structure of AT-III (22) Lys 125 and Arg 129 are located on one face of an α helix denoted helix D, whereas the region comprising Arg 132, Lys 133 and Lys 136 possesses a coil-like conformation.

Molecular dynamics studies, as well as CD spectrometry studies (23), indicate that helix-D has a tendency to elongate in the presence of pentasaccharide **2**. In this way the basic residues Lys 125, Arg 129, Arg 132, Lys 133 and Lys 136 become exposed to one face of the α-helix enhancing the interaction with pentasaccharide **2**.

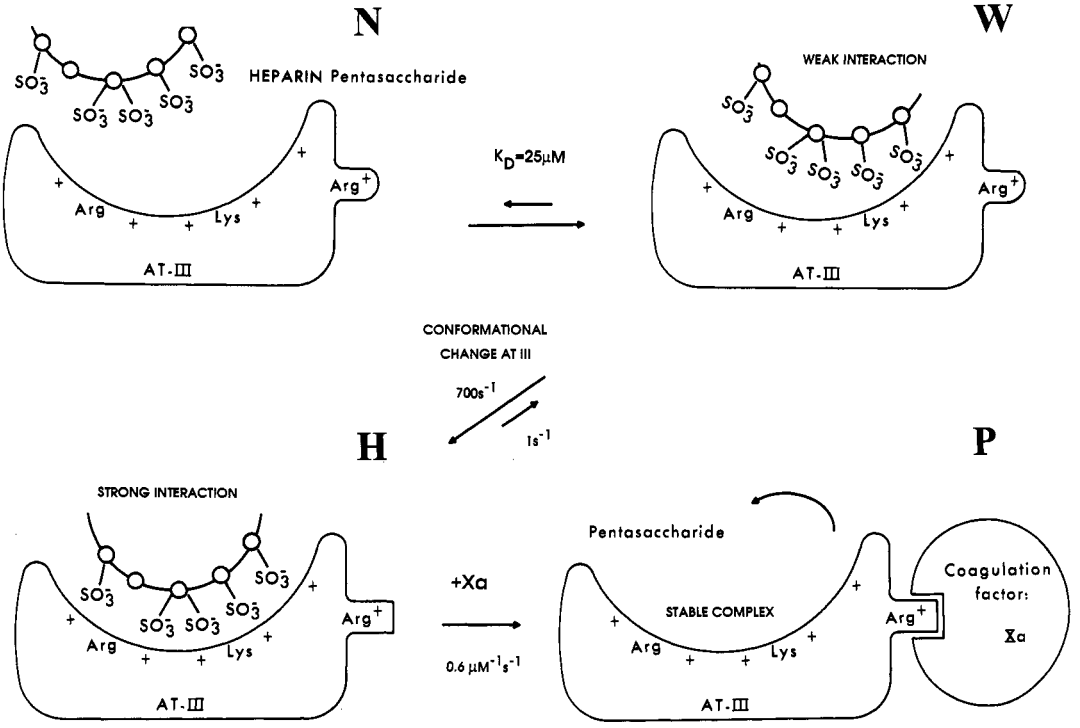

Figure 10. Kinetics of pentasaccharide mediated AT III activation.

It should be recalled that there is much experimental evidence indicating that heparin stabilizes α-helical conformations by reducing detrimental electrostatic interactions between Lys and/or Arg side chains (23,24). In this respect it is tempting to speculate that the pentasaccharide-induced elongation of helix D is the trigger for the well-known conformational change in AT-III.

In Figure 10 the role of the pentasaccharide in the AT-III mediated inactivation of factor Xa, as established by kinetic experiments, is shown schematically. Thus the pentasaccharide acts in a two-step process: first it binds to AT-III (state **N**) and achieves equilibrium quickly (formation of the weak complex, state **W**), then the protein undergoes a conformational change to give the activated form of AT-III (state **H**) (25). The latter state (**H**) is also known as the "high-affinity state" because after the conformational change the interaction of the pentasaccharide with AT-III strengthens. Subsequently the

activated state (**H**) of AT-III binds with factor Xa to give a stable AT III/Xa complex (state **P**), while simultaneously the pentasaccharide - the catalyst in this reaction - is released.

The dissociation constant K_D for the AT-III/pentasaccharide (**1b**) complex (state **H**) has been determined by several groups with values between 36 nM (pH=7.4) and 300 nM (pH=8.4) (7). The extra sulphated analogue **2** was found to bind about twentyfold stronger (K_D=1.3 and 22 nM respectively) (7).

The crystal structure of intact human AT-III (22) and studies on related serpins provide additional clues (26) as to how the pentasaccharide domain in heparin activates AT-III. An important observation is that the reactive center loop of native serpins is long and flexible and that the serpin's inhibitory activity can be modulated by a dynamic process of insertion and exposure of the loop. Upon proteolytic cleavage a large part of the loop becomes incorporated in the central beta-sheet (called A-sheet) yielding an inactive form of the serpin (27). Crystal structures reported so far reveal either a fully incorporated (in the A-sheet) reactive center loop (28,29), or full exposure (30). The structures, with the inserted loop consist of latent or cleaved serpins.

Serpins with a fully exposed reactive center loop were supposed only to occur in non-inhibitory serpin analogues, like ovalbumin, but recently Wei et al studied an antichymotrypsin mutant (30c) with inhibitory activity and which in its crystal structure shows a fully exposed reactive center loop. Analysis of mutations (31) and experiments with short peptides blocking insertion of the reactive center loop in the sheet (32), have established that the ability of the reactive center loop to insert in the central sheet is essential for inhibitory function. Complete insertion, on the other hand, leads to inactive, latent forms (28,32b). Functional serpins appear therefore to have structures in between no insertion and complete insertion. Several models of serpins with partially inserted reactive center loops have been proposed (32b,33,34).

The crystal structure (22) of the native human AT-III molecule shows that the reactive site is partially inserted into the A-sheet (see Fig. 11, native state **N**).

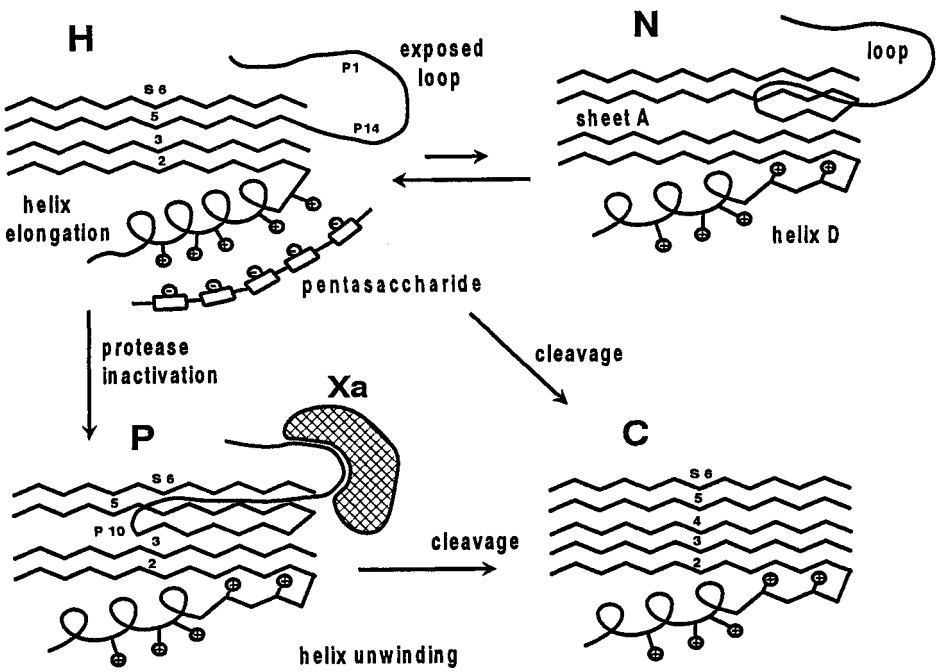

Figure 11. AT III functional forms and interconversions.

Two mechanisms have been proposed for heparin-mediated AT-III activation (formation of state **H**): (i) heparin causes partial insertion of the reactive center loop in the A-sheet (32b) or (ii) heparin assists the removal of the partially inserted reactive site loop from the A-sheet. Recent observations by Björk and Olson (32c,35) that both activation by heparin and insertion of synthetic exogenous peptide in the A-sheet leads to a similar substrate-like form of AT-III, are highly suggestive for mechanism (ii).

Consequently the reactive center loop of AT-III in the "high-affinity" state (**H**) adopts an exposed conformation meaning that non or only a single loop residue is incorporated into the A sheet. AT III in state **H** can either form a strong adduct with its target protease (state **P**) or can be cleaved (state **C**). During both processes AT-III undergoes a second conformational change that is accompanied with a decrease in affinity between pentasaccharide (or heparin) and AT-III. There is good evidence (27b,35) that in "low-affinity" states **P** and **C**, the reactive center loop is far inserted into the A-sheet. The fact that the three states where the reactive center loop is partially or completely inserted into the A-sheet (states **N, P, C**) have a low heparin affinity, is also in line with the mechanism (ii).
Stein and Chothia (27a) provide a clue as to how the processes of stabilization of helix D and the reactive center loop exposure may be connected. By comparing the structures of cleaved and uncleaved serpins, they concluded that serpin structures can be considered as two rigid bodies connected through a "flexible joint" comprised of helix-D (and E). Modulation of the serpin activity involves opening of the A-sheet with a movement of strands 2, 3 away from strands 5, 6 under simultaneous insertion of the hinge area of the reactive center loop (becoming strand 4). As strand 2 and helix-D are connected through a flexible region that incorporates the heparin binding residues Arg 132, Lys 133 and Lys 136, it is likely that the heparin mediated activation of AT-III involves also conformational changes in this area. Our model, schematically depicted in Fig. 11, reveals how this local conformational change may be connected to the reactive center loop mobility. Thus, interaction of the pentasaccharide domain of heparin with AT-III in the native state leads to a stabilization of helix-D (thus locking the "flexible joint") and closes the A-sheet under concomittant exposure of the reactive center loop (state **H**). Such closure of the A sheet is further supported by experiments of Evans et al. (36) showing that AT III polymerization, caused by the insertion of the reactive center of one molecule into the A-sheet of another, is significantly reduced in the presence of pentasaccharide. On the other hand re-insertion of the loop into the A-sheet (formation of states **P** or **C**) destabilizes helix-D (causing a "flexible joint") thereby releasing the heparin catalyst from AT-III.

The inhibition of thrombin by AT-III
As mentioned earlier the characteristic heparin pentasaccharide and larger heparin fragments containing this sequence accelerate reaction of AT-III with some serine proteases such as blood coagulation factor Xa. For the inactivation of thrombin, however, not only the presence of the unique pentasaccharide is a prerequisite but in addition the heparin chain should be about 18 saccharides in length (37). This phenomenon has been explained by a reaction that is composed of two distinct mechanisms: i) binding of the pentasaccharide with AT III (brought about by the unique pentasaccharide); and ii) the bridging of AT III and thrombin by the same heparin chain.
There is good evidence that the heparin chain interacts specifically and strongly with AT-III but only weakly and less specifically with thrombin (38).
Since the crystal structure of human thrombin has been published recently (39) in a complex with the inhibitor DPhe-Pro-Arg chloromethylketon (PPACK) and its putative heparin binding site amino acids have been indicated, we felt that now a first 3D model of a ternary complex consisting of AT III, thrombin and heparin can be constructed.
First the model of the complex between pentasaccharide and the crystal structure of AT III (vide supra) was used to dock on thrombin. Prior to docking the conformation of the reactive centre loop of AT III was changed into the canonical serpin conformation (40). The relative orientation of the protease and inhibitor in the X-ray structure of the trypsin-BPTI complex guided the final docking procedure.
The resulting complex (atoms) was energy-minimized and subjected to 10 picoseconds of molecular dynamics (Fig. 12). A possible interaction path for heparin was clearly revealed by a stretch of positively charged amino acids at the surfaces of AT III and thrombin, many of which are known to be essential for heparin activation.

We were pleased to find that in our model longer heparin fragments, constructed by elongation of the pentasaccharide with a regular sequence (41) at the non-reducing end, crossed the indicated path perfectly.
Indeed, the heparin binding site at thrombin is reached when the heparin chain in the model is comprised of 15 monosaccharide units.

Figure 12. AT III/thrombin/heparin ternairy complex.

It is to be noted that the orientation of heparin to the AT III-thrombin complex is determined by the orientation of the pentasaccharide to AT III, which could be established unambiguously due to an asymmetric distribution of known, complementary interaction points (vide infra, (21)).
In a recent paper of Gan et al. (42), the authors suggested a model of a thrombin -heparin complex in which the heparin chain interacts in the opposite fashion relative to our model.

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