Lysine−Heparin Interactions in Antithrombin. Properties of K125M and K290M,K294M,K297M Variants†

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ABSTRACT: Lysine residues in two different regions of antithrombin have been proposed to be involved in heparin binding and heparin-mediated acceleration of proteinase inhibition. Lysine 125 has been implicated as an essential heparin binding residue from chemical modification studies [Peterson, C. B., Noyes, C. M., Pecon, J. M., Church, F. C., & Blackburn, M. N. (1987) *J. Biol. Chem. 262,* 8061−8065] whereas lysines 290, 294, and 297 have been proposed from model building studies to constitute the heparin binding site [Villanueva, G. B. (1984) *J. Biol. Chem. 259,* 2531−2536]. To evaluate both of these proposals, we have prepared two variant human antithrombins, K125M and K290M,K294M,K297M, in which these lysines have been changed by site-directed mutagenesis to methionines. The K290M,-K294M,K297M variant had properties very similar to those of wild-type recombinant antithrombin in affinity for heparin, and in rates of inhibition of thrombin and factor Xa. In contrast, K125M antithrombin had reduced affinity for both heparin pentasaccharide and full-length heparin, corresponding to $\Delta\Delta G$s of 3.1 and 2.0 kcal mol^{-1}, respectively. However, this variant was still able to inhibit both thrombin and factor Xa. Whereas the rate of thrombin inhibition was similar to that of wild-type antithrombin, the rate of factor Xa inhibition was enhanced between 2- and 3-fold, suggesting a role for lysine 125 in the allosteric coupling between the heparin binding site and the reactive center region. At saturation with either heparin pentasaccharide or full-length high-affinity heparin, the rates of inhibition of both proteinases were similar to those of wild-type antithrombin for both the K125M and K290M,K294M,K297M variants. We conclude that lysine 125 plays an important role in the structure of the heparin binding region and in binding heparin with high affinity, but is not needed for the maximum heparin-induced acceleration of proteinase inhibition. We found no definitive evidence that lysines 290, 294, and 297 contribute to a heparin binding site, either as the primary site or involved in binding longer chain species.

Antithrombin is a member of the serpin superfamily of serine proteinase inhibitors and is an inhibitor of proteinases involved in regulation of proteinases of the blood coagulation cascade. The principal targets for inhibition by antithrombin are factor Xa and thrombin. However, for efficient rates of inhibition of these proteinases to occur, heparin must be present. The mechanisms by which heparin brings about enhancement of the rate of inhibition of these proteinases differ. For large enhancement of the rate of factor Xa inhibition, a critical heparin pentasaccharide, which binds to a specific site on antithrombin and causes a large conformational change, is necessary and sufficient (Olson et al., 1992). For enhancement of the rate of thrombin inhibition, a longer chain heparin species is required, that nevertheless also contains the same pentassacharide (Lindahl et al., 1980; Casu et al., 1981; Choay et al., 1983; Olson & Björk, 1991). Most of the rate enhancement results from simultaneous binding of both thrombin and antithrombin molecules to the same heparin chain and a consequent proximation effect on the rate. In this case, the requirement for the presence of the pentasaccharide is principally to ensure tight binding of the antithrombin (Olson et al., 1992).

Chemical modification and peptide studies as well as the properties of naturally occurring antithrombin variants have implicated basic residues of antithrombin, between residues 125 and 136 as well as further toward the amino terminus, as being critically involved in heparin binding (Pecon & Blackburn, 1984; Liu & Chang, 1987; Peterson et al., 1987; Chang, 1989; Lellouch & Lansbury, 1992; Carrell et al., 1994; Tyler-Cross et al., 1994). In particular, pyridoxal phosphate modification of lysine 125 was found to abolish the affinity of antithrombin for heparin and the ability of heparin to accelerate the rate of proteinase inhibition (Pecon & Blackburn, 1984; Peterson et al., 1987). A very plausible heparin binding site that includes all of these basic residues has recently been proposed (Carrell et al., 1994) on the basis of X-ray structures of two different antithrombin species (Mourey et al., 1983; Carrell et al., 1994). Although neither antithrombin species is complexed to heparin and neither structure is necessarily identical to the solution structure of native active antithrombin, they are probably very good starting structures for modeling the heparin binding site.

On the basis of secondary structure prediction and model building, Villanueva (1984) proposed lysines 290, 294, and 297 as constituting the heparin binding site. These residues were proposed to be on the outer face of an exposed α-helix

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and to be a likely site for interaction with the highly negatively charged heparin molecule. From circular dichroism studies, which indicated that pentasaccharide and longer chain heparin species induced different spectral changes, it was proposed that there are two sites of heparin binding, a primary and a secondary binding site, with the former involved in binding the pentasaccharide and the latter in binding to additional residues (Stone et al., 1982; Evans et al., 1992). An X-ray structure of cleaved bovine antithrombin showed the location of lysines 290, 294, and 297 as a continuation of basic residues closer to the amino terminus and thus raised the possibility that lysines 290, 294, and 297 constitute the secondary heparin binding site. The existence of such a secondary heparin binding region is, however, controversial and is not supported by a number of thermodynamic, kinetic, and NMR spectroscopic studies.

To quantitatively assess the role of lysine residues 125, 290, 294, and 297, we have prepared two variant antithrombins, K125M and K290M,K294M,K297M, by expressing them in baby hamster kidney (BHK)[1] cells and determining their functional properties. The lysine→methionine changes represent a structurally conservative substitution, since the two side chains are approximately isosteric, though the substitutions were deliberately nonconservative electrostatically. Alterations in heparin affinity or heparin rate acceleration should therefore primarily reflect the effect of loss of the positive amino moiety. This is in contrast to potential larger-scale structural alteration or accessibility changes resulting from the introduction of a bulky pyridoxal phosphate group.

We found that K125M antithrombin had significantly reduced affinity for both heparin pentasaccharide and long-chain high-affinity heparin and had a higher basal rate of factor Xa inhibition compared to wild-type antithrombin. The mechanisms of heparin-induced acceleration of the rates of inhibition of thrombin and factor Xa were not, however, affected. Replacement of the three lysines at positions 290, 294, and 297 by methionine resulted in an antithrombin with properties very similar to those of wild-type antithrombin. We conclude that lysine 125 plays an important role in heparin binding, but is not critical for acceleration of proteinase inhibition. Lysines 290, 294, and 297 do not appear to constitute a secondary heparin binding site.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis of human antithrombin cDNA to create the two lysine→methionine variants was carried out in M13mp19 as previously described (Gettins et al., 1993), using a single-stranded uracil-containing template and the antisense oligonucleotides 5′TCG GCA GTT CAG CAT GGC AAA GAA GAA GT 3′ and 5′G GGT GAG TTC CAT CTC CAC CAT GGC CAG GCT CAT CTC AGG CTT GG3′ for the K125M and K290M,K294M,K297M changes, respectively: the underlined triplets correspond to the lysine to methionine changes. The products of the *in vitro* extension and ligation reactions were used to transform *Escherichia coli* NK7085 or UT481 for K125M and K290M,K294M,K297M, respectively. Positive plaques were identified by hybridization with the oligonucleotides used for mutagenesis. Confirmation of the desired changes was obtained by dideoxy sequencing (Sanger et al., 1977) of single-strand phage DNA. Double-stranded M13mp19 containing the correctly mutated antithrombin cDNAs was digested with *SalI* and *XbaI*, and the products were separated by electrophoresis on an agarose gel. The *SalI*–*XbaI* fragment covering the whole of the coding region of human antithrombin (Gettins et al., 1993) was ligated into the expression vector pMAStop (Zettlmeissl, 1990) cut with the same restriction endonucleases to create a new variant antithrombin expression plasmids pMAAT3-K125M and pMAAT3-K290M,K294M,K297M. Identification of positive transformants was by restriction digestion with *SalI* and *XbaI*. The presence of the desired mutations in the expression plasmids was confirmed by sequencing of the double-stranded plasmid.

Expression and Purification of Recombinant Human BHK Cells. BHK cells were transfected with pMAAT3-K125M or pMAAT3-K290M,K294M,K297M and plasmids pRMH140 and pSV2dhfr, as previously described for wild-type antithrombin (Zettlmeissl et al., 1988). Stably transfected cells were selected by resistance to neomycin and methotrexate, as described (Zettlmeissl et al., 1988), and grown to confluence in roller bottles. Antithrombin was isolated from the growth medium of serum-free cycles by affinity chromatography on heparin–Sepharose using a linear salt gradient for elution. Further purification involved passage over G150 and, to remove any contaminating heparin, over DE52 ion-exchange resin. Isoforms, defined by differences in affinity for heparin, were kept separate and identified by SDS–PAGE and lectin staining. The purity of the preparations was judged to be >95% by SDS–PAGE. Antithrombin concentrations were determined spectrophotometrically using values of $E^{0.1\%}_{280nm} = 0.65$ and M_r 57 400 obtained for human plasma antithrombin (Nordenman et al., 1977), for both plasma antithrombin and all recombinant variants and isoforms, since it was considered unlikely that any of the lysine-to-methionine changes would have significantly altered the extinction coefficient.

Dissociation Constants of Heparin–Antithrombin Complexes. Dissociation constants (K_d) were determined at 25 °C in I0.15 buffer solution (20 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 0.1% PEG, and 1 mM EDTA) by titration of the heparin species into antithrombin at the concentration indicated and monitoring the change in endogenous tryptophan fluorescence at 340 nm with excitation at 280 nm. Heparin stock solutions were of appropriate concentration such that no more than 5% volume change occured during the titration. The antithrombin concentrations used for titrations of both pentasaccharide and full-length heparin with the K290M,K294M,K297M variant were 50 nM. For the K125M variant, antithrombin concentrations of 50 and 200 nM were used for titration with full-length heparin and with the weaker binding pentasaccharide, respectively. Data, corrected for dilution and assuming a 1:1 stoichiometry for complex formation, were fitted by nonlinear least-squares analysis using the program MINSQ II (Micromath Scientific Software, Salt Lake City, UT). The errors given in the text are the 95% confidence level from the least-squares analysis. Duplicate titrations were carried out for all heparin–antithrombin pairs and gave values that differed by no more than 15%.

[1] Abbreviations: BHK, baby hamster kidney; AT, antithrombin; K125M, variant in which lysine at position 125 has been replaced by methionine; K290M,K294M,K297M, variant in which lysines at positions 290, 294, and 297 have all been replaced by methionine.

Rate of Inhibition of Factor Xa and Thrombin. Rates of antithrombin inhibition of factor Xa and of thrombin were determined under psuedo-first-order reaction conditions by discontinuous assay of residual enzyme activity using the chromogenic substrates Spectrozyme Xa (100 μM final concentration) (American Diagnostica Inc., Greenwich, CT) and S2238 (100 μM final concentration), respectively. Fifty microliters of the antithrombin−proteinase incubation reaction was diluted to 1 mL. Measurements were made at 25 °C in 20 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 0.1% PEG, and 1 mM EDTA (I0.15) using a thermostated Shimadzu 2101PC spectrometer. The pseudo-first-order rate constants were determined from semi-log plots of residual enzyme activity against time. The incubations of antithrombin with proteinase used antithrombin concentrations of 0.8 or 1.0 μM, and proteinase concentrations (factor Xa and thrombin) of 20 nM. For reactions carried out in the absence of heparin, the second-order rate constant (k_{uncat}) was determined by dividing k_{obs} by the antithrombin concentration. The heparin-catalyzed second-order rate constant was determined from the heparin dependence of k_{obs}, by plotting k_{obs} against the heparin concentration, correcting for any effect of K_D on the concentration of heparin−antithrombin complex, and using the relationship between k_{obs}, k_{uncat}, and k_{cat} to determine k_{cat}/K_m (Björk et al., 1992). Measurements were made at three or more heparin concentrations. All rate constants were determined a minimum of 2 times. In the case of the unexpectedly high basal rate of inhibition of factor Xa by the K125M variant, five separate determinations were made to confirm the reproducibility of the enhanced rate of inhibition. The rate constants quoted are the median values together with the range.

Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide slab gels (7.5%) were run according to the procedure by Laemmli (1970) and stained with Coomassie R-250.

Characterization of Antithrombin Carbohydrate. Identification of the carbohydrate present on each isoform of antithrombin was carried out using a lectin staining kit (Boehringer Mannheim) according to the manufacturer's directions. The type and extent of glycosylation have been shown to be useful in identifying the particular isoform (Fan et al., 1993). Isoform III[2] lacks the fourth carbohydrate chain, probably at residue 135, whereas form II has all four chains, most of which are biantennary, as judged by the absence of staining for Gal→GlcNAc.

Materials. High-affinity heparin of molecular weight 9000, prepared by affinity chromatography on antithrombin−agarose, and containing 1.07 antithrombin binding sites per chain (determined by titration with antithrombin at a concentration well above the K_D), was a generous gift from Dr. Steven Olson (University of Illinois−Chicago). Synthetic high-affinity heparin pentasaccharide was a generous gift of Dr. Jean Choay (Sanofi Recherche).

RESULTS

Recombinant Variant Human Antithrombins. The presence of the desired mutations in the antithrombin cDNA was demonstrated at two stages of the mutagenesis−expression strategy. In addition to sequencing the mutated antithrombin cDNA in single-stranded M13mp19 phage prior to transfer to the expression plasmid, we wanted to eliminate the possibility of having reintroduced a wild-type sequence in the subsequent step of excission of the antithrombin cDNA and ligation into the expression vector by sequencing the expression plasmids pMAAT3-K125M and pMAAT3-K290M,K294M,K297M prior to transfection of the BHK cells. Dideoxy sequencing gels confirmed that the desired mutations were present in the plasmids used for transfection of the BHK cells for stable expression of the two variant antithrombins (data not shown).

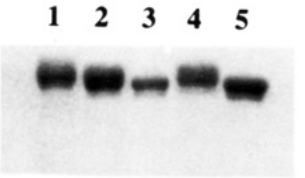

FIGURE 1: SDS−PAGE (7.5% gel) of wild-type and variant recombinant antithrombin isoforms. Lanes 1−3, wild-type antithrombin isoforms I, II, and III, respectively; lane 4, K290,294,-297M variant isoform II; lane 5, K125M variant antithrombin isoform III.

Identification of the Isoform of the Variant Antithrombins. We have previously shown that BHK-derived recombinant human antithrombin can exist in three distinct isoforms, based on affinity for heparin (Fan et al., 1993). It was therefore essential in the present study to identify each isoform of the variant antithrombins, to ensure that its properties were compared with those of the equivalent isoform of the wild-type inhibitor. Three criteria were used for this identification. One was the elution pattern from heparin−Sepharose as an indication of whether the mutation had altered the intrinsic affinity of each isoform for heparin. The second was the mobility on SDS−polyacrylamide gels, since isoform III, which is thought to bear only three carbohydrate chains rather than the four borne by isoforms I and II, has higher mobility than isoforms I and II. The third was the identity of the carbohydrate present in a given isoform.

The behavior of the K125M antithrombin variant during purification was consistent with a large reduction in the affinity of each isoform for heparin. Thus, the tightest binding antithrombin, expected to correspond to isoform III, eluted from the heparin−Sepharose column at about 0.8 M NaCl compared to 2.1 M NaCl for the equivalent wild-type isoform. Species corresponding to isoforms I and II were not isolated as pure proteins, since they eluted at much lower salt concentrations together with many contaminating proteins. In contrast, the K290M,K294M,K297M antithrombin variant behaved indistinguishably from wild-type antithrombin, suggesting that the amino acid substitutions had not altered the affinity for heparin−Sepharose. For this variant, the elution position of all three isoforms could be discerned. The two isoforms with higher affinity were purified to homogeneity, whereas the lowest affinity isoform was purified to near-homogeneity.

Confirmation of the isoform designation of the variant antithrombins was obtained from mobility on SDS−polyacrylamide gels and from lectin binding. Thus, the single isolated K125M isoform migrated at the same position as wild-type isoform III (Figure 1) and faster than wild-type isoforms I and II. K290M,K294M,K297M antithrombin migrated with mobilities equivalent to wild-type isoforms I, II, and III (form II shown in Figure 1). From lectin binding, it was seen that the K125M isoform contained significant

[2] Three isoforms of BHK-derived recombinant wild-type antithrombin have been characterized previously and designated I, II, and III, from lowest to highest affinity for heparin.

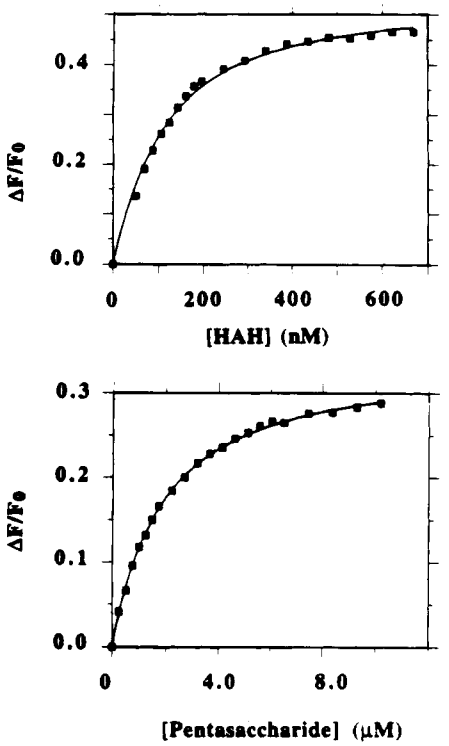

FIGURE 2: Enhancement of endogenous tryptophan fluorescence of K125M variant antithrombin upon binding heparin pentasaccharide and full-length high-affinity heparin demonstrating saturable binding by heparin and a normal enhancement of tryptophan fluorescence. The solid line is the best-fit line using the K_D values obtained from nonlinear least-squares fitting and reported in the text.

terminal mannose and very little terminal galactose (data not shown), consistent with the presence of high-mannose chains, but little highly branched complex carbohydrate, as expected for isoform III (Fan et al., 1993).

Dissociation Constants for Heparin–Antithrombin Complexes. Dissociation constants for the complexes of both K125M and K290M,K294M,K297M recombinant antithrombins with high-affinity heparin pentasaccharide and full-length high-affinity heparin were determined from the change in intrinsic fluorescence of the protein that accompanies binding of heparin. K125M variant antithrombin showed the normal enhancement of tryptophan fluorescence upon binding heparin (Figure 2). For K125M, the values of K_D were determined for the highest affinity form III isoform. For K290M,K294M,K297M antithrombin, K_D values were determined for the form II isoform, since more accurate determinations of the K_D can be made for this form than for the higher affinity form III isoform (Fan et al., 1993; Turko et al., 1993). Comparison of the K_D values of the variant and wild-type recombinant antithrombins confirmed the qualitative conclusion based on elution position from heparin–Sepharose. Thus, form II K290M,K294M,K297M isoform bound both pentasaccharide and full-length heparin indistinguishably from form II wild-type (Figure 3), with dissociation constants of 29 ± 12 and 10 ± 4 nM for heparin pentasaccharide and full-length heparin, respectively. In contrast, form III K125M had greatly reduced affinity compared to wild-type antithrombin form III (Figure 3), with dissociation constants of 1700 ± 220 and 70 ± 23 nM for complexes with pentasaccharide and full-length heparin, respectively, compared with values of 10 and 2.3 nM, respectively, for wild-type form III antithrombin (Figure 3). The different reductions in affinity for pentasaccharide and full-length heparin are significant and cannot be explained

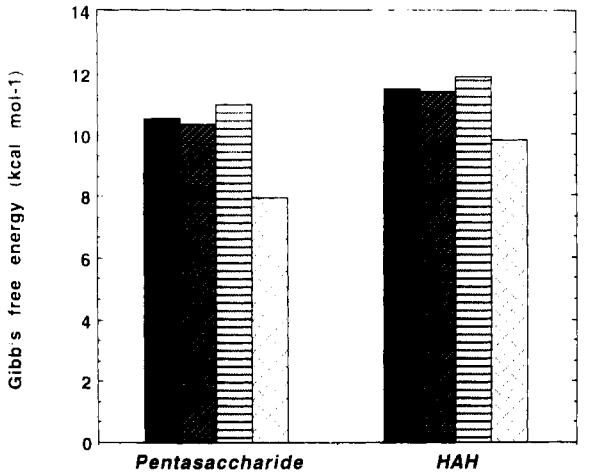

FIGURE 3: Gibb's free energies of binding of wild-type and variant antithrombins to heparin pentasaccharide and full-length heparin calculated from the dissociation constants for the heparin–antithrombin complexes. Left side, complex with synthetic pentasaccharide; right side, complex with full-length high-affinity heparin. Data are given for wild-type antithrombin isoforms II (solid) and III (horizontal stripes), for K290,294,297M isoform II (diagonal stripes) and for K125M isoform III (cross-hatched). Values for the K290,294,297M variant should be compared to the values for wild-type isoform II. Values for the K125M variant should be compared to the values for wild-type isoform III.

as resulting from an error in estimation of the very tight K_D for binding full-length heparin to wild-type isoform III, since the latter was estimated by extrapolation from accurately determined weaker K_Ds measured at higher ionic strengths (Fan et al., 1993).

Rates of Inhibition of Factor Xa and Thrombin in the Absence and Presence of Heparin. The rates of inhibition of factor Xa and thrombin were determined in the absence and presence of heparin for both variant antithrombins and for wild-type form II antithrombin (Table 1). With the exception of the inhibition of factor Xa by the K125M variant, the basal rates of proteinase inhibition (Figure 4) and the heparin-catalyzed rates of inhibition by the variant antithrombins (Figure 5) were similar, though not identical, to those of wild-type antithrombin. The rate of inhibition of factor Xa by K125M antithrombin was consistently found (five measurements) to be between 2- and 3-fold higher than by wild-type antithrombin. The higher rate did not appear to be due to any contamination by heparin, since the same rate was found in the presence of 100 μM polybrene. Despite this difference, the heparin-catalyzed rates of inhibition of factor Xa and thrombin by this variant were normal. It should be noted that the rate constants determined here for the full-length heparin-catalyzed enhancement of thrombin inhibition are lower than those reported by Olson et al. (1992). This may be due to different conditions used for determination of the rate constants. In the work of Olson et al., a fully-heparin-saturated antithrombin was used in a single-turnover reaction, whereas here measurements were made under conditions of catalytic activation by heparin. However, since rate constants were determined in the present study both for the variants and for wild-type antithrombin under equivalent conditions, comparison of the properties of normal and variant forms seems to be justified.

DISCUSSION

By comparing the properties of the K125M and K290,-294,297M variant antithrombins with those of wild-type

Table 1: Rates of Inhibition of Factor Xa and Thrombin by Wild-Type and Variant Antithrombins in the Absence and Presence of Full-Length and Pentasaccharide Heparins

antithrombin	factor Xa inhibition (M^{-1} s^{-1})			thrombin inhibition (M^{-1} s^{-1})	
	alone	+P-sacc	+HAH	alone	+HAH
wild-type	$(2.6 \pm 0.7) \times 10^3$	$(6.3 \pm 0.8) \times 10^5$	$(10 \pm 1.4) \times 10^5$	$(9.1 \pm 0.9) \times 10^3$	$(8.2 \pm 0.6) \times 10^6$
K290, 294, 297M	$(2.3 \pm 0.3) \times 10^3$	$(6.6 \pm 1.4) \times 10^5$	$(8 \pm 0.8) \times 10^5$	$(6.7 \pm 0.9) \times 10^3$	$(7.8 \pm 0.7) \times 10^6$
K125M	$(6.9 \pm 1.1) \times 10^3$	$(7.4 \pm 0.6) \times 10^5$	$(11 \pm 1.3) \times 10^5$	$(7.9 \pm 1.2) \times 10^3$	$(9.4 \pm 0.6) \times 10^6$

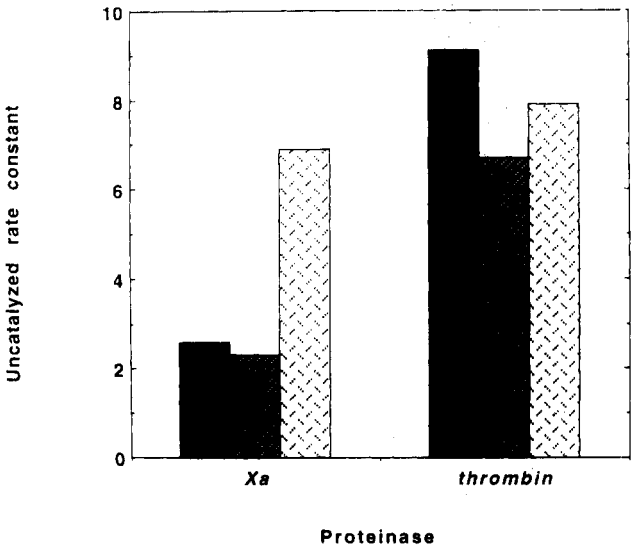

FIGURE 4: Rate constants for inhibition of factor Xa (left side) and thrombin (right side) by wild-type and variant antithrombins. Values are given in units of mM^{-1} s^{-1}. From left to right, wild-type antithrombin form II (solid bar); K290,294,297M form II (diagonal stripe); and K125M form III (cross-hatched).

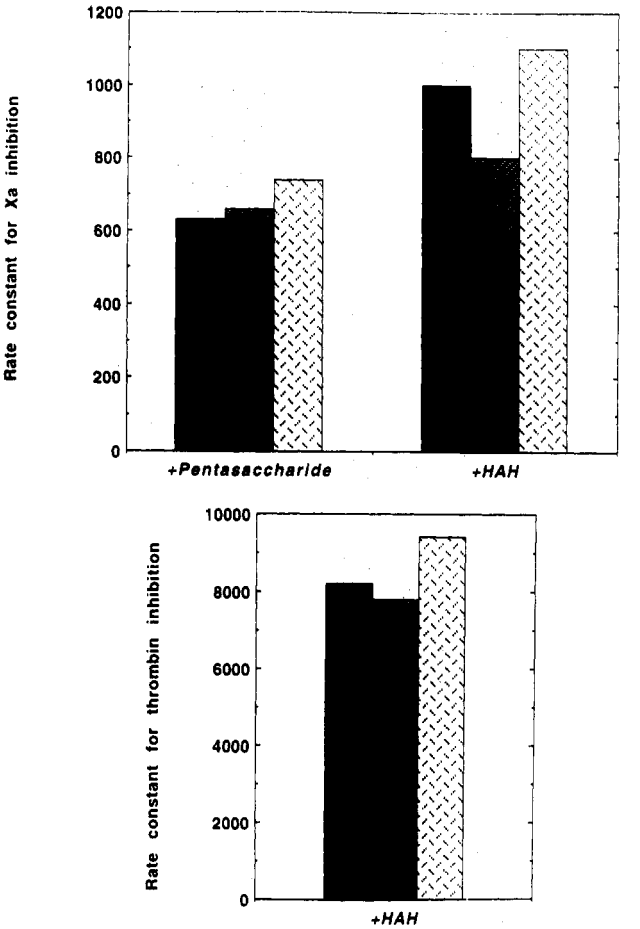

FIGURE 5: Rate constants for inhibition of factor Xa and thrombin by antithrombin–heparin complexes for wild-type and variant antithrombins. Values (units of mM^{-1} s^{-1}) are given for the fully saturated 1:1 heparin–antithrombin complex, obtained by extrapolation. Top panel: inhibition of factor Xa antithrombin–pentasaccharide complex (left) and antithrombin–full-length heparin complex (right). Bottom panel: inhibition of thrombin–antithrombin–full-length heparin complex. Wild-type antithrombin (solid); K290,294,297M (diagonal stripe); K125M (cross-hatched). Although the values for inhibition of thrombin by the antithrombin–full-length heparin complex are lower than reported in the literature for plasma antithrombin, comparison of the values for the variant antithrombins should be made to those reported here for the wild-type recombinant protein, which were determined under identical conditions to those for the variants.

recombinant antithrombin, we have been able to examine the roles of lysine 125 and of the trio of lysines 290, 294, and 297 in determining the affinity of antithrombin for heparin and in influencing the rates at which antithrombin inhibits factor Xa and thrombin. Whereas lysine 125 has a marked affect on the affinity of antithrombin for heparin, the three lysines at positions 290, 294, and 297 do not. Although the importance of lysine 125 had already been indicated by chemical modification studies (Pecon & Blackburn, 1984; Peterson et al., 1987), it had not been possible to quantitate the contribution of lysine 125 to the binding or to the rate of proteinase inhibition, due to the grossly-perturbing nature of the chemical modification, which involved introduction of a pyridoxyl phosphate group. We have now shown that lysine 125 also influences the basal rate of inhibition of factor Xa, though it is not required to permit attainment of the maximum rate of inhibition of factor Xa that is brought about by the heparin-induced conformational change. These findings are consistent with lysine 125 being in the binding site for the heparin pentasaccharide and affecting the conformation both of the heparin binding site and of the reactive center loop to which it is allosterically coupled (Gettins et al., 1993). In contrast, lysines 290, 294, and 297 appear not to be involved in heparin binding or, to any major extent, in the mechanism of heparin activation of antithrombin.

The effect of the lysine→methionine mutation at position 125 on the rates of the inhibition of factor Xa and thrombin is very interesting. It has been shown previously that the enhancement of the rate of proteinase inhibition brought about by heparin binding is almost all due to conformational change in the case of factor Xa (Olson et al., 1992) and almost all due to bridging in the case of thrombin inhibition (Olson & Björk, 1991). It has also been shown that binding of oligosaccharides in the heparin binding site causes a change in conformation of the antithrombin that is transmitted to the P1 arginine in the reactive center loop and that the resulting enhancement of factor Xa inhibition correlates quite well with the magnitude of the conformational change at P1 (Gettins et al., 1993). The higher basal rate of factor Xa inhibition by K125M variant antithrombin thus suggests an altered conformation of the P1 arginine. This in turn may result from a small alteration in conformation in the heparin binding site as a result of the lysine→methionine mutation,

that partially mimics the normal heparin-induced conformational change. The absence of an effect on the basal rate of thrombin inhibition as a result of such a small conformational change is understandable since the full heparin-induced conformational change results in only a 2-fold rate enhancement (Olson et al., 1992). Similarly, if the effect of the lysine→methionine mutation is to bring about part of the normal heparin-induced conformational change, it is not surprising that the maximal rates of factor Xa inhibition by K125M antithrombin–heparin complexes should be the same as for wild-type antithrombin–heparin complexes since the final antithrombin conformation would be the same in each case.

The magnitude of the reduction in binding energy of both pentasaccharide and full-length heparin upon replacement of lysine by methionine at position 125 is also consistent with an alteration in the conformation of the heparin binding site. Comparison of the affinity of equivalent heparin species to wild-type form III antithrombin and to K125M variant showed that the mutation caused a reduction in binding energy ($\Delta\Delta G$) of 3.1 and 2.0 kcal mol^{-1} for pentasaccharide and full-length heparin, respectively, which represent reductions of 27% and 17%, respectively, in binding energy. This compares with an anticipated reduction of only about 1 kcal mol^{-1} (5-fold increase in dissociation constant) for the loss of one ionic interaction (Olson et al., 1992). It is known from studies on plasma antithrombin that heparin binding is a two-step process involving initial ionic interaction followed by a step in which the major conformational change occurs (Olson et al., 1981). The lysine→methionine mutation may therefore affect both steps in this process, with loss of about 1 kcal mol^{-1} for the first ionic interaction step and a further loss of 1.0–2.1 kcal mol^{-1} in the second step as a result of the proposed alteration in antithrombin conformation caused by the mutation. It is not clear why there was a different reduction in affinity for the pentasaccharide compared to full-length heparin, since the latter contains the former, though this has been seen for another heparin binding site mutant antithrombin, W49K (Gettins et al., 1992).

In conclusion, this study has permitted a more complete analysis of the role of lysine residues in the functioning of antithrombin to be carried out than was possible by chemical modification alone. It was found that what had been anticipated to be a simple disruption of a single ionic interaction (resulting from substitution of charged lysine by uncharged but isosteric methionine) was more complicated. The study also showed that a residue that may be responsible for a quarter of the binding energy of heparin for antithrombin can still not be considered "essential" either for heparin binding per se or for the heparin enhancement of proteinase inhibition, since both of these processes still occur in the same way in the K125M variant, albiet requiring higher concentrations of heparin for the process to go to completion. This finding, together with other data that show that heparin species which do not contain the critical pentasaccharide can nevertheless give intermediate conformational changes and intermediate enhancements of the rate of factor Xa inhibition (Gettins et al., 1993), undermines the idea that the heparin-induced conformation change is an all-or-nothing phenomenon that has absolute structure requirements for both the heparin and antithrombin components. Rather, it appears as through a range of conformational changes may be possible with a corresponding range of factor Xa rate accelerations. This raises the possibility that there may be even more effective rate accelerators of factor Xa inhibition that the synthetic pentasaccharide of Choay and colleagues.

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