Behavior of Antithrombin III Isoforms on Immobilized Heparins

EVIDENCE THAT THE ISOFORMS BIND TO DIFFERENT NUMBERS OF LOW-AFFINITY HEPARIN SITES*

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Antithrombin III exists in plasma as major and minor isoforms differing in affinity for heparin. The nature of the binding of each purified isoform to immobilized heparins was investigated. Unfractionated, mixed-affinity heparin bound each isoform with both high affinity and concentration-dependent low affinity. The isoforms were resolved when filtered through low-affinity heparin (heparin repeatedly passed over immobilized antithrombin III) columns. Following chemical modification of a specific tryptophan residue required for heparin binding, each isoform failed to bind to either low-affinity or mixed-affinity heparin-agarose, but elution of the modified higher-affinity isoform was retarded on both gels. Because the modified lower-affinity isoform eluted with the similarly sized bovine serum albumin in these experiments, the difference in isoform affinity for heparin appears to be the result of a unique, secondary heparin-binding site in the higher-affinity isoform that can bind a heparin site with low affinity for antithrombin III. This interpretation was supported by the chromatographic behavior of the isoforms on mixed-affinity agarose during reverse gradient elution. Two other populations of each of the tryptophan-modified isoforms were identified. Since these isoforms bound tightly to mixed-affinity heparin-agarose but eluted at lower salt concentrations than the corresponding unmodified isoforms, both isoforms may contain additional secondary sites that interact weakly with heparin. A general model of heparin-antithrombin III interaction is proposed in which a high-affinity heparin site initially interacts with a primary site on antithrombin III. The subsequent conformational change leads to a cooperative, entropy-driven association between secondary sites on the protein and low-affinity sites on heparin, stabilizing antithrombin III in its activated form.

Antithrombin III (ATIII)† is required for the regulation of coagulation enzymes in vivo (1, 2). Considerable evidence exists that this requires the activation of ATIII by heparin-like substances (3, 4), and it is probable that the therapeutic effect of heparin in the treatment of thrombosis is primarily the result of an increase in the amount of activated ATIII (5, 6). The activation of ATIII by heparin is accompanied by tight electrostatic association of the two molecules (7, 8).

Two populations of ATIII with different affinities for heparin have been demonstrated in normal plasma (9–11). The major isoform, representing ≥90% of the ATIII antigen recovered by heparin-affinity chromatography (9–11), has a high affinity for heparin and has been designated high-affinity ATIII (ATH) (9). The second ATIII isoform binds heparin even more tightly than ATh (9–11) and has been designated very-high-affinity ATIII (AThv) (9). In addition to its greater affinity for heparin, the molecular mass of the latter isoform is 2 kDa less than ATh (9–11), and it is less negatively charged (9). The nature of the difference in ATIII-isoform binding to heparin has not been determined, although the structural basis for this difference appears to be the presence of a specific N-glycosidically linked carbohydrate side chain, as recently demonstrated by Brennan et al. (12).

The >1000-fold increase of ATIII activity in the presence of heparin requires intact lysine and arginine residues in the protein (6, 13), and it has been shown by several investigators that heparin binding is associated with a conformational change in ATIII (14–18) that increases heparin affinity (15, 16). In the presence of heparin, ATIII reaction with thrombin results in the formation of a ternary complex (19–23) in which heparin seems to act both to activate ATIII and to juxtapose enzyme and inhibitor (8, 19–25). A similar mechanism has been suggested for the reaction of ATIII with factors IXa (26) and XIa (26). For factor Xa inhibition, however, it is believed that only the activation of ATIII occurs (21, 26). A specific pentasaccharide that is the minimum structure in heparin necessary for binding and activating ATIII toward factor Xa has been determined (27) and its structure verified (28). On the other hand, some relationship between heparin size and the rate of factor Xa inhibition has recently been demonstrated (29, 30), suggesting a contribution to antifactor Xa activity by additional sites in heparin.

Crude heparin can be fractionated on immobilized ATIII

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‡ The abbreviations used are: ATIII, antithrombin III; ATh, antithrombin III with high affinity for heparin; AThv, antithrombin III with very high affinity for heparin; LAH, low-affinity heparin, MAH, mixed-affinity heparin; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; TCB, Tris-citrate buffer, HNB, hydroxybenzyl.
columns into high-affinity molecules, which contain the specific pentasaccharide with anti-factor Xa activity and low-affinity molecules which do not have this activity (7, 31).

Heparin molecules containing the specific ATIII-binding site displace those missing this site from immobilized ATIII (32).

However, a single specific site on the ATIII molecule that binds heparin tightly has not been located. Thus, when ATIII is digested with CNBr only, the largest fragment (residues 104-251) binds tightly to heparin (33). Several smaller fragments, including an area approximately 50 residues from the NH$_2$ terminus containing three amino acids (Pro$^8$, Arg$^9$, and Trp$^{10}$) required for normal heparin binding (34-36), have not been found to bind to immobilized heparin. These results suggest a more complicated reaction between ATIII and heparin than is possible if a single site on ATIII is involved in heparin binding. A reasonable explanation for this is that more than one site of interaction occurs between the two molecules, implying that ATIII binds heparin by a mechanism in which the protein and glycosaminoglycan act as complementary polymers, interacting at more than one site (37).

This type of mechanism has been suggested previously by others (18, 38), but additional evidence has not been reported. The multiple sites mechanism of ATIII-heparin association is consistent with data reported in this paper, obtained by studies of the interaction of the two isoforms of ATIII with immobilized heparins. Furthermore, these studies demonstrated that AT$_{3a}$ contains at least one site that interacts with heparin that is not present or expressed in AT$_{3h}$.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Studies on the Low-affinity Interactions between ATIII Isoforms and Immobilized Heparin**—It is well established that the chromatography of standard mixed-affinity-heparin (MAH) on immobilized ATIII leads to fractionation of the glycosaminoglycan into populations with high and low affinity for the protein (e.g. see 7, 31, 39). As pointed out above, it is possible that more than one site on ATIII interacts with heparin. Since ATIII and heparin generally associate in 1:1 complexes (see Ref. 31), this would require the simultaneous interaction with ATIII of both the high-affinity pentasaccharide and low-affinity sites in the same heparin molecule.

To determine if heparin immobilized on agarose can exhibit both high- and low-affinity interactions with ATIII isoforms, two kinds of experiments were initially done. First, AT$_{3h}$ was applied to a small MAH-Sepharose 4B column, and fractions were collected until the concentrations of the protein entering and eluting the column were equal. Under these circumstances, a steady state exists between AT$_{3h}$ in solution and AT$_{3h}$ bound to the immobilized heparin. From the differences between AT$_{3h}$ concentrations in the loading solutions and their concentrations in the fractions obtained between the approximate void-volume and column saturation, the total amount of AT$_{3h}$ associated with the column in the steady state was estimated. The amount of the AT$_{3h}$ which was loosely associated with the column in the steady state was estimated from the product of the AT$_{3h}$ concentrations and fraction volumes obtained after the void volume during washing of the column with buffer containing low salt. When a column was subjected to 80 mg of AT$_{3h}$ at 1.0 mg/ml in TCB/0.15 M NaCl, a little more than half of the protein associated with the immobilized heparin in the steady state was found to be loosely bound. Identical columns were loaded with equivalent amounts of AT$_{3h}$ at lower concentrations to determine if the amount of protein bound was concentration-dependent. As shown in Table I, when the concentrations of the AT$_{3h}$ in loading solutions were decreased to 0.5 and 0.25 mg/ml, the amount of low-affinity association, but not high-affinity binding, progressively decreased.

Also presented in Table I are the results of experiments in which the steady-state interactions of AT$_{3h}$ and AT$_{3h}$ were compared at equal loading concentrations. Both the amount of low-affinity and tightly bound protein were slightly greater for AT$_{3h}$ than AT$_{3h}$.

The second set of experiments done to investigate the low-affinity interaction of immobilized heparin with ATIII involved studies of the effect of salt concentration on the binding of AT$_{3h}$. In these studies, varying amounts of AT$_{3h}$ were added to 1.0-m1 volumes of a single heparin-Sepharose 4B preparation equilibrated with TCB/0.10 M NaCl. The AT$_{3h}$ was batch-absorbed by rocking gently and then centrifuged and the A$_{280}$ of the supernatants determined. Next, the supernatants were added back to the gel, and the ATIII was eluted in steps by addition of successive aliquots of 5.0 M NaCl. This procedure was repeated until sufficient NaCl had been added to elute all of the ATIII from the immobilized heparins. To determine the amount of ATIII eluted by the addition of each aliquot of NaCl, the A$_{280}$ of the supernatants was determined after the mixtures had been rocked and centrifuged as before.

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**Table I**

| Column | Isoform | Loading concentration | Steady-state association | Amount eluted |
|--------|---------|-----------------------|-------------------------|---------------|
|        |         | mg/ml                 | mg                      | 0.15 M NaCl | 3.0 M NaCl |
| 1$^*$  | AT$_{3h}$ | 1.0                   | 18.9                    | 10.4         | 8.9       |
| 2$^*$  | AT$_{3h}$ | 0.50                  | 15.6                    | 6.5          | 8.9       |
| 3$^*$  | AT$_{3h}$ | 0.25                  | 13.7                    | 4.9          | 9.2       |
| 4$^*$  | AT$_{3h}$ | 0.57                  | 20.8                    | 9.9          | 11.0      |
| 4$^*$  | AT$_{3h}$ | 0.57                  | 25.4                    | 11.7         | 14.5      |

$^*$ These were three separate 8.0-m1 columns prepared from a single batch of heparin-Sepharose 4B. At the time of usage, this preparation contained 0.6 mg of heparin/ml of Sepharose 4B. After all the ATIII (80 mg) had been loaded, each column was washed with 44 ml of TCB containing 0.15 M NaCl and then eluted with TCB containing 3.0 M NaCl.

$^*$ A single 8-m1 column was used twice. The heparin-Sepharose 4B was from a batch of material containing 1.3 mg of heparin/ml of Sepharose 4B. Before elution, each column was washed with TCB containing 0.15 M NaCl until the A$_{280}$ of the effluent was <0.02; 70 ml for AT$_{3h}$ and 78 ml for AT$_{3h}$.
Although the dilution of ATb in this procedure increases with increasing NaCl concentration, at salt concentrations <0.5 M where ATIII was found to bind to heparin in a concentration-dependent manner, dilution was less than 10%. These experiments showed that: 1) the quantity of ATIII initially bound was related to the concentration of protein added (Fig. 1A); 2) the ATIII-binding sites of heparin were saturated only at the highest ATIII concentration (Fig. 1B); and 3) a larger fraction of ATIII eluted at low salt concentrations from the saturated heparin-Sepharose 4B (Fig. 1, B and C).

In addition, it was observed that the salt concentrations at which all of the ATIII eluted (obtained by extrapolation of the curves to zero binding) were approximately 0.75 M NaCl for both the highest and medium amounts of ATIII. However, the lowest amount of ATIII required considerably more salt to completely elute. Since affinity for heparin is related to the salt concentration required to break the complex, these results suggested that at low ATIII:heparin ratios a small fraction of the protein bound more tightly than to the immobilized heparin than at higher ratios.

The results from the experiments in this section clearly demonstrate that both ATIII isoforms bind to heparin with variable affinities. Studies on ATb batch absorption by heparin-Sepharose 4B demonstrated that the low-affinity interaction, but generally not the high-affinity one, was concentration-dependent. Finally, at a low ATb to heparin-Sepharose 4B ratio, a small fraction of the protein was bound much more tightly than at higher rates.

Interactions of ATIII Isoforms with LAH-Sepharose 4B and HNB-ATb and HNB-ATvh, with LAH- and MAH-Sepharoses—To further characterize the participation of LAH sites in the binding of ATIII to immobilized MAH-Sepharose 4B, the behaviors of ATb and ATvh on an immobilized LAH column were first compared. The LAH, which had been prepared by passage of MAH over immobilized ATb, as described under “Experimental Procedures,” might still interact relatively strongly with the higher-affinity ATvh. To study this, a LAH column was loaded with ATb + ATvh, after equilibration with TCB/0.15 or 0.40 M NaCl, and then developed as described in Fig. 2. At the lower salt concentration, an initial sharp peak and a delayed and broadened secondary peak were obtained. SDS-PAGE of fractions from these peaks showed that the first peak was exclusively ATb, and the second ATvh. At 0.4 M NaCl, a single peak was obtained, but SDS-PAGE demonstrated some fractionation of the isoforms within this peak. Since Fig. 2 also compares the fractionation of ATb + ATvh with BSA filtered through the same column, it is evident that whereas the ATvh is greatly retarded at 0.15 M NaCl, ATb elutes only a little after BSA. Therefore, while neither isoform bound tightly to the LAH, ATvh interacted relatively strongly with LAH sites.

Because MAH includes both LAH and heparin molecules containing the high-affinity heparin pentasaccharide, the above results are consistent with the hypothesis that MAH contains, in addition to high-affinity ATIII-binding sites, sites that interact weakly with ATb and more strongly with ATvh. If this hypothesis is correct, the same heparin molecule could interact with at least two different ATIII molecules, because heparin molecules containing the high-affinity pentasaccharide presumably contain the low-affinity heparin structures at adjacent sites. Evidence that one heparin molecule can bind more than one ATIII molecule has been reported by Pletcher et al. (49). On the other hand, if the protein contains more than one heparin-binding site, the same heparin could bind...
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at least two sites on a single ATIII. In this case, a specific site on ATIII could interact with the high-affinity site in heparin, and one or more additional ATIII sites could interact with one or more low-affinity heparin sites. To rule out the possibility that a single site in ATIII interacts with both high- and low-affinity heparin sites, a tryptophan residue in ATIII known to be required for binding to MAH-agarose (36) and the corresponding tryptophan residue in ATVh were modified with dimethyl(2-hydroxy-5-nitrobenzyl)-sulfonium bromide and isolated as described under "Experimental Procedures." Each of these materials was subjected to chromatography on both LAH- and MAH-Sepharose 4B columns. If either ATs or ATv, contained more than one site which interacts with heparin, then that modified isoform should interact with the MAH column. If such an interaction also occurred with immobilized LAH, then multiple interactions could occur between the isoform and heparin. Fig. 3 shows the results obtained when Trp4'-modified ATs (HNB1-ATs) together with unmodified ATs were applied to the LAH-Sepharose 4B column equilibrated with TCB/0.15 M NaCl. In this case, HNB1-ATs eluted somewhat earlier than the unmodified inhibitor. Comparison of these results with Fig. 2 makes it clear that BSA and HNB1-ATs essentially coeluted from the LAH column. Therefore, it was suggested that either a low-affinity interaction between ATs and low-affinity heparin does not occur or if it does occur, it is dependent on an intact Trp4' or possibly the presence of high-affinity heparin, or both.

Next, an experiment like the one employing HNB1-ATs and LAH-Sepharose 4B was performed using HNB1-ATv, to examine the effect on ATv interaction with LAH of modification of the tryptophan residue in ATv, equivalent to Trp4' in ATs. Fig. 4 shows the results of an experiment in which HNB1-ATv, unmodified ATv, and unmodified ATs were applied together to the LAH column. SDS-PAGE (not shown) and the A405 demonstrated that ATs and HNB1-ATs, essentially coeluted in the first peak, whereas the unmodified ATv.
was significantly retarded. Therefore, in contrast to HNB1-
AH, HNB1-ATv retains approximately as much affinity for
Lah as unmodified ATv. These results indicated that modi-
fication or removal of a primary site reduces, but does not
completely abolish, ATv affinity for Lah.

The next set of studies was designed to determine if, in
contrast to the results obtained with Lah-Sepharose 4B, HNB1-ATv retained affinity for MAH. HNB1-ATv and
HNB1-ATv were applied separately to a MAH-Sepharose 4B
column of the same size and ionic strength as the Lah column
employed above. Filtration of the modified isoforms and BSA
were run. For each experiment, the column was developed at 16 ml/8 fractions/h. Points for fractions from either tail with base-line absorbance values or
which did not change significantly in the remainder of the chroma-
ograms are omitted.

To further investigate the possibility that more than one
site in ATv, ATv, or both ATv and ATv interact with heparin,
the HNB-ATIII molecules that were modified at tryptophan residues other than Tryp\(^3\) (HNB2-ATv and HNB2-ATv) were
isolated by elution from heparin-Sepharose 4B at high salt concen-
tration as described under “Experimental Procedures.” After
dialysis, HNB2-ATv and HNB2-ATv were applied sep-
ately to a MAH-Sepharose 4B column as described in the
legend to Fig. 6. Gradient elution resulted in partial separation
of three distinct fractions for each isoform. Of these, two
contained HNB, as shown by absorbance at 410 nm, and one
was unmodified ATv or ATv. Since the 4 tryptophan residues in
ATIII are widely separated (51), these results support the
hypothesis that both isoforms of ATIII contain several sites
that interact with heparin.

Behavior of ATIII Isoforms on MAH-Sepharose 4B during
Reverse Elution—The above results imply that ATv, differs
from ATv in a site outside their primary heparin-binding sites,
and this ATv site may interact with Lah sites, even when
the primary site has not interacted with the high-affinity
ATIII-binding site of heparin. Furthermore, as suggested by
the fractionation of isoforms on Lah columns at 0.4 M NaCl
this could occur at high ionic strength. Therefore, separation
on MAH-Sepharose 4B during NaCl gradient elution may be
the result of a weak interaction between secondary sites in
ATv and the relatively large number of Lah sites as the
isoforms pass through a MAH-Sepharose 4B column after
the salt concentration reaches a level that essentially neutralizes
a primary site interaction. To test this prediction, a MAH-
Sepharose 4B column was loaded with a mixture of ATv and
ATv. As shown in Fig. 7, when the column was eluted with a
linear NaCl gradient, applied in the same direction as the
sample had been loaded, the expected ATv and ATv peaks
were obtained. However, as also shown in Fig. 7, when the
same ATv and ATv mixture was loaded on the same column
deluted by a gradient applied to the column in the opposite
or reverse direction from which the sample had been applied
(buffer flow was into the bottom of the column and out from
the top), both of the isoforms eluted together at a salt concen-
tration that was lower than those found for either ATIII
isoform during the standard elution. These results were con-
sistent with the prediction about ATv and ATv behavior on
MAH columns and with the hypothesis that interactions occur
between heparin and one or more secondary sites on
ATv. Comparison of the elution position of ATv in the two
chromatograms suggested that this isoform also interacts
weakly with Lah sites after the high-affinity heparin-ATv
interaction is neutralized by the salt. However, as suggested
by the failure of HNB1-ATv to interact with MAH-Sepharose
4B (see Fig. 5), this weak interaction may be mainly the result
of an interaction between heparin and the ATv site containing
Tryp\(^3\).

**DISCUSSION**

Evidence is presented in this investigation that ATIII and
heparin each contain more than one site of interaction with
one another. Earlier work in other laboratories has shown
clearly that commercially available heparin preparations contain
populations with low and high affinities for immobilized
ATIII (7, 31). Here we have demonstrated that immobilized
heparin, containing both populations of the glycosaminogly-

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**Fig. 5.** Separate chromatography of type 1 HNB-modified ATv, type 1 HNB-modified ATv, and BSA on a MAH-Sepharose 4B column. A 1.5 \times 11.5-cm column was equilibrated with TCB + 0.15 M NaCl for each experiment. First, 2.0 mg/2.8 ml HNB1-
ATv (O = A\(_{290}\), \(\bullet = A\(_{410}\)), then 2.0 mg/2.8 ml HNB1-ATv (\(\square = A\(_{290}\))
\(\blacksquare = A\(_{410}\)), and last, 2.0 mg/2.8 ml BSA (\(\vartriangle = A\(_{290}\)) were run. For each
experiment, the column was developed at 16 ml/8 fractions/h. Points for fractions from either tail with base-line absorbance values or
which did not change significantly in the remainder of the chromat-
ograms are omitted.

**Fig. 6.** Separate chromatography on MAH-Sepharose 4B of type 2 HNB-modified ATv and type 2 HNB-modified ATv. The HNB2-ATv, resulting from modification of 100 mg of ATv, and the HNB2-ATv, resulting from modification of 4.15 mg of ATv were separately applied to the same 1.5 \times 28-cm column. After washing, the column was eluted with a 200-ml NaCl gradient from 0.15 to 3.0
M NaCl at 12.5 ml/3.12 fractions/h. The salt gradient was generated by a three-channel pump, and NaCl concentrations were determined
from conductivity measurements. O and \(\bullet\), A\(_{290}\) and A\(_{410}\) of ATv, respectively; \(\square\) and \(\blacksquare\), A\(_{290}\) and A\(_{410}\) of ATv, respectively. Points for fractions from either tail with base-line absorbance values are not
shown.
can, retains both of these kinds of sites (see Figs. 1 and 2 and Table 1).

Multiple sites of interaction with heparin are shown for AT<sub>3</sub>, by the studies on the behavior of the tryptophan-modified material shown in Figs. 4 and 5. In these studies, AT<sub>3</sub> with a modified tryptophan residue (36) in what seems to be a primary heparin-binding site (HN1-AT<sub>3</sub>) retained affinity for immobilized LAH, as well as for MAH. The data shown in Fig. 1 are consistent with the inference that AT<sub>3</sub> also contains one or more secondary heparin-binding sites, but this site(s) is exposed only after primary-site interaction occurs. In this experiment, at low ATIII-heparin ratios, the last of the bound AT<sub>3</sub> required nearly twice the limiting amount of salt as that required to break the association at higher ratios. For the small number of these very tightly bound molecules, the putative primary and secondary binding sites on the protein could be bound to the small fraction of heparin molecules containing more than one high-affinity ATIII-binding site (23, 50).

Further evidence that both ATIII isoforms interact with heparin at more than one site comes from modification of tryptophan residues other than residue 49 (HN2-ATIII). These derivatives bind tightly to immobilized heparin, but elute at lower salt concentrations than unmodified material. As shown in Fig. 6, two populations of both HNB-modified AT<sub>3</sub> and AT<sub>vh</sub> are obtained that bind heparin relatively tightly. From the elution patterns of HNB2-AT<sub>3</sub> and HNB2-AT<sub>vh</sub> (Fig. 6), it seems likely that none of the tryptophan residues modified are in a site that is different for AT<sub>3</sub> and AT<sub>vh</sub>, as a parallel difference in isoform affinity resulted for the corresponding population of each modified isoform. Therefore, it is indicated that AT<sub>3</sub> and AT<sub>vh</sub> could both contain at least two secondary heparin-binding sites. One or more additional binding sites are indicated for AT<sub>vh</sub> as discussed above.

Inspection of the amino acid sequence of AT<sub>3</sub> (51) reveals several sites in the NH<sub>2</sub>-terminal region of the molecule which are rich in lysine and arginine residues. These residues have been shown to be essential for binding to heparin (6, 13). Although the three-dimensional structure of ATIII could conceivably bring all of the necessary positively charged residues together in a single site, a concentration of positive charges at one site would most likely be unstable. This fact, the results reported in this paper, and the published data from other laboratories lead us to propose a model (see Fig. 8) of ATIII-heparin interaction in which protein and glycosaminoglycan are considered to interact at multiple sites. Thus, these macromolecules bind to one another by a mechanism analogous to that described by Tsuchida and Abe (37) for two different polymers containing multiple sites of interaction. The association of sites in such polymers is cooperative and is driven by the lower entropy of activation required for the association of sites subsequent to the initial interaction. At approximately equal molar concentrations, this type of binding leads to 1:1 associations. A feature of our model that is different from the interaction of complementary polymers (37) is that one binding site in each ATIII and one in heparin are qualitatively different from the others. This site in ATIII, herein called the primary site (near Trp<sup>49</sup>), is proposed to be the one associated with the change in conformation that accompanies binding of the protein to the high-affinity site in heparin (14, 18). This change in conformation is envisioned to allow multiple secondary ATIII-heparin interactions. In agreement with these features of the model, Olson et al. (16), using fast-flow kinetic data obtained from monitoring fluorescence changes in ATIII associated with heparin binding, demonstrated that the association of ATIII with heparin is a two step process.

The model in Fig. 8 also proposes that the secondary sites in ATIII are poorly exposed before the interaction of the primary site in the protein and the high-affinity heparin site. This is suggested by the large loss of heparin affinity of both AT<sub>3</sub> and AT<sub>vh</sub> when modified at Trp<sup>49</sup>. Presumably, the heparin sites which interact with the secondary binding sites of ATIII would generally be low-affinity sites, as relatively few high-affinity sites are present in heparin, and these are usually distributed on different molecules (50). The primary heparin-binding site on ATIII is proposed to interact too weakly with low-affinity heparin sites for the latter to be involved in the conformational change in ATIII.

Finally, the model illustrated in Fig. 8 proposes that the difference between the isoforms of ATIII is the result of at least one of the secondary sites on AT<sub>3</sub> being more free to interact with heparin before the conformational change occurs. The structural basis for this seems to be the additional glycosylation in AT<sub>vh</sub> vis-à-vis AT<sub>3</sub> demonstrated by others (10, 11). This is strongly indicated by the absence of glyco-
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Fig. 8. A model for the interactions of ATIII with heparin. a, the sequence of interactions between heparin and AT\(_{\alpha}\), the sequence of interactions between heparin and AT\(_{\alpha}\). At the top of each figure, the inhibitor is shown in a restricted conformation (I). A primary ( ) and several secondary ( ) heparin-binding sites are shown on each ATIII isoform; however, the secondary sites in AT\(_{\alpha}\) are more exposed for heparin interaction than those in AT\(_{\alpha}\). Heparin (H) is illustrated as containing one high-affinity ATIII-binding site (U) and many low-affinity ATIII-binding sites (V). Heparin can initially interact with either kind of site in AT\(_{\alpha}\) or AT\(_{\alpha}\), to form the I-H\(_{1}\) or I-H\(_{2}\) complexes. The I-H\(_{2}\) complex is favored for both isoforms, but even more highly for AT\(_{\alpha}\). When I-H\(_{2}\) changes conformations, secondary sites in AT\(_{\alpha}\) and AT\(_{\alpha}\) interact rapidly with low-affinity sites on heparin, resulting in stabilized ATIII-heparin complexes (II-H). The protease binding site (the darkened area on the surface of the molecule) is more exposed in II-H and can readily react with enzyme.

sialation at Asn\(^{276}\) in AT\(_{\alpha}\), that was recently demonstrated by Brennan et al. (12).

The proposed model seems to be contradicted by the significant increase in the anti-factor Xa activity of ATIII that occurs in the presence of the pentasaccharide that is the minimum heparin structure which binds to the inhibitor (28, 29). Presumably, the pentasaccharide could bind to the putative primary site on ATIII, suggesting that secondary interactions are not required for heparin to activate ATIII in this reaction. However, evidence that suggests secondary ATIII-heparin interactions are important in the heparin-catalyzed inhibition of factor Xa has been reported previously. For example, it has recently been shown that the enhancement of this reaction increases significantly with increasing heparin molecular weight, especially at lower heparin M\(_{r}\) values (29, 30). Further support for the importance of additional heparin sites comes from the earlier demonstration by Oosta et al. (26) of increasing affinity and anti-factor Xa activity of ATIII in the presence of high-affinity heparin fractions with from 6 to 16 monosaccharide units. Additional binding sites on heparin, distal from a high-affinity one, may also be involved in further stabilizing the heparin-ATIII complex. This was suggested by the work of Stone et al. (18) who found that heparin fragments of less than 18 monosaccharide units induced a chiral absorption spectrum different from that induced by larger high-affinity heparins. Therefore, it is possible both additional sites near the high-affinity region of heparin and some farther away may be involved in binding to ATIII.

The model proposed in Fig. 8 is also consistent with the results reported in Table I, with those shown in Fig. 1 for the stepwise elution of AT\(_{\alpha}\) from immobilized heparin at the highest AT\(_{\alpha}\)-heparin-Sepharose 4B ratio and also with recent observations made by Pletcher et al. (49). These latter investigators have used light scattering techniques to investigate the sizes of ATIII-heparin complexes produced over a range of ATIII and heparin concentrations. At low heparin:ATIII molar ratios, the size of the resulting complexes suggested an average of two ATIII molecules bound to each heparin. As the ratio of the concentrations of the molecules increased to near approximately 1:1 and above, the size of the complexes formed returned to 1:1. Both these results and those in Table I and Fig. 1 can be explained by the presence of multiple ATIII- and heparin-binding sites. At either low heparin:ATIII ratios (49) or high ATIII:heparin ratios (Table I and the highest AT\(_{\alpha}\)-heparin-Sepharose 4B ratio in Fig. 1), primary ATIII sites will interact with all the available high-affinity sites on heparin. When the subsequent conformational change in these ATIII molecules occurs, both secondary sites on the already bound ATIII molecules and primary sites on molecules which have not yet associated with heparin would compete for the low-affinity sites in the heparin molecule. This would result in the formation of ATIII-heparin complexes containing more than one ATIII. At more equal ATIII:heparin ratios, the secondary sites in the protein are likely to associate with sites on heparin molecules which have high-affinity ATIII-binding sites in adjacent locations, because at more equal ratios, polymers with multiple sites of interaction are driven by the low entropy of activation to associate in a 1:1 ratio (37).

In summary, the results reported here and data reported by other investigators are consistent with a model of ATIII-heparin binding in which a primary site on the inhibitor and the high-affinity site in heparin interact before a conformational change in the protein produces exposure of several secondary ATIII sites that subsequently bind to low-affinity sites on heparin. It is also demonstrated in this paper that AT\(_{\alpha}\) contains a site, not present or available in AT\(_{\alpha}\), that interacts weakly with a low-affinity site in heparin.

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Supplementary Material to

Behavior of Antiplatelet IIb/IIIa Receptors on Isolated Human Platelets

by

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EXPERIMENTAL PROCEDURES

Materials. Concanavalin A-Sepharose were purchased from Pharmacia and polyclonal typhoid A-F1 strain fish sera. 

Fractionation of platelets. Platelet rich plasma from heparinized blood was stored on ice and processed within 4 h. 

Preparation of the mixture of ATIII. Frozen slitted plasma (obtained from patients undergoing liver transplantation surgery) was thawed and centrifuged at 20,000 × g for 10 min. The supernatant was stored at -70°C until further analysis. 

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