Specificity of the Basic Side Chains of Lys114, Lys125, and Arg129 of Antithrombin in Heparin Binding†

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ABSTRACT: The anticoagulant polysaccharide heparin binds and activates the plasma proteinase inhibitor antithrombin through a pentasaccharide sequence. Lys114, Lys125, and Arg129 are the three most important residues of the inhibitor for pentasaccharide binding. To elucidate to what extent another positively charged side chain can fulfill the role of each of these residues, we have mutated Lys114 and Lys125 to Arg and Arg129 to Lys. Lys114 could be reasonably well replaced with Arg with only an ∼15-fold decrease in pentasaccharide affinity, in contrast to an $\sim 10^5$-fold decrease caused by substitution with an noncharged amino acid of comparable size. However, a loss of approximately one ionic interaction on mutation to Arg indicates that the optimal configuration of the network of basic residues of antithrombin that together interact with the pentasaccharide requires a Lys in position 114. Replacement of Lys125 with Arg caused an even smaller, ∼3-fold, decrease in pentasaccharide affinity, compared with that of ∼400-fold caused by mutation to a neutral amino acid. An Arg in position 125 is thus essentially equivalent to the wild-type Lys in pentasaccharide binding. Substitution of Arg129 with Lys decreased the pentasaccharide affinity an appreciable ∼100-fold, a loss approaching that of ∼400-fold caused by substitution with a neutral amino acid. Arg is thus specifically required in position 129 for high-affinity pentasaccharide binding. This requirement is most likely due to the ability of Arg to interact with other residues of antithrombin, primarily, Glu414 and Thr44, in a manner that appropriately positions the Arg side chain for keeping the pentasaccharide anchored to the activated state of the inhibitor.

Antithrombin is a plasma serine proteinase inhibitor that is a major regulator of blood clotting and thereby is essential for life (*1*, *2*). It belongs to the serpin superfamily of proteins (*3*) and inhibits a number of coagulation proteinases, most importantly, thrombin and factor Xa, by a mechanism specific for inhibitors of this family. The target proteinase initially recognizes and attacks a reactive bond, located in a surface-exposed loop of the serpin, as in a regular substrate. However, instead of this bond being fully hydrolyzed, the N-terminal part of the loop with the covalently linked proteinase inserts as a sixth strand in the middle of the major β-sheet of the serpin, the A sheet, at the acyl intermediate stage of normal proteolysis. As a consequence, the proteinase is transported along the surface of the serpin to the opposite pole of the latter, where it is inactivated due to its structure being extensively distorted (*4*−*9*).

The rate of antithrombin inhibition of target proteinases is greatly enhanced by the sulfated, highly negatively charged polysaccharide heparin, which in this manner acts as an effective anticoagulant (*4*, *5*, *10*). Heparin-like glycosaminoglycans on the surface of vascular endothelial cells may similarly function as physiological activators of antithrombin (*11*). The accelerating effect of heparin on antithrombin−proteinase reactions is exerted by two different mechanisms. In one of these, a specific pentasaccharide sequence, found in only approximately one-third of the chains in commercial heparin preparations, binds to and allosterically activates antithrombin by a conformational change. The binding occurs in two steps, an initial weak complex being formed in the first step, followed by the activating conformational change, which also tightens the interaction, in the second step (*12*−*15*). Heparin chains that contain the pentasaccharide sequence and are at least 18 saccharides long also increase the inhibition rate by an approximation mechanism, i.e., by their ability to bring the proteinase and the inhibitor together in a ternary bridging complex (*16*, *17*). The conformational change mechanism is most important for accelerating the inhibition of factor Xa, whereas the bridging mechanism is predominantly responsible for increasing the thrombin inhibition rate (*5*, *10*).

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Several studies of natural and recombinant antithrombin variants, as well as the X-ray structures of antithrombin, free and in complex with a pentasaccharide (*18*, *19*), have delineated the heparin binding site of the inhibitor (reviewed in ref *20*). A number of residues in the A and D helices and the N-terminal region, including Lys11, Arg13, Arg46, Arg47, Lys114, Lys125, and Arg129, form a basic site to which the negatively charged pentasaccharide binds. Lys114, Lys125, and Arg129 are the most important of these residues, with Lys114 contributing the most binding energy (*21*−*23*). The three residues constitute the core of a network of amino acids that act cooperatively in the binding, contributing both ionic and nonionic interactions. Lys125 is of appreciable importance for the first step of heparin binding, whereas Lys114 and Arg129 predominantly participate in the second step. The X-ray structures further reveal that the conformational change induced by the binding involves elongation and tilting of the D helix, formation of a new short P helix at the base of the D helix, and rearrangements of the N-terminal end of the A helix and the N-terminal region of the protein. These changes lead to contraction of the A β-sheet and expulsion of the reactive bond loop from this sheet, into which the loop is partially inserted in native antithrombin. As a result, the reactive bond becomes more accessible and the electrostatic surface surrounding the loop is altered, which together promote the binding of target proteinases (*18*, *19*, *24*, *25*).

Recent clinical trials have shown the synthetic heparin pentasaccharide to be more effective than other heparin forms for prevention of venous thromboembolism (*26*−*28*). Moreover, a synthetic hexadecasaccharide, containing a proteinase-binding region linked to the pentasaccharide, appears to have further improved anticoagulant properties (*29*). Development of even better such antithrombotic agents, as well as elucidation of the putative physiological role of vessel wall heparin-like glycosaminoglycans in preventing thrombus formation, requires a detailed understanding of the mechanism of binding of heparin to antithrombin. In the study presented here, we have introduced Arg to Lys and Lys to Arg substitutions of the three major heparin-binding residues of antithrombin, Lys114, Lys125, and Arg129, to clarify to what extent one basic side chain can substitute for another in the interaction. We find that Lys114 and Lys125 can be acceptably replaced with Arg, although at both sites, in particular in position 114, Lys is still optimal for heparin binding. In contrast, Arg129 cannot be similarly well replaced with Lys. The requirement for Arg in position 129 is presumably due to this residue specifically interacting not only with the pentasaccharide but also with other residues in antithrombin.

MATERIALS AND METHODS

Antithrombin Variants. Recombinant antithrombin variants with Lys114 to Arg, Lys125 to Arg, and Arg129 to Lys substitutions were produced by site-directed mutagenesis and expressed in a baculovirus system on an Asn135Ala background, as described previously (*21*, *22*, *30*−*32*). The K114R/N135A,[1] K125R/N135A, and R129K/N135A variants were purified by heparin affinity chromatography, as described in detail previously (*30*, *31*).

The purity of the antithrombin preparations was analyzed by SDS−PAGE with the Tris/Tricine buffer system (*33*). Concentrations of the antithrombin variants were determined from the absorbance at 280 nm with the use of a molar absorption coefficient of 37 700 M^{-1} cm^{-1} (*34*).

Proteinases and Saccharides. Human α-thrombin was a gift from J. Fenton (New York State Department of Health, Albany, NY), and human factor Xa was prepared as described previously (*35*). The concentrations of the enzymes were based on active site titrations, which showed that thrombin and factor Xa were >90 and ~70% active, respectively.

The synthetic antithrombin-binding heparin pentasaccharide (*12*) was a gift from M. Petitou (Sanofi-Synthélabo Recherche, Toulouse, France). Full-length heparin with a high affinity for antithrombin and with an average molecular mass of ~8000 Da (i.e., containing ~26 saccharide units) was isolated from commercial heparin, as previously described (*13*, *17*, *36*). Concentrations of both saccharides were determined by stoichiometric fluorescence titrations of plasma antithrombin with the saccharides (*13*).

Experimental Conditions. All experiments were performed at 25 ± 0.2 °C in 20 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 0.1% (w/v) poly(ethylene glycol) 8000. In the absence of added salt, the ionic strength of this buffer is 0.05. NaCl was added to total ionic strengths of up to 0.5.

Stoichiometries and Affinities of Heparin Binding. Stoichiometries and equilibrium binding constants, K_d, of pentasaccharide or full-length heparin binding to the antithrombin variants were measured by titrating the saccharides into a solution of antithrombin and monitoring the fluorescence enhancement induced by the interaction, as described previously (*13*, *17*, *36*). Stoichiometric titrations were done with full-length heparin at an ionic strength (I) of 0.15 with antithrombin concentrations, based on absorbance measurements, at least 20-fold above K_d. Affinity titrations were done with pentasaccharide or full-length heparin at an I of 0.1−0.5 with active antithrombin concentrations, i.e., those obtained from the heparin binding stoichiometries, from 5-fold below to 3-fold above K_d. The fluorescence increase was measured in an SLM 4800S spectrofluorometer (SLM Instruments, Rochester, NY) with excitation and emission wavelengths of 280 and 340 nm, respectively. The data were fitted to the equilibrium binding equation by nonlinear least-squares regression analysis (*36*).

Kinetics of Heparin Binding. Rate constants for pentasaccharide or full-length heparin binding to the antithrombin variants were measured by monitoring the increase in fluorescence accompanying the interaction in a stopped-flow instrument (SX-17MV; Applied Biophysics, Leatherhead, U.K.), essentially as described previously (*13*, *21*, *22*, *32*, *37*). The experiments were carried out at an ionic strength of 0.15 or 0.3 under pseudo-first-order conditions with heparin concentrations at least 10-fold higher than active antithrombin concentrations, derived from the heparin bind-

[1] Abbreviations: H26, full-length heparin with high affinity for antithrombin and containing ~26 saccharide units; H5, antithrombin-binding heparin pentasaccharide; K_d, dissociation equilibrium constant; k_{obs}, observed pseudo-first-order rate constant; k_{on}, overall association rate constant; k_{off}, overall dissociation rate constant; K114R, K125R, and R129K, substitution of Lys114, Lys125, and Arg129 with Arg, Arg, and Lys, respectively; N135A, substitution of Asn135 with Ala; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SEM, standard error of the mean.

Table 1: Dissociation Equilibrium Constants, Bimolecular Association Rate Constants, and Dissociation Rate Constants for Pentasaccharide and Full-Length Heparin Binding to the N135A, K114R/N135A, K125R/N135A, and R129K/N135A Antithrombin Variants at 25 °C, pH 7.4, and Ionic Strengths of 0.15 and 0.3[a]

ionic strength	heparin form	variant	K_d (nM)	k_{on} ($\times 10^6$ M^{-1} s^{-1})	k_{off} (s^{-1})	calcd K_d (nM)[b]	calcd k_{off} (s^{-1})[c]
0.15	H5	N135A	2 ± 1[d,e]	70 ± 2[e]	nd[f]		0.14 ± 0.07[e]
		K114R/N135A	29 ± 4	29 ± 1	1.9 ± 0.4	70 ± 20	0.8 ± 0.1
		K125R/N135A	6 ± 3[d]	69 ± 2	1 ± 1	15 ± 15	0.4 ± 0.2
		R129K/N135A	190 ± 20	52 ± 3	17 ± 2	330 ± 60	10 ± 2
0.3	H5	N135A	40 ± 4[e]	28 ± 1[e]	1.5 ± 0.4[e]	54 ± 16[e]	1.1 ± 0.2[e]
		K114R/N135A	280 ± 20	5 ± 0.1	2.6 ± 0.1	520 ± 30	1.4 ± 0.1
		K125R/N135A	160 ± 10	15 ± 0.5	3.6 ± 0.4	240 ± 30	2.4 ± 0.2
		R129K/N135A	2000 ± 900[d]	10 ± 1	29 ± 2	2900 ± 500	20 ± 10
	H26	N135A	7 ± 1[e]	23 ± 0.2[e]	0.3 ± 0.1[e]	13 ± 5[e]	0.16 ± 0.03[e]
		K114R/N135A	49 ± 4	7.5 ± 0.2	0.6 ± 0.1	80 ± 15	0.40 ± 0.04
		K125R/N135A	43 ± 2	14 ± 0.1	0.7 ± 0.1	50 ± 7	0.60 ± 0.03
		R129K/N135A	570 ± 20	11 ± 0.7	12 ± 1	1100 ± 200	6.3 ± 0.6

[a] Measured K_d values are averages ± SEM of three to six fluorescence titrations. Measured values of k_{on} and k_{off} ± SEM were obtained by linear regression of plots of k_{obs} vs heparin concentration comprising five to eight points in the concentration range of 0.1−1.2 μM. [b] From k_{on} and k_{off}. [c] From k_{on} and K_d. [d] Obtained by linear extrapolation of values measured at higher or lower ionic strengths. [e] Taken from ref *37*. [f] Not determined.

ing stoichiometries. The fluorescence was measured with an excitation wavelength of 280 nm and an emission cutoff filter with ~50% transmission at 320 nm. Observed pseudo-first-order rate constants, k_{obs}, were obtained by nonlinear regression fitting of the progress curves to a single-exponential function. Eight to sixteen fluorescence traces were averaged for each rate constant determination.

Stoichiometries and Kinetics of Proteinase Inactivation. Stoichiometries of inhibition of human α-thrombin by the antithrombin variants were determined as described in detail previously (*21*, *32*, *37*). Briefly, a series of samples at a constant thrombin concentration of 0.5 μM was incubated at an *I* of 0.15 with antithrombin at increasing concentrations, based on absorbance measurements. The residual activity of the enzyme was measured after 1−2 h from the initial rate of hydrolysis of the chromogenic substrate, S-2238 (Chromogenix, Mölndal, Sweden), monitored as the increase in absorbance at 405 nm. The inhibition stoichiometries were obtained from the intercept on the abscissa of plots of residual enzyme activity versus the molar ratio of inhibitor to enzyme (*36*).

Second-order association rate constants for inhibition of human α-thrombin or factor Xa by the antithrombin variants in the absence or presence of pentasaccharide or full-length heparin were measured under pseudo-first-order conditions at an *I* of 0.15, essentially as described in earlier work (*21*, *32*, *36*, *37*). The reaction mixtures contained 100 nM antithrombin, 10 nM proteinase, and 0−8 nM pentasaccharide or full-length heparin. The antithrombin concentrations were active concentrations, i.e., those derived from the thrombin inhibition stoichiometries. After various reaction times, aliquots of the reaction mixtures were quenched by dilution into 100 μM substrate, which was S-2238 for thrombin and Spectrozyme FXa (American Diagnostica, Greenwhich, CT) for factor Xa. The residual enzyme activity was determined from the initial rate of substrate hydrolysis, monitored by absorbance at 405 nm. The time dependence of enzyme activity loss was fitted by a single-exponential function with an end point of zero activity to give the observed pseudo-first-order rate constant, k_{obs} (*36*). Second-order association rate constants were obtained by dividing k_{obs} by the inhibitor concentration in the case of uncatalyzed reactions or were derived from the slope of linear regression fits of the dependence of k_{obs} on heparin concentration for heparin-catalyzed reactions (*32*, *38*).

RESULTS

Purification and Homogeneity of Antithrombin Variants. In this study, an antithrombin variant with an Asn135 to Ala mutation, equivalent to the β-form of plasma antithrombin (*32*), was used as a base molecule for additional Lys114 to Arg, Lys125 to Arg, and Arg129 to Lys mutations. The N135A substitution prevents glycosylation of Asn135, which leads to an increased heparin affinity of the inhibitor, as an oligosaccharide side chain in this position sterically hinders heparin binding (*30*, *32*). The mutation also eliminates the heterogeneity in heparin affinity arising from incomplete glycosylation at this site (*30*, *39*). Moreover, the baculovirus expression system that was used introduces shorter and structurally less divergent oligosaccharide side chains than mammalian expression systems, further decreasing heparin binding heterogeneity (*23*, *30*, *40*, *41*). All antithrombin variants were >95% homogeneous in SDS−PAGE under reducing conditions after heparin affinity chromatography and were therefore used without further purification.

The stoichiometry of binding of full-length heparin to the variants was 0.55 and 0.85 for the two preparations of K114R/N135A that were used, 0.6 for two preparations of K125R/N135A, and 0.75 for the single preparation of R129K/N135A. All mutants showed a 30−40% increase in tryptophan fluorescence in the stoichiometry titrations. The corresponding stoichiometries of thrombin binding to the variants were comparable with those of heparin binding, viz., 0.55−0.8, 0.65, and 0.65 for the K114R/N135A, K125R/N135A, and R129K/N135A preparations, respectively. The preparations thus contained some inactive, presumably latent, form of the inhibitor, as also seen in previous studies of recombinant antithrombin variants (*21*−*23*, *37*, *42*).

Affinity of Pentasaccharide and Full-Length Heparin Binding. Dissociation equilibrium constants, K_d, for pentasaccharide binding to the antithrombin variants were obtained at ionic strengths of both 0.15 and 0.3 (Table 1). However, these constants for the binding of full-length heparin could only be measured at an *I* of 0.3, as the interaction at an *I* of 0.15 was too tight to give meaningful data. Moreover, even the pentasaccharide bound too tightly

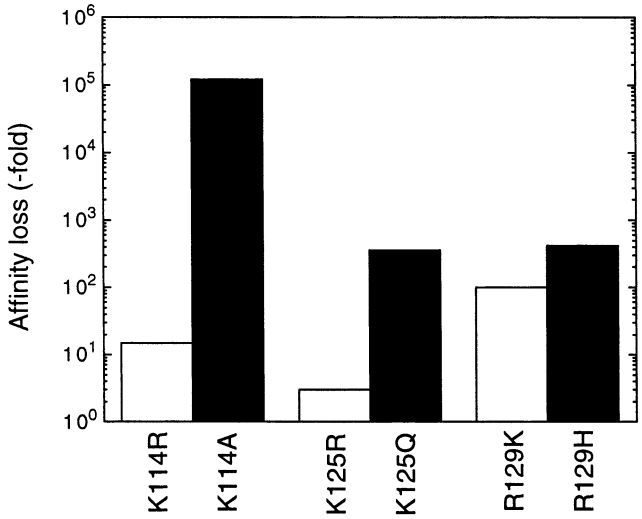

FIGURE 1: Losses of antithrombin affinity for the pentasaccharide at pH 7.4 and an *I* of 0.15 caused by substitutions of Lys114, Lys125, and Arg129 with other positively charged amino acids (white bars) compared with losses caused by substitutions of these residues with neutral amino acids (black bars). The affinity loss is expressed as K_d(mutated variant)/K_d(control variant). K_d values for the K114A, K125Q, and R129H variants are taken from previous works (*21*−*23*). All mutations are on an N135A background.

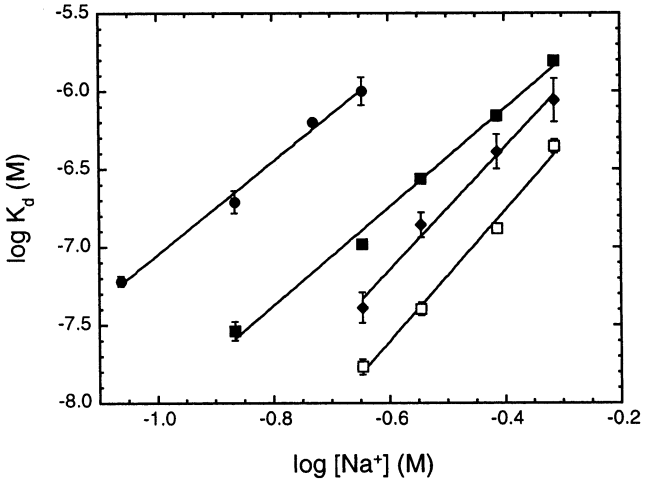

FIGURE 2: Sodium ion concentration dependence of dissociation equilibrium constants for pentasaccharide binding to the N135A, K114R/N135A, K125R/N135A, and R129K/N135A antithrombin variants at 25 °C and pH 7.4: (□) N135A, (■) K114R/N135A, (◆) K125R/N135A, and (●) R129K/N135A. Each value represents the average ± SEM of three to six determinations. Error bars not shown lie within the dimensions of the symbol. The solid lines represent linear regression fits. The data for the N135A variant are taken from ref *37*.

to the K125R/N135A variant, like to the N135A control variant in previous work (*37*), at an *I* of 0.15 for K_d to be determined directly, and this parameter was instead derived by extrapolation of the linear dependence of values measured at higher ionic strengths (see below). Conversely, the interaction of the pentasaccharide with the R129K/N135A variant at an *I* of 0.3 was too weak to be quantified directly, and K_d was therefore calculated from values obtained at lower ionic strengths. In agreement with previous studies of wild-type antithrombin and other mutants (*13*, *21*−*23*, *32*, *37*), all antithrombin variants had a somewhat higher affinity for full-length heparin than for the pentasaccharide (Table 1). Although the K114R/N135A, K125R/N135A, and R129K/N135A substitutions all preserved the positive charge of the original residue and introduced an approximately isosteric side chain, the three mutants bound both heparin forms more weakly than the N135A control (Table 1). The affinity losses varied with the mutation, being only moderate or small for the K114R/N135A and K125R/N135A variants (∼15- and ∼3-fold, respectively, for pentasaccharide binding at an *I* of 0.15) but appreciable for the R129K/N135A variant (∼100-fold). Comparable losses in affinity of each mutant for the pentasaccharide were observed at an *I* of 0.3, and the losses at this ionic strength were also similar to those for full-length heparin.

The effects of the K114R/N135A, K125R/N135A, and R129K/N135A mutations on the affinity of antithrombin for the pentasaccharide at an *I* of 0.15 can be compared with the corresponding effects, established in previous works (*21*−*23*), of mutation of these residues to noncharged side chains (Figure 1). Substitution of either Lys114 or Lys125 with Arg decreased the affinity to an appreciably smaller extent than substitution with a neutral residue. In contrast, substitution of Arg129 with Lys reduced the affinity nearly as much as substitution with a neutral residue.

Ionic and Nonionic Contributions to Pentasaccharide Binding. The ionic and nonionic contributions to the losses of pentasaccharide affinity caused by the K125R/N135A, K114R/N135A, and R129K/N135A antithrombin mutations were assessed from the dependence of the dissociation equilibrium constants on sodium ion concentration. Plots of log K_d versus log [Na^+] were linear for all antithrombin variants (Figure 2). According to polyelectrolyte theory (*13*, *32*, *43*), the slope of such plots is equal to $Z \times \Psi$, Z representing the number of ionic interactions participating in the binding and Ψ the number of sodium ions bound per heparin charge that are released on heparin binding [estimated to be 0.8 (*17*)]. In addition, the intercept on the ordinate of these plots gives log K_d', the logarithm of the dissociation equilibrium constant at 1 M Na^+, which is a measure of the nonionic contribution to the binding. As reported in previous work, the N135A control variant makes approximately five ionic interactions with the pentasaccharide (Table 2) (*32*, *37*). The K125R/N135A variant also made approximately five ionic interactions with the pentasaccharide, whereas the K114R/N135A and R129K/N135A variants made only approximately four such interactions. Log K_d' was similar, approximately −5, for the K114R/N135A and K125R/N135A variants as for the N135A control, but was appreciably increased to −4.1 for the R129K/N135A variant. Mutation of Lys114 to Arg or of Arg129 to Lys thus resulted in the loss of approximately one charge interaction with the

Table 2: Ionic and Nonionic Contributions to Pentasaccharide Binding to the N135A, K114R/N135A, K125R/N135A, and R129K/N135A Antithrombin Variants at 25 °C and pH 7.4[a]

variant	Z	log(K_d')	K_d' (mM)
N135A	5.3 ± 0.2[b]	−5.1 ± 0.1[b]	8 ± 2
K114R/N135A	4.0 ± 0.3	−4.8 ± 0.2	16 ± 6
K125R/N135A	4.9 ± 0.4	−4.8 ± 0.1	16 ± 4
R129K/N135A	3.7 ± 0.2	−4.1 ± 0.1	80 ± 20

[a] The number of ionic interactions established in the binding (Z) and the nonionic contribution to the binding [log(K_d')] were determined from the slopes and intercepts, respectively, of plots of log(K_d) vs log[Na^+] (Figure 2), as described in the Results. Errors represent the SEM obtained by linear regression. [b] Taken from ref *37*.

pentasaccharide, even though the positive charge of the side chain was maintained. The Arg129 to Lys substitution also led to a substantial ~10-fold loss in affinity of nonionic interactions, whereas the Lys114 to Arg substitution minimally affected these interactions. In contrast, mutation of Lys125 to Arg resulted in no appreciable changes of either type of interaction, consistent with the small overall affinity loss.

Kinetics of Heparin Binding. The kinetics of pentasaccharide or full-length heparin binding to the antithrombin variants were evaluated under pseudo-first-order conditions at an I of 0.15 or 0.3 by monitoring the increase in fluorescence accompanying the interaction in a stopped-flow instrument. The fluorescence change was monophasic for all interactions studied and was well fitted to a single-exponential function, giving the observed pseudo-first-order rate constant, k_{obs}. The bimolecular association rate constant, k_{on}, and the overall dissociation rate constant, k_{off}, for the binding were obtained from the slope and ordinate intercept, respectively, of the linear dependence of k_{obs} on saccharide concentration at low concentrations (0−1.2 μM). k_{on} and k_{off} for the interaction of the pentasaccharide with all antithrombin mutants were measured at ionic strengths of both 0.15 and 0.3 (Table 1). However, these parameters for the binding of full-length heparin were only determined at an I of 0.3 (Table 1), due to the tight binding of full-length heparin at an I of 0.15, resulting in k_{off} values that were too low to be experimentally accessible. All mutations only moderately affected k_{on}. At an I of 0.15, k_{on} for pentasaccharide binding was decreased ~2-fold for the K114R/N135A variant but was only negligibly altered for the K125R/N135A and R129K/N135A mutants. At an I of 0.3, the defects on k_{on} were somewhat larger, although still small, being 3−6-, ~2-, and 2−3-fold for both pentasaccharide and full-length heparin binding to the K114R/N135A, K125R/N135A, and R129K/N135A variants, respectively. k_{off} was also appreciably increased by the mutations. However, K_d calculated from the measured values of k_{on} and k_{off} was up to 3-fold higher than the measured K_d for all mutants (Table 1). As discussed in earlier works (*21*−*23*, *37*), such a disagreement between measured and calculated K_d values is most likely due to a small contribution of a preequilibrium pathway of heparin binding, in addition to the predominant induced-fit pathway. In the preequilibrium pathway, heparin binds to a small amount of antithrombin that is already conformationally activated and is in equilibrium with the unactivated inhibitor (*14*). A small contribution of this pathway can result in measured k_{off} values being anomalously high without k_{on} values being appreciably affected (*37*). The calculated k_{off} values therefore are presumably more accurate than the measured ones (Table 1) and will be taken to represent the true values. k_{off} for pentasaccharide binding at an I of 0.15 was moderately increased for the K114R/N135A and K125R/N135A mutants, by ~6- and ~3-fold, respectively, but was substantially, ~70-fold, higher than that of the control for the R129K/N135A mutant. The corresponding increases in k_{off} for the binding of both the pentasaccharide and full-length heparin at an I of 0.3 were somewhat smaller but comparable for the two saccharides, <2-, 2−4-, and 20−40-fold for the three variants, respectively. In all cases, the largest increase in k_{off} was thus observed for the Arg129 to Lys mutation.

Analyses of the pentasaccharide concentration dependence at an I of 0.15 of k_{obs} for pentasaccharide binding to the K114R/N135A variant, the only variant for which k_{on} was measurably increased at this ionic strength, were extended to higher saccharide concentrations. Under these conditions, k_{obs} varied hyperbolically with pentasaccharide concentration (Figure 3), as has been shown previously for plasma antithrombin, recombinant wild-type antithrombin, and other recombinant antithrombin variants (*13*, *21*−*23*, *32*, *37*). Such nonlinear behavior is in agreement with a two-step binding mechanism, in which a weak interaction with the pentasaccharide is established in a rapid equilibrium in the first step, followed by a conformational change leading to tight binding of the saccharide in the second step (Scheme 1) (*44*). In this

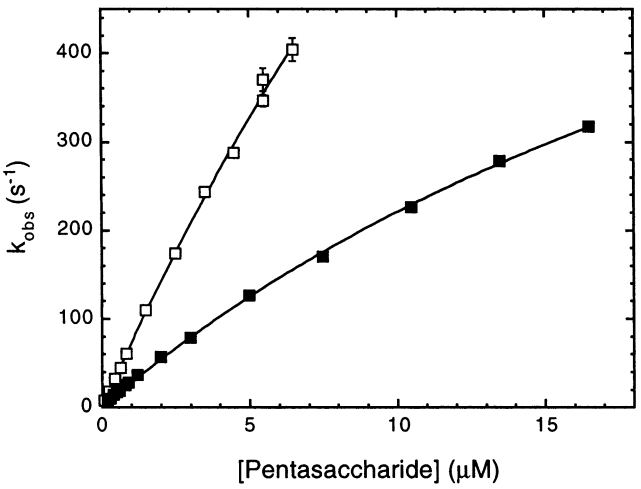

FIGURE 3: Pentasaccharide concentration dependence of observed pseudo-first-order rate constants for pentasaccharide binding to the N135A and K114R/N135A antithrombin variants at 25 °C, pH 7.4, and an ionic strength of 0.15: (□) N135A and (■) K114R/N135A. Values are averages ± SEM of 8−16 individual measurements. Error bars not shown lie within the dimensions of the symbols. The solid lines represent nonlinear regression fits of the data to the rectangular hyperbolic function characterizing the binding mechanism in Scheme 1 (*13*, *44*). The data for the N135A variant are taken from ref *37*.

Scheme 1

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

mechanism, K_1 is the dissociation equilibrium constant for the initial weak interaction step and k_{+2} and k_{-2} are the forward and reverse rate constants, respectively, for the second step (conformational change). Values of K_1 and k_{+2} were derived by nonlinear regression fits of the dependence of k_{obs} on pentasaccharide concentration to the rectangular hyperbolic function characterizing the mechanism in Scheme 1 (Figure 3) (*13*, *44*). The Lys114 to Arg mutation did not detectably affect K_1 for pentasaccharide binding but decreased k_{+2} by ~2-fold (Table 3). The decrease in k_{on} caused by the mutation was thus due only or predominantly to the decreased k_{+2}, as k_{on} is equal to k_{+2}/K_1 in the mechanism in Scheme 1 (*13*). k_{-2} for pentasaccharide binding, being equal to k_{off} in this mechanism (*13*) and taken to be the calculated value of k_{off} (Table 1; see above), was increased ~6-fold by the mutation.

Kinetics of Proteinase Inhibition. Second-order rate constants for the uncatalyzed or pentasaccharide- or full-length heparin-catalyzed inhibition of thrombin or factor Xa by the

Table 3: Kinetic Constants for the Two-Step Mechanism of Pentasaccharide Binding to the N135A and K114R/N135A Antithrombin Variants at 25 °C, pH 7.4, and an Ionic Strength of 0.15[a]

variant	K_1 (μM)	k_{+2} (s^{-1})	k_{-2} (s^{-1})
N135A	28 ± 4[b]	2100 ± 300[b]	0.14 ± 0.07[b]
K114R/N135A	36 ± 2	1000 ± 40	0.8 ± 0.1

[a] The dissociation equilibrium constants of the first step, K_1, and the forward rate constants of the second step, k_{+2}, were obtained by nonlinear regression fits of the data in Figure 3 to the equation describing the mechanism in Scheme 1 (*13*, *44*). The reverse rate constants of the second step, k_{-2}, were taken from Table 1 as the calculated value of k_{off}. Errors represent the SEM. [b] Taken from ref *37*.

K114R/N135A, K125R/N135A, and R129K/N135A antithrombin variants were determined by discontinuous assays of residual proteinase activity (Table 4). The pentasaccharide enhancement of the rate of thrombin inhibition is less than 2-fold for plasma and recombinant wild-type antithrombin (*13*, *32*) and therefore was not investigated. At most, small differences in the uncatalyzed or catalyzed rate constants for inhibition of the two proteinases by any of the antithrombin variants were evident.

DISCUSSION

Lys114, Lys125, and Arg129, which are all located in the same region in or close to the D helix in the antithrombin structure (Figure 4), are the three most important residues for binding of the heparin pentasaccharide (*20*). Lys125 and Arg129 are located in the middle and near the carboxy-terminal end of the D helix, respectively, whereas Lys114 is in the loop at the N-terminal end of the D helix in free antithrombin that forms the P helix in the complex with the pentasaccharide (*18*, *19*). Previous studies have shown that mutation of Lys114 to a neutral amino acid results in a substantial $\sim 10^5$-fold loss in pentasaccharide and heparin affinity at physiologic pH and ionic strength, corresponding to an ~50% loss of total binding energy (*22*). Corresponding mutations of Lys125 and Arg129 each result in smaller affinity losses of 400−500-fold, corresponding to ~30% losses of binding energy (*21*, *23*). It is apparent that the sum of these losses of binding energy, together with those caused by mutations of other amino acids involved in pentasaccharide binding, mainly, Lys11, Arg13, and Arg47 (*20*, *37*), appreciably exceeds the total binding energy. Moreover, the mutations of each of the residues Lys114, Lys125, and Arg129 to a neutral amino acid result in the loss of two to three ionic interactions with the pentasaccharide, despite only one positively charged side chain having been replaced (*21*−*23*). These observations indicate that these three amino acids act cooperatively in pentasaccharide binding, forming a core of a network of residues that contributes both ionic and nonionic interactions to the binding (*20*−*23*). The molecular details underlying such cooperativity, however, remain to be established.

To elucidate whether another positively charged side chain can fulfill the role of each of the three major heparin-binding residues of antithrombin in the interaction with the pentasaccharide and in forming the cooperative network, we have introduced Lys114 to Arg, Lys125 to Arg, and Arg129 to Lys mutations of the inhibitor in this work. Since Arg and Lys oligopeptides bind heparin with comparable affinities (*45*), any effects of interchanging these residues in antithrombin on pentasaccharide binding must reflect a specificity of the wild-type side chain in the binding. As in our previous studies of other mutants (*21*−*23*), the three mutants all exhibited the normal enhancement of tryptophan fluorescence on heparin binding and normal uncatalyzed and heparin-catalyzed rates of inhibition of thrombin and factor Xa. These findings indicate that only a side chain that is important for heparin binding was altered by the mutations and that the native and activated conformations of antithrombin were not affected.

The defect in antithrombin affinity for the pentasaccharide at pH 7.4 and an I of 0.15 caused by substitution of Lys114, the major heparin-binding residue, with an Arg residue was ~15-fold. This reduction in affinity is substantially lower than that of $\sim 10^5$-fold caused by substitution with a noncharged amino acid of comparable size (*22*). The positively charged side chain of Lys114 can thus be reasonably well replaced with that of Arg in heparin binding with only a modest affinity decrease. The Lys114 to Arg mutation resulted in the loss of approximately one ionic interaction but in no observable loss of nonionic interactions, whereas mutation to a neutral amino acid led to loss of both two to three ionic interactions and appreciable nonionic interactions (*22*). The loss of approximately one ionic interaction on mutation to Arg most likely is not due to the substitution eliminating the direct interaction of a positive charge in position 114 with the pentasaccharide, as this would be expected to lead to an appreciably larger affinity loss, approaching that caused by substitution with a neutral residue. Instead, this loss is presumably due to the mutation perturbing the network of charges interacting with the pentasaccharide. The optimal configuration of this network thus apparently requires a Lys in position 114. However, the nonionic interactions (i.e., hydrogen bonds and hydrophobic and van der Waals interactions) with the pentasaccharide contributed by the network are not appreciably altered by substitution to Arg. Previous studies have shown that Lys114 is not involved in the initial, weak interaction with heparin in the two-step binding mechanism but is the major residue of importance both for increasing the forward rate constant of the activating conformational change in the second step and for decreasing the reverse rate constant of this change, thereby locking the inhibitor in the activated state (*22*). This role is in agreement with the substantial conformational rearrangement of Lys114 on pentasaccharide binding indicated by the X-ray structures (Figure 4) (*18*, *19*). The Lys114 to Arg mutation resulted in an ~2-fold defect in k_{+2} at an I of 0.15, i.e., in promoting the conformational change step, as well as in an ~6-fold defect in k_{-2}, i.e., in locking the inhibitor in the activated state, to be compared with ~40- and ~1000-fold changes in these parameters, respectively, on mutation to a neutral amino acid (*22*). These moderate effects are apparently caused by the observed loss of approximately one ionic interaction with the pentasaccharide that is contributed by the interacting network. However, it is clear that an Arg residue can reasonably well replace Lys114 both in effecting the heparin-induced conformational change of antithrombin and in keeping the saccharide tightly bound to the activated inhibitor, although Lys is somewhat better in both respects.

Lys125 and Arg129 are the second most important residues of antithrombin for heparin binding, being responsible for

Table 4: Association Rate Constants for Uncatalyzed and Pentasaccharide- or Full-Length Heparin-Catalyzed Inhibition of Thrombin and Factor Xa by the N135A, K114R/N135A, K125R/N135A, and R129K/N135A Antithrombin Variants at 25 °C, pH 7.4, and an Ionic Strength of 0.15[a]

proteinase	variant	k_{uncat} ($\times 10^3$ M^{-1} s^{-1})	k_{H5} ($\times 10^5$ M^{-1} s^{-1})	k_{H26} ($\times 10^6$ M^{-1} s^{-1})
thrombin	N135A	9.4 ± 0.4[b]	nd[c]	9.0 ± 0.5[b]
	K114R/N135A	10.2 ± 0.3	nd[c]	6.5 ± 0.7
	K125R/N135A	9.3 ± 0.3	nd[c]	8.0 ± 0.6
	R129K/N135A	9.5 ± 0.9	nd[c]	7.8 ± 0.3
factor Xa	N135A	4.8 ± 0.2[b]	6.1 ± 0.2[b]	1.2 ± 0.04[b]
	K114R/N135A	5.3 ± 0.1	6.5 ± 0.3	1.0 ± 0.01
	K125R/N135A	5.4 ± 0.01	6.2 ± 0.2	1.1 ± 0.01
	R129K/N135A	4.5 ± 0.1	8.0 ± 0.1	1.1 ± 0.2

[a] Second-order association rate constants for uncatalyzed (k_{uncat}), pentasaccharide-catalyzed (k_{H5}), and full-length heparin-catalyzed (k_{H26}) reactions of the antithrombin variants with the proteinases were determined as described in Materials and Methods. Uncatalyzed rate constants are averages ± SEM of at least three determinations. Values of k_{H5} and k_{H26} ± SEM were obtained by linear regression of plots of k_{obs} vs pentasaccharide or full-length heparin concentration, comprising four points in the concentration range of 0.1−8 nM. Uncatalyzed rate constants for inhibition of thrombin were unaffected by the presence of 50 μg/mL Polybrene, showing that the antithrombin preparations were not contaminated by heparin. [b] Taken from ref *37*. [c] Not determined.

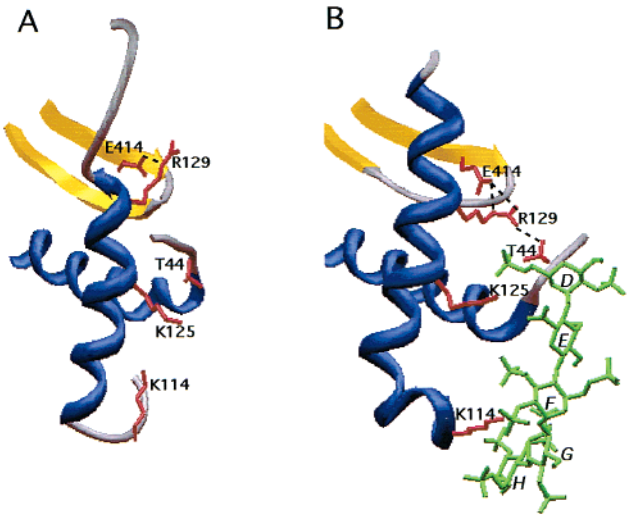

FIGURE 4: Closeup of the heparin binding site of (A) free antithrombin and (B) pentasaccharide-bound antithrombin. The N-terminal end of the A helix, the D helix, and the P helix are shown in blue. A segment of the B sheet is shown in yellow. The side chains of the amino acids mutated in this work are drawn in red. The pentasaccharide, denoted DEFGH from the nonreducing end, is shown in green. Dotted lines represent putative interactions of Arg129 with Glu414 and Thr44 with distances of 3−3.5 Å. Drawn from PDB structures 2ant and 1azx (*18*, *19*).

approximately equal energies of binding of the pentasaccharide (*21*, *23*). Mutation of Lys125 to Arg resulted in an only ~3-fold reduced pentasaccharide affinity at pH 7.4 and an *I* of 0.15, compared with a decrease of ~400-fold caused by mutation to a neutral amino acid (*23*). Lys125 can thus be even better replaced with Arg in binding of heparin than can Lys114, with essentially fully retained pentasaccharide affinity. Consistent with this conclusion, no definite effect of the Lys125 to Arg mutation on either the ionic or nonionic contribution to pentasaccharide binding could be ascertained. Lys125 is the only residue of antithrombin investigated so far that contributes to the first step of heparin binding by promoting the formation of the initial weak complex (*23*), in keeping with the minor conformational reorientation of Lys125 on pentasaccharide binding (Figure 4) (*18*, *19*). It also participates in the second, conformational change step, mainly by decreasing the reverse rate constant of this step, but also to some extent by increasing the forward rate constant. No effect or only a small effect of the Lys125 to Arg substitution on k_{on}, the overall forward rate constant for pentasaccharide binding, was observed, indicating that Arg can well replace Lys125 both in establishing the interactions necessary for the initial, weak complex to be formed and in promoting the forward rate of the conformational change. The kinetic constant that was most affected by the mutation was the reverse rate constant of the conformational change step, which was decreased ~3-fold at an *I* of 0.15, thereby accounting for most of the affinity loss. A Lys in position 125 is thus marginally more effective than an Arg in keeping antithrombin locked in the heparin-activated state.

Substitution of Arg129 with Lys led to an appreciable ~100-fold decrease in antithrombin affinity for the pentasaccharide at pH 7.4 and an *I* of 0.15, a loss only somewhat smaller than that of ~400-fold caused by substitution of Arg129 with a neutral amino acid (*21*). In contrast to Lys114 and Lys125, Arg129 thus cannot be well replaced with another basic amino acid in heparin binding. This finding is in agreement with a previous study, which showed that the decrease in affinity caused by mutation of Arg129 to His was similar at pH 6 and 7.4, i.e., regardless of the ionization state of the His residue (*21*). The Arg129 to Lys mutation was accompanied by the loss of approximately one ionic interaction as well as by an ~10-fold loss of affinity of nonionic interactions, to be compared with the loss of approximately two ionic interactions and an ~25-fold loss of affinity of nonionic interactions on mutation of Arg129 to a neutral residue (*21*). As the loss in pentasaccharide affinity caused by the Arg129 to Lys mutation approaches that on removal of the positive charge in position 129, the observed loss of approximately one ionic interaction may reflect the amino group of the Lys residue not being able to establish an appropriate charge interaction with the pentasaccharide. It is also apparent that Lys cannot make the same nonionic interactions as Arg in position 129. Previous work has shown that the role of Arg129 in heparin binding is in the second step, i.e., in increasing the rate constant of the conformational change and, to a greater extent, in locking the inhibitor in the activated state (*21*). This role is consistent with an appreciable change in the conformation of Arg129 on pentasaccharide binding (Figure 4) (*18*, *19*). The Arg129 to Lys mutation resulted in at most a small defect in the forward rate constant of the conformational change step, as

evidenced by no change or only a small change in k_{on}, but in an ~70-fold increased reverse rate constant of this step at an I of 0.15, accounting for virtually all of the loss in affinity. Lys in position 129 can thus well fulfill the role of Arg in promoting the heparin-induced conformational change of antithrombin, whereas an Arg residue is essential for keeping heparin bound to the activated state of the inhibitor.

The specific requirement of an Arg in position 129 of antithrombin for pentasaccharide binding may be due to the ability of the Arg side chain to form intramolecular interactions which are important for optimal interaction of this side chain with the pentasaccharide. The crystal structure of free antithrombin (*18*, *19*) shows that Arg129 interacts with Glu414, which stabilizes the conformation of Arg129 and holds it in a position that makes it more accessible to the pentasaccharide (Figure 4). The distance between Arg129 and Glu414 even decreases somewhat on pentasaccharide binding (*19*), possibly tightening the interaction between the two residues (Figure 4). The X-ray structures further show that Thr44, located near the N-terminus of the A helix, rearranges on pentasaccharide binding to approach and most likely establish an interaction with the guanidino group of Arg129. Such an interaction would be expected to aid the stabilization of the conformation of Arg129 in the pentasaccharide-bound form of antithrombin, thereby allowing this residue to appropriately interact with the non-reducing end D unit of the pentasaccharide (Figure 4) (*19*). In contrast, Lys125 and Lys114 do not participate in any intramolecular interactions, neither in the free nor in the pentasaccharide-bound form of antithrombin, but only interact directly with the pentasaccharide. This absence of intramolecular interactions may be the reason these two side chains are not as specifically required as Arg129 for high-affinity heparin binding.

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REFERENCES

1. Van Boven, H. H., and Lane, D. A. (1997) *Semin. Hematol. 34*, 188−204.
2. 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) *J. Clin. Invest. 106*, 873−878.
3. Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Gettins, P. G. W., Irving, J. A., Lomas, D. A., Luke, C. J., Moyer, R. W., Pemberton, P. A., Remold-O'Donnell, E., Salvesen, G. S., Travis, J., and Whisstock, J. C. (2001) *J. Biol. Chem. 276*, 33293−33296.
4. Gettins, P. G. W., Patston, P. A., and Olson, S. T. (1996) *Serpins: Structure, Function and Biology*, R. G. Landes, Austin, TX.
5. Björk, I., and Olson, S. T. (1997) in *Chemistry and Biology of Serpins* (Church, F. C., Cunningham, D. D., Ginsburg, D., Hoffman, M., Stone, S. R., and Tollefsen, D. M., Eds.) pp 17−33, Plenum Press, New York.
6. Stratikos, E., and Gettins, P. G. W. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 4808−4813.
7. Lawrence, D. A., Olson, S. T., Muhammad, S., Day, D. E., Kvassman, J. O., Ginsburg, D., and Shore, J. D. (2000) *J. Biol. Chem. 275*, 5839−5844.
8. Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) *Nature 407*, 923−926.
9. Calugaru, S. V., Swanson, R., and Olson, S. T. (2001) *J. Biol. Chem. 276*, 32446−32455.
10. Olson, S. T., and Björk, I. (1994) *Semin. Thromb. Hemostasis 20*, 373−409.
11. Marcum, J. A., Atha, D. H., Fritze, L. M., Nawroth, P., Stern, D., and Rosenberg, R. D. (1986) *J. Biol. Chem. 261*, 7507−7517.
12. Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B., and Gatti, G. (1983) *Biochem. Biophys. Res. Commun. 116*, 492−499.
13. Olson, S. T., Björk, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) *J. Biol. Chem. 267*, 12528−12538.
14. Desai, U. R., Petitou, M., Björk, I., and Olson, S. T. (1998) *J. Biol. Chem. 273*, 7478−7487.
15. Desai, U. R., Petitou, M., Björk, I., and Olson, S. T. (1998) *Biochemistry 37*, 13033−13041.
16. Danielsson, Å., Raub, E., Lindahl, U., and Björk, I. (1986) *J. Biol. Chem. 261*, 15467−15473.
17. Olson, S. T., and Björk, I. (1991) *J. Biol. Chem. 266*, 6353−6364.
18. Skinner, R., Abrahams, J. P., Whisstock, J. C., Lesk, A. M., Carrell, R. W., and Wardell, M. R. (1997) *J. Mol. Biol. 266*, 601−609.
19. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14683−14688.
20. Olson, S. T., Björk, I., and Bock, S. C. (2002) *Trends Cardiovasc. Med. 12*, 198−205.
21. Desai, U., Swanson, R., Bock, S. C., Björk, I., and Olson, S. T. (2000) *J. Biol. Chem. 275*, 18976−18984.
22. Arocas, V., Bock, S. C., Raja, S., Olson, S. T., and Björk, I. (2001) *J. Biol. Chem. 276*, 43809−43817.
23. Schedin-Weiss, S., Desai, U. R., Bock, S. C., Gettins, P. G. W., Olson, S. T., and Björk, I. (2002) *Biochemistry 41*, 4779−4788.
24. Huntington, J. A., McCoy, A., Belzar, K. J., Pei, X. Y., Gettins, P. G. W., and Carrell, R. W. (2000) *J. Biol. Chem. 275*, 15377−15383.
25. Chuang, Y. J., Swanson, R., Raja, S. M., and Olson, S. T. (2001) *J. Biol. Chem. 276*, 14961−14971.
26. Turpie, A. G. G., Gallus, A. S., and Hoek, J. A. (2001) *N. Engl. J. Med. 344*, 619−625.
27. Eriksson, B. I., Bauer, K. A., Lassen, M. R., and Turpie, A. G. G. (2001) *N. Engl. J. Med. 345*, 1298−1304.
28. Bauer, K. A., Eriksson, B. I., Lassen, M. R., and Turpie, A. G. G. (2001) *N. Engl. J. Med. 345*, 1305−1310.
29. Herbert, J. M., Hérault, J. P., Bernat, A., Savi, P., Schaeffer, P., Driguez, P. A., Duchaussoy, P., and Petitou, M. (2001) *Thromb. Haemostasis 85*, 852−860.
30. Ersdal-Badju, E., Lu, A., Peng, X., Picard, V., Zendehrouh, P., Turk, B., Björk, I., Olson, S. T., and Bock, S. C. (1995) *Biochem. J. 310*, 323−330.
31. Ersdal-Badju, E., Lu, A. Q., Zuo, Y. C., Picard, V., and Bock, S. C. (1997) *J. Biol. Chem. 272*, 19393−19400.
32. Turk, B., Brieditis, I., Bock, S. C., Olson, S. T., and Björk, I. (1997) *Biochemistry 36*, 6682−6691.
33. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
34. Nordenman, B., Nyström, C., and Björk, I. (1977) *Eur. J. Biochem. 78*, 195−203.
35. Bock, P. E., Craig, P. A., Olson, S. T., and Singh, P. (1989) *Arch. Biochem. Biophys. 273*, 375−388.
36. Olson, S. T., Björk, I., and Shore, J. D. (1993) *Methods Enzymol. 222*, 525−560.
37. Arocas, V., Bock, S. C., Olson, S. T., and Björk, I. (1999) *Biochemistry 38*, 10196−10204.
38. Björk, I., Ylinenjärvi, K., Olson, S. T., Hermentin, P., Conradt, H. S., and Zettlmeissl, G. (1992) *Biochem. J. 286*, 793−800.
39. Picard, V., Ersdal-Badju, E., and Bock, S. C. (1995) *Biochemistry 34*, 8433−8440.
40. Fan, B., Crews, B. C., Turko, I. V., Choay, J., Zettlmeissl, G., and Gettins, P. (1993) *J. Biol. Chem. 268*, 17588−17596.
41. Garone, L., Edmunds, T., Hanson, E., Bernasconi, R., Huntington, J. A., Meagher, J. L., Fan, B. Q., and Gettins, P. G. W. (1996) *Biochemistry 35*, 8881−8889.
42. Arocas, V., Turk, B., Bock, S. C., Olson, S. T., and Björk, I. (2000) *Biochemistry 39*, 8512−8518.
43. Record, M. T., Jr., Lohman, T. M., and de Haseth, P. (1976) *J. Mol. Biol. 107*, 145−158.
44. Olson, S. T., Srinivasan, K. R., Björk, I., and Shore, J. D. (1981) *J. Biol. Chem. 256*, 11073−11079.
45. Mascotti, D. P., and Lohman, T. M. (1995) *Biochemistry 34*, 2908−2915.

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