# The Oligosaccharide Side Chain on Asn-135 of α-Antithrombin, Absent in β-Antithrombin, Decreases the Heparin Affinity of the Inhibitor by Affecting the Heparin-Induced Conformational Change†

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ABSTRACT: The β-form of antithrombin, lacking a carbohydrate side chain on Asn-135, is known to bind heparin more tightly than the fully glycosylated α-form. The molecular basis for this difference in affinity was elucidated by rapid-kinetic studies of the binding of heparin and the antithrombin-binding heparin pentasaccharide to plasma and recombinant forms of α- and β-antithrombin. The dissociation equilibrium constant for the first step of the two-step mechanism of binding of both heparin and pentasaccharide to α-antithrombin was only slightly higher than that for the binding to the β-form. The oligosaccharide at Asn-135 thus at most moderately interferes with the initial, weak binding of heparin to α-antithrombin. In contrast, the rate constant for the conformational change induced by heparin and pentasaccharide in the second binding step was substantially lower for α-antithrombin than for β-antithrombin. Moreover, the rate constant for the reversal of this conformational change was appreciably higher for the α-form than for the β-form. The carbohydrate side chain at Asn-135 thus reduces the heparin affinity of α-antithrombin primarily by interfering with the heparin-induced conformational change. These and previous results suggest a model in which the Asn-135 oligosaccharide of α-antithrombin is oriented away from the heparin binding site and does not interfere with the first step of heparin binding. This initial binding induces conformational changes involving extension of helix D into the adjacent region containing Asn-135, which are transmitted to the reactive-bond loop. The resulting decreased conformational flexibility of the Asn-135 oligosaccharide and its close vicinity to the heparin binding site destabilize the activated relative to the native conformation. This effect results in a higher energy for inducing the activated conformation in α-antithrombin, leading to a decrease in heparin binding affinity.

Antithrombin is the major plasma inhibitor of serine proteinases of the blood coagulation cascade, thrombin and factor Xa being its most important target enzymes (Olson & Björk, 1994). It is a member of the serpin superfamily of proteins, to which most other plasma proteinase inhibitors and also several non-inhibitory proteins belong (Carrell & Travis, 1985; Huber & Carrell, 1989; Gettins et al., 1996). Serpins inactivate their target proteinases by forming stable, equimolar enzyme−inhibitor complexes. Complex formation is initiated by the enzyme attacking a specific reactive bond in an exposed loop of the serpin molecule, which induces a conformational change of the inhibitor that traps the enzyme in an inactive form. Considerable evidence indicates that this conformational change involves insertion of part of the reactive-bond loop into the major β-sheet of the inhibitor as a middle strand of this sheet. The trapping is essentially irreversible, but the complex slowly dissociates into a cleaved, inactive inhibitor and active enzyme (Olson & Björk, 1994; Schulze et al., 1994; Potempa et al., 1994; Stein & Carrell, 1995; Wright & Scarsdale, 1995; Gettins et al., 1996). Serpins thus inactivate their target enzymes in the manner of suicide inhibitors (Fish & Björk, 1979; Olson, 1985; Patston et al., 1991; Cooperman et al., 1993).

The rate by which antithrombin inactivates clotting proteinases is greatly accelerated by heparin and certain species of heparan sulfate (Olson & Björk, 1994). Such heparan sulfate molecules on the vessel wall presumably serve as physiological activators of antithrombin (Marcum & Rosenberg, 1984; Marcum et al., 1986; De Agostini et al., 1990). The acceleration is dependent on antithrombin binding with high affinity ($K_d = 1-2 \times 10^{-8}$ M at an $I$ of 0.15 and pH 7.4) to a specific pentasaccharide sequence in the glycosaminoglycan. The binding is a two-step process, involving formation of an initial complex in rapid equilibrium, followed by a conformational change. The altered conformation

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induced in the second step increases the affinity for heparin ~2000-fold and activates the inhibitor, presumably by making the reactive-bond loop more accessible to the proteinase (Olson et al., 1981, 1992; Gettins et al., 1993; Olson & Björk, 1994). Whereas the latter effect is primarily responsible for the acceleration of factor Xa and kallikrein inhibition, it negligibly affects the inhibition of thrombin or factor IXa. Instead, full acceleration of the inhibition of these enzymes requires that both antithrombin and proteinase bind to the same polysaccharide chain, the rate enhancement being due predominantly to an approximation, or bridging, effect (Olson & Björk, 1991, 1994; Olson et al., 1992).

Two major forms of antithrombin, α and $\beta$, circulate in blood (Carlson & Atencio, 1982; Peterson & Blackburn, 1985). The predominant α-form, which accounts for ~90% of the inhibitor in plasma, is glycosylated on all four potential glycosylation sites (Asn-96, Asn-135, Asn-155, and Asn-192), whereas the minor $\beta$-form lacks the carbohydrate side chain on Asn-135 (Brennan et al., 1987). The incomplete glycosylation is due to the presence of Ser instead of Thr in the recognition sequence for core oligosaccharide addition at this site (Picard et al., 1995). $\beta$-Antithrombin binds heparin with higher affinity than α-antithrombin (Peterson & Blackburn, 1985; Turko et al., 1993), presumably because the oligosaccharide at Asn-135 in the α-form, being located close to the putative heparin binding site (Carrell et al., 1994; van Boeckel et al., 1994), affects the interaction with the polysaccharide. Due to its stronger binding to surfaces, including the vessel wall, $\beta$-antithrombin has been suggested to be the physiologically most important antithrombin form (Witmer & Hatton, 1991; Frebelius et al., 1996).

In this work, we have elucidated the molecular basis for the higher heparin affinity of $\beta$- than of α-antithrombin by rapid-kinetic studies of the binding of heparin and the specific antithrombin-binding heparin pentasaccharide to plasma and recombinant forms of the two antithrombin variants. The tighter binding of $\beta$-antithrombin was shown to originate predominantly from the second step of the two-step binding mechanism and to be due to more favorable forward and reverse rate constants of this step. The oligosaccharide chain at Asn-135 thus reduces the heparin affinity of antithrombin primarily by interfering with the heparin-induced conformational change rather than by sterically hindering the initial, weak binding of heparin to the inhibitor.

## MATERIALS AND METHODS

*Proteins.* Human plasma α-antithrombin [α-AT(pl)[1]] was purified by affinity chromatography on heparin−agarose as described previously (Olson, 1988; Olson et al., 1993). Following the salt-gradient elution of α-antithrombin from the immobilized heparin in this procedure, plasma $\beta$-antithrombin [$\beta$-AT(pl)] was eluted in a broad peak with about 2 column volumes of 20 mM sodium phosphate buffer (pH 7.4) containing 3 M NaCl. Pooled $\beta$-antithrombin fractions were subsequently purified by Sephacryl S-200 (Pharmacia Biotech, Uppsala, Sweden) chromatography in the same manner as α-antithrombin (Olson, 1988; Olson et al., 1993).

Recombinant α- and $\beta$-antithrombin forms were expressed in a baculovirus system (Picard et al., 1995; Ersdal-Badju et al., 1995). The recombinant α-form [α-AT(rec)] was produced by replacement of Ser in the glycosylation site Asn-135−Ser-137 with Thr to induce full glycosylation of this site (Picard et al., 1995), whereas the recombinant $\beta$-form [$\beta$-AT(rec)] was produced by mutation of Asn-135 to Ala to prevent glycosylation of the same site (Ersdal-Badju et al., 1995). The expressed proteins were purified by affinity chromatography on immobilized heparin (Ersdal-Badju et al., 1995).

The antithrombin preparations were analyzed by SDS−PAGE (Laemmli, 1970). Protein concentrations of all antithrombin variants were determined by absorbance measurements at 280 nm with the use of a molar absorption coefficient of 37 700 $M^{-1}$ $cm^{-1}$ (Nordenman et al., 1977).

Human α-thrombin (>99% α-form) was a generous gift from J. Fenton (New York State Department of Health, Albany, NY). Human factor Xa was prepared as described elsewhere (Bock et al., 1989). Active-site titrations showed that the two enzyme preparations were >90 and ~70% active, respectively (Ersdal-Badju et al., 1995). All concentrations given below are active-site concentrations.

*Heparins.* Full-length heparin with high affinity for antithrombin and reduced polydispersity (H26) was isolated as described previously (Olson & Björk, 1991; Olson et al., 1992). The preparation had an average molecular weight of ~8000, as determined by gel chromatography (Olson & Shore, 1982), i.e. contained about 26 saccharide units, and was highly similar to that used in previous studies (Olson et al., 1992; Björk et al., 1992). The antithrombin-binding heparin pentasaccharide (H5), a generous gift from M. Petitou (Sanofi Recherche, Toulouse, France), was the same as in earlier work (Olson et al., 1992). Concentrations of both heparin and heparin pentasaccharide were determined by stoichiometric titrations with antithrombin (Olson et al., 1992).

*Fluorescence Titrations of Heparin Binding.* The binding of heparin or pentasaccharide to antithrombin was quantified by titrations, monitored by the fluorescence increase accompanying the interaction (Nordenman et al., 1978; Olson & Shore, 1981), at 25.0 ± 0.2 °C in an SLM 4800S spectrofluorimeter (SLM Instruments, Rochester, NY), as described previously (Olson & Björk, 1991; Olson et al., 1992). Stoichiometry titrations were done at antithrombin concentrations of 0.25−1 $\mu$M at an $I$ of 0.15, whereas concentrations comparable to the dissociation equilibrium constants were used in affinity titrations at an $I$ of 0.15−0.5. Binding stoichiometries and dissociation constants were determined by fitting the data to the equilibrium binding equation by nonlinear regression (Nordenman & Björk, 1978; Olson & Björk, 1991).

*Stopped-Flow Kinetics of Heparin Binding.* The kinetics of heparin or pentasaccharide binding to antithrombin were analyzed at 25.0 ± 0.1 °C in an SX-17MV stopped-flow apparatus (Applied Photophysics, Leatherhead, U.K.). The reactions were monitored by the increase of tryptophan fluorescence emission accompanying the interaction, measured with an excitation wavelength of 280 nm and an emission cutoff filter with ~50% transmission at 320 nm. The experiments were done under pseudo-first-order conditions with at least a 10-fold molar ratio of heparin to antithrombin. The progress curves recorded were fitted to

[1] Abbreviations: α-AT(pl), plasma α-antithrombin; $\beta$-AT(pl), plasma $\beta$-antithrombin; α-AT(rec), recombinant α-antithrombin; $\beta$-AT(rec), recombinant $\beta$-antithrombin; H26, full-length heparin with high affinity for antithrombin, containing 26 saccharide units; H5, the antithrombin-binding heparin pentasaccharide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

a single- exponential function by nonlinear regression, giving the observed pseudo-first-order rate constant, $k_{obs}$. All data given are averages of 10−25 individual measurements.

*Kinetics of Antithrombin Inhibition of Proteinases.* Second-order rate constants for reactions of antithrombin or the antithrombin−heparin complex with thrombin or factor Xa were determined under pseudo-first-order conditions at an *I* of 0.15 and 25 °C, essentially as in previous work (Olson et al., 1992; Björk et al., 1992). Briefly, reactions were initiated by mixing antithrombin (100−200 nM) plus high-affinity heparin or pentasaccharide, when present, with 5−10 nM proteinase in a 100 μL final reaction volume in a polyethylene glycol-coated polystyrene cuvette (Latallo & Hall, 1986). Polybrene was included at a concentration of 50 μg/mL in some experiments in the absence of heparin as a check of heparin contamination of the antithrombin preparations. The inhibitor concentrations were those obtained by stoichiometric titrations of thrombin with inhibitor in the absence of heparin (Olson et al., 1993). High-affinity heparin concentrations ranged from 0.25 to 1.5 nM for thrombin reactions and 2 to 4 nM for factor Xa reactions. Pentasaccharide was used in molar excess over antithrombin (200 nM) in the case of thrombin reactions but at catalytic levels ranging from 4 to 8 nM in the case of factor Xa reactions. Reactions were quenched after varying incubation times with 0.9 mL of chromogenic substrate [100 μM D-Phe-Pip-Arg-*p*-nitroanilide (Chromogenix, Mölndal, Sweden) for thrombin reactions or 100 μM Spectrozyme FXa (American Diagnostica, Greenwich, CT) for factor Xa reactions], and the residual enzyme activity was measured from the linear rate of *p*-nitroaniline formation at 405 nm. The time-dependent loss of enzyme activity was fitted by nonlinear regression to a single-exponential function with an end point of complete inactivation to give the observed pseudo-first-order rate constant, $k_{obs}$ (Olson et al., 1993).

Second-order rate constants for antithrombin−proteinase reactions in the absence of heparin were obtained by dividing $k_{obs}$ by the inhibitor concentration. Second-order rate constants for reactions of complexes between full-length heparin and antithrombin with proteinase were determined from the slope of plots of $k_{obs}$ vs heparin concentration, corrected for the extent of saturation of antithrombin with heparin by use of measured dissociation constants, according to the equation (Björk et al., 1992)

$$k_{obs} = k_H[H]_o \frac{[AT]_o}{(K_{AT,H} + [AT]_o)} + k_{uncat}[AT]_o \qquad (1)$$

where $k_{uncat}$ and $k_H$ are second-order rate constants for reactions of free antithrombin and the antithrombin−heparin complex with proteinase, respectively, $[H]_o$ and $[AT]_o$ are the total heparin and antithrombin concentrations, respectively, and $K_{AT,H}$ is the dissociation constant for heparin binding to antithrombin. Rate constants for reactions of antithrombin−pentasaccharide complexes with factor Xa were determined in the same manner. However, rate constants for reactions of such complexes with thrombin were determined by first subtracting $k_{obs}$ of the reaction with free antithrombin and then dividing by the concentration of the antithrombin-pentasaccharide complex, calculated from measured dissociation constants (Olson et al., 1992).

*Experimental Conditions.* All measurements were done in 20 mM sodium phosphate buffer (pH 7.4) containing 100 μM EDTA and 0.1% (w/v) polyethylene glycol 4000 or 8000. Sodium chloride was added to give ionic strengths of 0.15−0.5.

## RESULTS

*Homogeneity of Antithrombin Forms and Stoichiometry of Heparin and Thrombin Binding.* All antithrombin preparations were ≥95% homogeneous by SDS−PAGE under reducing conditions. The β-forms migrated slightly faster than the α-forms in these analyses, consistent with the absence of one oligosaccharide chain. Stoichiometric titrations, monitored by the increase in intrinsic fluorescence induced by the binding (Olson & Björk, 1991; Olson et al., 1992), of the variants with full-length heparin at an *I* of 0.15 gave heparin to antithrombin binding stoichiometries of ∼0.95, 0.60, 0.55−0.78, and 0.75−0.92 for α-AT(pl), β-AT(pl), α-AT(rec), and β-AT(rec), respectively. The range of values for the two recombinant antithrombins denote the binding stoichiometries for the two or three preparations used. Similar values were obtained for thrombin binding by stoichiometric titrations monitored by the loss of thrombin activity in the absence of heparin (Olson et al., 1993). These data indicate that plasma α-antithrombin was essentially fully active in binding to both heparin and thrombin, whereas the preparations of the plasma β-form and both recombinant forms contained some inactive protein. The antithrombin concentrations used in the studies of heparin and proteinase binding presented below are those obtained by the stoichiometric heparin and thrombin titrations, respectively.

*Affinity of Heparin Binding by Fluorescence Titrations.* Dissociation equilibrium constants for the interaction of full-length heparin or the antithrombin-binding heparin pentasaccharide with plasma α- and β-antithrombin and recombinant α-antithrombin were determined by fluorescence titrations at ionic strengths of 0.15 and 0.3 (Table 1). The values for recombinant β-antithrombin were taken from our recent characterization of this form (Ersdal-Badju et al., 1995). Some interactions at an *I* of 0.15 were too tight to be quantified with reasonable precision.

The dissociation constants measured for binding of the pentasaccharide to plasma α-antithrombin are in agreement with those determined previously (Olson et al., 1992). However, the corresponding values for the interaction with full-length heparin are somewhat higher, consistent with another heparin preparation having been used in this work. The dissociation constant for the binding of full-length heparin to plasma β-antithrombin at an *I* of 0.3 is in good agreement with one previous report (Peterson & Blackburn, 1985), whereas the values for both full-length heparin and pentasaccharide are higher than those obtained in another study (Turko et al., 1993). As shown previously for plasma α-antithrombin (Olson et al., 1992), full-length heparin bound more tightly than the pentasaccharide to all antithrombin forms. Moreover, both full-length heparin and pentasaccharide bound more tightly to the recombinant antithrombin forms than to the corresponding plasma forms. In agreement with previous work (Peterson & Blackburn, 1985; Turko et al., 1993), both full-length heparin and pentasaccharide also had a 3−10-fold higher affinity for both plasma and recombinant β-forms than for the corresponding α-forms.

*Kinetics of Heparin Binding.* The kinetics of binding of full-length heparin and pentasaccharide to plasma and

Table 1: Bimolecular Association Rate Constants, Dissociation Rate Constants, and Dissociation Equilibrium Constants for Heparin Binding to α- and β-Forms of Antithrombin at Different Ionic Strengths[a]

| ionic strength | heparin form | antithrombin form | $10^{-6}k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | measured $K_d$ (nM) | calculated $K_d$ (nM) |
|---|---|---|---|---|---|---|
| 0.15 | H26 | α-AT(pl) | 20 ± 1 | 0.41 ± 0.16 | 24 ± 1 | 20 ± 8 |
| | | β-AT(pl) | 72 ± 2 | ND | ND | |
| | | α-AT(rec) | 58 ± 3 | ND | ND | |
| | | β-AT(rec) | 131 ± 5 | ~0.03[b] | ~0.2[b] | |
| | H5 | α-AT(pl) | 32 ± 1 | 1.5 ± 0.4 | 63 ± 10 | 47 ± 14 |
| | | β-AT(pl) | 54 ± 1 | 0.96[c] | 18 ± 1 | |
| | | α-AT(rec) | 72 ± 1 | 0.57[c] | 8 ± 1 | |
| | | β-AT(rec) | 74 ± 1 | ~0.07[b] | ~1[b] | |
| 0.3 | H26 | α-AT(pl) | 2.9 ± 0.02 | 0.82 ± 0.02 | 300 ± 18 | 280 ± 10 |
| | | β-AT(pl) | 13 ± 0.8 | 0.5 ± 0.5 | 54 ± 7 | 40 ± 40 |
| | | α-AT(rec) | 8.5 ± 0.2 | 0.5 ± 0.1 | 53 ± 6 | 60 ± 13 |
| | | β-AT(rec) | 20 ± 0.5 | 0.1 ± 0.3 | 6 ± 2[b] | 5 ± 14 |
| | H5 | α-AT(pl) | 9.5 ± 0.4 | 8.9 ± 0.4 | 610 ± 40 | 940 ± 90 |
| | | β-AT(pl) | 19 ± 0.4 | 3.6 ± 0.3 | 190 ± 7 | 190 ± 18 |
| | | α-AT(rec) | 20 ± 0.3 | 5.3 ± 0.2 | 250 ± 24 | 260 ± 14 |
| | | β-AT(rec) | 22 ± 0.5 | 0.7 ± 0.2 | 24 ± 2[b] | 32 ± 10 |
| 0.4 | H26 | α-AT(pl) | 1.6 ± 0.1 | 1.4 ± 0.6 | | 900 ± 440 |
| | | β-AT(pl) | 5.6 ± 0.3 | 1.0 ± 0.3 | | 180 ± 60 |
| | | α-AT(rec) | 4.8 ± 0.2 | 1.3 ± 0.2 | | 270 ± 40 |
| | | β-AT(rec) | 10.7 ± 0.3 | 0.3 ± 0.1 | 20 ± 5[b] | 28 ± 15 |
| | H5 | α-AT(pl) | 5.3 ± 0.2 | 11.3 ± 0.9 | | 2100 ± 260 |
| | | β-AT(pl) | 11.1 ± 0.2 | 4.9 ± 0.3 | | 440 ± 40 |
| | | α-AT(rec) | 10.3 ± 0.3 | 8.9 ± 0.6 | | 860 ± 80 |
| | | β-AT(rec) | 14.4 ± 0.7 | 1.1 ± 0.5 | 72 ± 10[b] | 80 ± 40 |
| 0.5 | H26 | α-AT(pl) | 0.62 ± 0.03 | 2.6 ± 0.2 | | 4200 ± 400 |
| | | β-AT(pl) | 3.1 ± 0.1 | 1.5 ± 0.1 | | 480 ± 40 |
| | | α-AT(rec) | 2.2 ± 0.05 | 1.6 ± 0.1 | | 730 ± 50 |
| | | β-AT(rec) | 4.7 ± 0.2 | 0.45 ± 0.26 | 79 ± 4[b] | 96 ± 60 |
| | H5 | α-AT(pl) | 2.5 ± 0.1 | 14.5 ± 0.3 | | 5800 ± 250 |
| | | β-AT(pl) | 5.9 ± 0.3 | 6.1 ± 0.7 | | 1030 ± 180 |
| | | α-AT(rec) | 4.9 ± 0.4 | 11.1 ± 1.3 | | 2300 ± 460 |
| | | β-AT(rec) | 6.8 ± 0.2 | 1.8 ± 0.3 | 200 ± 6[b] | 260 ± 60 |

[a] Bimolecular association rate constants ($k_{on}$), dissociation rate constants ($k_{off}$), and dissociation equilibrium constants ($K_d$) were determined at different ionic strengths at pH 7.4, and 25 °C, as detailed in Materials and Methods. Dissociation equilibrium constants were also calculated from measured association and dissociation rate constants (i.e. as $k_{off}/k_{on}$). The values for $k_{on}$ and $k_{off}$ were obtained as the slope and intercept, respectively, of linear plots of observed pseudo-first-order rate constants vs heparin concentration. These plots were based on measurements at five to nine heparin concentrations in a range which varied from 0.1−1 μM at an $I$ of 0.15 to 0.5−5 μM at an $I$ of 0.5. Measured $K_d$ values are averages of three or four measurements at antithrombin concentrations comparable to $K_d$. All errors represent ±SE. [b] Taken from Ersdal-Badju et al. (1995). [c] Calculated from measured values for $K_d$ and $k_{on}$.

recombinant α- and β-antithrombin were investigated by stopped-flow fluorescence under pseudo-first-order conditions. The observed fluorescence change was monophasic for all interactions and was satisfactorily fitted to a single-exponential function at all concentrations studied. The measurements were done in two heparin concentration ranges.

*(a) Low Heparin Concentrations.* Analyses at low heparin or pentasaccharide concentrations were done at ionic strengths between 0.15 and 0.5, the saccharide concentration range being varied from 0.1−1 μM at an $I$ of 0.15 to 0.5−5 μM at an $I$ of 0.5. Under these conditions, the observed pseudo-first-order rate constants increased linearly with heparin or pentasaccharide concentration, the slope of such plots giving the bimolecular association rate constant, $k_{on}$, and the intercept on the ordinate giving the overall dissociation rate constant, $k_{off}$ (Olson et al., 1981, 1992). The association rate constants and most dissociation rate constants could be measured with good precision (Table 1). However, some dissociation rate constants at the lower ionic strengths, in particular for the β-forms, were too low to be experimentally accessible or could only be measured with considerable error. Instead, these values were calculated from measured dissociation equilibrium constants and bimolecular association rate constants, when such values were available (Table 1). In those cases where both the association and dissociation rate constants could be measured, the overall dissociation equilibrium constant was calculated from these values (Table 1). These calculated dissociation constants agreed reasonably well with those measured by fluorescence titrations.

Similar to previous results for plasma α-antithrombin (Olson et al., 1992), the association rate constants decreased and the dissociation rate constants increased with increasing ionic strength for all interactions (Table 1). The values for pentasaccharide binding to α-antithrombin were in fair agreement with those measured previously (Olson et al., 1992), whereas the values for full-length heparin differed somewhat, again reflecting another heparin preparation. As shown for plasma α-antithrombin (Olson et al., 1992), both the association and dissociation rate constants in general were lower for full-length heparin than for the pentasaccharide, the only exception being the association rate constants for the β-forms at an $I$ of 0.15. Moreover, the association rate constants for the interactions with the recombinant antithrombin forms were higher and the dissociation rate constants lower than for the same interactions with the plasma forms. Most importantly, the association rate constants for the binding of both full-length heparin and pentasaccharide to β-antithrombin were higher and the dissociation rate constants lower than the values for the

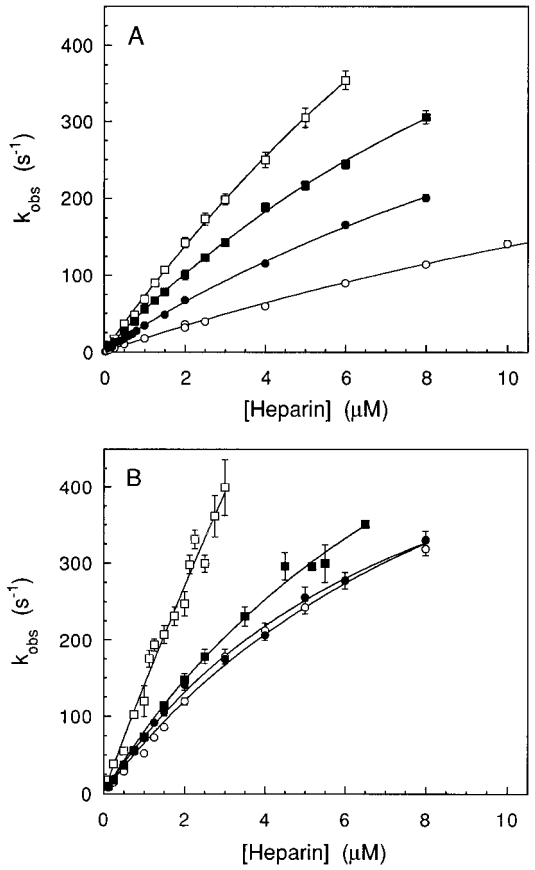


FIGURE 1: Heparin concentration dependence of observed pseudo-first-order rate constants, $k_{obs}$, for heparin binding to α- and β-antithrombin. (A) Plasma antithrombin and (B) recombinant antithrombin. (○) Full-length heparin−α-antithrombin, (●) pentasaccharide−α-antithrombin, (□) full-length heparin−β-antithrombin, and (■) pentasaccharide−β-antithrombin. Average values ± SE were measured by stopped-flow fluorescence at an $I$ of 0.15 and pH 7.4 and 25 °C, as detailed in Materials and Methods. Error bars not shown lie within the dimensions of the symbol. The solid lines represent nonlinear regression fits to eq 2. Data for the binding of full-length heparin to plasma α-antithrombin were collected up to a heparin concentration of 20 μM; the data above 10 μM are not shown but were included in the fit.

binding to α-antithrombin. These findings are consistent with the observed differences in dissociation equilibrium constants presented above.

*(b) Higher Heparin Concentrations.* Analyses of the kinetics of binding of full-length heparin and the pentasaccharide to the four antithrombin forms at an $I$ of 0.15 were extended to heparin concentrations of 6−10 μM for most interactions. However, the fast reaction between full-length heparin and recombinant β-antithrombin limited the accessible heparin concentrations to below ∼3 μM, as the dead time of the stopped-flow instrument precluded measurements of rate constants higher than ∼400 $s^{-1}$. For all interactions that could be studied in the full concentration range, the observed pseudo-first-order rate constants increased hyperbolically with increasing saccharide concentration (Figure 1), in agreement with previous studies of the binding of full-length heparin or pentasaccharide to plasma α-antithrombin (Olson et al., 1981, 1992). This behavior has been shown to arise from a two-step process, involving an initial, weak binding in rapid equilibrium, followed by a protein conformational change responsible for the fluorescence change, as illustrated in the following scheme:

Table 2: Kinetic Constants for the Two-Step Mechanism in Scheme 1 for Heparin Binding to α- and β-Forms of Antithrombin at an Ionic Strength of 0.15[a]

| heparin form | antithrombin form | $K_1$ (μM) | $k_{+2}$ ($s^{-1}$) |
|---|---|---|---|
| H26 | α-AT(pl) | 37 ± 4 | 650 ± 50 |
| | β-AT(pl) | 21 ± 2 | 1600 ± 140 |
| | α-AT(rec) | 11 ± 1 | 770 ± 70 |
| | β-AT(rec) | ≥10 | ≥3000 |
| H5 | α-AT(pl) | 22 ± 2 | 750 ± 50 |
| | β-AT(pl) | 16 ± 1 | 910 ± 40 |
| | α-AT(rec) | 8.3 ± 0.7 | 660 ± 30 |
| | β-AT(rec) | 11 ± 2 | 920 ± 100 |

[a] The dissociation equilibrium constant for the first step ($K_1$) and the forward rate constant for the second step ($k_{+2}$) of Scheme 1 were obtained by fitting the data of Figure 1 to eq 2 by nonlinear least-squares regression. All errors represent ± SE.

Scheme 1

$$AT + H \underset{}{\overset{K_1}{\rightleftharpoons}} AT{-}H \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} AT^*{-}H$$

In this scheme, AT is antithrombin, H is heparin, $K_1$ is the dissociation equilibrium constant of the first binding step, leading to the initial complex AT−H, and $k_{+2}$ and $k_{-2}$ are the forward and reverse rate constants of the conformational change, producing the final complex AT*−H. For this mechanism, the observed pseudo-first-order rate constant, $k_{obs}$, varies with heparin concentration, $[H]_o$, according to the equation (Olson et al., 1981, 1992)

$$k_{obs} = \frac{k_{+2}[H]_o}{K_1 + [H]_o} + k_{-2} \quad (2)$$

Moreover, the bimolecular association rate constant, $k_{on}$, and the overall dissociation rate constant, $k_{off}$, measured at low heparin concentrations as described above, are equal to $k_{+2}/K_1$ and $k_{-2}$, respectively (Olson et al., 1992). All hyperbolic curves could be well fitted to eq 2 by nonlinear regression analysis, giving the values for $K_1$ and $k_{+2}$ shown in Table 2. In these fits, $k_{-2}$ was set to the value measured for the equivalent parameter, $k_{off}$ (Table 1), or to zero when this value was not measurable. In contrast to this hyperbolic dependence of $k_{obs}$ on heparin concentration, a dependence that was linear within experimental error was seen for the reaction between full-length heparin and recombinant β-antithrombin, which could be investigated only up to ∼3 μM heparin (Figure 1). On the basis of the assumption that this dependence is the initial, approximately linear part of a hyperbolic dependence at higher heparin concentrations, limits for the values of $K_1$ and $k_{+2}$ were estimated for this interaction (Table 2).

The values of $K_1$ and $k_{+2}$ for the binding of the pentasaccharide to plasma α-antithrombin (Table 2) agreed well with those measured previously (Olson et al., 1992), but the values for the new preparation of full-length heparin used were slightly different, consistent with results presented above. In the case of most variants, both $K_1$ and $k_{+2}$ were higher for full-length heparin than for the pentasaccharide, a difference particularly apparent for $k_{+2}$ of the β-forms. However, $k_{+2}$ for the binding of full-length heparin to plasma α-antithrombin was somewhat lower than that for the binding of the pentasaccharide, in agreement with earlier studies of

this antithrombin form (Olson et al., 1992). Furthermore, $K_1$ for the interactions of full-length heparin and pentasaccharide with the recombinant antithrombin forms in general was lower than that for the interactions with the plasma forms. Corresponding differences in $k_{+2}$ were inconsistent, except in the case of full-length heparin binding to β-antithrombin, in which case $k_{+2}$ was appreciably higher for the recombinant form. The values of $K_1$ for the interactions of full-length heparin and pentasaccharide with plasma β-antithrombin were somewhat lower than those for the corresponding interactions with the plasma α-form, whereas a similar difference was not apparent in the case of the recombinant antithrombins. Most notably, however, $k_{+2}$ for binding of full-length heparin to the two β-antithrombin forms was considerably higher than that for the binding to the corresponding α-forms, and this difference, although smaller, was also evident for pentasaccharide binding.

*Ionic and Nonionic Contributions to Heparin Binding. (a) Overall Binding.* The ionic and nonionic contributions to the overall binding of full-length heparin and pentasaccharide to α- and β-antithrombin were evaluated from the dependence of the observed dissociation equilibrium constants on sodium ion concentration. According to polyelectrolyte theory (Record et al., 1976, 1978; Olson & Björk, 1991; Olson et al., 1991, 1992), the binding of a polyelectrolyte, heparin, to a protein, antithrombin, in the presence of sodium ions can be described by the equation

$$\log K_d = \log K_d' + Z\psi \log[Na^+] \qquad (3)$$

where $K_d$ is the observed dissociation equilibrium constant, $K_d'$ is the dissociation constant at 1 M $Na^+$, $Z$ is the number of ionic interactions involved in the binding, and $\psi$ is the fraction of $Na^+$ which is bound per ionic charge of heparin and which is released on antithrombin binding. This equation thus assumes that anion release from antithrombin can be neglected. In eq 3, the contribution of ionic interactions to the heparin−antithrombin binding energy is represented by the second term, the product $Z\psi$ reflecting the number of sodium ions displaced from heparin in the binding process, whereas the contribution of nonionic interactions is given by the first term. As measured and calculated equilibrium constants agreed well at each ionic strength (Table 1), the values obtained by the two procedures were combined to enable analyses in the range of $I$ from 0.15 to 0.5 (see the legend to Figure 2). Plots of log $K_d$ vs log[$Na^+$] were linear for all heparin−antithrombin interactions (Figure 2), the slopes and intercepts of these plots giving $Z\psi$ and log $K_d'$, respectively (Table 3).

As observed previously for plasma α-antithrombin (Olson et al., 1992), about 4 sodium ions were found to be displaced in the overall binding of full-length heparin to all antithrombin variants, as shown by the values of $Z\psi$. The extent of sodium ion release was lower on pentasaccharide binding, also in accord with the previous work, although this difference was not as readily evident for the recombinant forms (Table 3). The nonionic contribution to the binding to all antithrombin forms, represented by log $K_d'$, was similar for full-length heparin and pentasaccharide, in further agreement with earlier results obtained with plasma α-antithrombin (Olson et al., 1992). No consistent differences in the number of sodium ions released on heparin or pentasaccharide binding or in the nonionic contribution to this binding were apparent between the plasma and recombinant antithrombin forms. Comparisons between α- and β-antithrombin also showed no consistent differences in the number of sodium ions released on binding of either full-length heparin or pentasaccharide. However, the nonionic contribution to the binding of both heparin forms was appreciably higher for β- than for α-antithrombin.

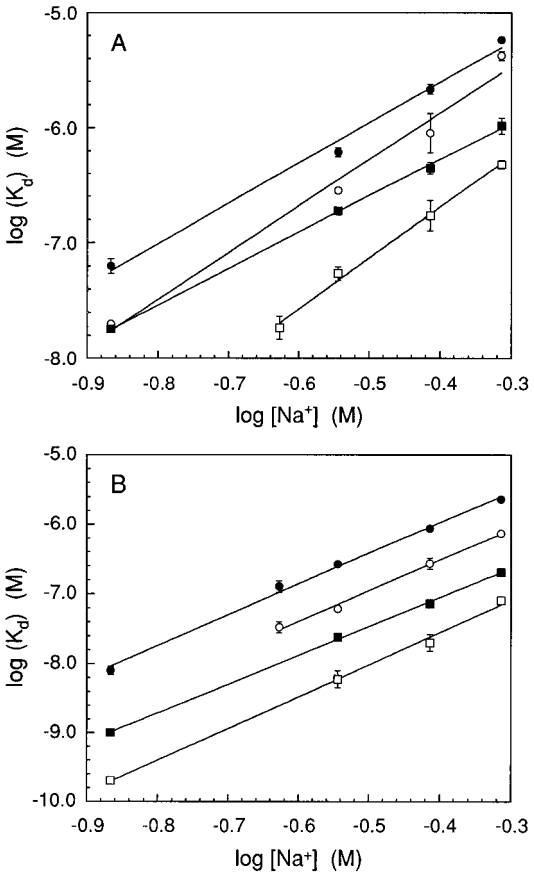


FIGURE 2: NaCl concentration dependence of dissociation equilibrium constants, $K_d$, for heparin binding to α- and β-antithrombin. (A) Plasma antithrombin and (B) recombinant antithrombin. (○) Full-length heparin−α-antithrombin, (●) pentasaccharide−α-antithrombin, (□) full-length heparin−β-antithrombin, and (■) pentasaccharide−β-antithrombin. Most values are from Table 1. Measured dissociation constants were used at an $I$ of 0.15 and 0.3, whereas calculated values were used at an $I$ of 0.4 and 0.5, except in the case of recombinant β-antithrombin, for which measured values were available also at these ionic strengths (Table 1). In those cases where the dissociation constants could not be measured at an $I$ of 0.15 (Table 1), such constants (not included in Table 1) were instead determined at an $I$ of 0.25 by additional fluorescence titrations. Error bars represent ±SE. Error bars not shown lie within the dimensions of the symbol, except for recombinant β-antithrombin at an $I$ of 0.15, in which case errors were lacking. The solid lines represent linear regression fits.

*(b) Initial Binding Step.* The ionic contribution to the initial step of the two-step mechanism for binding of full-length heparin and pentasaccharide to α- and β-antithrombin (Scheme 1) was assessed in an analogous manner from the dependence of the bimolecular association rate constants on sodium ion concentration. Previous studies with α-antithrombin have shown that $k_{+2}$, the rate constant for the conformational change of the two-step mechanism, is unaffected by salt for both full-length heparin and pentasaccharide (Olson et al., 1992). As it is reasonable to assume that this finding is valid also for the other antithrombin forms, the salt dependence of the bimolecular association rate constant,

Table 3: Ionic and Nonionic Contributions to The Overall Binding of Heparin to α- and β-Forms of Antithrombin and Ionic Contributions to the First Binding Step[a]

| | | overall binding | | | first binding step | |
|---|---|---|---|---|---|---|
| heparin form | antithrombin form | $Z\psi$ | $Z$ | log $K_d'$ | $Z_1\psi$ | $Z_1$ |
| H26 | α-AT(pl) | 4.0 ± 0.4 | 5.0 ± 0.5 | -4.3 ± 0.2 | 2.6 ± 0.2 | 3.3 ± 0.2 |
| | β-AT(pl) | 4.4 ± 0.2 | 5.5 ± 0.3 | -4.9 ± 0.1 | 2.5 ± 0.1 | 3.1 ± 0.1 |
| | α-AT(rec) | 4.4 ± 0.2 | 5.5 ± 0.3 | -4.8 ± 0.1 | 2.5 ± 0.1 | 3.1 ± 0.1 |
| | β-AT(rec) | 4.6 ± 0.2 | 5.8 ± 0.2 | -5.7 ± 0.1 | 2.6 ± 0.1 | 3.2 ± 0.1 |
| H5 | α-AT(pl) | 3.5 ± 0.2 | 4.4 ± 0.3 | -4.2 ± 0.2 | 1.9 ± 0.2 | 2.4 ± 0.3 |
| | β-AT(pl) | 3.1 ± 0.1 | 3.9 ± 0.1 | -5.0 ± 0.1 | 1.7 ± 0.2 | 2.1 ± 0.2 |
| | α-AT(rec) | 4.4 ± 0.2 | 5.5 ± 0.2 | -4.2 ± 0.1 | 2.1 ± 0.2 | 2.6 ± 0.2 |
| | β-AT(rec) | 4.2 ± 0.1 | 5.2 ± 0.1 | -5.4 ± 0.1 | 1.8 ± 0.2 | 2.2 ± 0.3 |

[a] The number of sodium ions released in the overall binding of heparin to antithrombin, $Z\psi$, and in the first binding step, $Z_1\psi$ (eq 3), was obtained from the slopes of double-logarithmic plots of observed dissociation equilibrium constants (Figure 2) and bimolecular association rate constants (not shown), respectively, vs sodium concentration. The number of ionic interactions involved in the overall binding, $Z$, and in the first binding step, $Z_1$, was calculated from a value for $\psi$ of 0.8, computed from reported values of the axial charge density of heparin (Olson & Björk, 1991; Olson et al., 1991, 1992). The nonionic contribution to the overall binding, log $K_d'$, was obtained from the intercepts on the ordinates of the plots of Figure 2. The slopes and intercepts ± SE were calculated by linear regression.

Table 4: Association Rate Constants for Uncatalyzed and Heparin-Catalyzed Reactions of α- and β-Forms of Antithrombin with Proteinases at an Ionic Strength of 0.15[a]

| proteinase | antithrombin form | $k_{uncat}$($M^{-1}$ $s^{-1}$) | $k_{H26}$ ($M^{-1}$ $s^{-1}$) | $k_{H5}$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| thrombin | α-AT(pl) | $(7.4 \pm 0.2) \times 10^3$ | $(1.0 \pm 0.1) \times 10^7$ | $(1.5 \pm 0.1) \times 10^4$ |
| | β-AT(pl) | $(8.5 \pm 0.5) \times 10^3$ | $(8.6 \pm 1.3) \times 10^6$ | $(1.6 \pm 0.1) \times 10^4$ |
| | α-AT(rec) | $(8.4 \pm 0.1) \times 10^3$ [c] | $(8.2 \pm 0.3) \times 10^6$ | $1.7 \times 10^4$ [b] |
| | β-AT(rec) | $(6.9 \pm 0.2) \times 10^3$ | $(6.1 \pm 0.6) \times 10^6$ | $(1.1 \pm 0.1) \times 10^4$ |
| factor Xa | α-AT(pl) | $(2.3 \pm 0.2) \times 10^3$ | $(8.6 \pm 0.2) \times 10^5$ | $(5.9 \pm 0.3) \times 10^5$ |
| | β-AT(pl) | $(4.6 \pm 0.1) \times 10^3$ | $(8.3 \pm 0.3) \times 10^5$ | $(5.7 \pm 0.1) \times 10^5$ |
| | α-AT(rec) | $(2.3 \pm 0.1) \times 10^3$ | $(6.4 \pm 0.1) \times 10^5$ | $(3.8 \pm 0.2) \times 10^5$ |
| | β-AT(rec) | $(4.3 \pm 0.4) \times 10^3$ | $(9.2 \pm 0.2) \times 10^5$ | $(5.0 \pm 0.2) \times 10^5$ |

[a] Second-order association rate constants for uncatalyzed ($k_{uncat}$), full-length heparin-catalyzed ($k_{H26}$), and pentasaccharide-catalyzed ($k_{H5}$) reactions of antithrombin with proteinases were determined at an $I$ of 0.15 and pH 7.4 and 25 °C, as detailed in Materials and Methods. All measurements were done at least twice, except where noted, with errors representing ± range (for $n = 2$) or SE (when $n > 2$). [b] Single measurement. [c] Measured in the presence of 50 μg/mL polybrene. A 1.9-fold greater rate constant was measured in the absence of polybrene, indicating a minor heparin contamination equivalent to 0.1 nM high-affinity heparin. Since this level of contamination was expected to insignificantly (<5%) affect rate constants for reactions with factor Xa and since polybrene appreciably (15−30%) enhanced the rate of the antithrombin−factor Xa reaction, the corresponding rate constant for factor Xa was measured without polybrene. The rates of reactions of thrombin with the other antithrombin forms were indistinguishable with and without polybrene, showing that these forms were free of heparin contamination.

$k_{on}$, which is equal to $k_{+2}/K_1$, therefore should reflect the salt dependence of $K_1$, the dissociation equilibrium constant for the initial binding step (Olson et al., 1992). Double-logarithmic plots of log $k_{on}$ vs log[$Na^+$] were linear, within experimental error, for all heparin−antithrombin interactions (not shown) and gave the values for $Z_1\psi$, the number of sodium ions displaced from heparin in the first binding step, shown in Table 3. In this case, the intercept on the ordinate is not a direct measure of the nonionic contribution to the binding energy, as this intercept is a function of both $K_1$ and $k_{+2}$, the latter being different for the various heparin−antithrombin interactions and undetermined for full-length heparin binding to recombinant β-antithrombin (Table 2).

The data indicate that an average of about 2.5 sodium ions are displaced in the first step of binding of full-length heparin to all antithrombin variants, in agreement with earlier studies with plasma α-antithrombin (Olson et al., 1992), whereas only about 2 such ions are displaced in this step during the binding of the pentasaccharide (Table 3). No definitive differences in the number of sodium ions released in the first binding step were observed between the plasma and recombinant antithrombin forms or between α- and β-antithrombin.

*Kinetics of Inactivation of Thrombin and Factor Xa.* Second-order association rate constants were determined for the inhibition of thrombin and factor Xa by antithrombin alone and by the complexes between antithrombin and full-length heparin or pentasaccharide (Table 4). The rate constants for the unaccelerated inhibition of thrombin by the four antithrombin forms were undistinguishable. However, the corresponding rate constants for the inhibition of factor Xa were about 2-fold higher for both plasma and recombinant β-forms than for the α-forms, as was also observed in an earlier comparison of recombinant β-antithrombin with plasma α-antithrombin (Ersdal-Badju et al., 1995). In agreement with previous evidence for α-antithrombin (Choay et al., 1983; Olson et al., 1992), the pentasaccharide accelerated the inhibition of thrombin by all antithrombin forms to a much lower extent than full-length heparin, whereas the acceleration of factor Xa inhibition by all antithrombin forms was comparable for full-length heparin and pentasaccharide. Only minor differences, with no apparent trends, in the rate constants for inhibition of thrombin or factor Xa were evident for complexes of the four antithrombin forms with full-length heparin or for such complexes with the pentasaccharide.

## DISCUSSION

The results of previous studies comparing the binding of full-length heparin and the specific heparin pentasaccharide to plasma α-antithrombin (Olson et al., 1992) were confirmed also for the other antithrombin forms investigated in this work. Full-length heparin thus bound more tightly than the pentasaccharide to all antithrombin forms, the higher affinity predominantly being due to a lower dissociation rate constant.

Moreover, full-length heparin appeared to interact more weakly than the pentasaccharide with all antithrombin forms in the initial complex of the two-step binding reaction, as is made evident by a somewhat higher dissociation equilibrium constant of the first binding step, $K_1$. This behavior may be due to a weak repulsion between negatively charged groups on full-length heparin outside the pentasaccharide region and acidic groups on antithrombin adjacent to the heparin binding site. As was also shown for plasma α-antithrombin (Olson et al., 1992), more sodium ions were released in the overall binding of full-length heparin than in the binding of the pentasaccharide to all antithrombin variants, although this difference was less clear for the recombinant forms. A likely interpretation of the data, also made in the previous work (Olson et al., 1992), is that about five ionic interactions contribute to the binding of full-length heparin to all antithrombin forms, whereas about one less such interaction participates in the binding of the pentasaccharide (Table 2). The additional ionic interaction established by full-length heparin was suggested to be weak, contributing little to the binding energy and the conformational change, and made with a positively charged group outside the pentasaccharide binding area of antithrombin (Olson et al., 1992). A notable difference from the earlier studies is that more sodium ions were found to be released in the first step of binding of full-length heparin to all antithrombin variants than in the first step of pentasaccharide binding. The data may be interpreted as three ionic interactions being established in the first binding step for full-length heparin but only two such interactions in the first step for the pentasaccharide (Table 2). The additional weak ionic interaction contributed by full-length heparin may thus be made in the first binding step. However, these interpretations are based on the assumption that the value for $\psi$, the fraction of $Na^+$ bound per ionic charge of heparin and displaced on antithrombin binding (see eq 3), that was calculated from reported axial charge densities of heparin (Table 2; Olson et al., 1991) is valid for both full-length heparin and pentasaccharide. An alternative possibility is that counterion binding to the pentasaccharide is reduced, because of the lower charge density at the ends of this short heparin chain, resulting in a lower value of $\psi$ (Record & Lohman, 1978). This would lead to a decreased counterion release on binding of the pentasaccharide than on binding of full-length heparin to antithrombin, even if the two heparins make the same number of ionic interactions with the protein. Because of the lack of suitable model studies allowing evaluation of this effect, a clear distinction between the two alternatives cannot be made. In contrast to these differences in ionic contributions, the nonionic contributions to the binding of full-length heparin and pentasaccharide were similar for all antithrombin forms, as demonstrated previously for the plasma α-form (Olson et al., 1992).

Comparisons showed that all recombinant antithrombin forms bound full-length heparin and pentasaccharide somewhat more tightly than the corresponding plasma forms. This higher affinity was due both to a higher bimolecular association rate constant and to a lower dissociation rate constant. The higher association rate constant was caused at least partly by a tighter binding of the two heparin forms in the initial binding step, a higher rate constant for the conformational change also possibly contributing. These modest differences were seen for both α- and β-antithrombin and are thus largely independent of the presence of an oligosaccharide chain on Asn-135. Instead, they are presumably caused mainly by differences between the plasma and recombinant antithrombins in the structure of the other oligosaccharide side chains, the only known distinguishing property of the two forms. The recombinant antithrombin forms thus have short, nonsialylated carbohydrate side chains, some of which are of the high-mannose or hybrid type (Ersdal-Badju et al., 1995). In contrast, the side chains of the plasma forms are longer, sialylated and all of the complex type (Franzén et al., 1980; Mizuochi et al., 1980). The overall glycosylation properties of antithrombin thus appear to affect the heparin binding ability of the inhibitor to some extent. In keeping with this conclusion, deletion of any one of the three oligsaccharide chains other than that at Asn-135 from antihrombin expressed in a mammalian system increases the heparin affinity of the protein (Olson et al., 1997).

The main goal of this work was to elucidate the molecular basis for the previously demonstrated higher affinity of β-antithrombin than of α-antithrombin for heparin (Carlson & Atencio, 1982; Peterson & Blackburn, 1985; Brennan et al., 1987; Turko et al., 1993). The oligosaccharide side chain at Asn-135 in the α-form that is absent in the β-form is located close to the putative heparin binding site (Carrell et al., 1994; van Boeckel et al., 1994). A likely possibility therefore would be that this side chain, by virtue of its size or negative charge, interferes with the weak binding of heparin to α-antithrombin in the initial step of the two-step binding reaction (Olson et al., 1981, 1992), thereby reducing the overall heparin affinity of the α-form. However, the results of this work show that such an effect contributes at most moderately to the decreased affinity. Although the dissociation equilibrium constant, $K_1$, for the initial binding of full-length heparin and pentasaccharide was somewhat higher for plasma α- than for plasma β-antithrombin, consistent with a slightly weaker initial binding to the α-form, the corresponding difference was less apparent for the two recombinant forms. This small effect on $K_1$ indicates that the interactions established in the initial step involve regions of antithrombin some distance away from the oligosaccharide at Asn-135. Instead, all data indicate that this oligosaccharide reduces the heparin affinity of α-antithrombin predominantly by affecting the heparin-induced conformational change that results in activation of the inhibitor. The rate constant for the conformational change step, $k_{+2}$, was thus considerably lower for the binding of full-length heparin to both plasma and recombinant α-antithrombin than for the binding to the β-forms, and this difference, although less pronounced, was also seen for the pentasaccharide. Together, the differences in $K_1$ and $k_{+2}$ resulted in appreciably higher bimolecular association rate constants for the binding of the two heparin forms to β-antithrombin. Further evidence that the Asn-135 oligosaccharide primarily affects the heparin-induced conformational change is provided by the higher overall dissociation rate constant, identical with the reverse rate constant for the conformational change step, $k_{-2}$, for both full-length heparin and pentasaccharide binding to α- than to β-antithrombin. Moreover, the lower nonionic contribution to the binding of both heparin forms to plasma and recombinant α-antithrombin than to the binding to the β-variants is consistent with the conformational change in the second binding step being affected by the oligosaccharide.

However, the possibility that the additional nonionic interactions established in the interaction with β-antithrombin are all made in the first binding step cannot be excluded, although this alternative is less likely. No differences in the number of ionic interactions made by full-length heparin and pentasaccharide, either in the first binding step or in the overall binding, were observed between α- and β-antithrombin. The Asn-135 oligosaccharide thus does not shield any charged groups on antithrombin from interaction with heparin or pentasaccharide.

The data are consistent with the Asn-135 oligosaccharide side chain in α-antithrombin predominantly affecting the extent of the heparin-induced conformational change activating the inhibitor. The lower $k_{+2}$ and higher $k_{-2}$ for the binding of heparin to α-antithrombin than for the binding to the β-form thus result in a higher equilibrium constant of the second step, $K_2$ ($=k_{-2}/k_{+2}$), for α-antithrombin. For instance, this equilibrium constant is $\sim 2 \times 10^{-3}$ and $\sim 1 \times 10^{-3}$ for pentasaccharide binding to plasma and recombinant α-antithrombin, respectively, at an $I$ of 0.15 and $\sim 1 \times 10^{-3}$ and $\sim 1 \times 10^{-4}$, respectively, for the binding to the corresponding β-forms. The equilibrium is thus slighly less shifted toward the conformational change in the case of α-antithrombin. Nevertheless, it is apparent that the equilibrium is strongly in favor of the activated conformation for both the α- and β-forms. As a consequence, the extent of heparin activation of the inhibitor is not measurably affected, as is evident from the finding that the rate constants for the inhibition of factor Xa at saturation of antithrombin with heparin or pentasaccharide were indistinguishable for the α- and β-forms. In contrast to these similar heparin-activated rate constants, the uncatalyzed rate of factor Xa inactivation by β-antithrombin was enhanced about 2-fold over that by α-antithrombin. This observation suggests that the Asn-135 oligosaccharide to a small extent may affect a conformational equilibrium between the native and activated conformations of antithrombin that exists also in the absence of heparin, albeit highly shifted in favor of the native conformation. This proposal thus implies that the Asn-135 oligosaccharide disfavors the conformational change both in the absence and in the presence of heparin.

Together, these results suggest a model in which the Asn-135 oligosaccharide of α-antithrombin functions as a negative effector of antithrombin activation (Figure 3). In this model, the Asn-135 oligosaccharide is oriented away from the heparin binding site in the native antithrombin conformation and consequently does not interfere with the initial binding of heparin. As a result of this initial binding, conformational changes are induced in both antithrombin and the pentasaccharide region of heparin, leading to subsequent tighter binding of the polysaccharide. These conformational changes may involve an extension of helix D into the polypeptide chain segment adjacent to its C-terminal end, which connects helix D to β-sheet A and contains Asn-135 (van Boeckel et al., 1994). This conformational change has been suggested to align positively charged residues on helix D for optimal interaction with heparin and to transmit additional conformational changes to the reactive-bond loop, leading to inhibitor activation (van Boeckel et al., 1994). An equilibrium involving such a loop extension, although highly unfavorable, may also occur in the absence of heparin, as discussed above. The change in the Asn-135 loop from an extended to a helical conformation would be expected to

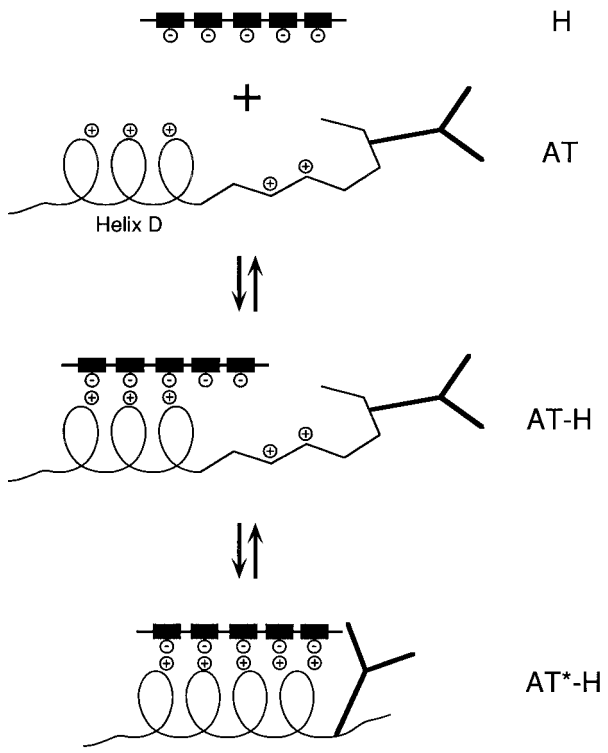


FIGURE 3: Schematic model for the effect of the Asn-135 carbohydrate side chain in α-antithrombin on heparin binding. H, heparin, represented by the antithrombin-binding pentasaccharide region; AT, antithrombin; AT−H, the initial antithrombin−heparin complex, in which the pentasaccharide region binds weakly to helix D; and AT*−H, the final, tight antithrombin−heparin complex, in which a conformational change in the form of an elongation of helix D by one turn has been induced in the inhibitor. The carbohydrate sidechain on Asn-135 is denoted by the Y-shaped symbol.

reduce the conformational flexibility of the Asn-135 oligosaccharide and orient it closer to the pentasaccharide binding site. This decreased flexibility of the oligosaccharide presumably destabilizes the activated, helix D-extended conformation relative to the native conformation for both the free and the heparin-bound inhibitor. Moreover, the close proximity of the oligosaccharide to the heparin binding site may interfere with the binding of the pentasaccharide region in the second binding step. Both of these effects would be expected to result in a higher energy for inducing the activated antithrombin conformation, leading to a decrease in heparin binding energy. According to this model, the Asn-135 oligosaccharide of α-antithrombin thus reduces heparin affinity because heparin binding energy is used to overcome less favorable interactions of the oligosaccharide with the main body of the protein and with the pentasaccharide region of heparin in the activated than in the native antithrombin conformation.

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