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Contribution of 3-O- and 6-O-Sulfated Glucosamine Residues in the Heparin-Induced Conformational Change in Antithrombin III†

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ABSTRACT: The role of 3-O- and 6-O-sulfated glucosamine residues within the heparin octasaccharide critical for biological activity, iduronic acid[(1)]→*N*-acetylglucosamine 6-*O*-sulfate[(2)]→glucuronic acid[(3)]→N-sulfated glucosamine 3,6-di-*O*-sulfate[(4)]→iduronic acid 2-*O*-sulfate[(5)]→N-sulfated glucosamine 6-*O*-sulfate[(6)]→iduronic acid 2-*O*-sulfate[(7)]→anhydromannitol 6-*O*-sulfate[(8)], was determined by comparing its ability to bind antithrombin, induce a conformational change in this protease inhibitor as monitored by the enhancement of intrinsic fluorescence, and accelerate (*at saturation*) the interaction of this protein with human factor Xa. The octasaccharide produced a maximum 48% increase in intrinsic fluorescence at 37 °C and a rate of factor Xa inhibition of 6×10^5 M^{-1} s^{-1} as measured by stopped-flow fluorometry at 25 °C. The basal rate of the antithrombin–factor Xa interaction observed in the absence of oligosaccharide was 2×10^3 M^{-1} s^{-1}. The synthetic pentasaccharide, consisting of residues 2–6, produced fluorescence enhancement and rate of inhibition equal to those of the octasaccharide. However, a similar pentasaccharide, identical in all respects except that it lacked the 3-*O*-sulfate on residue 4, produced less than a 5% fluorescence enhancement and a rate of factor Xa inhibition of 8×10^3 M^{-1} s^{-1}. The tetrasaccharide consisting of residues 2–5 produced a 35% fluorescence enhancement and a rate of factor Xa inhibition of 3×10^5 M^{-1} s^{-1}. The tetrasaccharide consisting of residues 3–6 produced a 33% fluorescence enhancement and a rate of factor Xa inhibition of 6×10^4 M^{-1} s^{-1}. Thus, the loss of either the 6-O-sulfated residue 2 or the 3-*O*-sulfate of residue 4 results in a 30–95% loss in the ability of the pentasaccharide to enhance the intrinsic fluorescence of antithrombin and a 10–76-fold reduction in the rate of factor Xa inhibition. These two residues must, necessarily, represent the major contributors to a conformational change in antithrombin, which is linked to the biological activity of the octasaccharide. In addition, nonproportional changes in the intrinsic fluorescence enhancement and the acceleration of factor Xa neutralization indicate that multiple conformational stages can occur in antithrombin when complexed to these oligosaccharides.

Heparin (H) is a highly sulfated polysaccharide that functions by binding to antithrombin (AT) and accelerating the rate at which this protease inhibitor neutralizes proteolytic enzymes of the hemostatic mechanism (Rosenberg, 1977). Only a small fraction of all heparin preparations exhibits high affinity for antithrombin and is responsible for virtually all of the anticoagulant properties of the polysaccharide (Lam et al., 1976). This fraction of anticoagulantly active heparin was found to contain an antithrombin binding domain with the unique tetrasaccharide sequence iduronic acid→*N*-acetylglucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 6-*O*-sulfate (Rosenberg et al., 1978; Rosenberg & Lam, 1979; Lindahl et al., 1979). Subsequently, Leder (1980) isolated a sulfatase that specifically removes 3-*O*-sulfate groups from nonreducing end glucosamine residues of heparin and postulated that this unique substituent is present within the antithrombin binding domain of heparin. Data provided by Lindahl et al. (1980) and Casu et al. (1981) confirmed this supposition and located the 3-*O*-sulfate on the glucosamine moiety at the reducing end of the unique tetrasaccharide sequence. Choay et al. (1980), Casu et al. (1981), Oosta et al. (1981), Riesenfeld et al. (1981), and Atha et al. (1984b) have isolated octasaccharide fragments from deaminative as well as enzymatic cleavage products of heparin that contain this tetrasaccharide binding region. These oligosaccharides exhibit a high affinity for antithrombin as well as the capacity to accelerate the formation of the factor Xa–protease inhibitor complex. Studies by Choay et al. (1981) suggested that the major features of the antithrombin binding site of heparin are

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contained within a pentasaccharide sequence. This hypothesis was confirmed by the synthesis of a pentasaccharide that exhibited a significant affinity for antithrombin as well as the ability to accelerate factor Xa inhibition (Choay et al., 1983).

Although progress has been made in determining the overall structure of the major antithrombin binding domain of heparin, relatively little is known about the comparative contribution of individual monosaccharide units within the octasaccharide sequence to interactions with antithrombin and the subsequent acceleration of factor Xa neutralization. Studies performed by Riesenfeld et al. (1981), Thunberg et al. (1982), and Lindahl et al. (1983) have provided evidence for an important role of certain sulfate groups. In previous studies we used a pentasaccharide devoid of the unique 3-*O*-sulfate as well other heparin fragments produced synthetically and enzymatically to show that the 6-*O*-sulfate group of residue 2 and the 3-*O*-sulfate group of residue 4 are the major contributors to the binding energy of the octasaccharide (Atha et al., 1984a, 1985). In the present study, we have examined synthetic pentasaccharides and tetrasaccharides in which the 3-O- and 6-O-sulfated glucosamine moieties are present or absent to determine the effect of these residues on the acceleration of factor Xa inhibition. We have used these data to evaluate the requirement for these residues within the octasaccharide sequence with respect to the binding-induced conformational change in antithrombin and the acceleration of protease inhibitor function.

Materials and Methods

Preparation of Octasaccharide. Porcine mucosal heparin (7.5 g of batch no. 41681 at 169 USP units/mg, Diosynth Inc., Chicago, IL) was dissolved in 150 mL of cold 0.2 M citric acid and the pH adjusted to 1.5 with concentrated sulfuric acid. Sodium nitrite was added to a final concentration of 0.05 M. The reaction proceeded for 9 min at 0 °C and was quenched with excess ammonium sulfamate (0.075 M). The reaction mixture was precipitated with a final concentration of 80% (v/v) cold ethanol, centrifuged at 10000*g* for 15 min, dissolved in a minimum volume, and gel filtered at a flow rate of 270 mL/h on a polyacrylamide P-10 (Bio-Rad) column (4.7 × 200 cm) in 0.5 M ammonium bicarbonate. The octasaccharide fraction was selected from well-resolved peaks ranging from disaccharide to dodecasaccharide as monitored by absorbance at 254 nm and colorimetric assay of uronic acid (Bitter & Muir, 1962) and rechromatographed on the same column to remove traces of residual hexasaccharide and decasaccharide (Oosta et al., 1981). The octasaccharides from four preparations as described above were combined for a total yield of ~1.5 g.

Affinity Fractionation. Bovine antithrombin (1.5 g) was added to the octasaccharide (1.3 g) at final concentrations of 450 μM and 10 mM, respectively, in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) containing 0.15 M NaCl, pH 7.5. The solution, in four 13-mL additions, was applied to polyacrylamide P-100 columns (2.5 × 100 cm), equilibrated in the same buffer, and gel filtered at 30 mL/h. The column effluent was monitored by absorbance at 280 nm and colorimetric assay of uronic acid (Bitter & Muir, 1962). The antithrombin–octasaccharide complex, which completely separated from the free mucopolysaccharide, was freeze-dried, redissolved in 0.01 M Tris-HCl containing 3 M NaCl, pH 7.5, and loaded on identical columns equilibrated in the high-salt buffer. The octasaccharide was desalted on polyacrylamide P-2 columns (2.5 × 100 cm) equilibrated with 0.5 M ammonium bicarbonate. The affinity-fractionated fragment was rechromatographed on a polyacrylamide P-10 column (1.6 × 200 cm) equilibrated in 0.5 M ammonium bicarbonate, and peak fractions were pooled and freeze-dried. The active octasaccharide pool (~50 mg) had an anti factor Xa activity of 200 USP units/mg as measured by chromogenic assay compared to 2 USP units/mg for the octasaccharide pool before affinity fractionation. The structure was confirmed by high-resolution proton and carbon-13 NMR and further affinity fractionated as described (Atha et al., 1984b).

Preparation of Synthetic Tetra- and Pentasaccharides. Synthetic tetra- and pentasaccharides were prepared from D-glucose and D-glucosamine by a chemical synthesis that permits the sulfation of hydroxyl groups as required (Sinäy et al., 1984). The structures were confirmed by comparison of the high-resolution proton NMR spectra with those of a number of synthetic model compounds (Choay et al., 1983; Sinäy et al., 1984) and by spin decoupling (Torri et al., 1985). The extent of O-sulfation was determined by the downfield shift of 0.6–1 ppm for adjacent protons of the glucosamine residues. The specific 3-O-sulfation of the central glucosamine of the pentasaccharides was measured by a downfield shift in the H-3 resonance from 3.70 to 4.37 ppm (Torri et al., 1985). Integration of the anomeric signals in the different tetra- and pentasaccharides yielded an equimolar amount of all of the monosaccharide residues and demonstrated that the synthetic oligosaccharides are >90% pure. In addition, the homogeneity of the oligosaccharides was confirmed by high-pressure ion-exchange chromatography as described below.

Measurement of Protein or Mucopolysaccharide Concentration. Antithrombin and factor Xa concentrations were determined by absorbance measurements at 280 nm with absorptivity values $A^{1\%,1cm}$ of 6.5 and 11.6, respectively (Nordenman et al., 1977; Discipio et al., 1977). Mucopolysaccharide concentrations were measured colorimetrically by assay of uronic acid and were found to agree with oligosaccharide concentrations based on dry weight (Bitter & Muir, 1962).

Proteins. Human and bovine antithrombins were prepared in homogeneous form by chromatography on heparin–Sepharose and DEAE-cellulose (Damus & Rosenberg, 1976; Jordan et al., 1982). Human activated factor X was isolated as described in a previous publication from this laboratory (Jordan et al., 1980a).

High-Performance Liquid Chromatography (HPLC). HPLC was performed on a Waters system including two 6000A pumps, a U6K injector, and a 660 solvent programmer. Ion exchange was conducted on a Whatman Partisil PXS-1025 SAX column at a flow rate of 1 mL/min with a gradient of potassium phosphate, pH 4.3, from 40 mM to 1 M.

Fluorescence Measurements. Fluorescence measurements were made with a Perkin-Elmer MP-44A spectrofluorometer equipped with a thermostated sample compartment (4-mm path) and a differential corrected spectra accessory. Heparin oligosaccharide at a concentration of 10 mM was added by using a 10-μL syringe to 0.3 mL of human antithrombin at a concentration of 1 mM in 0.01 M Tris, pH 7.5, and 0.15 M NaCl. The solution was carefully stirred with the syringe needle after each addition. The binding of the oligosaccharide to antithrombin was monitored by measurement of the tryptophan fluorescence relative to the fluorescence determined in the absence of mucopolysaccharide, ΔF. The wavelengths of excitation and emission were 280 and 330 nm, respectively.

Stopped-Flow Measurements. The rate of inhibition of human factor Xa by antithrombin was measured by fluorescent probe displacement as previously outlined for thrombin (T) (Evans et al., 1982; Olson & Shore, 1982). A Durrum

Table I: Activity of Heparin Fragments

residues	saccharide[a]	$[AT]_0$[b] (μM)	k_{obsd}[c] (s^{-1})	k_{assoc}[d] (M^{-1} s^{-1})	ΔF_{max}[e] (%)
1–8	ID→GLNAc-6-*O*-SO_3→GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3 →ID-2-*O*-SO_3→AMN-6-*O*-SO_3	8	1.6	5.5×10^5	48
2–6	GLNS-6-*O*-SO_3→GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3	8	1.7	5.8×10^5	44
		100	6.9	2×10^5	
2–6	GLNS-6-*O*-SO_3→GLU→GLNS-6-*O*-SO_3→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3	500	1.5	8×10^3	(<5)
2–5	GLNS-6-*O*-SO_3→GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3	8	0.88	3.0×10^5	35
3–6	GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3	8	0.17	5.8×10^4	33
control	(no oligosaccharide)	8	0.018	6.2×10^3	0
		300	0.2	2×10^3	

[a] ID = iduronic acid; GLU = glucuronic acid; GLNS = N-sulfated glucosamine; GLNAc = *N*-acetylglucosamine; AMN = anhydromannitol. [b] $[AT]_0$ is the antithrombin concentration (after mixing). [c] k_{obsd} determined at 25 °C from data of Figure 1. [d] k_{assoc} calculated by using eq 2 (see Materials and Methods). [e] ΔF_{max} determined at 37 °C from data given in Atha et al. (1985); value in parentheses was determined at 25 °C in this study from the data of Figure 2.

stopped-flow fluorescence spectrophotometer (Model D-110) and a NEC Model 9801E microcomputer were used to record changes in fluorescence after equal volumes of a solution containing 2 μM factor Xa and 400 μM *p*-aminobenzamidine were mixed with a solution of 16 μM–1 mM human antithrombin and 0.1–1 mM oligosaccharide. The displacement of the probe from the active site of factor Xa resulted in an exponential decrease in the residual fluorescence with rate constant k_{obsd}. Olson and Shore (1982) used equilibrium expressions for dissociation of the thrombin–*p*-aminobenzamidine and thrombin–antithrombin complexes to show that the amplitude of the residual fluorescence will decay at a pseudo-first-order rate proportional to the rate of formation of the T–AT* complex and with an observed rate constant equal to

$$k_{obsd} = \frac{k[AT]_0}{K_{TAT}(1 + [P]_0/K_{TP}) + [AT]_0} \quad (1)$$

where $[AT]_0$ and $[P]_0$ are the initial antithrombin and probe concentrations and K_{TP} is the dissociation constant of the probe (Olson & Shore, 1982). When $[AT]_0 \ll K_{TAT}(1 + [P]_0/K_{TP})$, k_{obsd} varies linearly with $[AT]_0$ and fits a bimolecular process with a rate constant $k_{assoc} = k/K_{TAT}$. Under these conditions the above equation can be reduced to

$$k_{obsd} = \frac{k[AT]_0}{K_{TAT}(1 + [P]_0/K_{TP})} \quad (2)$$

According to eq 1, when the antithrombin concentration $[AT]_0$ approaches and exceeds $K_{TAT}(1 + [P]_0/K_{TP})$, the observed rate of the probe displacement from factor Xa, k_{obsd}, approaches the first-order rate constant k. This condition has been observed in stopped-flow measurements of probe displacement from thrombin by antithrombin both in the presence and in the absence of high-affinity heparin, M_r 7900 ± 800 (Olson & Shore, 1982). These measurements yielded values of K_{TAT} for uncomplexed and heparin-complexed antithrombin of 1.1–1.6 mM and 3–5 μM, respectively. The values of k for uncomplexed and complexed antithrombin were 10 and 5 s^{-1}, respectively (Olson & Shore, 1982). The authors have noted that the nearly 3 orders of magnitude difference in K_{TAT}, which is a measure of the stability of the intermediate TAT complex, accounts for the large difference in the observed rate of thrombin inactivation in the presence and absence of heparin. The authors suggest that the much smaller difference in the limiting rate constant, k, may be accounted for by conformational changes in antithrombin. However, the authors cannot exclude the possibility that conformational changes in antithrombin produced by its interaction with heparin also have a large effect on the stability of the intermediate TAT complex and thereby produce large changes in K_{TAT}.

The dissociation constant of the probe was measured for factor Xa (K_{XP}) by using fluorescence titration as described by Evans et al. (1982) and resulted in a value of 115 μM. Both the stopped-flow and the fluorescence titration measurements were made at 25 °C in 0.01 M Tris, pH 7.5, containing 0.15 M NaCl. In a separate experiment dialysis was performed on a mixture of *p*-aminobenzamidine and active octasaccharide in order to detect a possible interaction between the two under the conditions of the stopped-flow experiments. The rate of equilibration of the probe, measured by fluorescence, was not effected by the presence of octasaccharide. Therefore, it was concluded that these components do not interact to any significant extent.

Results

We have compared the ability of a number of individual heparin fragments to induce a conformational change in antithrombin and accelerate the inhibition of factor Xa. This has allowed us to assess the relative functional contribution of individual residues within the antithrombin binding domain. We have utilized intrinsic fluorescence to determine conformational changes and stopped-flow spectrophotometry to ascertain the rate of factor Xa inhibition.

Measurement of Acceleration of Factor Xa Inhibition. Figure 1 depicts the quantitation of the rate of inhibition of factor Xa by antithrombin in the presence of saturating concentrations of octa-, penta-, and tetrasaccharides. These measurements were performed by monitoring the fluorescence decrease that accompanies the displacement of the active-site probe *p*-aminobenzamidine from factor Xa. Evans et al. (1982) and Olson and Shore (1982) initially developed this methodology to monitor the rapid inactivation of thrombin by antithrombin. Their protocol permits an accurate determination of the neutralization of protease at concentrations of antithrombin and heparin that render the protease–protease inhibitor interaction too fast to monitor by previously described methods (Jordan et al., 1980b). Under conditions where the antithrombin and probe are much higher in concentration than factor Xa, the reaction is pseudo first order and can be closely fit to a single rate constant, k_{obsd}. The observed rate constants, k_{obsd}, and the corresponding bimolecular rate constants, k_{assoc}, which have been calculated by using eq 2 and the data of Figure 1, are given in Table I.

Parts A and B of Figure 1 show that both the octasaccharide and the 3-O-sulfated pentasaccharide accelerate the rate of factor Xa neutralization about 100-fold compared to the basal rate in the absence of oligosaccharide (Figure 1G). However, non-3-O-sulfated pentasaccharide produces only a 4-fold acceleration in the rate of factor Xa neutralization as compared to the control measurement in the absence of oligosaccharide

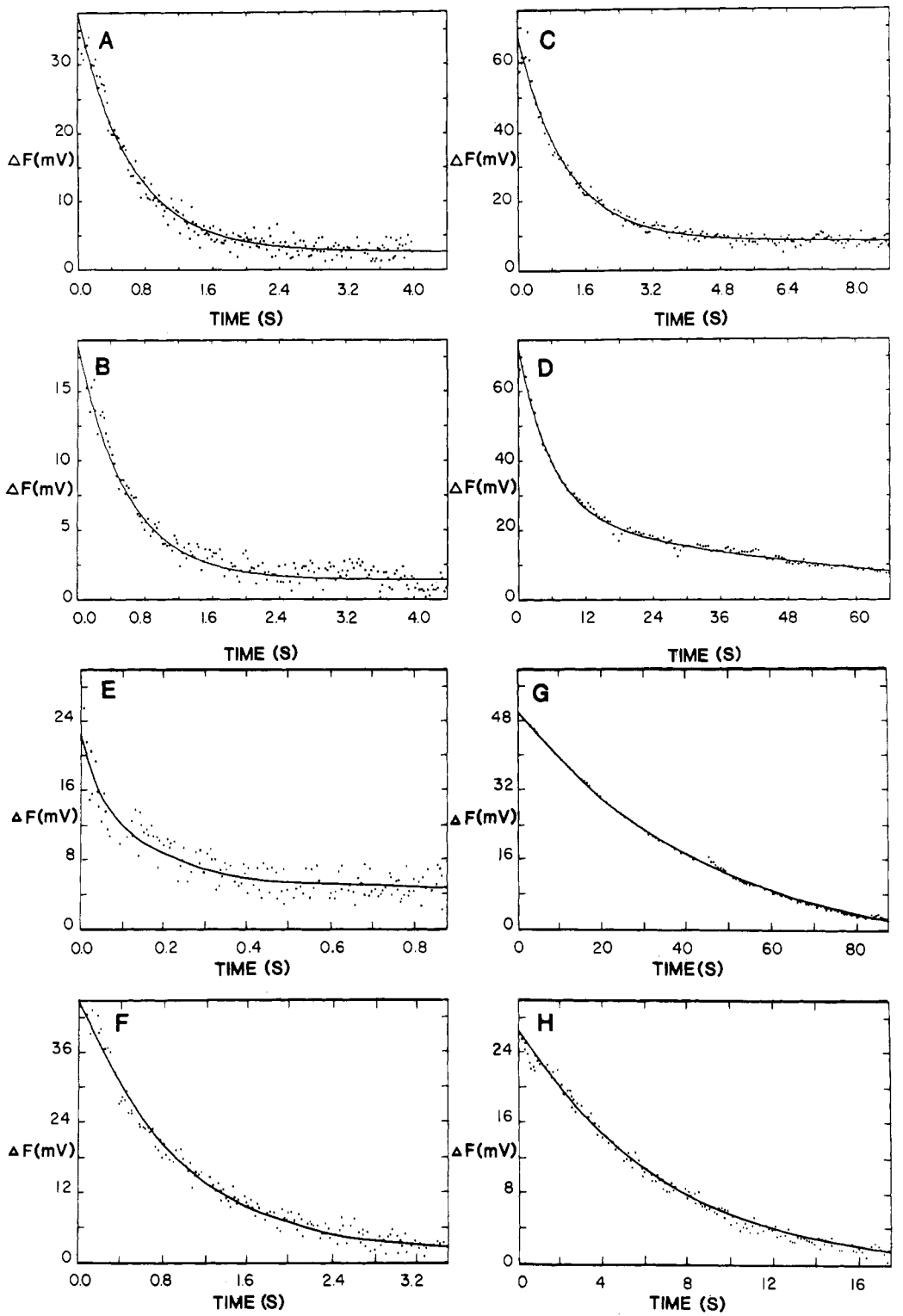

FIGURE 1: Comparison of oligosaccharide acceleration of factor Xa inhibition by antithrombin. The time course of the inhibition of human factor Xa by human antithrombin was monitored under the following conditions: (A) 50 μM octasaccharide (residues 1–8), 8 μM antithrombin (100% saturation); (B) 50 μM pentasaccharide (residues 2–6), 8 μM antithrombin (99% saturation); (C) 100 μM tetrasaccharide (residues 2–5), 8 μM antithrombin (95% saturation); (D) 500 μM tetrasaccharide (residues 3–6), 8 μM antithrombin (90% saturation); (E) 100 μM pentasaccharide (residues 2–6), 100 μM antithrombin (100% saturation); (F) 500 μM non-3-O-sulfated pentasaccharide, 500 μM antithrombin (40% saturation); (G) control (no oligosaccharide), 8 μM antithrombin; (H) control (no oligosaccharide), 300 μM antithrombin. Measurements were made at 25 °C in 0.01 M Tris, pH 7.5, containing 0.15 M NaCl with factor Xa and *p*-aminobenzamidine concentrations of 1 and 200 μM, respectively. The excitation wavelength was 320 nm, and the emission was monitored above 340 nm with a cutoff filter. The magnitude of the fluorescence change, measured in arbitrary voltage units, was dependent on instrument settings, which varied for each set of experiments. The solid lines represent least-squares fits of the data to the expression for probe displacement (see Materials and Methods). The resulting rate constants, k_{obsd}, and the bimolecular rate constants, k_{assoc}, are given in Table I.

(Figure 1F,H). The latter measurements were performed at an antithrombin concentration of 300–500 μM in order to achieve the highest possible level of saturation of the antithrombin with the available low-affinity non-3-O-sulfated oligosaccharide (40%). Both tetrasaccharides show a reduction in the rate of inhibition. As compared to the basal rate in the absence of oligosaccharide, the acceleration in the rate of factor Xa neutralization is approximately 50-fold in the case of the

tetrasaccharide that contains the 6-O-sulfated residue 2. It is approximately 10-fold in the case of the non-6-O-sulfated tetrasaccharide (Figure 1C,D).

Previous kinetic measurements of probe displacement from factor Xa by heparin-complexed antithrombin are linearly dependent on antithrombin concentration up to a concentration of 90 μM and do not show any indication of an approach to a first-order rate constant (Craig et al., 1986). On the basis of the maximum observed rate of 25 s^{-1}, these authors estimate values for K_{XATH} and k for heparin-complexed antithrombin of >300 μM and >200 s^{-1}, respectively. This showed that the intermediate antithrombin–factor Xa–heparin complex is at least 100-fold less stable than the TATH complex. In the present study, the kinetic measurements of probe displacement from factor Xa by antithrombin in the presence of pentasaccharide also does not show a significant indication of an approach to a first-order rate constant at an antithrombin concentration of 100 μM. In fact, the bimolecular rate is relatively constant in the range of 10–100 μM. At 100 μM antithrombin, our observed rate of 7 s^{-1} for the pentasaccharide is about 4-fold lower than that observed for Craig et al. (1986) for low molecular weight heparin. The fact that we have also observed about a 4-fold increase in the rate using low molecular weight heparin (M_r ~6000) at lower concentrations of heparin and antithrombin (10 μM) indicates that this effect is due to conformational differences in antithrombin rather than a nonspecific interaction of low molecular weight heparin with factor Xa.

Measurement of Fluorescence Enhancement. Heparin binding to antithrombin is accompanied by an approximate increase of 30–50% in intrinsic fluorescence. This change has previously been shown to be a reflection of an alteration in the conformation of antithrombin (Olson & Shore, 1981; Olson et al., 1981). In the present study the intrinsic fluorescence enhancement of totally complexed antithrombin, ΔF_{max}, has been used as a probe of the extent of the conformational change. We have been able to monitor the conformational alterations in antithrombin that are produced when this inhibitor binds our synthetic oligosaccharides and to determine the extent to which this change in conformation is associated with key residues within the heparin octasaccharide.

Figure 2 depicts an intrinsic fluorescence emission spectrum of antithrombin measured in the presence and absence of a saturating concentration of octasaccharide. The data demonstrate an increase of almost 50% in fluorescence, ΔF_{max}, at 330 nm. However, in the presence of non-3-O-sulfated pentasaccharide at a 50% saturation there is less than a 5% increase in fluorescence as calculated from the equilibrium dialysis measurements. This result and those obtained from previous determinations of ΔF_{max} of various other heparin fragments are given in Table I. As in the case of the kinetic measurements of factor Xa neutralization, the intrinsic fluorescence determinations indicate that the 3-O-sulfated pentasaccharide is responsible for a conformational change in the antithrombin molecule equivalent to that produced by the octasaccharide. However, both the 6-O-sulfated and the non-6-O-sulfated tetrasaccharides produce substantial reductions in ΔF_{max}. In addition, the non-3-O-sulfated pentasaccharide shows essentially no indication of inducing a conformational change in antithrombin: this is in agreement with the kinetic measurements of the factor Xa neutralization process.

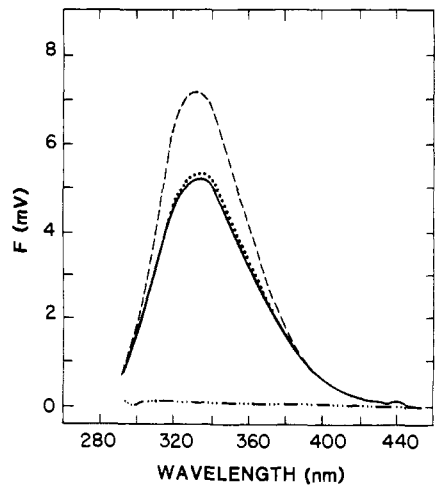

FIGURE 2: Comparison of oligosaccharide-induced fluorescence enhancement of antithrombin. Intrinsic fluorescence spectra of human antithrombin were determined under the following conditions: (—) no oligosaccharide, 750 μM antithrombin; (---) 750 μM octasaccharide (residues 1–8), 750 μM antithrombin (100% saturation); (···) 750 μM non-3-O-sulfated pentasaccharide, 750 μM antithrombin (50% saturation); (-···-) 750 μM octasaccharide only, no antithrombin. Measurements were made at 25 °C in 0.01 M Tris, pH 7.5, containing 0.15 M NaCl. The fluorescence was recorded in arbitrary voltage units at an excitation wavelength of 280 nm.

Discussion

The precise role of specific monosaccharide residues of heparin in inducing a conformational change in antithrombin that allows the protease inhibitor to function as a potent anticoagulant has not been established. The experimental complexities that have prevented a resolution of this problem are the potential interactions of multiple domains of heparin with both coagulation serine protease and antithrombin as well as the microheterogeneity of the glycosaminoglycan. We have chosen to circumvent these difficulties by examining the structure–function relationships of small heparin oligosaccharides of known sequence with regard to the alteration of antithrombin conformation and glycosaminoglycan-dependent acceleration of factor Xa neutralization.

The kinetics of neutralization of factor Xa by antithrombin and heparin have previously been examined by our laboratory (Jordan et al., 1980b). These studies demonstrate that the initial rate of factor Xa neutralization is proportional to the fraction of antithrombin bound to heparin with no evidence of a reduction in the velocity of enzyme inhibition at polysaccharide concentrations as high as 10^{-4} M. Factor Xa is known to be fully complexed with heparin at these concentrations. Thus, the heparin-induced acceleration of factor Xa–antithrombin interactions is not dependent on the binding of free enzyme to polysaccharide complexed with protease inhibitor, the "approximation effect", which is known to occur during thrombin–antithrombin and factor IXa–antithrombin interactions. This conclusion is consistent with the measurements of Craig et al. (1986), which indicated that the intermediate antithrombin–factor Xa–heparin complex is at least 100 times less stable than the antithrombin–thrombin–heparin complex.

Previous studies of Oosta et al. (1981) have shown that a heparin octasaccharide of known sequence accelerates factor Xa inhibition but does not enhance the inactivation of other hemostatic enzymes. In addition, the investigations of Choay et al. (1983) have demonstrated that a heparin pentasaccharide of known sequence behaves in a similar fashion. Furthermore, examination of heparin tetrasaccharides of known sequence carried out in the present work reveals that these fragments are able to accelerate factor Xa neutralization at high concentrations. We have used these oligosaccharides in our investigations of factor Xa neutralization by antithrombin. This

has permitted us to unambiguously examine the effect of specific monosaccharide residues on the conformational change in antithrombin and the resulting acceleration of factor Xa neutralization without regard to the complications introduced by either enzyme approximation effects or microheterogeneity of the mucopolysaccharide.

The octasaccharide, as delineated in Table I, binds antithrombin with an affinity of 2×10^{-8} M (Atha et al., 1985). The 3-O-sulfated pentasaccharide was shown to have a binding constant of 1×10^{-7} M. This represents a binding affinity approximately 10-fold lower than that of the octasaccharide. Thus, residues 6–8 of the octasaccharide play a significant role in binding to antithrombin. This pentasaccharide, however, produced a value of ΔF_{max} which is 92% that of the octasaccharide and a rate of factor Xa inhibition identical with that observed with the larger fragment. Our data show that although residues 6–8 of the octasaccharide bind to antithrombin, they have essentially no effect on the inhibitor's conformational change and biologic activity. The synthetic tetrasaccharide consisting of residues 2–5, delineated in Table I, has an approximately 1000-fold lower affinity for antithrombin as compared to the octasaccharide. However, at saturation, this molecular species produced a rate of factor Xa inhibition that is within a factor of 2 of that of the octasaccharide. These results indicate that the oligosaccharides which contain both the 6-O-sulfated residue 2 and the 3-O-sulfated residue 4 are capable of inducing substantial conformational changes in antithrombin. The synthetic tetrasaccharide, residues 3–6, which lacks the 6-O-sulfated residue 2, has an antithrombin binding affinity 5–10-fold lower than that of the synthetic tetrasaccharide, residues 2–5, which contains this residue (Atha et al., 1985). The tetrasaccharide consisting of residues 3–6 produced a 5-fold lower rate of factor Xa inhibition as compared to the tetrasaccharide of residues 2–5 containing the 6-O-sulfated residue 2. The tetrasaccharide of residues 3–6 produced a rate only about 10-fold higher than the basal rate of factor Xa inhibition measured in the absence of oligosaccharide. The non-3-O-sulfated pentasaccharide, delineated in Table I, binds antithrombin with an affinity of 5×10^{-4} M and shows essentially no capacity for effecting a conformational change in the protease inhibitor (Atha et al., 1985). This oligosaccharide did, however, produce a small but significant acceleration in the rate of factor Xa inhibition. These data further support our previously reported observation that the 6-*O*-sulfate group of residue 2 is linked to the 3-*O*-sulfate group of residue 4 by a conformational change in antithrombin such that the loss of either residue results in a substantial reduction in affinity for the protease inhibitor.

Both synthetic tetrasaccharides produced an approximately 30% lower value of ΔF_{max} than that obtained when antithrombin was complexed to the octasaccharide. This indicates that oligosaccharides with affinities below that of the 3-O-sulfated pentasaccharide do not induce a complete conformational change in antithrombin. The factor of 2 drop in the rate of factor Xa inhibition observed with the synthetic tetrasaccharide of residues 2–5 supports this conclusion. The fact that the tetrasaccharide of residues 3–6 produced a substantial fluorescence enhancement indicates that even this low-affinity oligosaccharide is capable of inducing a conformational change in antithrombin. Indeed, the ΔF_{max} produced by antithrombin binding the tetrasaccharide of residues 3–6 is almost identical with that produced by binding the tetrasaccharide of residues 2–5. This result indicates that multiple conformational states are produced in binding these oligosaccharides which do not produce proportional changes in ΔF_{max} and k_{assoc}. A simple two-state model in which antithrombin exists in equilibrium between inactive and active forms would yield instead proportional changes in ΔF_{max} and k_{assoc} as the equilibrium is perturbed by binding the different oligosaccharides. Our data also show that the kinetic measurements of the neutralization of factor Xa are particularly sensitive to conformational changes in antithrombin which are produced when both the 6-O-sulfated residue 2 and the 3-O-sulfated residue 4 interact with the protease inhibitor. When either residue is absent, the acceleration is minimal. In contrast, the intrinsic fluorescence measurements are sensitive to conformational changes in antithrombin which require only that the 3-O-sulfated residue 4 interacts with the protease inhibitor. The large decreases in k_{assoc} observed in the absence of either the 6-O-sulfated residue 2 or the 3-O-sulfated residue 4 may be caused by the particular effect that the resulting conformational changes in antithrombin have on the stability of the intermediate antithrombin–factor Xa complex. Although the first-order rate constant, k, would be expected to reflect changes in antithrombin conformation at the protease binding site, these conformational changes may also significantly alter the stability of the intermediate antithrombin–factor Xa complex, thereby affecting the value of K_{XATH}.

The results outlined above support a model in which multiple sites on the heparin oligosaccharide interact with discrete residues on antithrombin. Villanueva (1984) describes the heparin binding site of antithrombin as an unstable helical segment composed of residues 281–292. From available NMR and X-ray data of the repeating units of heparin, three lysine residues within the α-helix of this segment (Lys-282, Lys-286, and Lys-289) can be matched for maximal interaction with three sulfate groups in the octasaccharide (6-*O*-sulfate of residue 2, *N*-sulfate of residue 4, and the *N*-sulfate of residue 6). A recent study by Rosenfeld and Danishefsky (1986), however, indicates that heparin may also interact with the region of antithrombin contained within the polypeptide with residues 104–251. Although the above residues on the octasaccharide have been qualitatively shown to be important for binding, the data of the present study demonstrate that the biologically critical interaction depends on the presence of the 3-*O*-sulfate residue 4, which appears to be out of range of Lys-286. The 3-*O*-sulfate may, instead, interact on a different helical segment. In doing so, it might alter the conformation of antithrombin in a way that greatly affects its intrinsic fluorescence and ability to neutralize factor Xa. This is consistent with the studies of Blackburn et al. (1984) in which modification of Trp-49 near the NH_2-terminal end of antithrombin prevents the heparin-accelerated inhibition of thrombin or factor Xa. In addition, fluorescence-transfer studies by Pecon and Blackburn (1984) indicate that one or two lysine residues which are essential to heparin binding are located near Trp-49.

We have diagrammed the interaction of antithrombin with heparin in Figure 3 to illustrate the different conformational stages that can be observed when the protease inhibitor is complexed with the oligosaccharides. As illustrated in reaction I, antithrombin is shown to interact with a heparin oligosaccharide which contains 6-O-sulfated residue 2, 3-O-sulfated residue 4, and N-sulfated residues 4 and 6. This resulted in a conformational change in antithrombin that greatly affects its intrinsic fluorescence and ability to neutralize factor Xa. As observed in reaction II, the loss of the 6-*O*-sulfate of residue 2 resulted in a 10-fold reduction in the rate of inactivation of factor Xa. Thus, this residue must also play a major role in the biologic activity of antithrombin. However, the fact that

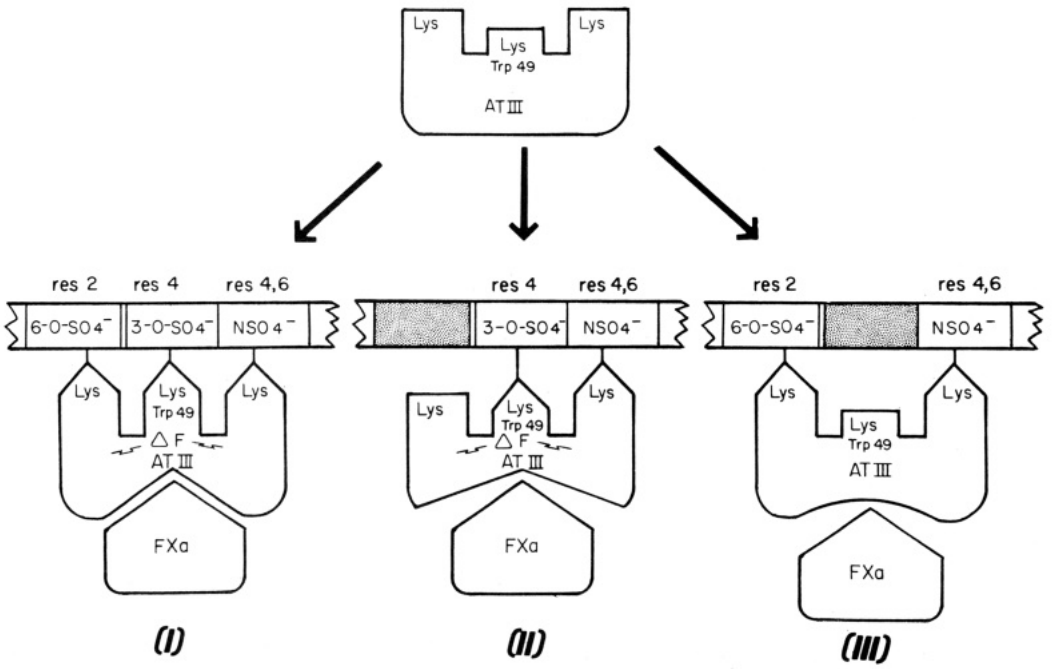

FIGURE 3: Diagram of multiple conformational states of antithrombin. Conformational changes in antithrombin (ATIII) that affect its ability to neutralize factor Xa are shown to occur after it forms complexes with three different heparin oligosaccharides. (I) Antithrombin complexed to the heparin oligosaccharide containing 6-O-sulfated residue 2, 3-O-sulfated residue 4, and N-sulfated residues 4 and 6. Lysine residues at positions 282, 286, and 289 in an α-helical segment and lysine residues near tryptophan-49 may be involved (see Discussion). This reaction produces a conformational change in antithrombin that results in maximal neutralization of factor Xa and maximal intrinsic fluorescence enhancement (ΔF). (II) Antithrombin complexed to heparin oligosaccharide lacking 6-O-sulfated residue 2. This reaction produces a reduced conformational change in antithrombin that results in minimal neutralization of factor Xa but produces a large fluorescence enhancement (ΔF). (III) Antithrombin complexed to heparin oligosaccharide lacking 3-O-sulfated residue 4. This reaction produces a significant conformational change in antithrombin that results in minimal neutralization of factor Xa and minimal intrinsic fluorescence enhancement.

the loss of this residue causes neither a major reduction in the enhancement of intrinsic fluorescence nor a total loss of the acceleration of factor Xa neutralization indicates that a limited conformational change can still occur in antithrombin without this particular interaction. Therefore, the intrinsic fluorescence measurements are an incomplete monitor of conformational changes in antithrombin, especially those that affect the neutralization of factor Xa. As shown in reaction III, the absence of the 3-*O*-sulfate of residue 4 results in nearly a complete loss of intrinsic fluorescence enhancement as well as a very significant reduction in the acceleration of factor Xa neutralization. The fact that the fluorescence enhancement is preserved in reaction II but is nearly eliminated in reaction III suggests that the interaction between the 3-*O*-sulfate and a lysyl site near Trp-49 may be responsible for the fluorescence enhancement.

We have previously demonstrated that the sum of the individual contributions of the 6-O-sulfated residue 2 and the 3-O-sulfated residue 4 to the binding energy of the octasaccharide greatly exceeds that of the nonreducing end tetrasaccharide which contains both of these residues (Atha et al., 1985). We believe that this difference in binding energy is due to a conformational change in antithrombin which occurs only when heparin oligosaccharides containing both of these residues interact with the protease inhibitor. In the present study, we have observed that the same two residues are functionally linked with regard to the acceleration of factor Xa inhibition by antithrombin and suggest that in a coordinated manner they must trigger a conformational change in the protease inhibitor which leads to the acceleration of factor Xa inhibition. On the basis of available evidence, we conclude that heparin oligosaccharides which possess the correctly aligned 6-O-sulfated residue 2 as well as the 3-O-sulfated residue 4 are able to bind to antithrombin and induce a conformational change in the protease inhibitor that both strengthens the interaction of the polysaccharide with the protein and is responsible for the acceleration of factor Xa neutralization.

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Sulfhydryl Modification and Activation of Phenylalanine Hydroxylase by Dinitrophenyl Alkyl Disulfide

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ABSTRACT: A new family of asymmetric thiol–disulfide exchange reagents, the dinitrophenyl alkyl disulfides (DNPSSR), was used to modify rat liver phenylalanine hydroxylase. The results indicate that the enzyme has two different types of reactive sulfhydryl (SH) residues per subunit. One SH residue was modified selectively by a DNPSSR having a neutral and hydrophilic alkyl group, and this modification was accompanied by appreciable activation of enzyme; the other SH residue was modified only by an anionic DNPSSR, and this modification did not result in activation. The catalytic properties of phenylalanine hydroxylase activated by DNPSSR were similar to those of the *N*-ethylmaleimide- (NEM-) modified enzyme, but the process of activation by DNPSSR was quite different from modification with NEM. An analysis of the reaction kinetics of the modification and of catalysis by the modified enzyme suggests that DNPSSR modification causes a change in the subunit interaction leading to a loss of the negative cooperativity normally seen with phenylalanine hydroxylase.

Specific sulfhydryl (SH)[1] residues in some proteins have important roles in the function and/or the structure of that protein. There have been many reports of SH modifications inactivating various enzymes or weakening the subunit interaction in the proteins but only a few reports on the activation of enzyme by SH modification (Sekine et al., 1962, 1984;

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[1] Abbreviations: DNPS⁻, dinitrothiophenolate anion; DNPSCl, dinitrobenzenesulfonyl chloride; DNPSSR, dinitrophenyl alkyl disulfides; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; SH, sulfhydryl.

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