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Conformational Activation of Antithrombin by Heparin Involves an Altered Exosite Interaction with Protease*

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Background: Exosites are known to mediate heparin allosteric activation of antithrombin.
Results: Mutagenesis revealed that an exosite differentially contributes to antithrombin reactivity with factors Xa/IXa in unactivated and heparin-activated states.
Conclusion: Heparin allosteric activation of antithrombin results from alterations in an exosite interaction with protease induced by core conformational changes.
Significance: The findings support our recently proposed model of antithrombin allosteric activation.

Heparin allosterically activates antithrombin as an inhibitor of factors Xa and IXa by enhancing the initial Michaelis complex interaction of inhibitor with protease through exosites. Here, we investigate the mechanism of this enhancement by analyzing the effects of alanine mutations of six putative antithrombin exosite residues and three complementary protease exosite residues on antithrombin reactivity with these proteases in unactivated and heparin-activated states. Mutations of antithrombin Tyr^{253} and His^{319} exosite residues produced massive 10–200-fold losses in reactivity with factors Xa and IXa in both unactivated and heparin-activated states, indicating that these residues made critical attractive interactions with protease independent of heparin activation. By contrast, mutations of Asn^{233}, Arg^{235}, Glu^{237}, and Glu^{255} exosite residues showed that these residues made both repulsive and attractive interactions with protease that depended on the activation state and whether the critical Tyr^{253}/His^{319} residues were mutated. Mutation of factor Xa Arg^{143}, Lys^{148}, and Arg^{150} residues that interact with the exosite in the x-ray structure of the Michaelis complex confirmed the importance of all residues for heparin-activated antithrombin reactivity and Arg^{150} for native serpin reactivity. These results demonstrate that the exosite is a key determinant of antithrombin reactivity with factors Xa and IXa in the native as well as the heparin-activated state and support a new model of allosteric activation we recently proposed in which a balance between attractive and repulsive exosite interactions in the native state is shifted to favor the attractive interactions in the activated state through core conformational changes induced by heparin binding.

Antithrombin, a member of the serine protease inhibitor (serpin) superfamily, acts as a critical anticoagulant regulator of hemostasis through its ability to rapidly inhibit the blood coagulation proteases, thrombin, factor Xa, and factor IXa (1). The physiologic importance of this anticoagulant protein is indicated from the increased risk of thrombotic diseases associated with acquired or inherited deficiencies of the protein in humans (2) and the lethality and coagulopathy associated with complete antithrombin deficiency in mice (3) and zebrafish (4). Antithrombin circulates in the blood in a repressed state capable of slow inhibition of its target coagulation proteases and becomes activated at sites of injury by extravascular heparin and heparan sulfate glycosaminoglycans to localize and prevent the dissemination of blood clots (5, 6). Heparin activation of antithrombin involves both an allosteric component wherein a specific pentasaccharide sequence in heparin induces activating conformational changes in the serpin that promotes its interaction with protease and a bridging component in which the extended heparin chain in the antithrombin-heparin complex provides a site for the protease to bind next to the serpin to promote the serpin-protease interaction (7–9). The allosteric mechanism activates antithrombin reactivity with the target proteases, factors Xa and IXa, but only minimally with thrombin, whereas the bridging mechanism activates antithrombin reactivity with all three proteases (10).

The mechanism of allosteric activation of antithrombin has been the subject of numerous studies. Early models based on x-ray structures of native and heparin-activated antithrombin suggested the importance of releasing the reactive center protease binding loop (RCL)[2] from a constrained interaction with sheet A in the native state, first as a means of making the critical P1 Arg recognition determinant accessible to target proteases (11) and later as a means to gain access to exosites outside the RCL to augment the protease interaction (12–15). Although a critical role for exosites in augmenting the antithrombin RCL interaction with factors Xa/IXa in the allosterically activated state is now well established, a role for RCL expulsion from sheet A in making the exosites available for interaction with protease is not supported by much available data.

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[2] The abbreviation used is: RCL, reactive center protease binding loop.

In particular, the observation that mutations of a critical Tyr^{253} exosite residue cause large losses in antithrombin reactivity with factors Xa and IXa in both unactivated and heparin-activated states (12) has suggested that the exosite contributes to antithrombin reactivity in both native and activated states and therefore that release of the RCL from its interaction with sheet A is not required to engage the exosite. This and other data suggested an alternative model in which the antithrombin exosite is available to interact with RCL-bound proteases in the native RCL-constrained state, but the interaction is less favorable due to repulsive interactions counteracting the attractive interactions (16). Allosteric activation in this model enhances antithrombin reactivity by mitigating the repulsive interactions and augmenting the attractive interactions with protease. More recently, it was found that an antithrombin variant with mutations designed to promote the allosteric-activating changes in the heparin binding site was fully activated as an inhibitor of factors Xa and IXa without the need for heparin (17). Significantly, this activation was not accompanied by the signature tryptophan fluorescence changes that report RCL expulsion from sheet A, although such changes could be induced by heparin, consistent with the idea that RCL expulsion is not required for allosteric activation.

To provide a more rigorous test of the new model of allosteric activation and in particular to provide evidence for the proposed different modes of exosite interaction in native and allosterically activated states, we chose to mutate six antithrombin residues that comprise the critical exosite in x-ray structures of heparin-antithrombin-protease Michaelis complexes (13, 14) and assess their importance as determinants of antithrombin reactivity with factors Xa, IXa, and thrombin in unactivated and heparin-activated states of the serpin. Complementary mutations of three active-site loop residues in factor Xa that interact with the antithrombin exosite in the Michaelis complex structures (18, 19) were done to assess the dependence of the exosite interaction on these protease residues. Our results demonstrate the importance of antithrombin and protease exosite residues for reactivity in both native and heparin-activated states of the serpin and thus show that RCL expulsion is not required to engage the exosite. Significantly, they support a new model of allosteric activation in which a balance between attractive and repulsive exosite interactions in the native state designed to repress reactivity at an otherwise attractive P1 Arg residue is shifted to favor the attractive interactions in the activated state through core conformational changes induced by heparin binding.

EXPERIMENTAL PROCEDURES

Proteins and Heparins—Recombinant antithrombin variants were constructed on an N135Q background to prevent glycosylation of Asn^{135} and consequent heparin binding heterogeneity (20) as described in previous studies (21). Gene mutagenesis was carried out by PCR using specially designed oligonucleotides from Integrated DNA Technologies and *Pfu* Turbo DNA polymerase from Stratagene according to the manufacturer's instructions. All mutations were confirmed by DNA sequencing. Antithrombin was expressed in Hi5 insect cells using the baculovirus expression system from Invitrogen and purified from culture media by a combination of heparin affinity and ion exchange chromatography, as previously described (12, 21). Protein concentration was determined from absorbance at 280 nm using an extinction coefficient of 37,700 M^{-1} cm^{-1} (22). Coagulation factors IXa and Xa from human plasma were purchased from Enzyme Research Laboratories, and human plasma thrombin was purchased from U.S. Biochemical Corp. Recombinant Gla-domainless wild-type and mutant human factor Xas were expressed in mammalian cells and activated and purified as described (18). Active protease concentrations were determined from standard substrate assays previously calibrated with active site-titrated enzyme. A synthetic heparin pentasaccharide corresponding to the antithrombin binding sequence in heparin was provided by Sanofi-Aventis (Toulouse, France). A full-length heparin (50-mer) containing the pentasaccharide binding sequence was prepared by size and affinity fractionation of commercial heparin as described (23).

SDS-PAGE—The purity of recombinant antithrombins as well as plasma and recombinant proteases and their ability to form antithrombin-protease covalent complexes was analyzed by SDS-PAGE electrophoresis using the Laemmli discontinuous buffer system (24).

Experimental Conditions—Antithrombin-protease reaction stoichiometries and kinetics were analyzed in ionic strength (*I*) 0.15, pH 7.4, buffers at 25 °C. Reactions with plasma factor Xa and factor IXa were done in 100 mM Hepes, 93.5 mM NaCl, 5 mM $CaCl_2$, 0.1% PEG 8000, whereas reactions with plasma thrombin and recombinant factor Xa were conducted in 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000 because the latter were not affected by calcium.

Stoichiometry of Antithrombin-Protease Reactions—The stoichiometries of antithrombin inhibition of proteases were determined in the absence and presence of pentasaccharide and full-length heparins by end point assays essentially as described in past studies (12). Fixed concentrations of protease and heparin (~100 nM) were incubated with increasing molar ratios of antithrombin to protease (up to 2:1) for a time sufficient to complete the reaction based on measured rate constants. Residual protease activity was then assayed by dilution of an aliquot into 100 μM chromogenic or 50 μM fluorogenic substrate solution and the initial linear absorbance (405 nm) or fluorescence (λ_{ex} 380 nm, λ_{em} 440 nm) increase was measured for several minutes. Plots of residual protease activity as a function of the molar ratio of antithrombin to protease were linear and yielded the stoichiometry of inhibition from the abscissa intercept. The substrates used were S2238 for thrombin (Chromogenix), Spectrozyme FXa for factor Xa (American Diagnostica), and Pefachrome or Pefafluor for factor IXa (Centerchem). Substrates were made up in buffers consisting of 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4, for S2238, 100 mM Hepes, 100 mM NaCl, 1 mM EDTA, 0.1% PEG 8000, pH 7.4, for Spectrozyme FXa, and 100 mM Hepes, 100 mM NaCl, 10 mM $CaCl_2$, 33% ethylene glycol, 0.1% PEG 8000, pH 8, for Pefachrome and Pefafluor. 50 μg/ml of Polybrene was included in all substrates to neutralize heparin when present.

Kinetics of Antithrombin-Protease Reactions—The kinetics of antithrombin inhibition of proteases was studied under

pseudo first-order conditions with antithrombin concentrations at least 10-fold higher than that of the protease as in previous studies (12). For reactions without heparin or in the presence of heparin pentasaccharide concentrations sufficient to saturate antithrombin (20–100% molar excess) and with measured rate constants up to $10^5\ \text{M}^{-1}\ \text{s}^{-1}$, full reaction time courses of the loss of protease activity were measured by assaying residual protease activity in aliquots of reaction mixtures as a function of time as described in measurements of reaction stoichiometries. Progress curves were fit by a single exponential decay function with zero end point for thrombin and factor Xa reactions and nonzero end point for factor IXa reactions to account for low levels of degraded protease less susceptible to inhibition (<10%) to obtain the observed pseudo first-order rate constant (k_{obs}). Apparent second-order association rate constants were calculated by dividing k_{obs} by the concentration of antithrombin. For reactions in the presence of pentasaccharide or full-length heparin that were faster than $10^5\ \text{M}^{-1}\ \text{s}^{-1}$, reactions were conducted for a fixed time in the presence of increasing concentrations of heparin at catalytic levels and residual protease activity was then assayed. The loss in protease activity was in this case fit by an exponential function with the heparin concentration instead of time as the independent variable (10). The apparent second-order rate constant was obtained by dividing the exponential rate constant by the fixed reaction time. Because antithrombin concentrations greatly exceeded dissociation constants for antithrombin-heparin interactions at *I* 0.15 for wild-type and variant antithrombins (>20-fold), no corrections for unbound heparin were required. Corrected second-order association rate constants reflecting reaction along the inhibitory pathway (k_a) were obtained by multiplying apparent second-order rate constants by inhibition reaction stoichiometries.

Quantitation of Antithrombin-Heparin Affinities—Equilibrium dissociation constants for antithrombin-heparin interactions were measured by titrations of wild-type and mutant antithrombins (100 nM) with the heparin pentasaccharide monitored from the ~40% enhancement in tryptophan fluorescence that accompanies heparin binding as in previous studies (9). Titrations were performed in *I* 0.35 buffer containing 20 mM sodium phosphate, 0.3 M NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4, to weaken heparin affinity and allow accurate measurements of K_D. K_D and the maximal fluorescence change were obtained by computer fitting data with the quadratic equilibrium binding equation (9). Stoichiometric titrations of antithrombin variants with heparin pentasaccharide were performed in *I* 0.15 sodium phosphate buffer to ensure that recombinant proteins were fully functional in binding the saccharide.

RESULTS

Evaluation of Putative Antithrombin Exosite Residues—The importance of six putative antithrombin exosite residues that interact with factors Xa and IXa in x-ray structures of heparin-activated antithrombin-factor Xa/IXa Michaelis complexes (13, 14) (Fig. 1) for allosteric activation of the serpin was assessed by mutagenesis. Antithrombin variants with alanine mutations of Asn^{233}, Arg^{235}, Glu^{237}, Tyr^{253}, Glu^{255}, and His^{319} residues of strands 2, 3, and 4 of sheet C were made alone and in combination. Although His^{319} was previously not implicated as part of the exosite, intramolecular interactions of this residue with Glu^{237} and Tyr^{253} exosite residues suggested a potential role in exosite function. The variant antithrombins were evaluated for their effects on antithrombin reactivity with factors Xa and IXa in the absence and presence of specific pentasaccharide and full-length ~50-saccharide heparins containing the pentasaccharide sequence. The heparin pentasaccharide is sufficient to allosterically activate the inhibitor, whereas the full-length heparin augments the allosteric activating effect by

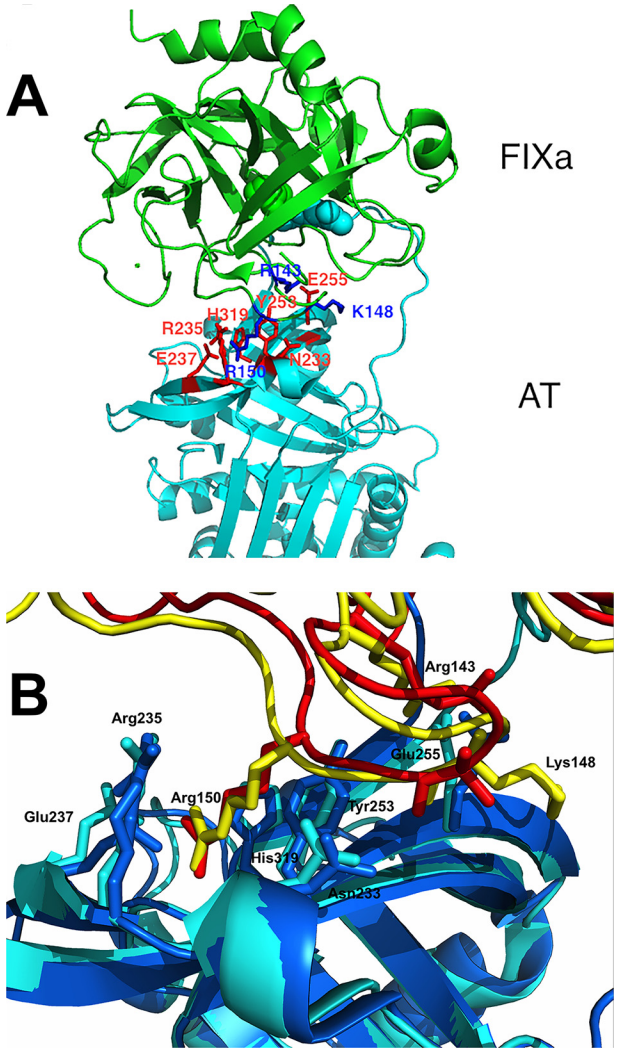

FIGURE 1. **Putative exosite residues in the x-ray structures of heparin-antithrombin-S195A factor Xa/IXa Michaelis complexes.** *A*, the antithrombin-factor IXa Michaelis complex (Protein Data Bank code 3KCG) is shown in ribbon with antithrombin colored *cyan* and the factor IXa catalytic domain colored *green*. Putative antithrombin exosite residues, Asn^{233}, Arg^{235}, Glu^{237}, Tyr^{253}, Glu^{255}, and His^{319} in strands 2, 3, and 4 of sheet C are highlighted in *red stick*. Complementary factor IXa exosite residues Arg^{143}, Lys^{148}, and Arg^{150} in the active-site autolysis loop are shown in *blue stick*. The antithrombin P1 Arg and factor IXa active-site Ala^{195} residues are shown in space-filling. *B*, closeup view of antithrombin exosite interactions in aligned Michaelis complexes of heparin-activated antithrombin with factor IXa and Xa (Protein Data Bank code 2GD4) are shown in ribbon with antithrombin in *cyan* and *blue* in the two structures, factor Xa in *yellow*, and factor IXa in *red*. Antithrombin exosite residues and the complementary factor Xa and IXa exosite residues indicated above are shown in stick.

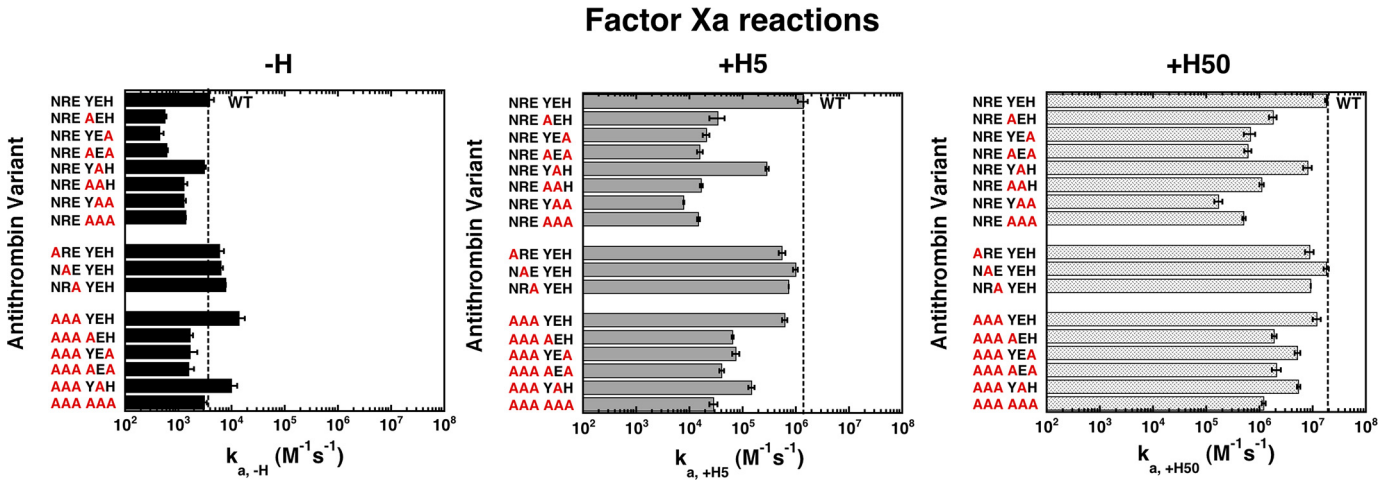

FIGURE 2. **Effects of mutating antithrombin exosite residues on association rate constants for unactivated and heparin-activated antithrombin reactions with factor Xa.** Bar graph comparison of rate constants measured for mutant antithrombin reactions with factor Xa from Table 1 without heparin activation (*left panel*), with heparin pentasaccharide activation (*middle panel*), and with full-length heparin activation (*right panel*). Antithrombins with single or multiple mutations of Asn^{233}, Arg^{235}, Glu^{237}, Tyr^{253}, Glu^{255}, and His^{319} residues (NRE YEH) to Ala are designated by the *single letter* amino acid code with mutated residues shown in *red*. The *dashed line* indicates control wild-type rate constants.

providing an additional bridging interaction site for the protease in the ternary Michaelis complex (9). Because thrombin does not engage the exosite and its reactivity with antithrombin is solely enhanced by bridging heparin activation (25, 26), the effects of the mutations on antithrombin reactivity with thrombin served as an important control for any nonspecific effects of the mutations on serpin reactivity.

All antithrombin mutants were expressed and purified similar to wild-type and yielded amounts of protein comparable with wild-type. All mutants bound the heparin pentasaccharide with binding stoichiometries close to 1:1 and inhibited thrombin, factor Xa, and factor IXa in the absence and presence of heparin with stoichiometries of 1–2 mol of inhibitor/mol of protease that were comparable with wild-type values. The effect of the mutations on antithrombin reactivity with the three proteases was assessed by measuring the apparent second-order rate constants for the reactions under pseudo first-order conditions as in past studies (12). These were corrected for the fraction of inhibitor reacting through a competing substrate pathway by multiplying by the inhibition stoichiometries to yield the second-order association rate constant (k_a) for reaction along the inhibitory pathway.

Tyr^{253} and His^{319} Are Critical Exosite Residues in Both Native and Heparin-activated States—Mutations of Tyr^{253} and His^{319} had profound effects on antithrombin reactivity with factors Xa and IXa in native as well as heparin-activated states (Figs. 2 and 3 and Tables 1 and 2). Y253A antithrombin inhibited factor Xa with a 7-fold lower k_a than wild-type in the absence of heparin and more substantial 40- and 10-fold lower k_a in the presence of pentasaccharide and bridging heparins, respectively. The mutant antithrombin inhibited factor IXa with an even greater 69-fold reduction in k_a from wild-type in the unactivated state but lesser 32- and 14-fold reductions in k_a in pentasaccharide and bridging heparin-activated states, although the latter reductions were comparable with those observed in corresponding reactions with factor Xa. Similarly, H319A antithrombin inhibited factor Xa with a 9-fold lower k_a than wild-type in the absence of heparin and greater 67- and 27-fold reduced values in the presence of pentasaccharide and bridging heparins. As observed with the Y253A mutant, H319A antithrombin inhibited factor IXa with the most dramatic 230-fold reduction in k_a from wild-type in the absence of heparin compared with the lesser 50- and 20-fold reductions in k_a in the presence of pentasaccharide and bridging heparins, although the latter reductions were again comparable with those observed with factor Xa. Interestingly, combining the Tyr^{253} and His^{319} mutations caused losses in antithrombin reactivity with the two proteases that were similar to or only modestly greater than those caused by mutation of either residue alone in unactivated or heparin-activated states. The double Y253A/H319A antithrombin mutant thus showed 6-, 88-, and 30-fold reductions in k_a for reactions with factor Xa in the absence and presence of pentasaccharide and full-length heparins, respectively, and 200-, 89-, and 72-fold reductions in k_a for reactions with factor IXa in the absence and presence of the corresponding heparin activators. Mutating either residue of the Tyr^{253}/His^{319} pair after the other residue has been mutated thus produces little or no further loss in antithrombin reactivity with either protease, indicating that the contributions of each of these residues to antithrombin reactivity are strongly coupled (Figs. 4 and 5).

Contrasting these results, Y253A and H319A single and double antithrombin mutants inhibited thrombin at rates modestly altered from those of wild-type, k_a being reduced 1.7-fold in the absence of heparin and increased 1.3–3.5-fold in the presence of the two heparin activators (Fig. 6 and Table 3). Tyr^{253} and His^{319} are thus critically important and interdependent contributors to antithrombin reactivity with factors Xa and IXa in both unactivated and heparin-activated inhibitor states although these residues make minor contributions to reactivity with thrombin.

Glu^{255} Contributes to the Exosite in a Context-dependent Manner—Mutation of Glu^{255} produced significant losses in antithrombin reactivity with factors Xa and IXa, again both in native and heparin-activated states, although these losses were less than those resulting from Tyr^{253} and His^{319} mutations

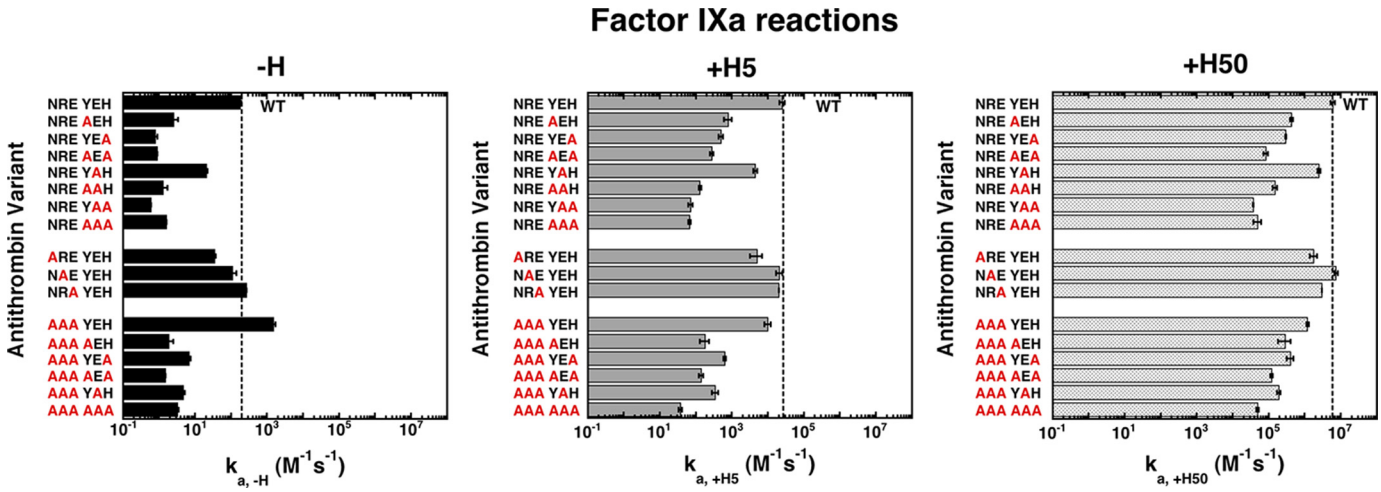

FIGURE 3. **Effects of mutating antithrombin exosite residues on association rate constants for unactivated and heparin-activated antithrombin reactions with factor IXa.** Bar graph comparison of rate constants measured for mutant antithrombin reactions with factor IXa from Table 2 without heparin activation (*left panel*), with heparin pentasaccharide activation (*middle panel*), and with full-length heparin activation (*right panel*) as described in the legend to Fig. 2.

TABLE 1

Second-order association rate constants for reactions of wild-type (WT) and exosite mutant antithrombins with factor Xa in the absence ($k_{a,-H}$) and presence of pentasaccharide ($k_{a,+H5}$) and full-length heparins ($k_{a,+H50}$)

Association rate constants for reactions of antithrombin exosite mutants with factor Xa were measured under pseudo first-order conditions in *I* 0.15, pH 7.4, Hepes buffer containing 5 mM Ca^{2+} at 25 °C and then corrected for variable inhibition reaction stoichiometries (SIs) as described under "Experimental Procedures." Reported errors are ranges or S.E. from two or more experiments. Values in parentheses express rate constants relative to wild-type. Heparin allosteric and bridging activation rate enhancements were calculated from the rate constant ratios in the right-most columns.

Antithrombin	$k_{a,-H}$	$k_{a,+H5}$	$k_{a,+H50}$	$k_{a,+H5}/k_{a,-H}$	$k_{a,+H50}/k_{a,+H5}$
NRE YEH (WT)	$(3.9 \pm 0.8) \times 10^3$ (1)	$(1.4 \pm 0.3) \times 10^6$ (1)	$(1.8 \pm 0.1) \times 10^7$ (1)	360	13
NRE AEH	$(5.7 \pm 0.4) \times 10^2$ (0.15)	$(3.5 \pm 1.1) \times 10^4$ (0.025)	$(1.8 \pm 0.3) \times 10^6$ (0.10)	61	51
NRE YEA	$(4.5 \pm 0.9) \times 10^2$ (0.12)	$(2.1 \pm 0.3) \times 10^4$ (0.015)	$(6.7 \pm 1.6) \times 10^5$ (0.037)	47	32
NRE AEA	$(6.2 \pm 0.2) \times 10^2$ (0.16)	$(1.6 \pm 0.2) \times 10^4$ (0.011)	$(6.1 \pm 0.9) \times 10^5$ (0.034)	26	38
NRE YAH	$(3.1 \pm 0.2) \times 10^3$ (0.79)	$(2.9 \pm 0.2) \times 10^5$ (0.21)	$(8.1 \pm 1.5) \times 10^6$ (0.45)	94	28
NRE AAH	$(1.3 \pm 0.2) \times 10^3$ (0.33)	$(1.7 \pm 0.1) \times 10^4$ (0.012)	$(1.1 \pm 0.1) \times 10^6$ (0.061)	13	65
NRE YAA	$(1.3 \pm 0.1) \times 10^3$ (0.33)	$(7.9 \pm 0.2) \times 10^3$ (0.006)	$(1.7 \pm 0.3) \times 10^5$ (0.009)	6.1	22
NRE AAA	$(1.4 \pm 0.0) \times 10^3$ (0.36)	$(1.5 \pm 0.1) \times 10^4$ (0.011)	$(5.1 \pm 0.4) \times 10^5$ (0.028)	11	34
ARE YEH	$(6.0 \pm 1.3) \times 10^3$ (1.5)	$(5.6 \pm 0.8) \times 10^5$ (0.40)	$(8.7 \pm 1.6) \times 10^6$ (0.48)	93	16
NAE YEH	$(6.5 \pm 0.5) \times 10^3$ (1.7)	$(1.0 \pm 0.1) \times 10^6$ (0.71)	$(1.8 \pm 0.2) \times 10^7$ (1.0)	150	18
NRA YEH	$(7.9 \pm 0.0) \times 10^3$ (2.0)	$(7.5 \pm 0.0) \times 10^5$ (0.54)	$(9.1 \pm 0.0) \times 10^6$ (0.51)	95	12
AAA YEH	$(1.4 \pm 0.4) \times 10^4$ (3.6)	$(6.3 \pm 0.7) \times 10^5$ (0.45)	$(1.2 \pm 0.2) \times 10^7$ (0.67)	45	19
AAA AEH	$(1.7 \pm 0.2) \times 10^3$ (0.44)	$(6.6 \pm 0.3) \times 10^4$ (0.047)	$(1.9 \pm 0.2) \times 10^6$ (0.11)	39	29
AAA YEA	$(1.7 \pm 0.6) \times 10^3$ (0.44)	$(7.6 \pm 1.2) \times 10^4$ (0.054)	$(5.2 \pm 0.6) \times 10^6$ (0.29)	45	68
AAA AEA	$(1.6 \pm 0.4) \times 10^3$ (0.41)	$(4.1 \pm 0.4) \times 10^4$ (0.029)	$(2.1 \pm 0.4) \times 10^6$ (0.12)	26	51
AAA YAH	$(1.0 \pm 0.3) \times 10^4$ (2.6)	$(1.5 \pm 0.2) \times 10^5$ (0.11)	$(5.4 \pm 0.4) \times 10^6$ (0.30)	15	36
AAA AAA	$(3.1 \pm 0.4) \times 10^3$ (0.79)	$(2.9 \pm 0.5) \times 10^4$ (0.021)	$(1.2 \pm 0.1) \times 10^6$ (0.067)	9.4	41

(Figs. 2 and 3 and Tables 1 and 2). E255A antithrombin inhibited factor Xa with a 1.3-fold lower k_a than wild-type in the absence of heparin and 4.8- and 2.2-fold lower k_a in the presence of pentasaccharide and bridging heparins, whereas the mutant antithrombin inhibited factor IXa with an 8.6-fold lower k_a in the absence of heparin and 5.6- and 2.4-fold lower k_a in the presence of pentasaccharide and full-length heparin activators. Combining the E255A mutation with Y253A/H319A mutations revealed that the contribution of Glu^{255} to antithrombin reactivity depended on residues Tyr^{253} and His^{319} and the activation state of the inhibitor (Figs. 4 and 5). In the absence of heparin, adding the E255A mutation to Y253A/H319A mutant antithrombins enhanced rather than depressed reactivity with factor Xa by 2–3-fold and only marginally affected reactivity with factor IXa compared with that of the Y253A/H319A mutants. By contrast, in the presence of pentasaccharide or bridging heparins, the addition of the E255A mutation to Y253A/H319A antithrombin variants produced additional decreases in reactivity with factor Xa of up to 4-fold and with factor IXa of 2–8-fold over the reactivity losses of the corresponding Y253A/H319A mutants. These reactivity decreases were in most cases less than those observed when Glu^{255} was mutated alone in reactions with factor Xa but comparable with or somewhat greater than those of the Glu^{255} single mutant in reactions with factor IXa. Glu^{255} thus positively contributed to antithrombin reactivity in the unactivated inhibitor in the context of wild-type Tyr^{253} and His^{319} residues, but this contribution became negative or marginally positive when Tyr^{253} and His^{319} were mutated. In the heparin-activated inhibitor, Glu^{255} made significant positive contributions to reactivity that were reduced by Tyr^{253} and His^{319} mutations in factor Xa reactions but were less affected by Tyr^{253}/His^{319} mutations in factor IXa reactions. The effects of Tyr^{253}/His^{319} mutations on Glu^{255} contributions to reactivity were reciprocal, the positive contributions of Tyr^{253}/His^{319} residues being reduced in the context of the Glu^{255} mutation for factor Xa

TABLE 2

Second-order association rate constants for reactions of wild-type (WT) and exosite mutant antithrombins with factor IXa in the absence ($k_{a,-H}$) and presence of pentasaccharide ($k_{a,+H5}$) and full-length heparins ($k_{a,+H50}$)

Association rate constants for reactions of antithrombin exosite mutants with factor IXa were measured in *I* 0.15, pH 7.4, Hepes buffer containing 5 mM Ca^{2+} at 25 °C and corrected for variable inhibition reaction stoichiometries as described in the legend to Table 1. Errors, relative rate constants, and heparin allosteric and bridging activation rate enhancements are given as in Table 1.

Antithrombin	$k_{a,-H}$	$k_{a,+H5}$	$k_{a,+H50}$	$k_{a,+H5}/k_{a,-H}$	$k_{a,+H50}/k_{a,+H5}$
NRE YEH (WT)	$(1.8 \pm 0.1) \times 10^2$ (1)	$(2.5 \pm 0.4) \times 10^4$ (1)	$(6.0 \pm 0.8) \times 10^6$ (1)	140	240
NRE AEH	$(2.6 \pm 0.8) \times 10^0$ (0.014)	$(7.9 \pm 1.9) \times 10^2$ (0.032)	$(4.3 \pm 0.4) \times 10^5$ (0.072)	300	540
NRE YEA	$(0.8 \pm 0.1) \times 10^0$ (0.004)	$(5.0 \pm 0.7) \times 10^2$ (0.020)	$(3.0 \pm 0.1) \times 10^5$ (0.050)	620	600
NRE AEA	$(0.9 \pm 0.0) \times 10^0$ (0.005)	$(2.8 \pm 0.3) \times 10^2$ (0.011)	$(8.3 \pm 1.1) \times 10^4$ (0.014)	310	300
NRE YAH	$(2.1 \pm 0.1) \times 10^1$ (0.12)	$(4.5 \pm 0.6) \times 10^3$ (0.18)	$(2.5 \pm 0.2) \times 10^6$ (0.42)	210	560
NRE AAH	$(1.3 \pm 0.4) \times 10^0$ (0.007)	$(1.3 \pm 0.1) \times 10^2$ (0.005)	$(1.5 \pm 0.2) \times 10^5$ (0.025)	100	1200
NRE YAA	$(0.6 \pm 0.0) \times 10^0$ (0.003)	$(7.1 \pm 1.0) \times 10^1$ (0.003)	$(3.7 \pm 0.1) \times 10^4$ (0.006)	120	520
NRE AAA	$(1.6 \pm 0.0) \times 10^0$ (0.009)	$(6.7 \pm 0.5) \times 10^1$ (0.003)	$(5.0 \pm 1.2) \times 10^4$ (0.008)	42	750
ARE YEH	$(3.5 \pm 0.2) \times 10^1$ (0.19)	$(5.0 \pm 1.8) \times 10^3$ (0.20)	$(1.8 \pm 0.4) \times 10^6$ (0.30)	140	360
NAE YEH	$(1.1 \pm 0.3) \times 10^2$ (0.61)	$(2.1 \pm 0.4) \times 10^4$ (0.84)	$(7.4 \pm 0.8) \times 10^6$ (1.2)	190	350
NRA YEH	$(2.7 \pm 0.0) \times 10^2$ (1.5)	$(2.0 \pm 0.0) \times 10^4$ (0.80)	$(3.0 \pm 0.0) \times 10^6$ (0.50)	74	150
AAA YEH	$(1.5 \pm 0.2) \times 10^3$ (8.3)	$(1.0 \pm 0.2) \times 10^4$ (0.40)	$(1.2 \pm 0.1) \times 10^6$ (0.20)	6.7	120
AAA AEH	$(1.9 \pm 0.6) \times 10^0$ (0.011)	$(1.8 \pm 0.5) \times 10^2$ (0.007)	$(2.9 \pm 1.1) \times 10^5$ (0.048)	95	1600
AAA YEA	$(6.8 \pm 0.7) \times 10^0$ (0.038)	$(6.4 \pm 0.5) \times 10^2$ (0.026)	$(4.0 \pm 0.8) \times 10^5$ (0.067)	94	620
AAA AEA	$(1.5 \pm 0.0) \times 10^0$ (0.008)	$(1.4 \pm 0.2) \times 10^2$ (0.006)	$(1.2 \pm 0.1) \times 10^5$ (0.020)	93	860
AAA YAH	$(4.8 \pm 0.5) \times 10^0$ (0.027)	$(3.5 \pm 0.7) \times 10^2$ (0.014)	$(1.9 \pm 0.2) \times 10^5$ (0.032)	73	540
AAA AAA	$(3.3 \pm 0.2) \times 10^0$ (0.018)	$(3.7 \pm 0.4) \times 10^1$ (0.001)	$(4.9 \pm 0.4) \times 10^4$ (0.008)	11	1300

reactions and less dependent on the Glu^{255} mutation for factor IXa reactions (Figs. 4 and 5).

Contrasting these results, Glu^{255} made a negative contribution to antithrombin reactivity with thrombin both in the absence and presence of heparin (Fig. 6 and Table 3). The E255A antithrombin mutation thus increased the k_a for inhibition of thrombin 4-fold in unactivated and ~2-fold in heparin-activated states. Adding the E255A mutation to Y253A/H319A mutant antithrombins somewhat augmented or did not affect the increase in k_a in the absence or presence of pentasaccharide heparin and slightly diminished the increase in the presence of the bridging heparin, consistent with the latter mutations modulating the negative contribution of Glu^{255}.

Asn^{233}, Arg^{235}, and Glu^{237} Modulate Exosite Function—Single mutations of Asn^{233}, Arg^{235}, and Glu^{237} residues of the putative exosite resulted in modest changes in antithrombin reactivity with factors Xa and IXa in the absence or presence of the two heparin activators (1–2.5-fold), except for the Asn^{233} mutation, which caused more significant 3.3–5.3-fold losses in antithrombin reactivity with factor IXa with or without heparin activation (Figs. 2 and 3 and Tables 1 and 2). The reactivity of these antithrombin variants with thrombin was also only modestly altered from wild-type (<3-fold) (Fig. 6 and Table 3). To assess whether these residues made any contribution to the factor Xa/IXa-specific exosite in antithrombin, we investigated the effect of mutating Asn^{233}, Arg^{235}, and Glu^{237} residues together as a block. Surprisingly, the combined N233A/R235A/E237A (NRE) mutations caused substantial enhancements of antithrombin reactivity with factors Xa and IXa of 3.6- and 8.3-fold, respectively, in the absence of heparin, whereas the mutations caused losses in reactivity of 1.5–5-fold in the presence of pentasaccharide or bridging heparin activators. That this pattern of reactivity changes was unique to factors Xa and IXa was indicated from the insignificant effect of the NRE block mutations on antithrombin reactivity with thrombin in the absence or presence of pentasaccharide heparin and modest 1.8-fold gain in reactivity in the presence of the bridging heparin (Fig. 6 and Table 3). Asn^{233}, Arg^{235}, and Glu^{237} thus together make a large negative contribution to antithrombin reactivity with factors Xa and IXa in the unactivated state and this negative contribution becomes positive upon heparin activation of the serpin. By contrast, the three residues make no or small contributions to antithrombin reactivity with thrombin in unactivated and heparin-activated states.

Coupling between the Asn^{233}/Arg^{235}/Glu^{237} block mutations and Tyr^{253}, Glu^{255}, and His^{319} exosite residues was evaluated by adding Y253A, E255A, and H319A mutations to the NRE block antithrombin mutant as single, double, or triple mutations and measuring the effects on antithrombin reactivity with the three target proteases. Notably, in the absence of heparin, where the NRE block mutations resulted in significantly enhanced reactivities with factors Xa and IXa, Tyr^{253}, Glu^{255}, and His^{319} (YEH) mutations produced equal or greater reactivity losses in NRE mutant than in wild-type backgrounds (Figs. 4 and 5). These augmented losses in reactivity were modest for factor Xa reactions (maximally 1.6-fold greater) but large for factor IXa reactions (5–36-fold greater) and reflected NRE suppression of the positive contributions of YEH residues. In the presence of heparin where NRE block mutations reduced antithrombin reactivity with factors Xa and IXa, YEH mutations produced smaller losses in reactivity in the NRE mutant than in wild-type backgrounds, reflecting NRE enhancement of the positive contributions of YEH residues. The contribution of YEH residues to antithrombin reactivity with factors Xa and IXa was thus significantly attenuated by the NRE triad in the unactivated inhibitor, whereas the YEH contribution was enhanced by the NRE triad with pentasasccharide and bridging heparin activation of the serpin.

Net Exosite Contribution to Antithrombin Reactivity—The overall effect of mutating all six putative antithrombin exosite residues was to produce a minor 1.3-fold loss in antithrombin reactivity with factor Xa in the absence of heparin, but substantial 48- and 15-fold losses in reactivity in the presence of pentasaccharide and bridging heparins, respectively (Table 1). Notably, the effect of the six mutations on antithrombin reactivity with factor IXa was more pronounced, *i.e.* a 55-fold

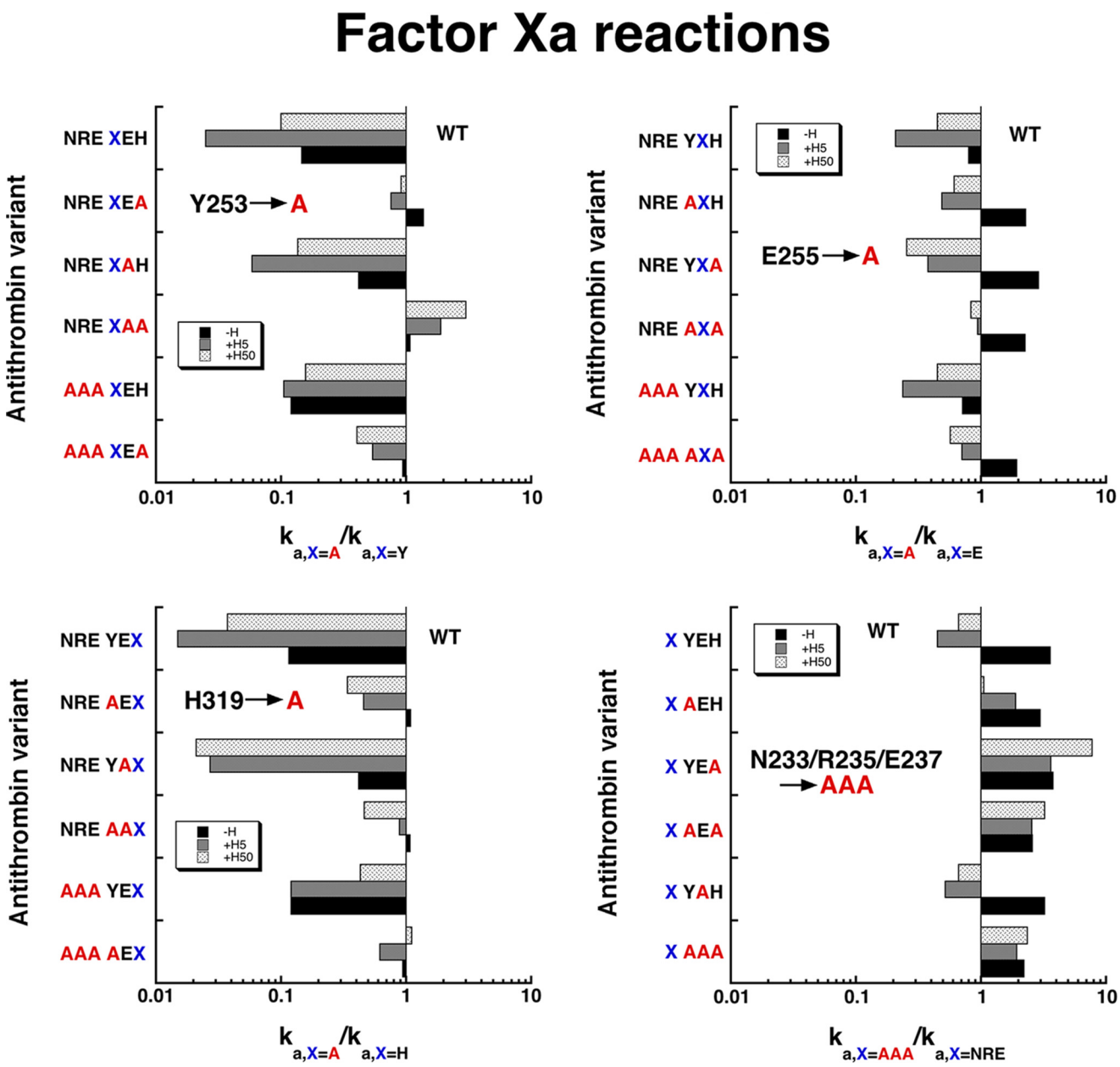

FIGURE 4. **Context dependence of the effects of exosite residue mutations on antithrombin reactivity with factor Xa.** Bar graph showing the effects of mutating Tyr^{253}, His^{319}, Glu^{255}, or $Asn^{233}/Arg^{235}/Glu^{237}$ triad exosite residues in different exosite mutant contexts on second-order rate constants for antithrombin inhibition of factor Xa in the absence of heparin (*black bars*) and in the presence of pentasaccharide (*gray bars*) and bridging heparins (*stippled bars*). Each *bar* represents the ratio of rate constants for reactions of antithrombin variants in which the indicated residue in the panel is mutated to that in which the residue is not mutated both in the context of the indicated antithrombin exosite mutants given on the vertical axis. Each antithrombin variant is designated as described in the legends to Figs. 2 and 3 with the mutated residue indicated by *X* in *blue*.

decrease in the absence of heparin and 680- and 120-fold decreases in the presence of pentasaccharide and bridging heparin activators, respectively (Table 2). The greater antithrombin reactivity losses in the heparin-activated than the unactivated state for both factor Xa and IXa reactions was accounted for by the negative contributions of NRE residues in the unactivated state being alleviated in the heparin-activated state and the positively contributing YEH residues in both states being enhanced by heparin activation. By contrast, the six exosite residue mutations resulted in relatively small 2–3-fold enhancements in reactivity with thrombin in the absence or presence of heparin that reflected a dominant-negative contribution of YEH residues (Table 3).

Role of factor Xa Arg^{150}—To ascertain the contribution of the complementary protease exosite residue, Arg^{150} of factors Xa and IXa, to allosteric activation of antithrombin (Fig. 1), we mutated this residue in factor Xa and determined the effect on reactivity with wild-type and exosite mutant antithrombins relative to wild-type factor Xa (Fig. 7 and Table 4). These studies were done with recombinant factor Xa that lacks the Gla domain. The reactivity of the recombinant Gla-domainless factor Xa with antithrombin resembles that of plasma factor Xa measured in the presence of calcium (Tables 1 and 5). The R150A mutation reduced the reactivity of factor Xa with wild-type antithrombin by 2.2-fold in the absence of heparin and 20- and 4.3-fold in the presence of pentasaccharide and bridging

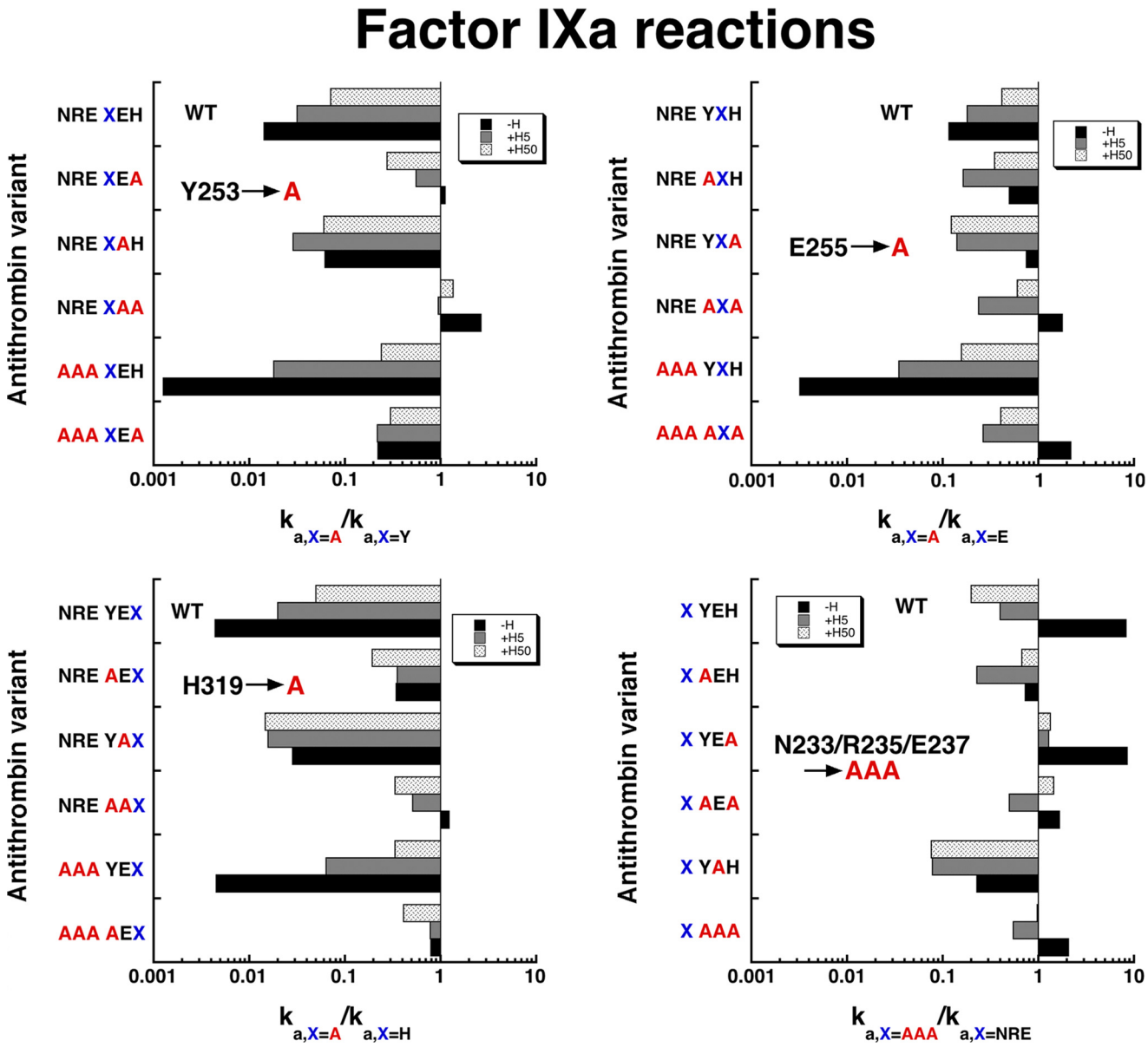

FIGURE 5. **Context dependence of the effects of exosite residue mutations on antithrombin reactivity with factor IXa.** Bar graph showing the effects of mutating the indicated antithrombin exosite residues in different exosite mutant contexts on association rate constants for antithrombin inhibition of factor IXa in the absence and presence of pentasaccharide and bridging heparins as described in the legend to Fig. 4.

heparin activators, respectively, confirming an important positive contribution of Arg^{150} to reactivity that was greatest in the allosterically activated inhibitor (Fig. 7 and Table 4). Significantly, this positive reactivity contribution was abolished or transformed to a negative contribution in reactions of factor Xa with antithrombin possessing mutations of the critical Tyr^{253} or His^{319} exosite residues, as judged by the comparable or greater reactivities of the antithrombin exosite mutants with R150A factor Xa relative to wild-type factor Xa in the absence or presence of heparin. Moreover, the positive contribution of Arg^{150} of factor Xa was reduced by Glu^{255} or NRE block mutations in antithrombin, based on the observed lessened effect of the Arg^{150} mutation on factor Xa reactivity with these mutant antithrombins relative to the wild-type serpin. Notably, the NRE mutations retained a strong negative effect on antithrombin reactivity with R150A factor Xa in the unactivated state as with wild-type factor Xa, but in contrast to the wild-type factor Xa reaction, this negative contribution was not alleviated by heparin activation. The overall effect of mutating all six exosite residues was to enhance antithrombin reactivity with R150A factor Xa by 4-fold in the absence of heparin and marginally decrease reactivity by 1.1- and 1.6-fold in the presence of pentasacharide and bridging heparins, respectively (Table 4). These effects contrasted sharply with the neutral effect of the exosite residue mutations on reactivity with factor Xa in the absence of heparin and large 48- and 15-fold losses in reactivity in the presence of pentasaccharide and bridging heparins, respectively (Table 1). The exosite residue contributions to antithrombin reactivity with wild-type factor Xa are thus lost as a result of the R150A mutation in factor Xa.

Roles of factor Xa Arg^{143} and Lys^{148}—The importance of two other factor Xa autolysis loop residues, Arg^{143} and Lys^{148}, that

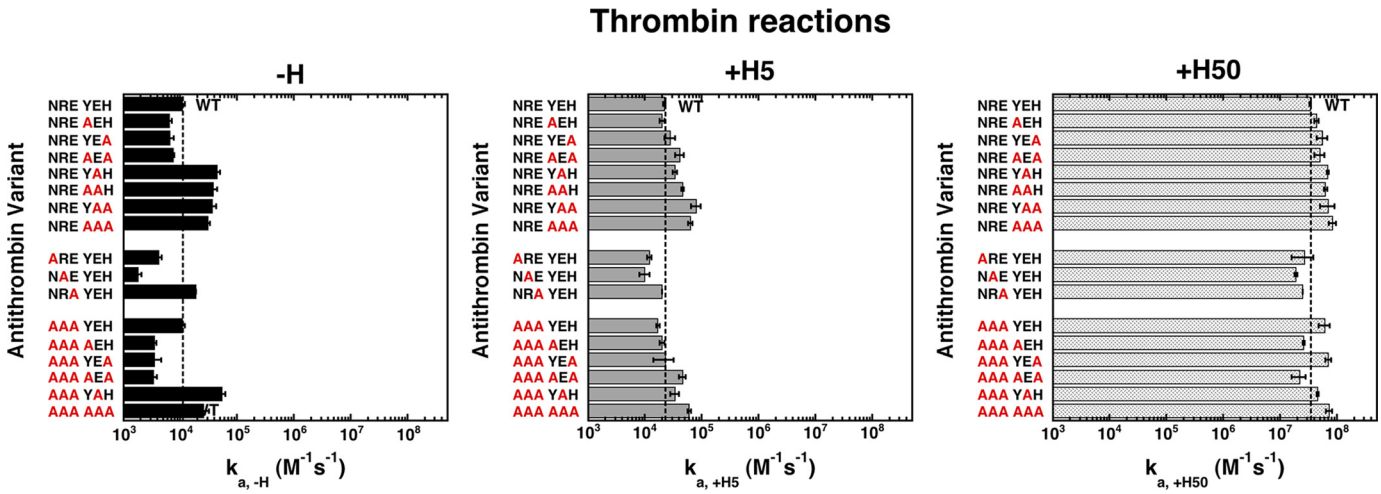

FIGURE 6. **Effects of mutating antithrombin exosite residues on the association rate constants for unactivated and heparin-activated antithrombin reactions with thrombin.** Bar graph comparison of rate constants measured for mutant antithrombin reactions with thrombin from Table 3 without heparin activation (*left panel*), with heparin pentasaccharide activation (*middle panel*), and with full-length heparin activation (*right panel*) as described in the legend to Fig. 2.

TABLE 3

Second-order association rate constants for reactions of wild-type (WT) and exosite mutant antithrombins with thrombin in the absence ($k_{a,-H}$) and presence of pentasaccharide ($k_{a,+H5}$) and full-length heparins ($k_{a,+H50}$)

Association rate constants for reactions of antithrombin exosite mutants with thrombin were measured in *I* 0.15, pH 7.4, phosphate buffer at 25 °C and corrected for variable inhibition reaction stoichiometries as described in the legend to Table 1. Errors, relative rate constants, and heparin allosteric and bridging activation rate enhancements are given as in Table 1.

Antithrombin	$k_{a,-H}$	$k_{a,+H5}$	$k_{a,+H50}$	$k_{a,+H5}/k_{a,-H}$	$k_{a,+H50}/k_{a,+H5}$
NRE YEH (WT)	$(1.1 \pm 0.1) \times 10^4$ (1)	$(2.2 \pm 0.1) \times 10^4$ (1)	$(3.4 \pm 0.1) \times 10^7$ (1)	2.0	1500
NRE AEH	$(6.4 \pm 0.7) \times 10^3$ (0.58)	$(2.0 \pm 0.2) \times 10^4$ (0.91)	$(4.4 \pm 0.4) \times 10^7$ (1.3)	3.1	2200
NRE YEA	$(6.6 \pm 1.1) \times 10^3$ (0.60)	$(2.8 \pm 0.6) \times 10^4$ (1.3)	$(5.6 \pm 1.2) \times 10^7$ (1.6)	4.2	2000
NRE AEA	$(7.6 \pm 0.3) \times 10^3$ (0.69)	$(4.1 \pm 0.7) \times 10^4$ (1.9)	$(5.0 \pm 1.0) \times 10^7$ (1.5)	5.4	1200
NRE YAH	$(4.5 \pm 0.5) \times 10^4$ (4.1)	$(3.4 \pm 0.3) \times 10^4$ (1.5)	$(6.9 \pm 0.3) \times 10^7$ (2.0)	0.76	2000
NRE AAH	$(3.8 \pm 0.7) \times 10^4$ (3.5)	$(4.6 \pm 0.2) \times 10^4$ (2.1)	$(6.3 \pm 0.4) \times 10^7$ (1.9)	1.2	1400
NRE YAA	$(3.7 \pm 0.6) \times 10^4$ (3.4)	$(8.0 \pm 1.6) \times 10^4$ (3.6)	$(7.0 \pm 2.0) \times 10^7$ (2.1)	2.2	880
NRE AAA	$(3.1 \pm 0.2) \times 10^4$ (2.8)	$(6.3 \pm 0.5) \times 10^4$ (2.9)	$(8.4 \pm 1.2) \times 10^7$ (2.5)	2.0	1300
ARE YEH	$(4.2 \pm 0.5) \times 10^3$ (0.38)	$(1.2 \pm 0.1) \times 10^4$ (0.55)	$(2.7 \pm 1.1) \times 10^7$ (0.79)	2.9	2200
NAE YEH	$(1.8 \pm 0.3) \times 10^3$ (0.16)	$(1.0 \pm 0.2) \times 10^4$ (0.45)	$(1.9 \pm 0.1) \times 10^7$ (0.56)	5.6	1900
NRA YEH	$(1.9 \pm 0.0) \times 10^4$ (1.7)	$(2.0 \pm 0.0) \times 10^4$ (0.91)	$(2.5 \pm 0.0) \times 10^7$ (0.74)	1.1	1200
AAA YEH	$(1.1 \pm 0.1) \times 10^4$ (1.0)	$(1.7 \pm 0.1) \times 10^4$ (0.77)	$(6.1 \pm 1.3) \times 10^7$ (1.8)	1.5	3600
AAA AEH	$(3.5 \pm 0.3) \times 10^3$ (0.32)	$(2.0 \pm 0.2) \times 10^4$ (0.91)	$(2.6 \pm 0.1) \times 10^7$ (0.76)	5.7	1300
AAA YEA	$(3.5 \pm 1.1) \times 10^3$ (0.32)	$(2.3 \pm 0.9) \times 10^4$ (1.0)	$(7.1 \pm 0.8) \times 10^7$ (2.1)	6.6	3100
AAA AEA	$(3.4 \pm 0.5) \times 10^3$ (0.31)	$(4.6 \pm 0.6) \times 10^4$ (2.1)	$(2.2 \pm 0.6) \times 10^7$ (0.65)	14	480
AAA YAH	$(5.4 \pm 0.8) \times 10^4$ (4.9)	$(3.4 \pm 0.6) \times 10^4$ (1.5)	$(4.6 \pm 0.2) \times 10^7$ (1.4)	0.63	1400
AAA AAA	$(2.6 \pm 0.6) \times 10^4$ (2.4)	$(6.0 \pm 0.4) \times 10^4$ (2.7)	$(7.3 \pm 0.9) \times 10^7$ (2.1)	2.3	1200

interact with the antithrombin exosite in the x-ray structures of antithrombin-factor Xa/IXa Michaelis complexes (Fig. 1) was similarly evaluated by mutating these residues and determining the effect on factor Xa reactivity with wild-type and a subset of the exosite mutant antithrombins. R143A factor Xa was inhibited by wild-type antithrombin ~3-fold faster than wild-type factor Xa in the absence of heparin, whereas the mutant factor Xa was inhibited 3–5-fold slower than wild-type factor Xa in the presence of the two heparin activators. Similarly, K148A factor Xa was inhibited by wild-type antithrombin 2-fold faster than wild-type factor Xa in the absence of heparin, but 2–10-fold slower than wild-type factor Xa in the presence of the heparin activators (Fig. 8 and Table 5). Arg^{143} and Lys^{148} thus both make negative contributions to factor Xa reactivity with unactivated antithrombin but positive contributions with the heparin-activated serpin. Similar to wild-type antithrombin, exosite mutant antithrombins showed 2–4-fold enhanced reactivities with R143A and K148A mutant factor Xa relative to wild-type factor Xa in the absence of heparin, indicating that the negative contributions of the protease basic residues were independent of antithrombin exosite residues. In the presence of pentasaccharide or bridging heparins, by contrast, antithrombins with mutations in the critical Tyr^{253}, Glu^{255}, and His^{319} residues exhibited reactivities with R143A and K148A factor Xa mutants more comparable with those with wild-type factor Xa, implying that the positive contributions of Arg^{143} and Lys^{148} in the heparin-activated state depended on an interaction with the antithrombin exosite.

Effects of Exosite Mutations on Heparin Binding—The exosite mutations not only affected antithrombin reactivity with proteases, but also had significant effects on heparin affinity. This was assessed by equilibrium binding titrations of mutant antithrombin-heparin pentasaccharide interactions monitored from the ~40% tryptophan fluorescence enhancement that accompanies the binding interaction (Table 6). Mutations of Tyr^{253} or His^{319} alone significantly weakened heparin affinity 3- and 8-fold, respectively, with the double mutant producing an intermediate 6-fold affinity loss. By contrast, mutation of

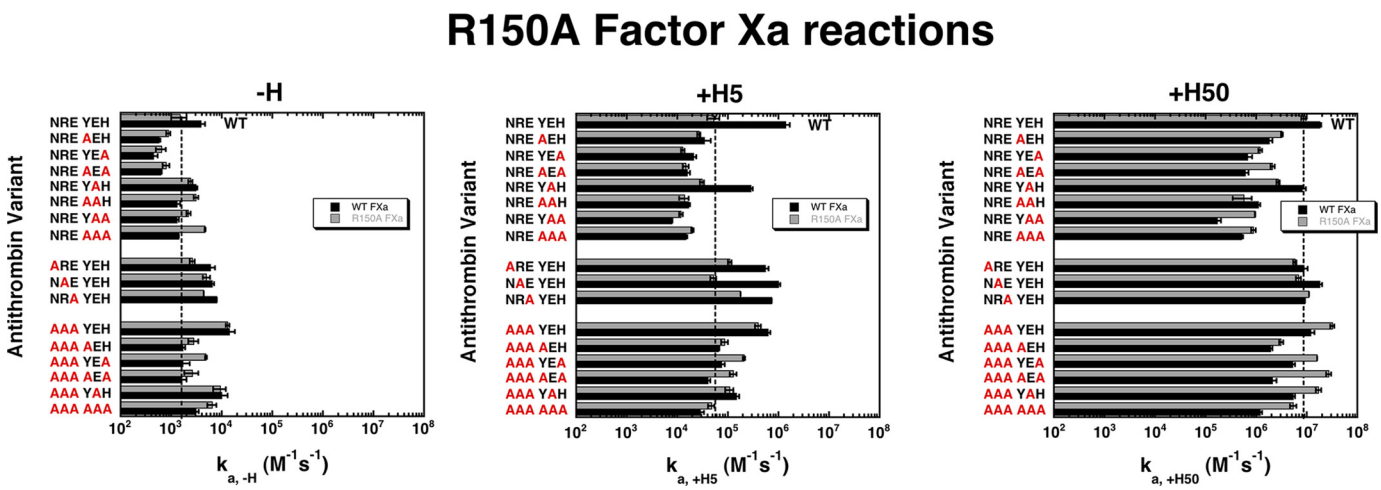

FIGURE 7. **Effects of mutating factor Xa Arg^{150} on factor Xa reactivity with wild-type and exosite mutant antithrombins.** Bar graph comparison of rate constants measured for wild-type and mutant antithrombin reactions with R150A factor Xa (*gray bars*) from Table 4 relative to the corresponding reactions with wild-type factor Xa (*black bars*) from Table 1. Antithrombin reactions were performed without heparin activation (*left panel*), with heparin pentasaccharide activation (*middle panel*) and full-length heparin activation (*right panel*). Antithrombin variants are designated as described in the legend to Fig. 2. *Dashed lines* indicate values for reactions of wild-type antithrombin with R150A factor Xa.

TABLE 4

Second-order association rate constants for reactions of wild-type (WT) and exosite mutant antithrombins with R150A factor Xa in the absence ($k_{a,-H}$) and presence of pentasaccharide ($k_{a,+H5}$) and full-length heparins ($k_{a,+H50}$)

Association rate constants for reactions of antithrombin exosite mutants with R150A factor Xa were measured in *I* 0.15, pH 7.4, phosphate buffer at 25 °C and corrected for variable inhibition reaction stoichiometries as described in the legend to Table 1. Errors, relative rate constants and heparin allosteric and bridging activation rate enhancements are given as in Table 1.

Antithrombin	$k_{a,-H}$	$k_{a,+H5}$	$k_{a,+H50}$	$k_{a,+H5}/k_{a,-H}$	$k_{a,+H50}/k_{a,+H5}$
NRE YEH (WT)	$(1.5 \pm 0.5) \times 10^3$ (1)	$(5.4 \pm 1.5) \times 10^4$ (1)	$(8.6 \pm 0.9) \times 10^6$ (1)	36	160
NRE AEH	$(8.8 \pm 0.8) \times 10^2$ (0.59)	$(2.7 \pm 0.2) \times 10^4$ (0.50)	$(3.2 \pm 0.1) \times 10^6$ (0.37)	31	120
NRE YEA	$(6.4 \pm 1.4) \times 10^2$ (0.43)	$(1.3 \pm 0.1) \times 10^4$ (0.24)	$(1.2 \pm 0.1) \times 10^6$ (0.14)	20	92
NRE AEA	$(8.0 \pm 1.2) \times 10^2$ (0.53)	$(1.5 \pm 0.2) \times 10^4$ (0.28)	$(2.1 \pm 0.2) \times 10^6$ (0.24)	19	140
NRE YAH	$(2.4 \pm 0.2) \times 10^3$ (1.6)	$(3.1 \pm 0.3) \times 10^4$ (0.57)	$(2.7 \pm 0.2) \times 10^6$ (0.31)	13	87
NRE AAH	$(3.1 \pm 0.3) \times 10^3$ (2.1)	$(1.4 \pm 0.3) \times 10^4$ (0.26)	$(5.7 \pm 2.3) \times 10^5$ (0.066)	4.5	41
NRE YAA	$(2.2 \pm 0.2) \times 10^3$ (1.5)	$(1.2 \pm 0.1) \times 10^4$ (0.22)	$(9.5 \pm 0.1) \times 10^5$ (0.11)	5.5	79
NRE AAA	$(4.7 \pm 0.1) \times 10^3$ (3.1)	$(2.0 \pm 0.1) \times 10^4$ (0.37)	$(8.8 \pm 0.9) \times 10^5$ (0.10)	4.3	44
ARE YEH	$(2.6 \pm 0.3) \times 10^3$ (1.7)	$(1.1 \pm 0.1) \times 10^5$ (2.0)	$(5.8 \pm 0.4) \times 10^6$ (0.67)	42	53
NAE YEH	$(5.0 \pm 0.8) \times 10^3$ (3.3)	$(5.2 \pm 0.7) \times 10^4$ (0.96)	$(6.8 \pm 0.8) \times 10^6$ (0.79)	10	130
NRA YEH	$(4.4 \pm 0.0) \times 10^3$ (2.9)	$(1.8 \pm 0.0) \times 10^5$ (3.3)	$(1.1 \pm 0.0) \times 10^7$ (1.3)	41	61
AAA YEH	$(1.3 \pm 0.1) \times 10^4$ (8.7)	$(4.0 \pm 0.5) \times 10^5$ (7.4)	$(3.2 \pm 0.3) \times 10^7$ (3.7)	31	80
AAA AEH	$(2.8 \pm 0.6) \times 10^3$ (1.9)	$(8.8 \pm 1.3) \times 10^4$ (1.6)	$(3.1 \pm 0.3) \times 10^6$ (0.36)	31	35
AAA YEA	$(4.9 \pm 0.2) \times 10^3$ (3.3)	$(2.1 \pm 0.1) \times 10^5$ (3.9)	$(1.6 \pm 0.0) \times 10^7$ (1.9)	43	76
AAA AEA	$(2.6 \pm 0.8) \times 10^3$ (1.7)	$(1.3 \pm 0.2) \times 10^5$ (2.4)	$(2.7 \pm 0.3) \times 10^7$ (3.1)	50	210
AAA YAH	$(9.4 \pm 2.6) \times 10^3$ (6.3)	$(1.1 \pm 0.2) \times 10^5$ (2.0)	$(1.7 \pm 0.2) \times 10^7$ (2.0)	12	160
AAA AAA	$(6.5 \pm 1.3) \times 10^3$ (4.3)	$(4.8 \pm 0.7) \times 10^4$ (0.89)	$(5.4 \pm 0.8) \times 10^6$ (0.63)	7.4	110

Glu^{255} somewhat enhanced heparin affinity and combining this mutation with Tyr^{253} and His^{319} mutations attenuated the losses in heparin affinity produced by the latter residues. The block of NRE mutations enhanced heparin affinity to an even greater extent of 5-fold, largely accountable for by smaller additive affinity enhancements from each individual residue mutation. Adding Tyr^{253} and His^{319} mutations countered this affinity enhancement to approach wild-type affinities. Adding the Glu^{255} mutation together with Tyr^{253} and His^{319} to the NRE mutant slightly reduced the negative effect of the latter residues on affinity. Notably, the observed fluorescence enhancements reporting allosteric activation showed no clear pattern of change, yielding values ranging from 37 to 48% that only marginally differed from wild-type.

DISCUSSION

Antithrombin Exosite Residues Are Critical for Reactivity in Both Native and Allosterically Activated States—Our studies have shown that six putative antithrombin exosite residues that interact with protease in heparin-activated antithrombin-factor Xa/IXa Michaelis complex structures are significant contributors to antithrombin reactivity with factors Xa and IXa not only in the heparin-activated state but more importantly also in the unactivated state. Tyr^{253} and His^{319} in particular were found to be the most crucial determinants of the exosite, mutations to Ala causing ~10–200-fold reactivity losses in the unactivated state and 10–90-fold losses in heparin-activated states in reactions with factors Xa and IXa. Notably, the greatest reactivity losses were observed in unactivated antithrombin reactions with factor IXa. Such findings argue most strongly that Tyr^{253} and His^{319} are key determinants of antithrombin reactivity in both native and heparin-activated states. The alternative possibility that these residues only contribute to reactivity in the heparin-activated state would otherwise imply that the observed major effects of mutating these residues on unactivated antithrombin reactivity arise from a minor fraction of activated antithrombin in conformational equilibrium with native antithrombin. However, estimates of this fraction based

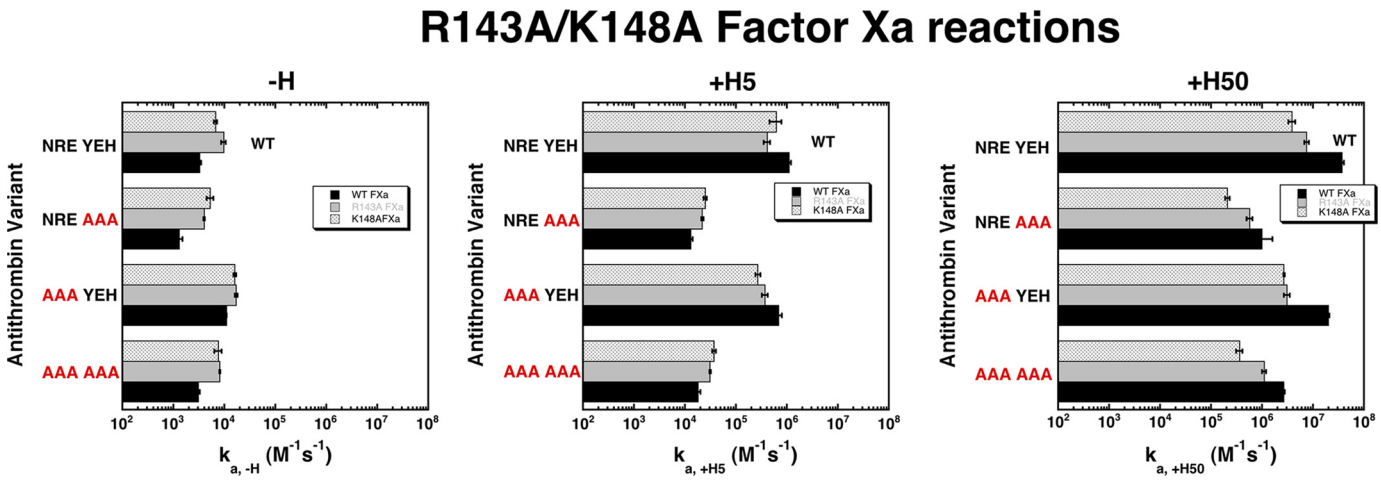

FIGURE 8. **Effects of mutating factor Xa Arg^{143} and Lys^{148} on factor Xa reactivity with wild-type and exosite mutant antithrombins.** Bar graph comparison of rate constants measured for wild-type and selected mutant antithrombin reactions with R143A factor Xa (*gray bars*) and K148A factor Xa relative to the corresponding reactions with wild-type recombinant factor Xa (*black bars*), all from Table 5. Antithrombin reactions were performed without heparin activation (*left panel*), with heparin pentasaccharide activation (*middle panel*), and with full-length heparin activation (*right panel*).

on the reactivities and heparin affinities of antithrombin variants locked in native or heparin-activated states (27, 28) indicate an amount (~0.1%) that is insufficient to account for the major reactivity losses observed.[3] Moreover, the observed 3–8-fold decreases in heparin affinity caused by the Tyr^{253}/His^{319} mutations (Table 6) suggest that the activated fraction in these mutants is considerably reduced from wild-type.[4] The observed 1 to 2 order of magnitude losses in wild-type antithrombin reactivity produced by Tyr^{253} and His^{319} mutations in the absence of heparin thus imply that the mutations are almost exclusively affecting an intrinsic native state reactivity and not just the reactivity of a minor equilibrium fraction of activated serpin.

Alternative Modes of Exosite Interaction in Native and Heparin-activated Antithrombin—The finding that Tyr^{253} and His^{319} are key determinants of the exosite was unexpected based on x-ray structures showing that Asn^{233}, Arg^{235}, Glu^{237}, and Tyr^{253} residues together comprise an exosite binding pocket that interacts with Arg^{150} of factors Xa and IXa in the allosterically activated state (13, 14) (Fig. 1). His^{319} was not previously thought to be part of the exosite because it makes no direct interaction with Arg^{150}. However, a close examination of the Michaelis complex structures shows that the His^{319} aromatic ring closes a gap between Tyr^{253} and Glu^{237} residues of the exosite binding pocket by making an orthogonal interaction with the aromatic ring of Tyr^{253} and a potential ionic interaction with Glu^{237} when His^{319} is in the charged state (Fig. 1). The effects of mutating Tyr^{253} and His^{319} alone or together revealed that these residues function together as a cooperative unit, implying that a His^{319}-Tyr^{253} interaction may function to position Tyr^{253} in the exosite binding pocket to interact with the protease Arg^{150} residue in both native and activated conformational states. The importance of Tyr^{253} and His^{319} for exosite function is supported by the ability to create a factor Xa/IXa-specific exosite in a P1 Arg variant of the related serpin, α_1-protease inhibitor, in which His^{319} is conserved, by substituting Tyr in the homologous strand 3C 253 position (29).

Although Tyr^{253}/His^{319} function together to promote the exosite binding pocket interaction in both native and activated states, the interaction must differ in the two conformational states based on our finding that the three other residues of the binding pocket, Asn^{233}, Arg^{235}, and Glu^{237}, together make substantial negative contributions to antithrombin reactivity in the native state, but positive contributions in the heparin-activated state in reactions with both factors Xa and IXa. Moreover, coupling between Asn^{233}/Arg^{235}/Glu^{237} (NRE) and Tyr^{253}/His^{319} mutations revealed that NRE residues suppress the positive contributions of the Tyr^{253}/His^{319} pair in the unactivated state but augment Tyr^{253}/His^{319} positive contributions in heparin-activated states (Figs. 4 and 5). NRE residues in the exosite binding pocket thus appear to modulate the Tyr^{253} interaction with protease in a manner dependent on heparin activation.

That the contribution of antithrombin exosite binding pocket residues to reactivity results from a specific interaction with Arg^{150} of the protease is supported by previous studies (18, 19, 29) together with our present finding that the exosite contribution is abolished in antithrombin reactions with an R150A mutant factor Xa in both unactivated and heparin-activated states (Table 4). Notably, the Arg^{150} mutation produced large reductions in factor Xa reactivity with wild-type and exosite mutant antithrombins only when the

[3] R. Roth and S. T. Olson, unpublished observations. The 0.1% estimate is in keeping with the <0.3% activated fraction possible based on unactivated wild-type antithrombin reactivities with factors Xa and IXa that are 0.3 and 0.7% of allosterically activated reactivities. An activated fraction of 0.1% would imply intrinsic native state antithrombin reactivities with factors Xa and IXa less than 2-fold lower than those observed, *i.e.* k_a values of ~2500 M^{-1} s^{-1} and ~150 M^{-1} s^{-1}, respectively. If Tyr^{253}/His^{319} mutations only affected the 0.1% activated fraction, then the observed reactivities of these exosite mutant antithrombins in the absence of heparin should not be less than ~2500 M^{-1} s^{-1} and ~150 M^{-1} s^{-1}.

[4] Assuming the changes in heparin affinity of the exosite mutants reflect changes in the conformational equilibrium between native and activated states of antithrombin in the unactivated serpin and given the observation that the observed wild-type heparin affinity significantly exceeds that of the native state (28), the fold-change in the fraction of activated antithrombin caused by the exosite mutations can be approximated by the ratio, $K_{D,WT}/K_{D,mut}$. Assuming ~0.1% activated fraction in wild-type, this would imply an activated fraction in Tyr^{253}/His^{319} mutants of 0.01–0.03%.

TABLE 5

Second-order association rate constants for reactions of wild-type (WT) and exosite mutant antithrombins with wild-type and mutant Gla-domainless factor Xas (GD-FXa) in the absence ($k_{a,-H}$) and presence of pentasaccharide ($k_{a,+H5}$) and full-length heparins ($k_{a,+H50}$)

Association rate constants for reactions of antithrombin exosite mutants with wild-type and mutant factor Xas were measured in *I* 0.15, pH 7.4, phosphate buffer at 25 °C and corrected for variable inhibition reaction stoichiometries as described in the legend to Table 1. Errors and heparin allosteric and bridging activation rate enhancements are given as in Table 1. Rate constants are expressed relative to values for the wild-type antithrombin reaction with wild-type GD-factor Xa (in parentheses).

Antithrombin	GD-FXa	$k_{a,-H}$	$k_{a,+H5}$	$k_{a,+H50}$	$k_{a,+H5}/k_{a,-H}$	$k_{a,+H50}/k_{a,+H5}$
NRE YEH (WT)	WT	$(3.3 \pm 0.2) \times 10^3$ (1)	$(1.1 \pm 0.1) \times 10^6$ (1)	$(3.7 \pm 0.3) \times 10^7$ (1)	330	34
	R143A	$(9.7 \pm 1.0) \times 10^3$ (2.9)	$(4.1 \pm 0.6) \times 10^5$ (0.37)	$(7.5 \pm 0.7) \times 10^6$ (0.20)	42	18
	K148A	$(6.7 \pm 0.5) \times 10^3$ (2.0)	$(6.2 \pm 1.6) \times 10^5$ (0.56)	$(3.9 \pm 0.6) \times 10^6$ (0.11)	93	6.3
NRE AAA	WT	$(1.3 \pm 0.2) \times 10^3$ (0.39)	$(1.3 \pm 0.1) \times 10^4$ (0.012)	$(1.0 \pm 0.6) \times 10^6$ (0.027)	10	77
	R143A	$(4.0 \pm 0.1) \times 10^3$ (1.2)	$(2.2 \pm 0.1) \times 10^4$ (0.020)	$(5.7 \pm 0.7) \times 10^5$ (0.015)	5.5	26
	K148A	$(5.3 \pm 0.8) \times 10^3$ (1.6)	$(2.5 \pm 0.2) \times 10^4$ (0.023)	$(2.1 \pm 0.2) \times 10^5$ (0.006)	4.7	8.4
AAA YEH	WT	$(1.1 \pm 0.0) \times 10^4$ (3.3)	$(6.9 \pm 1.1) \times 10^5$ (0.63)	$(2.0 \pm 0.1) \times 10^7$ (0.54)	63	29
	R143A	$(1.7 \pm 0.1) \times 10^4$ (5.2)	$(3.7 \pm 0.5) \times 10^5$ (0.34)	$(3.1 \pm 0.4) \times 10^6$ (0.084)	22	8.4
	K148A	$(1.6 \pm 0.1) \times 10^4$ (4.8)	$(2.7 \pm 0.3) \times 10^5$ (0.25)	$(2.7 \pm 0.1) \times 10^6$ (0.073)	17	10
AAA AAA	WT	$(3.1 \pm 0.2) \times 10^3$ (0.94)	$(1.8 \pm 0.2) \times 10^4$ (0.016)	$(2.7 \pm 0.1) \times 10^6$ (0.073)	5.8	150
	R143A	$(8.1 \pm 0.2) \times 10^3$ (2.5)	$(3.1 \pm 0.1) \times 10^4$ (0.028)	$(1.1 \pm 0.1) \times 10^6$ (0.030)	3.8	35
	K148A	$(7.6 \pm 1.3) \times 10^3$ (2.3)	$(3.7 \pm 0.3) \times 10^4$ (0.034)	$(3.6 \pm 0.5) \times 10^5$ (0.010)	4.9	9.7

TABLE 6

Heparin affinities of antithrombin exosite mutants

Equilibrium dissociation constants (K_D) and relative maximal fluorescence changes ($\Delta F_{max}/F_o$) associated with heparin pentasaccharide binding and allosteric activation of antithrombin were measured in *I* 0.35 phosphate buffer, pH 7.4, at 25 °C by fluorescence titrations of antithrombin with the saccharide as described under "Experimental Procedures." Errors in K_D are ranges or S.E. from at least 2 titrations. An R393A antithrombin variant that lacked inhibitor activity was engineered, expressed, and purified similar to other variants and its heparin affinity measured as with the exosite mutants.

Antithrombin	K_D	$\Delta F_{max}/F_o$
	*n*M	%
NRE YEH	43 ± 3	44 ± 1
NRE AEH	124 ± 15	41 ± 1
NRE YEA	331 ± 20	44 ± 2
NRE AEA	256 ± 6	37 ± 5
NRE YAH	31 ± 2	45 ± 3
NRE AAH	35 ± 3	39 ± 1
NRE YAA	267 ± 30	45 ± 8
NRE AAA	128 ± 17	42 ± 1
ARE YEH	26 ± 2	45 ± 4
NAE YEH	28 ± 1	48 ± 0
NRA YEH	15 ± 3	42 ± 1
AAA YEH	8.6 ± 0.7	44 ± 1
AAA AEH	22 ± 1	45 ± 0
AAA YEA	49 ± 1	39 ± 1
AAA AEA	43 ± 11	41 ± 0
AAA YAH	10 ± 0	46 ± 0
AAA AAA	31 ± 3	43 ± 3
R393A	9.4 ± 1.2	44 ± 1

critical Tyr^{253} and His^{319} residues were present and otherwise enhanced reactivity (Fig. 7). The observation that NRE residues made a negative contribution to reactivity with Arg^{150} mutant factor Xa in the unactivated state that was not alleviated by heparin allosteric activation further suggests an important role for Arg^{150} in mitigating this negative contribution in the heparin-activated state.

The antithrombin Glu^{255} residue outside the exosite binding pocket made more modest positive contributions to antithrombin reactivity that were dependent on the critical Tyr^{253}/His^{319} residues of the exosite binding pocket. This contribution required factor Xa Arg^{143} and Lys^{148} residues only in the heparin-activated state, consistent with Michaelis complex structures showing that Arg^{143} and Lys^{148} of factors Xa and IXa interact with Glu^{255} of antithrombin in the heparin-activated state and that this interaction occurs in concert with the Arg^{150} interaction with the exosite binding pocket (13, 14) (Fig. 1). The colocalization of Arg^{143}, Lys^{148}, and Arg^{150} on the autolysis loop of factors Xa and IXa explains why the interactions of these protease residues with the exosite in heparin-activated antithrombin are strongly linked. In unactivated antithrombin, Arg^{143} and Lys^{148} were found to make a modest negative contribution to factor Xa reactivity, implying that Glu^{255} does not interact with these residues in the native state and instead interacts with other protease residues. Together, these findings support different modes of interaction of the antithrombin exosite with protease in unactivated and heparin-activated states.

Interestingly, the exosite residues are strongly conserved or conservatively replaced in all vertebrate antithrombins except for Arg^{235} and Glu^{237}, which are replaced by uncharged residues in fish (30). Surprisingly, Glu^{237} is the only exosite residue for which a natural antithrombin mutation (to Lys) that is associated with thrombosis has been reported (31). However, the thrombosis association appears to result from a reduced antigenic level of the circulating serpin rather than a reduced anticoagulant activity, in keeping with the slightly enhanced anticoagulant activity of Glu^{237} variants found in this and other studies (12, 32, 33). Of greater relevance is the report of a natural antithrombin variant associated with recurrent thrombosis with a mutation in Met^{251}, a buried residue contiguous to the exosite (34). This residue is highly conserved in vertebrate antithrombins and its mutation to Ile reduces anticoagulant activity to a much greater extent than it affects the antigenic level of circulating antithrombin. Examination of antithrombin-protease Michaelis complex structures reveals that the Met^{251} side chain forms the bottom of the exosite binding pocket for the protease Arg^{150} residue, suggesting that the Met^{251} mutation may produce a defect in anticoagulant function by perturbing the structure of the exosite binding pocket through altered core packing interactions.

Exosite Contribution to Heparin Rate Enhancement—The positive and negative contributions of the six exosite residues to antithrombin reactivity in the unactivated state were found to be equally balanced in reactions with factor Xa (1.3-fold reactivity increase) but weighted toward the positive in reactions with factor IXa (55-fold reactivity increase). In allosterically activated and bridging heparin-activated states, the exosite made major positive contributions to antithrombin reactivity

of 48- and 15-fold, respectively, in reactions with factor Xa and 680- and 120-fold in reactions with factor IXa (Tables 1 and 2). The reduced exosite contribution to antithrombin reactivity upon bridging heparin activation most likely reflects constraints on the ability of the protease to engage the antithrombin exosite when it must at the same time engage a bridging site on heparin in the heparin-antithrombin-protease Michaelis complex. Importantly, mutations of the six antithrombin exosite residues produced modest 2–3-fold enhancements in antithrombin reactivity with thrombin independent of heparin activation that reflected a dominant-negative contribution of Glu^{255}, consistent with the fact that thrombin lacks the Arg^{150} residue of the autolysis loop and is unable to make a specific interaction with the antithrombin exosite (25, 26).

The differential contribution of exosite residues to antithrombin reactivity in unactivated and heparin-activated states was responsible for large rate enhancements of 360-fold with factor Xa[5] and 140-fold with factor IXa upon allosteric activation by the heparin pentasaccharide. These rate enhancements were abrogated to 9- and 11-fold, respectively, when all six exosite residues were mutated (Tables 1 and 2). Mutation of the Tyr^{253}/Glu^{255}/His^{319} triad (YEH) alone accounted for much of this abrogation with factor Xa (to 11-fold) but only a small part with factor IXa (to 42-fold), suggesting a greater differential contribution of YEH to antithrombin reactivity in unactivated and activated states with factor Xa than factor IXa. Significantly, mutation of the NRE triad alone caused a marked reduction of the allosteric activation rate enhancement to 45-fold with factor Xa and more substantial reduction to 7-fold with factor IXa, a result of NRE residues mitigating YEH reactivity in the unactivated state and enhancing this reactivity in the activated state. This underscores the important role for NRE exosite residues in repressing antithrombin reactivity in the absence of heparin but enhancing reactivity upon heparin activation especially in the reaction with factor IXa. Although bridging heparin activation of antithrombin reduced the exosite contribution to antithrombin reactivity, this was compensated for by a large gain in reactivity due to heparin bridging interactions with protease promoting the Michaelis complex interaction. Notably, the heparin bridging rate enhancement was observed to increase for almost all mutant antithrombin reactions with factors Xa and IXa relative to the wild-type reaction (Tables 1 and 2). This would be consistent with the impaired exosite interaction in the mutant antithrombins allowing the protease to more effectively engage the heparin bridging site and thereby increase the bridging rate enhancement.

Structural Basis for Alternative Modes of Exosite Interaction—The different modes of protease interaction with the antithrombin exosite in native and allosterically activated states of the serpin can be understood based on available x-ray structures. Of particular note in this regard is the x-ray structure of native antithrombin, which shows an intramolecular interaction of the RCL P1 Arg with the exosite (33). This interaction stabilizes the native state based on previous observations that mutations or modification of the P1 Arg shift the native-activated state conformational equilibrium toward the activated state (35, 36). Our present findings support a P1 Arg native state stabilizing interaction with the exosite based on our finding that the exosite mutations perturb heparin affinity in a manner similar to that of the P1 Arg mutations. Interestingly, the mode of the P1 Arg interaction with the exosite is entirely different from that of the protease Arg^{150} residue interaction with the exosite in heparin-activated antithrombin-factor Xa/IXa Michaelis complex structures (Fig. 9). The P1 Arg-exosite interaction is promoted by Asn^{233}/Arg^{235}/Glu^{237} residues and antagonized by Tyr^{253}/His^{319} residues based on the observed effects of mutating these residues on heparin affinity in this study and previous studies in which Glu^{237} was mutated (12, 33). The x-ray structure shows that the P1 Arg side chain interacts with Glu^{237}. However, the Asn^{233}/Arg^{235}/Glu^{237} residues appear to function together to promote this interaction because their combined mutation is required to produce an increase in heparin affinity comparable with that caused by a P1 Arg to Ala mutation, which is expected to abolish the P1 Arg interaction (Table 6). The antagonism by Tyr^{253}/His^{319} appears to result from the native structure constraining the entry of the P1 Arg into the exosite pocket so that the nonpolar stem of the Arg side chain is unable to interact with the Tyr^{253}/His^{319} aromatic side chains.

Contrasting this constrained P1 Arg interaction with the exosite, the factor Xa and factor IXa Arg^{150} side chain interactions with the exosite in heparin-activated antithrombin Michaelis complex structures is presumably the most optimal in that interactions with all binding pocket residues are favorable in this state. Indeed, the structure shows that the nonpolar stem of Arg^{150} aligns with the Tyr^{253}/His^{319} aromatic side chains and the charged end of Arg^{150} makes an ionic interaction with Glu^{237} and multiple backbone interactions (13, 14). Glu^{255} interactions with protease Arg^{143} and Lys^{148} residues presumably promote this favorable mode of interaction based on the observed linkage between these interactions. These favorable interactions are made possible by the major subdomain movements that are involved in allosteric activation (28, 32). This suggests a model for the less favorable mode of the factor Xa/IXa Arg^{150} interaction with the exosite in native antithrombin in which the protease Arg^{150} is constrained by the native subdomain structure to enter the exosite binding pocket in a manner that favors interaction of the nonpolar stem of the Arg side chain with Tyr^{253}/His^{319} residues but does not allow the charged end of the side chain to neutralize the repulsive interactions of Asn^{233}/Arg^{235}/Glu^{237} residues. The altered mode of the Arg^{150}-exosite binding pocket interaction in this state precludes the neighboring Arg^{143} and Lys^{148} protease residues from interacting with Glu^{255} and forces Glu^{255} to make alternative less favorable interactions with protease.

Implications for the Mechanism of Heparin Allosteric Activation—Together our findings support a new model for heparin allosteric activation of antithrombin that we recently proposed (16). In this model, factors Xa and IXa engage the antithrombin exosite in the Michaelis complex in the native

[5] The allosteric activation rate enhancements for antithrombin-factor Xa reactions reported here are larger than those reported previously because rate constants were measured in calcium buffer in the present study but in phosphate buffer lacking calcium in past studies.

unactivated state, but this interaction becomes more favorable in the allosterically activated state. The model is supported by our findings that in unactivated antithrombin, Tyr^{253}, Glu^{255}, and His^{319} exosite residues make attractive interactions with factors Xa and IXa in the Michaelis complex but these are attenuated by repulsive interactions of Asn^{233}, Arg^{235}, and Glu^{237} with the proteases that serve to down-regulate antithrombin reactivity. Heparin allosteric activation mitigates the repulsive interactions and makes the attractive interactions more favorable so that antithrombin reactivity with factors Xa and IXa is up-regulated.

This new model significantly differs from an earlier model in which allosteric activation was proposed to make the antithrombin exosite available for interaction with RCL-bound factors Xa and IXa (12–14, 28). This was thought to occur through the hallmark structural change that accompanies allosteric activation, the disruption of the native state stabilizing RCL-sheet A interaction. The RCL-sheet A interaction was believed to constrain the RCL in the native state and prevent bound proteases from engaging the exosite. A key observation supporting our new model, that factors Xa and IXa are able to engage the antithrombin exosite in the unactivated RCL-constrained state, indicates that the release of the RCL from its interaction with sheet A is not necessary for this engagement and therefore that the earlier model cannot be correct.

Role of RCL Expulsion from Sheet A in Allosteric Activation—In keeping with this, recent findings suggest that the changes in the exosite accompanying allosteric activation do not require a disruption of the RCL-sheet A interaction. Mutations in the heparin binding site were thus found to induce antithrombin to undergo the full allosteric activating changes in reactivity with factors Xa and IXa in the absence of heparin without the signature fluorescence changes that report RCL expulsion from sheet A although with large CD and NMR perturbations (17). This implies that the activating conformational changes in the hydrophobic core of antithrombin are sufficient to mitigate the repulsive interactions and enhance the attractive interactions in the exosite without RCL expulsion from sheet A. Such changes in the hydrophobic core are evident from x-ray structures of so-called intermediate antithrombin-heparin complexes that have undergone all the allosteric activating changes except for RCL expulsion (28, 37). Although the exosite itself does not appear to undergo a structural change upon allosteric activation (28), movements of the subdomain in which the exosite resides could relax the structural constraints between the flexible RCL and serpin body in the native conformation and allow Arg^{150} of factors Xa and IXa to adopt a more productive mode of interaction with the exosite without the need for disrupting the RCL-sheet A interaction.

It is worth noting that heparin binding site mutations that were found to activate antithrombin without disrupting the RCL-sheet A interaction did not affect the ability of the mutant serpin to bind heparin and induce the accompanying fluorescence changes that report RCL expulsion from sheet A (17). Heparin binding to this mutant was accompanied by a small 3–4-fold additional increase in antithrombin reactivity with factors Xa and IXa. Such a reactivity enhancement resembles

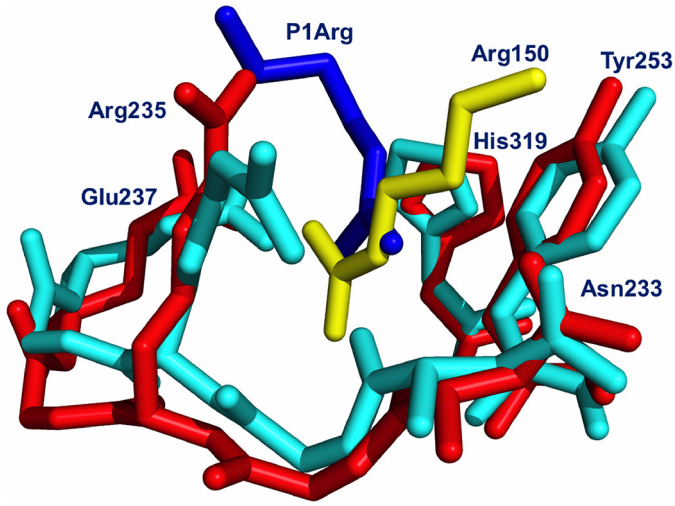

FIGURE 9. **Comparison of antithrombin exosite interactions in x-ray structures of native antithrombin and the heparin-activated antithrombin-factor Xa Michaelis complex.** Alignments of the antithrombin exosite residues Asn^{233}-Glu^{237}, Tyr^{253}, and His^{319} in native antithrombin (Protein Data Bank code 1T1F) and in the heparin-antithrombin-S195A factor Xa Michaelis complex (Protein Data Bank code 2GD4) are shown in *cyan* and *red stick* representation, respectively. The P1 Arg residue is shown in *blue stick* interacting with the exosite in native antithrombin, whereas Arg^{150} of factor Xa is shown in *yellow stick* interacting with the exosite in the Michaelis complex. The P1 Arg and Arg^{150} have in common hydrogen bond/ion pair interactions with Glu^{237} and a hydrogen bond interaction with the main chain of Asn^{233}, whereas Arg^{150} makes additional main chain hydrogen bonds and nonpolar interactions with Tyr^{253}.

the enhancements observed upon allosteric activation of wild-type and exosite mutant antithrombins in reactions of the serpin with thrombin and likely reflects a lessening of unfavorable interactions between the RCL-bound protease and the serpin core when the RCL extends away from the core. RCL expulsion and extension away from the serpin surface may thus contribute, albeit in a minor way, to enhancing the exosite interaction in the allosterically activated state by reducing repulsive interactions between RCL-bound factors Xa and IXa and residues of the serpin body outside the exosite. This would explain the residual allosteric activation rate enhancement observed in reactions of the mutant antithrombin lacking all exosite residues with factors Xa and IXa. Such a role for RCL expulsion was postulated in the new model of allosteric activation we recently proposed (16).

Conclusions—Together, our findings provide important new insights into the role of an exosite in the mechanism of heparin allosteric activation of antithrombin. Our studies have unequivocally shown that the exosite is a crucial determinant of antithrombin reactivity with factors Xa and IXa in both unactivated and allosterically activated states and thus that allosteric activation does not involve making the exosite available to protease as previously suggested. They have further demonstrated the key importance of Tyr^{253} and His^{319} exosite residues in promoting the interaction with the protease Arg^{150} residue in both native and activated states and how Glu^{255} augments this interaction. Most importantly, they have shown that the exosite down-regulates reactivity in the native state through repulsive interactions of Asn^{233}, Arg^{235}, and Glu^{237} exosite residues and up-regulates reactivity upon allosteric activation by mitigating this repulsion, in accordance with a model we hypothesized

previously. Finally, they suggest a structural basis for the different modes of exosite interaction with protease in native and activated states. Collectively, these findings provide a new detailed molecular understanding of the antithrombin allosteric activation mechanism.

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REFERENCES

1. Olson, S. T., Richard, B., Izaguirre, G., Schedin-Weiss, S., and Gettins, P. G. (2010) Molecular mechanisms of antithrombin-heparin regulation of blood clotting proteinases: a paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. *Biochimie* **92,** 1587–1596
2. van Boven, H. H., and Lane, D. A. (1997) Antithrombin and its inherited deficiency states. *Semin. Hematol.* **34,** 188–204
3. Ishiguro, K., Kojima, T., Kadomatsu, K., Nakayama, Y., Takagi, A., Suzuki, M., Takeda, N., Ito, M., Yamamoto, K., Matsushita, T., Kusugami, K., Muramatsu, T., and Saito, H. (2000) Complete antithrombin deficiency in mice results in embryonic lethality. *J. Clin. Invest.* **106,** 873–878
4. Liu, Y., Kretz, C. A., Maeder, M. L., Richter, C. E., Tsao, P., Vo, A. H., Huarng, M. C., Rode, T., Hu, Z., Mehra, R., Olson, S. T., Joung, J. K., and Shavit, J. A. (2014) Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function. *Blood* **124,** 142–150
5. Marcum, J. A., Atha, D. H., Fritze, L. M., Nawroth, P., Stern, D., and Rosenberg, R. D. (1986) Cloned bovine aortic endothelial cells synthesize anticoagulantly active heparin sulfate proteoglycan. *J. Biol. Chem.* **261,** 7507–7517
6. de Agostini, A. I., Watkins, S. C., Slayter, H. S., Youssoufian, H., and Rosenberg, R. D. (1990) Localization of anticoagulantly active heparan sulfate proteoglycans in vascular endothelium: antithrombin binding on cultured endothelial cells and perfused rat aorta. *J. Cell Biol.* **111,** 1293–1304
7. Petitou, M., Casu, B., and Lindahl, U. (2003) 1976–1983, a critical period in the history of heparin: the discovery of the antithrombin binding site. *Biochimie* **85,** 83–89
8. Olson, S. T., and Björk, I. (1991) Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin-thrombin reaction. Elucidation from salt concentration effects. *J. Biol. Chem.* **266,** 6353–6364
9. Olson, S. T., Björk, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions: resolution of the antithrombin conformational change contribution to heparin rate enhancement. *J. Biol. Chem.* **267,** 12528–12538
10. Bedsted, T., Swanson, R., Chuang, Y.-J., Bock, P. E., Björk, I., and Olson, S. T. (2003) Heparin and calcium ions dramatically enhance antithrombin reactivity with factor IXa by generating new interaction exosites. *Biochemistry* **42,** 8143–8152
11. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) The anticoagulant activation of antithrombin by heparin. *Proc. Natl. Acad. Sci.* **94,** 14683–14688
12. Izaguirre, G., and Olson, S. T. (2006) Residues Tyr-253 and Glu-255 in strand 3 of β-sheet C of antithrombin are key determinants of an exosite made accessible by heparin activation to promote rapid inhibition of factors Xa and IXa. *J. Biol. Chem.* **281,** 13424–13432
13. Johnson, D. J., Li, W., Adams, T. E., and Huntington, J. A. (2006) Antithrombin-S195A factor Xa-heparin structure reveals the mechanism of antithrombin activation. *EMBO J.* **25,** 2029–2037
14. Johnson, D. J., Langdown, J., and Huntington, J. A. (2010) Molecular basis of factor IXa recognition by heparin-activated antithrombin revealed by a 1.7-Å structure of the ternary complex. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 645–650
15. Chuang, Y.-J., Swanson, R., Raja, S. M., and Olson, S. T. (2001) Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. *J. Biol. Chem.* **276,** 14961–14971
16. Gettins, P. G., and Olson, S. T. (2009) Activation of antithrombin as a factor IXa and Xa inhibitor involves mitigation of repression rather than positive enhancement. *FEBS Lett.* **583,** 3397–3400
17. Dementiev, A., Swanson, R., Roth, R., Isetti, G., Izaguirre, G., Olson, S. T., and Gettins, P. G. (2013) The allosteric mechanism of activation of antithrombin as an inhibitor of factor IXa and factor Xa: heparin-independent full activation through mutations adjacent to helix D. *J. Biol. Chem.* **288,** 33611–33619
18. Manithody, C., Yang, L., and Rezaie, A. R. (2002) Role of basic residues of the autolysis loop in the catalytic function of factor Xa. *Biochemistry* **41,** 6780–6788
19. Yang, L., Manithody, C., Olson, S. T., and Rezaie, A. R. (2003) Contribution of basic residues of the autolysis loop to the substrate and inhibitor specificity of factor IXa. *J. Biol. Chem.* **278,** 25032–25038
20. Turk, B., Brieditis, I., Bock, S. C., Olson, S. T., and Björk, I. (1997) The oligosaccharide side chain on Asn-135 of α-antithrombin, absent in β-antithrombin, decreases the heparin affinity of the inhibitor by affecting the heparin-induced conformational change. *Biochemistry* **36,** 6682–6691
21. Izaguirre, G., Zhang, W., Swanson, R., Bedsted, T., and Olson, S. T. (2003) Localization of an antithrombin exosite that promotes rapid inhibition of factors Xa and IXa dependent on heparin activation of the serpin. *J. Biol. Chem.* **278,** 51433–51440
22. Nordenman, B., Nyström, C., and Björk, I. (1977) The size and shape of human and bovine antithrombin III. *Eur. J. Biochem.* **78,** 195–203
23. Olson, S. T., Björk, I., and Shore, J. D. (1993) Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. *Methods Enzymol.* **222,** 525–559
24. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685
25. Li, W., Johnson, D. J., Esmon, C. T., and Huntington, J. A. (2004) Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nat. Struct. Mol. Biol.* **11,** 857–862
26. Dementiev, A., Petitou, M., Herbert, J.-M., and Gettins, P. G. (2004) The ternary complex of antithrombin-anhydrothrombin-heparin reveals the basis of inhibitor specificity. *Nat. Struct. Mol. Biol.* **11,** 863–867
27. Futamura, A., and Gettins, P. G. (2000) Serine 380 (P14)→ glutamate mutation activates antithrombin as an inhibitor of Factor Xa. *J. Biol. Chem.* **275,** 4092–4098
28. Langdown, J., Belzar, K. J., Savory, W. J., Baglin, T. P., and Huntington, J. A. (2009) The critical role of hinge-region expulsion in the induced-fit heparin binding mechanism of antithrombin. *J. Mol. Biol.* **386,** 1278–1289
29. Izaguirre, G., Rezaie, A. R., and Olson, S. T. (2009) Engineering functional antithrombin exosites in α1-proteinase inhibitor that specifically enhnace the inhibiiton of factor Xa and factor IXa. *J. Biol. Chem.* **284,** 1550–1558
30. Backovic, M., and Gettins, P. G. (2002) Insight into the residues critical for antithrombin function from analysis of an expanded database of sequences that includes frog, turtle, and ostrich antithrombins. *J. Proteome Res.* **1,** 367–373
31. Graham, J. A., Daly, H. M., and Carson, P. J. (1992) Antithrombin III deficiency and cerebrovascular accidents in young adults. *J. Clin. Pathol.* **45,** 921–922
32. Whisstock, J. C., Pike, R. N., Jin, L., Skinner, R., Pei, X. Y., Carrell, R. W., and Lesk, A. M. (2000) Conformational changes in serpins: II. the mechanism of activation of antithrombin by heparin. *J. Mol. Biol.* **301,** 1287–1305
33. Johnson, D. J., Langdown, J., Li, W., Luis, S. A., Baglin, T. P., and Huntington, J. A. (2006) Crystal structure of monomeric native antithrombin reveals a novel reactive center loop conformation. *J. Biol. Chem.* **281,** 35478–35486
34. Millar, D. S., Wacey, A. I., Ribando, J., Melissari, E., Laursen, B., Woods, P., Kakkar, V. V., and Cooper, D. N. (1994) Three novel missense mutations

in the antithombin III (AT3) gene causing recurrent venous thrombosis. *Hum. Genet.* **94,** 509–512
35. Chuang, Y.-J., Swanson, R., Raja, S. M., Bock, S. C., and Olson, S. T. (2001) The antithrombin P1 residue is important for target proteinase specificity but not for heparin acvtivation of the serpin. *Biochemistry* **40,** 6670–6679
36. Pike, R. N., Potempa, J., Skinner, R., Fitton, H. L., McGraw, W. T., Travis, J., Owen, M., Jin, L., and Carrell, R. W. (1997) Heparin-dependent modification of the reactive center arginine of antithrombin and consequent increase in heparin binding affinity. *J. Biol. Chem.* **272,** 19652–19655
37. Johnson, D. J., and Huntington, J. A. (2003) Crystal structure of antithrombin in a heparin-bound intermediate state. *Biochemistry* **42,** 8712–8719