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Role of Arginine 129 in Heparin Binding and Activation of Antithrombin*

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The contribution of Arg^{129} of the serpin, antithrombin, to the mechanism of allosteric activation of the protein by heparin was determined from the effect of mutating this residue to either His or Gln. R129H and R129Q antithrombins bound pentasaccharide and full-length heparins containing the antithrombin recognition sequence with similar large reductions in affinity ranging from 400- to 2500-fold relative to the control serpin, corresponding to a loss of 28–35% of the binding free energy. The salt dependence of pentasaccharide binding showed that the binding defect of the mutant serpin resulted from the loss of ~2 ionic interactions, suggesting that Arg^{129} binds the pentasaccharide cooperatively with other residues. Rapid kinetic studies showed that the mutation minimally affected the initial low affinity binding of heparin to antithrombin, but greatly affected the subsequent conformational activation of the serpin leading to high affinity heparin binding, although not enough to disfavor activation. Consistent with these findings, the mutant antithrombin was normally activated by heparin for accelerated inhibition of factor Xa and thrombin. These results support an important role for Arg^{129} in an induced-fit mechanism of heparin activation of antithrombin wherein conformational activation of the serpin positions Arg^{129} and other residues for cooperative interactions with the heparin pentasaccharide so as to lock the serpin in the activated state.

Antithrombin, a plasma glycoprotein belonging to the serpin (serine proteinase inhibitor) superfamily of proteins, is a critical regulator of the proteolytic enzymes of blood coagulation, especially thrombin and factor Xa (1, 2). Antithrombin inactivates these enzymes by trapping them in equimolar, SDS-stable complexes through a novel mechanism shared by other serpin family proteinase inhibitors. The inactivation is initiated by the proteolytic enzyme recognizing a reactive bond in an exposed loop of the serpin as a regular substrate. The proteinase proceeds to attack this bond and generate an acyl-intermediate in which the P1′ residue of the bond has been released and the proteinase is joined in acyl linkage to the P1 residue through its active-site serine (3–6). The cleavage of the reactive-bond loop is believed then to trigger a massive conformational change in which the loop, with the proteinase covalently-linked, is inserted into the center of β-sheet A of the serpin, resulting in the translocation of the enzyme to the opposite end of the inhibitor and its consequent trapping as a stable acyl-intermediate complex (1, 7, 8).

Unlike most other serpins, which inhibit their target enzymes at diffusion limited rates, the rates of antithrombin inhibition of factor Xa and thrombin are several orders of magnitude slower than the diffusion limit. However, these rates can be accelerated several thousandfold to approach the diffusion limit by the highly sulfated polysaccharide, heparin (1, 9). This property of heparin is responsible for its widespread clinical use as an anticoagulant drug. The ability of heparin to accelerate antithrombin-proteinase reactions is dependent on the binding to the inhibitor of a unique pentasaccharide sequence in the polysaccharide chain (10–12). Heparin chains containing this sequence bind the fully glycosylated α-form of antithrombin with high affinity (K_D of 10–20 nM at physiological ionic strength and pH) and induce an activating conformational change in the inhibitor. Whereas this conformational change is sufficient to activate antithrombin to rapidly inhibit factor Xa, the acceleration of antithrombin inhibition of thrombin requires in addition a longer heparin chain to bridge the inhibitor and proteinase in a ternary complex. The x-ray crystal structures of free and heparin-complexed antithrombin (13–15) together with molecular modeling and biochemical studies (16, 17) have suggested that heparin activates antithrombin by expelling the reactive bond loop from the center of β-sheet A of the protein core in which it is partially buried in the native structure. This results in the complete exposure of the loop in a manner similar to that found in other native serpin structures and presumably in a conformation optimal for proteinase interaction (15).

Studies of natural and recombinant antithrombin variants with defects in heparin binding have localized the heparin pentasaccharide binding site of antithrombin to basic residues of helix A, helix D, and the polypeptide N terminus (1, 14, 18–20). The recent x-ray crystal structure of an antithrombin-pentasaccharide complex identified the interacting basic residues as Lys^{11}, Arg^{13}, Arg^{46}, Arg^{47}, Lys^{114}, Lys^{125}, and Arg^{129} (15). However, the relative contributions of these residues to pentasaccharide binding and activation of antithrombin has not been established in most cases. Our recent studies showed that Arg^{46} and Arg^{47} make very different contributions to the

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binding, with Arg^{47} being much more important than Arg^{46} in this respect (21). Lys^{125} has been shown to be an even more important contributor to heparin binding than Arg^{47} (20). However, neither Arg^{47} nor Lys^{125} was found to be essential for activation of the serpin, although Arg^{47} did contribute to stabilizing the activated conformation (20, 21).

The present report concerns the role of Arg^{129} in the heparin activation mechanism. The importance of this residue was first suggested from chemical modification studies (22) and later more firmly established by the isolation of an abnormal antithrombin from a patient with thrombosis, in which Arg^{129} was mutated to Gln (18, 23). However, the importance of Arg^{129} in heparin activation was difficult to assess in this study since the variant antithrombin was found to be unstable (18, 24). To determine the role of Arg^{129} in heparin binding and activation of antithrombin, we have engineered recombinant antithrombins in which this residue was mutated to His to allow modulation of the charge of this residue in the neutral pH range and thereby to assess the importance of charge, or changed to Gln to replicate the natural mutation. Our findings clearly establish that Arg^{129} is critical for high affinity binding of the heparin pentasaccharide and much more important in this binding than Arg^{47}. Arg^{129} is further shown to play an important role in heparin activation of antithrombin by binding the pentasaccharide cooperatively with other antithrombin residues only in the activated state, and thereby stabilizing the serpin in the activated conformation.

MATERIALS AND METHODS

Expression and Purification of Recombinant Antithrombins—Recombinant antithrombins were expressed in baculovirus-infected insect cells as described previously (19, 25). The previously characterized N135A antithrombin variant served as the base molecule for the mutations (25, 26). R129H/N135A and R129Q/N135A antithrombins were constructed by additional Arg → His and Arg → Gln mutations, respectively, of the N135A antithrombin cDNA. Mutations were verified both prior to recombination of cDNAs into viral DNA as well as after viral integration.

All recombinant antithrombins were purified by heparin affinity chromatography at pH 7.4 on 5-ml HiTrap Heparin (Amersham Pharmacia Biotech, Uppsala, Sweden) or EconoPac Heparin (Bio-Rad) columns, as described previously (19, 25). The R129H/N135A and R129Q/N135A antithrombin variants were further purified by ion exchange chromatography on a 1-ml MonoQ column (Amersham Pharmacia Biotech), eluted with a 30–45-ml gradient from 0 to 0.6 M NaCl in 20 mM sodium phosphate, pH 7.4, containing 0.1 mM EDTA and 0.1% (w/v) polyethylene glycol 8000. The purity of antithrombins was assessed by SDS-PAGE[1] and nondenaturing PAGE using the Laemmli discontinuous buffer system (27) or by the SDS-Tricine gel system (28). Molar concentrations of the three variants were calculated from absorbance measurements at 280 nm using the molar extinction coefficient of 37,700 M^{-1} cm^{-1} determined for plasma antithrombin (29).

Proteinases—Human α-thrombin was a generous gift of Dr. John Fenton (New York State Department of Health, Albany, NY). Human factor Xa (predominantly α-form) was prepared as described previously (30). The concentrations of the proteinases were based on active-site titrations, which indicated that the enzymes were >90% (thrombin) and ~70% (factor Xa) active, respectively (25).

Heparins—Full-length heparin with high-affinity for antithrombin and with a highly reduced polydispersity and average molecular mass of ~8,000 Da (~26 saccharide units) was isolated from commercial heparin by size and affinity fractionation as described previously (12). The synthetic antithrombin-binding pentasaccharide, DEFGH, and the truncated variants, DEFG* and EFGH′ (31) (see Fig. 3), were generously provided by Maurice Petitou (Sanofi Recherche, Toulouse, France). The asterisk and prime notations on the tetrasaccharides follow the nomenclature in Ref. 31 and denote slight structural modifications of the saccharides, which do not adversely affect their binding to antithrombin, as shown in Ref. 31. Concentrations of full-length, pentasaccharide, and tetrasaccharide DEFG* heparins were determined from the end points of stoichiometric fluorescence titrations of plasma antithrombin with the saccharides (12). The concentration of tetrasaccharide EFGH′ was determined by weight.

Experimental Conditions—All experiments were conducted at 25 °C and in 20 mM sodium phosphate buffer containing 0.1 mM EDTA and 0.1% (w/v) polyethylene glycol 8000, adjusted to either pH 7.4 or pH 6.0. In the absence of added salt, the ionic strength (I) of the buffer was 0.05 (pH 7.4) or 0.025 (pH 6.0). Sodium chloride was added to achieve higher ionic strengths at either pH.

Stoichiometries of Thrombin Inhibition—Recombinant antithrombin was added to identical samples of thrombin (50–500 nM) to give molar ratios of inhibitor to proteinase ranging from 0 to ~2. Following incubation for 5–18 h, residual thrombin activity was determined by dilution of aliquots 100-fold into 100 μM S-2238 substrate (Chromogenix, Franklin, OH) in I 0.15, pH 7.4 buffer and measurement of the initial rate of substrate hydrolysis at 405 nm. Linear regression fitting of the residual thrombin activities as a function of the molar ratio of inhibitor to enzyme yielded the apparent inhibition stoichiometry from the abscissa intercept (32).

Equilibrium Binding of Heparin—Binding of oligosaccharide or full-length heparins to recombinant antithrombins was measured by titrating the saccharides into a solution of antithrombin and monitoring the increase in intrinsic protein fluorescence which signals the binding interaction, as described previously (12, 32–34). Measurements were made at 25 °C with an SLM 8000C or 4800S spectrofluorometer (SLM Instruments, Rochester, NY) with excitation and emission wavelengths of 280 and 340 nm, respectively. Stoichiometric titrations of antithrombin variants with full-length heparin were done at pH 6.0, I 0.025 for R129Q/N135A antithrombin, at pH 7.4, I 0.05 for R129H/N135A and N135A antithrombins and also at pH 7.4, I 0.15 for N135A antithrombin. Titrations were done at least in duplicate with 0.1–0.5 μM antithrombin, based on absorbance measurements. Equilibrium titrations at pH 6 and 7.4 and different ionic strengths were performed at least in duplicate at antithrombin concentrations (based on heparin binding or thrombin inhibition titrations) less than or equal to K_D and were carried out to a saturation of >95% with the saccharide in each case. The fluorescence titration data was fit by nonlinear regression analysis to the quadratic equilibrium binding equation for a 1:1 binding interaction to yield K_D and the maximal fluorescence change (12, 32, 34).

Rapid Kinetics of Heparin Binding—The rates of binding of pentasaccharide or full-length high affinity heparins to recombinant R129H/N135A antithrombin were measured in pH 7.4, I 0.15 buffer at 25 °C in an SX-17MV stopped-flow fluorometer (Applied Photophysics, Leatherhead, United Kingdom), as described previously (12, 26, 35). Pseudo-first-order conditions in which molar concentrations of saccharide and antithrombin (the latter based on thrombin inhibition titrations) were maintained at a ratio of at least 5:1 and in most cases ≥10:1 were employed. The interaction was monitored from the increase in intrinsic protein fluorescence with an excitation wavelength of 280 nm and an emission cutoff filter with ~50% transmission of light at 320 nm. The fluorescence traces were acquired for about 10 half-lives and fit by a single exponential function to give the observed pseudo-first-order rate constant (k_{obs}). Typically, 12–18 fluorescence traces were acquired for each saccharide concentration and k_{obs} values from these traces averaged.

Kinetics of Proteinase Inactivation—Second-order association rate constants for antithrombin or antithrombin-heparin complex inhibition of thrombin or factor Xa were measured under pseudo-first-order conditions at pH 7.4, I 0.15, 25 °C with catalytic levels of heparin, as described previously (21, 26, 32). Reactions containing antithrombin (25–100 nM), heparin (0–5 nM), and proteinase (5–10 nM) were quenched at varying times by dilution into 100 μM S-2238 for thrombin or 100–200 μM Spectrozyme FXa (American Diagnostica, Greenwich, CT) for factor Xa and residual enzyme activity determined from the initial rate of substrate cleavage at 405 nm. The time dependence of enzyme activity loss was fit by a single exponential function with an end point of zero activity to give k_{obs}. Second-order association rate constants were obtained by dividing k_{obs} by the inhibitor concentration in the case of the uncatalyzed reactions or determined from the slope of linear regression fits of the dependence of k_{obs} on the heparin concentration for heparin-catalyzed reactions, as described (26, 31). The inhibitor concentrations used were those determined from thrombin stoichiometric titrations described above.

RESULTS

Purification of N135A, R129H/N135A, and R129Q/N135A Antithrombins—As in our previous studies of heparin binding

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-tris(hydroxymethyl)methylglycine.

TABLE I

Dissociation equilibrium constants, association rate constants, and dissociation rate constants for full-length heparin and pentasaccharide binding to N135A, R129Q/N135A, and R129H/N135A antithrombins at 25 °C, pH 7.4, and ionic strength 0.15

K_D values are the averages ± S.E. ($n > 2$) or range ($n = 2$) of at least two fluorescence titrations. k_{on} and k_{off} were obtained by linear regression analysis of plots, shown in Fig. 2 (*panel A*), of k_{obs} *vs.* heparin concentration in the heparin concentration range ≤ 1.5 μM.

Heparin form	Antithrombin variant	K_D	$10^{-6} \times k_{on}$	k_{off}	Calculated K_D[a]	Calculated k_{off}[b]
		nM	$M^{-1}\ s^{-1}$	s^{-1}	*nM*	s^{-1}
H26	N135A[c]	~0.2	154 ± 1	ND[d]		~0.03
	R129Q/N135A	540 ± 110	ND	ND		
	R129H/N135A	110 ± 10	29 ± 2	7.6 ± 1.1	260 ± 60	3.2 ± 0.5
H5	N135A[c]	2 ± 1	70 ± 2	ND		0.14 ± 0.07
	R129Q/N135A	1800 ± 300	ND	ND		
	R129H/N135A	820 ± 110	23 ± 4	19 ± 3	830 ± 280	19 ± 6

[a] From k_{on} and k_{off}.
[b] From K_D and k_{on}.
[c] Values taken from Ref. 21.
[d] ND, not determined.

site variants of antithrombin, N135A antithrombin served as the reference because this variant lacks the Asn^{135} carbohydrate chain adjacent to the heparin binding site and as a result exhibits enhanced affinity for heparin (25, 26, 36). The N135A mutation also eliminates possible glycosylation differences affecting heparin affinity (25, 37–40). We have previously shown that the properties of N135A antithrombin resemble those of the natural β-form of antithrombin, which similarly lacks an oligosaccharide chain at Asn^{135} (25, 26).

R129H/N135A and R129Q/N135A antithrombins showed a significant heparin binding defect, based on the significantly lower salt concentration required to elute the variants from a heparin affinity column (~0.5 M) as compared with that needed to elute the reference N135A antithrombin (~2 M). Nevertheless, reasonably pure preparations of both Arg^{129} variants were obtained after the affinity chromatography. Further purification of the variants on Mono Q yielded an essentially homogeneous preparation in the case of the R129H/N135A variant, whereas some residual impurities were still detectable in the R129Q/N135A variant, based on SDS-PAGE analysis of the two variants. The electrophoretic mobilities of R129H/N135A and R129Q/N135A were indistinguishable from that of N135A on SDS-PAGE. Nondenaturing PAGE at alkaline pH showed an increased mobility of the Arg^{129} variants relative to the reference N135A antithrombin, consistent with the mutation of Arg^{129} resulting in the loss of a positive charge. The latter gel further showed that the preparations were free of inactive polymeric forms of the serpin (41). R129H/N135A and N135A antithrombins additionally showed equivalent abilities to form SDS-stable complexes with thrombin on SDS-PAGE, indicating that these recombinant antithrombins exhibited the functional properties characteristic of the properly folded serpin.

Stoichiometry of Heparin and Thrombin Binding—The stoichiometry of full-length heparin binding to the mutant antithrombins was assessed by fluorescence titrations at low ionic strength and at antithrombin concentrations greatly exceeding K_D (>20-fold). R129Q/N135A antithrombin gave low heparin binding stoichiometries ranging from ~0.2 to 0.31 ± 0.02 mol of heparin/mol of antithrombin for two preparations. By contrast, the R129H/N135A variant showed much higher heparin binding stoichiometries of 0.78 ± 0.01 to 0.86 ± 0.02 for two preparations, values comparable to those observed for several preparations of the N135A reference antithrombin (0.79–0.85) (25, 26), indicating that the preparations were nearly fully active. Confirming these findings, three preparations of R129H/N135A antithrombin inhibited thrombin with close to equimolar stoichiometries (1.2–1.3 mol of inhibitor/mol of enzyme), comparable to those measured for N135A antithrombin, indicating a similar fraction of active inhibitor (77–83%). Fluorescence enhancements were somewhat low in titrations of R129Q/N135A antithrombin (24–25%), whereas they approached normal values for titrations of the R129H/N135A variant (31–34%) when compared with the reference N135A antithrombin (34–43%). These results suggest that the R129Q/N135A variant contained a substantial fraction of inactive, probably latent inhibitor, formed by the spontaneous insertion of the reactive center loop into β-sheet A (14, 41). A natural R129Q mutation of antithrombin was similarly isolated largely as the inactive, latent form of the serpin (18, 24). Such findings imply a reduced stability of R129Q/N135A antithrombin relative to R129H/N135A and N135A antithrombins, although no losses in functional activity were detected upon incubation of the control and two mutant inhibitors at temperatures up to 37 °C for 2 h. Concentrations of antithrombins employed for subsequent analyses were based on the functional concentrations determined from heparin (R129Q/N135A) or thrombin (R129H/N135A) binding titrations.

Heparin Affinity—Dissociation constants for the binding of pentasaccharide and full-length heparins to R129Q/N135A or R129H/N135A variant antithrombins were measured at pH 7.4, I 0.15, 25 °C by fluorescence titrations of the variants with heparin at antithrombin concentrations approximating K_D (Table I). Because of the tight binding of both heparins to N135A antithrombin under these conditions, K_D values for control antithrombin-heparin interactions were measured at higher ionic strengths but at the same pH and temperature and the K_D at I 0.15 obtained by extrapolating the ionic strength dependence of K_D to I 0.15. As previously reported, N135A antithrombin bound full-length heparin somewhat more tightly than pentasaccharide (26), and a similar differential affinity of full-length and pentasaccharide heparins for the two variant antithrombins was observed. Mutation of Arg^{129} in R129Q/N135A antithrombin produced comparable large reductions in heparin affinity of ~2500 for full-length heparin and ~900-fold for pentasaccharide when compared with the affinities of control N135A antithrombin. The reductions in heparin affinity were somewhat less but still dramatic for R129H/N135A antithrombin, being ~500-fold for full-length heparin and ~400-fold for the pentasaccharide. Because of the limited amounts of the R129Q/N135A variant and the presence of significant inactive inhibitor in the preparation, all further analyses of the heparin binding defect produced by mutating Arg^{129} of antithrombin were confined to the R129H/N135A antithrombin variant.

Ionic Strength Dependence of Heparin Pentasaccharide Binding—To determine whether the reduced affinity of heparin for R129H/N135A antithrombin was due to a loss of ionic or nonionic interactions, dissociation constants for heparin pentasaccharide binding to R129H/N135A mutant and N135A control

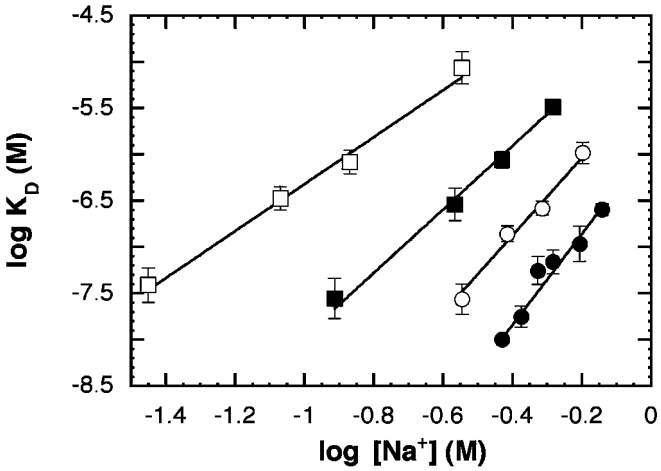

FIG. 1. **Sodium ion concentration dependence of dissociation equilibrium constants for pentasaccharide binding to N135A and R129H/N135A antithrombins at 25 °C, pH 7.4 and pH 6.0.** Pentasaccharide, N135A antithrombin at pH 6.0 (*closed circles*) or pH 7.4 (*open circles*); pentasaccharide, R129H/N135A antithrombin at pH 6.0 (*closed squares*) or pH 7.4 (*open squares*). Average values ± S.E. or range of at least two determinations are shown. *Solid lines* are linear regression fits of data.

antithrombins were measured as a function of sodium chloride concentration at pH 7.4, 25 °C. Plots of log K_D *versus* log [Na^+] were linear for both interactions (Fig. 1), in accordance with the expected salt concentration dependence of the protein-heparin interactions (12, 26, 42, 43). The slope of such plots is equal to $Z\Psi$, the number of charge-charge interactions involved in the association (Z) times the fraction of sodium ions bound per heparin charge that are released upon antithrombin binding (Ψ) (42), Ψ having a value of 0.8 for heparin (43). The ordinate intercept of the plots (at 1 M Na^+) gives log K_D', the logarithm of the dissociation constant due to nonionic interactions (42). Linear regression analyses of the plots of Fig. 1 showed that the pentasaccharide made ~5 ionic interactions with N135A antithrombin, in agreement with past studies (21, 26), whereas only ~3 interactions were made with R129H/N135A antithrombin (Table II). Mutation of Arg^{129} thus resulted in the loss of two ionic interactions. The nonionic dissociation constant was also ~30-fold higher for R129H/N135A than for N135A (Table II), indicating that the Arg^{129} mutation additionally reduced the number of nonionic interactions between the pentasaccharide and antithrombin. Mutation of Arg^{129} of antithrombin thus reduces heparin affinity by eliminating multiple ionic and nonionic interactions with the heparin pentasaccharide.

pH Dependence of Heparin Pentasaccharide Binding—To determine whether the heparin binding interactions lost due to mutation of Arg^{129} to His could be restored by converting the largely neutral His residue at pH 7.4 to a positively charged residue by protonation at low pH, we measured pentasaccharide binding to N135A and R129H/N135A antithrombins at pH 6, 25 °C. The plots of log K_D *versus* log [Na^+] for normal and mutant antithrombin interactions with pentasaccharide under these conditions were also linear (Fig. 1), allowing the values of Z and log K_D' to be determined (Table II). Pentasaccharide binding to N135A antithrombin at pH 6 was of much higher affinity than at pH 7.4 due to ~1 additional charge-charge interaction as well as to a ~5-fold increase in the nonionic binding affinity, similar to what was previously found for the plasma antithrombin-pentasaccharide interaction (31). Compared with the N135A antithrombin interaction, binding of the heparin pentasaccharide to R129H/N135A antithrombin at pH 6 was associated with a loss of ~2 ionic interactions and ~30-fold lower nonionic binding affinity, *i.e.* indistinguishable from the changes observed at pH 7.4. As a consequence, the Arg^{129} mutation produced similar decreases in pentasaccharide affinity for antithrombin at pH 6 as at pH 7.4 when compared at the same ionic strength. These results indicate that the heparin binding defect could not be restored by increasing the positive charge on the mutant His residue at lower pH and thus that the pentasaccharide interaction with antithrombin is specific for an Arg side chain at position 129.

TABLE II

Ionic and nonionic contributions to pentasaccharide binding to N135A and R129H/N135A antithrombins at 25 °C and pH 7.4 and pH 6.0

The number of ionic interactions involved in the binding of pentasaccharide to antithrombin variants (Z) and the nonionic contribution to the binding (log K_D') were obtained from the slopes and intercepts, respectively, of the linear plots of Fig. 1, as described in the text. Errors represent ± S.E.

pH	Antithrombin variant	Z	log K_D'	K_D'
				μM
7.4	N135A	5.2 ± 0.2	−5.2 ± 0.2	6.3
	R129H/N135A	3.2 ± 0.3	−3.8 ± 0.3	160
6.0	N135A	6.1 ± 0.3	−5.9 ± 0.1	1.3
	R129H/N135A	4.3 ± 0.4	−4.5 ± 0.1	32

Rapid Kinetics of Heparin Binding—The kinetics of pentasaccharide and full-length heparin binding to N135A and R129H/N135A antithrombins was studied at pH 7.4, I 0.15, 25 °C by continuously monitoring the intrinsic protein fluorescence changes accompanying this binding by stopped-flow fluorimetry under pseudo-first-order conditions, as in past studies (12, 21, 26, 31, 35). The binding kinetics conformed to a monophasic exponential process for both mutant and control inhibitor interactions with either heparin, as was previously found for N135A and plasma antithrombin interactions with the two heparins (12, 26, 35). The kinetics at low heparin concentrations (≤1 μM) were first analyzed to obtain the overall k_{on} and k_{off} for heparin binding. In this concentration range, k_{obs} increased linearly with increasing heparin concentration, yielding k_{on} and k_{off} from the slope and intercept, respectively, of this linear dependence (Fig. 2A and Table I). As shown previously, k_{off} could not be determined precisely for control antithrombin-heparin interactions because the intercept values were indistinguishable from zero (21, 26). Instead, k_{off} values were calculated in this case from the product, $K_D \times k_{on}$. Comparison of k_{on} and k_{off} for mutant and control antithrombin interactions with the two heparins showed that the reduction in heparin affinity caused by the Arg^{129} mutation was due to both modest (3–5-fold) decreases in k_{on} as well as major (140–250-fold) increases in k_{off}. For the two mutant antithrombin-heparin interactions for which both k_{on} and k_{off} could be accurately determined, the ratio of k_{off}/k_{on} agreed well with the measured K_D for the pentasaccharide interaction but was somewhat higher than the measured K_D for the full-length heparin interaction. The latter discrepancy may reflect experimental error or be due to a small contribution of a preequilibrium pathway for conformational activation of the mutants, as was found previously with a R47H/N135A variant antithrombin (21). In this pathway, heparin binds preferentially to a small amount of already conformationally activated antithrombin in preequilibrium with native, unactivated antithrombin.

Extension of the binding kinetics studies to higher heparin concentrations showed the expected approach of k_{obs} to a limiting value as the heparin concentration was increased both for control and mutant inhibitor interactions (Fig. 2*B*), as has been previously shown for both plasma and N135A antithrombin interactions with the two heparins (12, 21, 26, 31, 35). How-

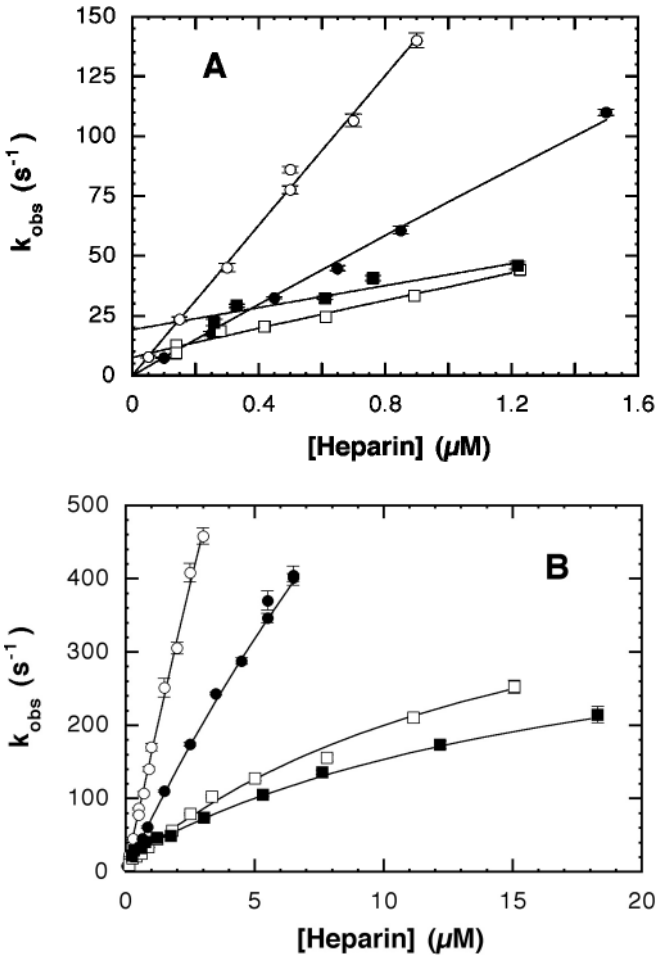

FIG. 2. **Heparin concentration dependence of observed pseudo-first-order rate constants for the binding of full-length heparin and pentasaccharide to N135A and R129H/N135A antithrombins at 25 °C, pH 7.4, and ionic strength 0.15.** *Open circles*, full-length heparin, N135A; *closed circles*, pentasaccharide, N135A; *open squares*, full-length heparin, R129H/N135A; *closed squares*, pentasaccharide, R129H/N135A. *Panel A* shows data obtained at low heparin concentrations, which were used to determine values of k_{on} and k_{off} (Table I); *panel B* shows data obtained at high heparin concentrations, which were used to determine values of K_1 and k_{+2} (Table III). N135A antithrombin data are taken from Ref. 21. *Error bars* represent ± S.E. from at least 12 kinetic traces. Error bars not shown lie within the dimensions of the symbol. *Solid lines* in *panel B* are nonlinear regression fits of data to Equation 1. In these fits, k_{-2} was fixed at k_{off} values determined from linear regression fits of data in *panel A*.

ever, the deviation from linearity was minimal for the full-length heparin interaction with N135A antithrombin, as we previously reported (21, 26). Such nonlinear behavior is indicative of a two-step binding mechanism in which an initial weak, rapid equilibrium binding of heparin (H) to antithrombin (AT) induces a subsequent conformational change in the inhibitor (AT*), which causes the fluorescence change and leads to tight heparin binding (35).

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

SCHEME 1

In this binding mechanism, K_1 is the dissociation constant for the initial rapid equilibrium binding step and k_{+2} and k_{-2} are the forward and reverse rate constants for the subsequent conformational activation step. According to this mechanism,

TABLE III

Kinetic constants for the two-step binding of full-length heparin and pentasaccharide to N135A and R129H/N135A antithrombins (Scheme 1) at 25 °C, pH 7.4 and ionic strength 0.15

The dissociation equilibrium constant for the first step (K_1) and the forward rate constant for the second step (k_{+2}) of the two-step binding of heparins to antithrombin variants were determined from nonlinear regression fits of the data of Fig. 2 (*panel B*) to Equation 1. In these fits, k_{-2} was fixed at k_{off} values determined from linear regression fits of the data of Fig. 2 (*panel A*). Errors represent ± S.E.

Heparin form	Antithrombin variant	K_1	k_{+2}	k_{+2}/k_{-2}[a]
		μM	s^{-1}	
H26	N135A[b]	≥10	≥3000	≥100,000
	R129H/N135A	16 ± 2	500 ± 40	70
H5	N135A[b]	28 ± 4	2100 ± 300	15,000
	R129H/N135A	19 ± 2	390 ± 20	20

[a] Calculated using values of k_{-2}, equal to k_{off}, given in Table I.
[b] Taken from Ref. 21.

the dependence of k_{obs} on the total heparin concentration ($[H]_o$) is given by the rectangular hyperbolic function (35) shown in Equation 1.

$$k_{obs} = \frac{k_{+2}[H]_o}{K_1 + [H]_o} + k_{-2} \quad \text{(Eq. 1)}$$

k_{on} and k_{off} in this case are given by k_{+2}/K_1 and k_{-2}, respectively. Nonlinear regression fitting of the data in Fig. 2*B* to Equation 1 yielded values for K_1 and k_{+2} for the two-step binding of pentasaccharide and full-length heparins to either control or mutant inhibitors (Table III). Comparison of these kinetic parameters showed that mutation of Arg^{129} had no significant effect on K_1, whereas it reduced k_{+2} for both heparin interactions. For the pentasaccharide interaction, k_{+2} was reduced ~5-fold by the Arg^{129} mutation, this change accounting entirely for the reduced k_{on}. For the full-length heparin interaction, the effect of the mutation on k_{+2} could not be quantified since only a lower limit for k_{+2} was measurable for the control inhibitor (21, 26). Nevertheless, this lower limit fully accounted for the ~5-fold lower k_{on} for the mutant inhibitor interaction, consistent with the reduction in k_{+2} being the predominant effect of the mutation and any effects on K_1 being relatively small. The primary effects of the mutation on k_{+2} and k_{-2} (k_{off}) and minimal effect on K_1 suggest that the interaction of Arg^{129} of antithrombin with pentasaccharide or full-length heparins does not occur in the initial binding step but rather in the subsequent conformational activation step.

Kinetics of Proteinase Inhibition—Second-order rate constants for the inhibition of thrombin and factor Xa by free or heparin-complexed R129H/N135A or N135A antithrombins were measured by discontinuous assays of the loss of enzyme activity at pH 7.4, *I* 0.15, 25 °C, as in previous studies (Table IV) (21, 26, 31). Reactions of pentasaccharide-complexed antithrombin with thrombin were not studied because the pentasaccharide enhancement of this reaction is small (<2-fold) (12). Association rate constants for mutant inhibitor reactions with the two proteinases were indistinguishable from those of the corresponding control N135A antithrombin reactions, both for the unactivated inhibitor reactions and for the reactions of inhibitor activated by pentasaccharide or full-length heparins (Table IV).

Binding of Truncated Pentasaccharides—To determine which end of the pentasaccharide is involved in interacting with Arg^{129}, we measured the binding to N135A and R129H/N135A antithrombins of two tetrasaccharides, DEFG* and EFGH′, lacking the reducing and nonreducing end saccharide unit of the pentasaccharide, respectively (Fig. 3). These tetrasaccharides are also slightly modified from the parent pen-

tasaccharide, DEFGH, in other saccharide units, but in groups not essential for binding (31, 44). We have previously shown that these tetrasaccharides, although binding more weakly than the pentasaccharide, DEFGH, compete with the latter for binding to plasma antithrombin and induce an activating conformational change in the serpin equivalent to that induced by DEFGH (31). The affinities of these tetrasaccharides for control and mutant inhibitors were measured by fluorescence titrations at pH 7.4, *I* 0.15, 25 °C (Table V). The fluorescence enhancements induced in mutant and control antithrombins by either tetrasaccharide at saturation were similar (27–33% for DEFG* and 35–43% for EFGH′),[2] consistent with normal conformational activation of the mutant inhibitor by the tetrasaccharides. The affinity of DEFG* for R129H/N135A antithrombin was greatly reduced ~120-fold relative to the control N135A antithrombin, whereas the affinity of EFGH′ was only slightly lower (~2-fold) for the mutant than for the control inhibitor. Elimination of the reducing-end saccharide H thus resulted in retention of the heparin binding defect of R129H/N135A antithrombin, consistent with residue H of the pentasaccharide not interacting with Arg[129]. In contrast, elimination of the nonreducing-end saccharide, D, resulted in a loss of the binding defect, suggesting that the nonreducing-end residue of the pentasaccharide is primarily responsible for the interaction with Arg[129].

TABLE IV

Association rate constants for reactions of free or heparin-complexed N135A and R129H/N135A antithrombins with thrombin or factor Xa at 25 °C, pH 7.4, and ionic strength 0.15

Second-order association rate constants for reactions of uncomplexed (k_{uncat}), full-length heparin-complexed (k_{H26}), and pentasaccharide-complexed (k_{H5}) antithrombin variants with proteinases were calculated from k_{obs} values measured by nonlinear regression fitting of the exponential decay of enzyme activity under pseudo-first-order reaction conditions as described under "Materials and Methods." Errors represent ± S.E. or the range from at least two determinations for k_{uncat} · k_{H26} and k_{H5} ± S.E. were determined from the slopes of linear regression fits of plots of k_{obs} *vs.* heparin concentration for at least three heparin concentrations in the range 0.25–3 nM.

Proteinase	Antithrombin variant	$10^{-3} \times k_{uncat}$	$10^{-6} \times k_{H26}$	$10^{-6} \times k_{H5}$
		$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$
Thrombin	N135A	11 ± 1[a]	13 ± 3	ND[b]
	R129H/N135A	14 ± 1[a]	22 ± 4	ND
Factor Xa	N135A	4.0 ± 0.5	1.3 ± 0.1	0.51 ± 0.07
	R129H/N135A	4.3 ± 0.4	1.6 ± 0.3	0.59 ± 0.02

[a] Indistinguishable values were obtained for reactions conducted in the absence or presence of 100 μg/ml Polybrene, indicating no detectable contamination of the recombinant inhibitor with heparin.

[b] ND, not determined.

DISCUSSION

In the present study, we have elucidated the contribution of Arg[129] of antithrombin to heparin binding and activation of the serpin. The importance of Arg[129] in heparin binding was first suggested from chemical modification studies (22) and later more convincingly shown by the identification of a natural Arg[129] to Gln antithrombin variant with decreased affinity for heparin (18, 24). However, the quantitative contribution of this residue to heparin binding was unclear from the latter study, since the variant antithrombin was isolated largely in a latent, inactive conformation known to reduce heparin affinity (14, 41). The recent x-ray crystal structure of a complex of antithrombin with the heparin pentasaccharide binding sequence confirmed that Arg[129] is in direct contact with the pentasaccharide in the complex and thus contributes to binding the

[2] The fluorescence enhancements induced by DEFG* in mutant and control antithrombins are indistinguishable from those induced by pentasaccharide DEFGH. The slightly higher enhancements observed for EFGH′ reflect an additional structural change induced in antithrombin by the tetrasaccharide, but one which does not affect the properties of the activated inhibitor (31).

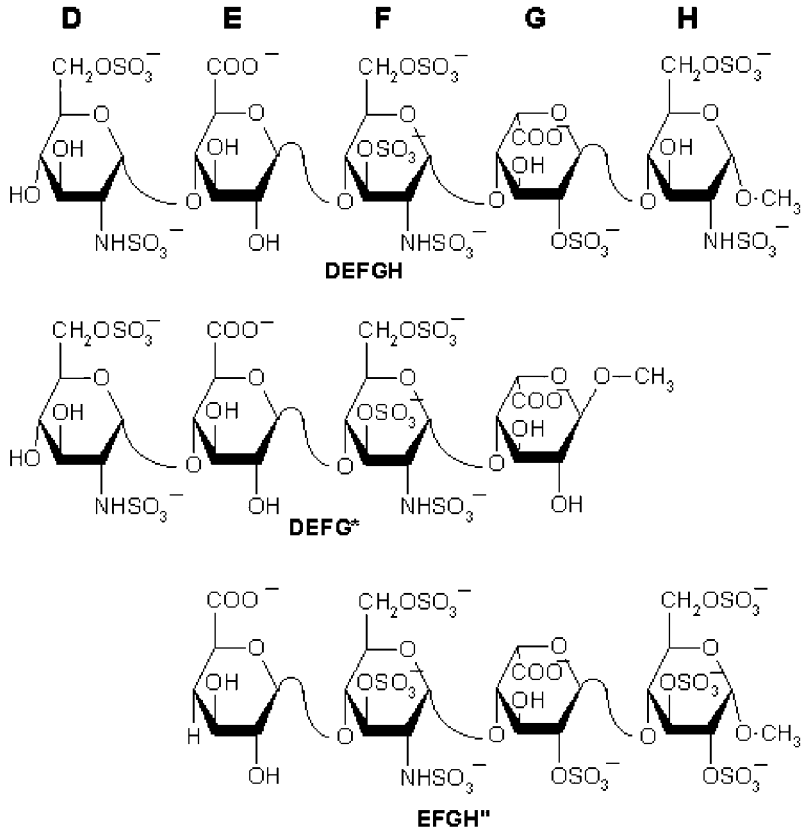

FIG. 3. **Structures of pentasaccharide DEFGH and tetrasaccharides DEFG* and EFGH′.**

TABLE V

Dissociation equilibrium constants for the binding of truncated pentasaccharides to N135A and R129H/N135A antithrombins at 25 °C, pH 7.4, and ionic strength 0.15

Dissociation constants for the binding to antithrombin variants of pentasaccharides truncated on the reducing end (DEFG*) or the nonreducing end (EFGH′) were determined by nonlinear regression fitting of fluorescence titrations to the quadratic equilibrium binding equation assuming a 1:1 binding stoichiometry. The asterisk and prime notations on the tetrasaccharides denote modifications in groups not essential for binding, as given in Ref. 31.

Tetrasaccharide	Antithrombin variant	K_D
		nM
DEFG*	N135A	210 ± 40
	R129H/N135A	26,500 ± 4300
EFGH′	N135A	860 ± 120
	R129H/N135A	2100 ± 400

activator (15). To determine the contribution of Arg[129] to heparin binding, we analyzed the effect of mutating this residue in an N135A recombinant antithrombin which lacks the carbohydrate chain that sterically interferes with heparin binding (25, 26, 39, 40). As was previously found with the natural Arg → Gln antithrombin variant (24), the isolated recombinant R129Q/N135A antithrombin contained substantial inactive, presumably latent inhibitor. Nevertheless, the active fraction of the mutant clearly showed a ~900–2500-fold reduced affinity for pentasaccharide and full-length heparins. An alternative recombinant R129H/N135A antithrombin, which was isolated almost completely in the active state, enabled us to confirm that the Arg[129] mutation results in a substantial, although somewhat less, ~400–500-fold loss in heparin affinity for the two heparins. The observed losses in affinity due to the Arg[129] mutations correspond to 3.6–4.7 kcal/mol (15–20 kJ/mol) in binding free energy or 28–35% of the total binding free energy. Such a loss in binding energy is about twice that previously found for mutation of Arg[47] (15%) (21) and significantly greater than the loss reported for mutation of Lys[125] (17–27%) (20). Arg[129] is thus the most important residue identified so far in binding either pentasaccharide or full-length heparins. Like the Arg[47] and Lys[125] mutations, mutation of Arg[129] did not significantly affect the uncatalyzed rates of antithrombin inhibition of thrombin or factor Xa. Moreover, it did not affect the ability of heparin to induce protein fluorescence changes indicative of normal conformational activation of the serpin or to fully accelerate antithrombin inhibition of thrombin or factor Xa. These observations indicate that the mutation affects heparin binding affinity but not the native or activated conformations of the serpin.

The large decrease in heparin affinity arising from the Arg[129] → His mutation at pH 7.4, where the His should be largely uncharged, involved the loss of multiple ionic interactions (~2) as well as nonionic interactions, the two types of interactions each contributing about 30-fold to the decrease in affinity at ionic strength 0.15. The nonionic interactions involved presumably reflect hydrogen bonding, hydrophobic, or van der Waals interactions. An equivalent loss of ionic and nonionic interactions was found at pH 6, which should have resulted in protonation of the major fraction of the His residue and its acquisition of a positive charge. Arg[129] thus appears to make highly specific ionic and nonionic interactions with the heparin pentasaccharide, which cannot be replicated by substituting a positively charged His side chain. These interactions appear to involve nonreducing-end residue D of pentasaccharide DEFGH, based on the finding that the binding defect of the mutant inhibitor is lost when residue D is deleted from the pentasaccharide. This is in agreement with the x-ray crystal

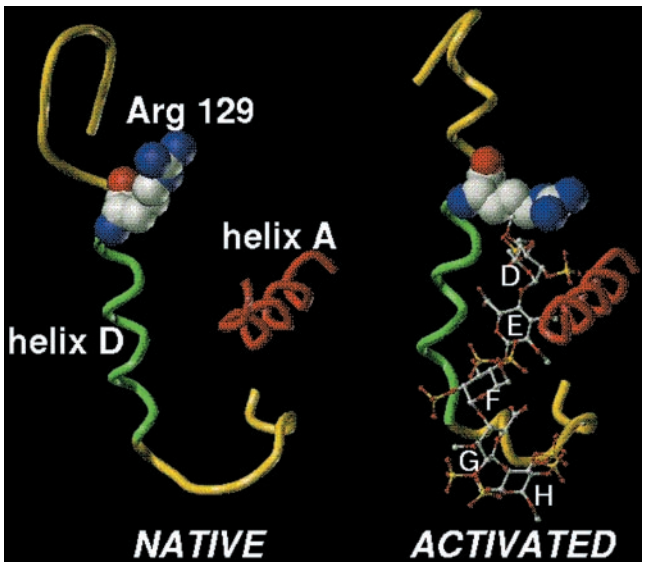

FIG. 4. **Structural changes induced in the heparin binding site of antithrombin upon interaction with pentasaccharide heparin.** Shown are backbone representations of the A helix (*red*), D helix (*green*), and connecting loop (*yellow*) regions of antithrombin, which make up the heparin binding site in the structures of uncomplexed native antithrombin (*left*, Protein Data Bank code 2ant) and the antithrombin-pentasaccharide complex (*right*, Protein Data Bank code 1azx). The backbone and side chain atoms of Arg[129] are shown as a space-filling model with oxygen colored *red*, nitrogen *blue*, and carbon *white*. The pentasaccharide, DEFGH, is shown as a ball-and-stick representation with carbon atoms in *white*, sulfur in *yellow*, and oxygen in *red*. Individual saccharides are denoted by *letters*. The Arg[129] side chain is seen to move downward in going from the native to the pentasaccharide-activated conformation of antithrombin to allow interaction with the nonreducing-end residue (*D*) of the pentasaccharide. The figure was made with Sybyl 6.5 molecular modeling software (Tripos Associates, San Diego, CA) using helix A as reference.

structure of the antithrombin-pentasaccharide complex, which shows an interaction of Arg[129] with the essential 6-O-sulfate of the D saccharide (Fig. 4) (15, 45, 46). The specific Arg[129] interaction with the D saccharide appears necessary to allow at least one other antithrombin residue to make an ionic interaction and possibly also nonionic interactions with the pentasaccharide, *i.e.* the binding of Arg[129] and one or more other residues of antithrombin to heparin would seem to be highly cooperative. Such behavior contrasts with our previous findings of the effect of mutating Arg[47] of antithrombin to His (21). The decreased heparin binding affinity of this latter mutant was thus found to be mostly due to the loss of a single ionic interaction, although some loss of nonionic interactions was also observed. Additionally, the loss of ionic binding energy was largely restored by lowering the pH from 7.4 to 6, presumably due to the His side chain being able to substitute for the Arg side chain when it is positively charged at the lower pH. Arg[47] thus differs from Arg[129] in binding the pentasaccharide independently of other antithrombin-pentasaccharide interactions and with less specificity for the arginine side chain.

Rapid kinetic studies showed that the loss of heparin binding energy due to replacement of Arg[129] with His resulted from a decrease in k_{on} as well as an increase in k_{off} for the binding. R129H/N135A antithrombin was shown to bind pentasaccharide and full-length heparins by the same induced-fit binding mechanism previously established for N135A and plasma antithrombins (12, 26, 35). In this mechanism, an initial weak interaction of heparin with antithrombin induces an activating conformational change in the inhibitor, leading to a tight interaction. Resolution of the two-step binding of pentasaccharide and full-length heparins to mutant and control inhibitors

showed that the effects of the Arg^{129} mutation on k_{on} and k_{off} minimally involved the initial binding step and were entirely localized in the second conformational activation step. These effects involved both a reduced rate of conformational activation and a higher rate of reversal of this activation. Arg^{129} thus does not participate in binding heparin in the initial interaction step but is instead engaged to bind in the subsequent conformational activation step.

The effects of the Arg^{129} mutation on both forward and reverse rates of conformational activation of antithrombin indicate that Arg^{129} not only facilitates the conversion of the native conformation to the activated conformation but, more importantly, stabilizes the activated conformation by allowing multiple interactions with heparin to be established in the second step. The lack of participation of Arg^{129} in the initial binding step was somewhat surprising in view of our previous findings. Thus, deletion of residue D of the pentasaccharide was previously shown to greatly weaken the initial binding interaction, indicating that the D residue, which interacts with Arg^{129} in the second step, also interacts with antithrombin in the first step (31). Residue D was further shown to contribute 6.2 kcal/mol binding free energy to the overall pentasaccharide interaction at pH 7.4, *I* 0.15, and 25 °C, which significantly exceeds the 3.6–4.0 kcal/mol contribution found for Arg^{129} in the present study. It would thus appear that part of the binding energy of residue D is used to form the initial weak complex and the remainder to bind Arg^{129} and possibly other residues following the conformational activation step. The binding of Arg^{129} to residue D only after conformational activation of antithrombin is consistent with the structural changes induced in antithrombin upon pentasaccharide binding as seen in the x-ray structures of free and pentasaccharide-complexed antithrombins (13–15). These structures reveal that the Arg^{129} side chain points away from the pentasaccharide binding site in the uncomplexed inhibitor, but swings toward the site to allow its engagement with the 6-O-sulfate of residue D in the inhibitor complex with pentasaccharide (Fig. 4). This movement is induced by a one-and-a-half turn extension of the C-terminal end of helix D at which Arg^{129} is positioned and by the tilting of the helix. This significant structural change is in keeping with Arg^{129} not making contact with pentasaccharide in the initial weak complex but requiring conformational activation to position the side chain for interaction with residue D.

Whereas mutation of Arg^{129} of antithrombin weakens heparin affinity in the second conformational activation step, the mutant inhibitor was nevertheless fully activable by pentasaccharide or full-length heparins for rapid factor Xa inhibition and normally activated by the full-length heparin for accelerated inhibition of thrombin. The normal activation of the mutant inhibitor is a consequence of the high degree to which the activated conformation is favored over the native conformation when the pentasaccharide is bound. The equilibrium constant for this conversion is thus ~15,000 for the control antithrombin at *I* 0.15, pH 7.4, 25 °C[3] and is reduced ~800-fold to a value of ~20 for the mutant inhibitor. The activated conformation therefore remains favored for the mutant inhibitor despite the significant destabilization of this conformation, thereby accounting for the observed full activation of the inhibitor by heparin and normal heparin acceleration of proteinase inhibition by either allosteric or bridging mechanisms.

In summary, the present study has demonstrated an important role for Arg^{129} of antithrombin in the high affinity binding and activation of the serpin by a sequence-specific heparin pentasaccharide. Whereas Arg^{129} does not participate in the initial docking of pentasaccharide to antithrombin, it is critical for inducing the serpin into the activated state and for stabilizing this state. The stabilization arises from the positioning of Arg^{129} and other antithrombin residues for specific cooperative interactions with the nonreducing-end saccharide D and possibly other residues of the pentasaccharide. These cooperative interactions combine with the noncooperative interactions between Arg^{47} and the reducing-end disaccharide GH to further stabilize the activated conformation, as shown by our previous studies (21). It is thus clear from our present and past studies that Arg^{129} and Arg^{47} are not involved in the mechanism for switching antithrombin between native and activated states, in contrast to what was recently found for residues in the linker region connecting helix D with strand 2A (47). Instead, these residues act to stabilize antithrombin in the activated state through preferential binding of heparin to this state.

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[3] The equilibrium constant for the native to activated transition for pentasaccharide-complexed plasma α-antithrombin is ~800 under the same conditions, consistent with the presence of the Asn^{135} oligosaccharide side chain in the predominant glycoform of the serpin decreasing heparin affinity through a primary effect on this equilibrium (26).

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