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Interaction of Factor Xa with Heparin Does Not Contribute to the Inhibition of Factor Xa by Antithrombin III-Heparin†

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ABSTRACT: Factor Xa modified by reductive methylation (>92%) loses the capacity to bind heparin as determined both by gel chromatography and by sedimentation equilibrium ultracentrifugation. The kinetic properties of methylated factor Xa differ, with respect to K_M and V_{max} for a synthetic tripeptide substrate and for antithrombin III inhibition rate constants, from those of the unmodified enzyme. The 10000-fold rate enhancement elicited by the addition of heparin to the antithrombin III inhibition reaction, however, is the same. The observed second-order rate constants (k''_{obs}) for antithrombin III inhibition of factor Xa and methylated factor Xa are 3000 and 340 M^{-1} s^{-1}, respectively, whereas k''_{obs} values for the inhibition of factor Xa or methylated factor Xa with antithrombin III-heparin are 4×10^7 and 3×10^6 M^{-1} s^{-1}, respectively. These findings provide direct evidence that the interaction of factor Xa with heparin is not involved in the heparin-enhanced inhibition of this enzyme.

Heparin is a sulfated glycosaminoglycan found in the basophilic granules of mast cells and various mammalian tissue (Jorpes et al., 1937; Nader & Dietrich, 1989). The degree and type of sulfation and oligosaccharide sequence and length are determinants for binding to the glycoprotein antithrombin III (Andersson et al., 1976; Atha et al., 1985; Lindahl et al., 1980), a member of the serpin superfamily of protease inhibitors. Complement C1s, trypsin, plasmin, kallikrein, and the clotting factors XIIa, XIa, Xa, IXa, and thrombin are inhibited by antithrombin III via the formation of a stable covalent bond between the inhibitor and the active site of the enzyme (Rosenberg & Damus, 1973). Heparin bound to antithrombin III increases the rate of inhibition by as much as 50000-fold in the case of thrombin and to variable, but smaller degrees, for the other enzymes (Ogston et al., 1976; Rosenberg, 1977; Jordan et al., 1980; Holyaerts et al., 1984).

The mechanism of the rate enhancement induced by heparin has proven problematic; inherent in this system is the difficulty in attributing either heparin-inhibitor or heparin-protease interactions to change in the rate of inhibition. Substantial evidence indicates a requirement for both interactions for the

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inhibition of thrombin. Particularly, 18 monosaccharides is the minimal length that enhances the rate of thrombin inhibition, which increases with increasing polysaccharide chain length (Laurent et al., 1978; Holmer et al., 1981; Danielsson et al., 1986; Nesheim et al., 1986; Holyaerts et al., 1984). Furthermore, inhibition of thrombin by antithrombin–heparin is more sensitive to ionic strength than would be predicted solely by an effect on heparin binding to antithrombin III (Olson et al., 1986a). Finally, Hoylaerts et al. (1984) found that active-site-blocked thrombin blocks inhibition of thrombin by covalent antithrombin–heparin complexes.

The contribution of a protease–heparin interaction to the inhibition of activated factor X by antithrombin III and heparin remains unknown. The conclusion that an interaction between factor Xa and heparin is not required is based on kinetic arguments (Jordan et al., 1980) and the findings that, in contrast to thrombin, the inhibition of factor Xa by antithrombin–heparin is less dependent on the length of the heparin polysaccharide and is less sensitive to ionic strength (Holmer et al., 1981; Danielsson et al., 1986; Olson et al., 1986a). In the present study, the role of heparin binding to factor Xa in the reaction with antithrombin–heparin is investigated by using a modified form of the enzyme that does not bind heparin. The results provide direct evidence that a heparin–factor Xa interaction is not required in the heparin-enhanced inhibition of this enzyme.

Materials and Methods

Materials

Heparin, bovine albumin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),[1] Tris (TRIZMA), dithiothreitol, iodoacetamide, lyophilized *Vipera russelli* venom, and DEAE-Sephadex were purchased from Sigma Chemical Co., St. Louis, Mo. Formaldehyde was purchased from Curtin Matheson Scientific, Houston, TX; guanidinium chloride, ultrapure grade, was from ICN Biomedicals, Costa Mesa, CA; trinitrobenzenesulfonic acid and $NaCNBH_3$ were from Aldrich; sodium dodecyl sulfate and trifluoroacetic acid were from Pierce, Rockford, IL; Spectrozyme FXa (MeO-CO-D-CHG-Gly-Arg-pNA AcOH) was from American Diagnostica, New York, NY; (*p*-amidinophenyl)methanesulfonylfluoride (aPMSF) was from Chemicon, Los Angles, CA; azure A was from MCB, Gibbstown, NJ; acetonitrile (UV-grade) was from Burdick and Jackson, Muskegon, MI; and lysyl endopeptidase was from Wako Chemicals, USA, Richmond, VA.

Proteins. Bovine factor X was isolated as described previously (Owen, 1975; Lollar et al., 1984) and purified further and concentrated on an additional QAE-Sephadex column with gradient elution from 0.15–0.55 M NaCl/0.02 M Tris-HCl, pH 7.5. The purified factor X preparation (1.5 mg/mL) was dialyzed in 0.1 M NaCl/0.05 M HEPES-HCl, pH 7.2, and stored at −70 °C. Bovine antithrombin III was purified as previously described (Thaler & Schmer, 1975) but with an additional further purification and concentration step on DEAE-Sephadex with gradient elution from 0.1–0.6 M NaCl/0.02 M Tris-HCl, pH 7.5. The purified antithrombin III preparation (1.5 mg/mL) was dialyzed into 0.1 M NaCl/0.05 M HEPES-HCl, and stored at −70 °C. The factor X clotting protein (X-CP) was purified from lyophilized *Vipera russelli* venom (Kisiel et al., 1976).

For some experiments, factor Xa was inactivated with aPMSF. When used in molar excess at pH 7, factor Xa activity was lowered to below detection limits within less than 5 min at room temperature.

Methods

$NaCNBH_3$ was recrystallized according to the procedure of Jentoft and Dearborn (1979), dried, and stored desiccated at room temperature.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was applied to samples dialyzed in 0.1 M NaCl/0.05 HEPES-HCl, pH 7.5; gradient gels (8–25%) were developed by silver staining (PhastSystem, Pharmacia). Reverse-phase HPLC was performed with an Isco System Model 2350. Samples (100 μL) were injected onto a Vydac C_3 (4.6 mm × 7.5 cm) column and were eluted with 0.05% trifluoroacetic acid and a two-step gradient: a 10-min linear gradient of 2–40% acetonitrile followed by a 30-min linear gradient of 40–70% acetonitrile. Column effluent was monitored at 214 nm, and data were analyzed with a ChemResearch chromatography software package (Microsoft Corp.).

Modification and Characterization of Methylated Factor X. Factor X was modified with formaldehyde as the methylating reagent (Jentoft & Dearborn, 1983). A typical reaction mixture contained 1 mL of factor X (1.5 mg/mL) in 0.05 M HEPES-HCl (pH 7.0), 0.62 mg of $NaCNBH_3$ and 2 μL of formaldehyde. A maximal modification yield was obtained with a 10-fold molar excess of alkylating reagents over lysine residues. No significant increase in the extent of methylation occurred after 2 h, at which point samples were removed and dialyzed in 0.05 M HEPES-HCl (pH 7.2)/0.1 M NaCl.

The extent of modification was estimated by the trinitrobenzenesulfonic acid method (Habeeb, 1966) with unmodified factor X as a standard. To confirm this estimate and further establish the extent of modification, methylated protein was digested with lysyl endopeptidase. The samples in 6 M guanidinium chloride were reduced and carboxymethylated (Edelman et al., 1968), then diluted by half with 0.1 M HEPES-HCl (pH 7.0), and digested with lysyl endopeptidase (10 μg/mL) for 12 h at 30 °C. Activated factor X or methylated factor Xa was pretreated with aPMSF to prevent autolysis during sample preparation. Digested samples were analyzed by electrophoresis and C_3 reverse-phase chromatography.

Heparin Binding. The binding of heparin to activated native or methylated factor X was assayed by gel chromatography using an FPLC (Pharmacia) liquid chromatography system. Samples (50 μL) were injected onto a Superose 6 HR 10/30 column and were eluted with 0.05 M NaCl/0.02 M Tris-HCl, pH 7.5. Detection of column effluent was at 280 nm, and 0.5-mL fractions were collected. The chromatographic profile of free heparin was determined by assaying fractions for azure A binding (Nesheim, 1983).

Enzyme Kinetics. Activated factor X activity was measured by the release of *p*-nitroaniline from Spectrozyme FXa. The assay buffer consisted of 0.05 M NaCl, 0.1 M Tris-HCl, and 0.1 mg/mL albumin, pH 7.5. Inhibition of activated factor X by antithrombin III or antithrombin III and heparin was assayed by adding enzyme (2 μL) and inhibitor with or without heparin (10 μL) to a reaction vessel containing buffer (388 μL) and a magnetic stir-bar. The components were allowed to react for a specific time after which 50 μL of 5 M NH_4Cl (0.5 M final concentration) and 50 μL of 1 mM Spectrozyme FXa (0.1 mM final concentration) were added in rapid succession. Vigorous stirring was maintained throughout. The

[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; aPMS, (*p*-amidinophenyl)methanesulfonyl; Tris, tris(hydroxymethyl)aminomethane; X-CP, factor X activator from *Vipera russelli* venom.

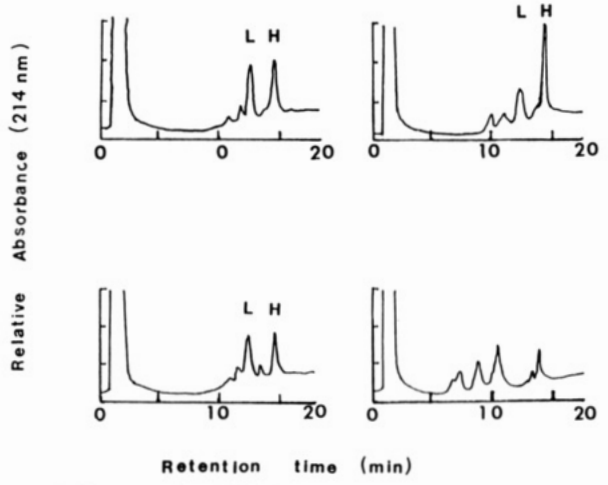

FIGURE 1: Reverse phase (C_3) chromatography of lysyl endopeptidase digests of methylated or native factor Xa. (Upper left) Methylated factor Xa, untreated; (lower left) methylated factor Xa (750 μg/mL), digested with lysyl endopeptidase (10 μg/mL) for 12 h, 30 °C; (upper right) native factor Xa; (lower right) factor Xa (750 μg/mL), digested with lysyl endopeptidase (10 μg/mL) for 12 h, 30 °C. Factor Xa preparations were inactivated with aPMSF and then reduced and carboxymethylated.

contents of the tube were transferred immediately to a cuvette, and the change of absorbance at 405 nm with time was determined.

Ultracentrifugation. Sedimentation equilibrium analysis at 17 °C was carried out with a 6-place (AN-H) rotor in a Beckman Prep-Scanner analytical ultracentrifuge. Preparations of factor Xa or methyl-factor Xa inactivated with aPMSF were desalted by chromatography on Sephadex G-50 equilibrated in 0.13 M NaCl/0.02 M HEPES-HCl, pH 7.5. Heparin was added to the protein preparations by dilution (1:100) from aqueous concentrates. Low-speed sedimentation equilibrium in long columns (0.2 mL) was attained in 16 h by an initial 2.5-h overspeed (32000 rpm) and then equilibration at 9000 rpm. Each run comprised two samples with heparin, two without, and one cell containing 0.25 mM tris(hydroxymethyl)methylammonium salicylate (A_{280} = 0.7) to verify linearity of the scanner. Scans (λ_{max} = 280 nm) were digitized and stored in a Bascom-Turner electronic recorder where, if necessary, base lines were set to zero, and then the data were transferred to a PC-DOS file with a program obtained from On-Line Instrument Systems, Jefferson, GA. The data were analyzed as suggested by Johnson et al. (1981) with a robust fitting procedure described by Matheson (1990). The fits were implemented with an 80286/386 microcomputer program obtained from On-Line Instrument Systems. Partial specific volumes used in calculations were 0.47 for heparin (Barlow et al., 1961), 0.71 (calculated) for factor Xa (Cohn & Edsall, 1943), and 0.66 (weight-averaged) for factor Xa–heparin. Molecular weight distributions in heparin fractions after Biogel P-60 gel chromatography were determined as described previously (Nesheim et al., 1986) by meniscus depletion sedimentation equilibrium (Yphantis, 1964) in a Beckman Model E analytical ultracentrifuge.

RESULTS

A 10-fold molar excess of alkylating reagents to lysine results in greater than 92% methylation of the lysine residues of bovine factor X as determined by trinitrobenzenesulfonic acid. To confirm the extent of methylation and to determine if any resistant lysine residues remained, the methylated derivative and native factor X, or the activated enzymes, were denatured and treated with lysyl endopeptidase, which cleaves

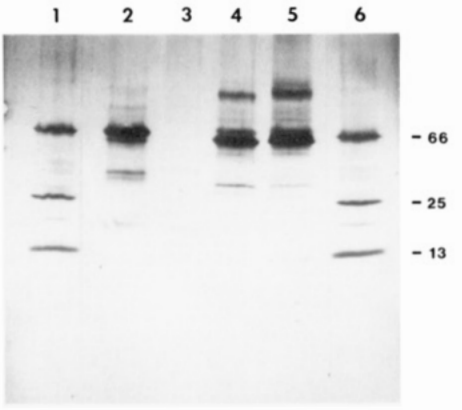

FIGURE 2: Gel electrophoresis in dodecyl sulfate of lysyl endopeptidase digest of methylated or native factor X. Lanes 1 and 6, molecular weight markers, molecular weight as indicated ($\times 10^{-3}$); lane 2, factor X; lane 3, factor X (750 μg/mL) digested with lysyl endopeptidase (10 μg/mL), 12 h, 30 °C; lane 4, methylated factor X; lane 5, methylated factor X (750 μg/mL) digested with lysyl endopeptidase (10 μg/mL), 12 h, 30 °C.

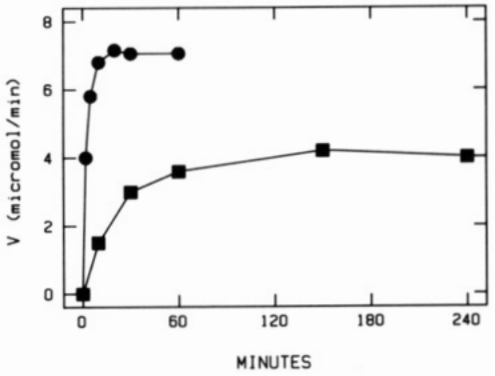

FIGURE 3: Activation of factor X and methylated factor X. Native (●) or methylated (■) factor X (1.5 mg/mL) was incubated with X-CP (15 μg/mL) in 10 mM $CaCl_2$, 0.1M NaCl, and 0.05 M HEPES-HCl, pH 7.5, at room temperature. At the times indicated, the activation mixture was sampled, and Spectrozyme F-Xa hydrolysis was measured.

specifically at unmodified lysine residues. Reverse-phase (C_3) chromatography of lysyl endopeptidase digested native and methylated factor X revealed intact heavy and light chains in the case of the methylated derivative, but the unmodified protein was degraded to peptides (Figure 1). Analysis by gel electrophoresis in dodecyl sulfate (Figure 2) showed the same results for lysyl endopeptidase treated methylated (lanes 4 and 5) and native factor Xa (lanes 2 and 3). Accordingly, analysis of the digests both by electrophoresis and by C_3 chromatography indicates that reductive alkylation does not yield a detectable subpopulation of modified protein with specific resistant lysine residues.

Methylated factor X can be activated completely by the factor X clotting protein purified from Russell's viper venom. The activation rate is 0.03 of that of native enzyme (Figure 3 and Table I). Complete activation of methylated factor X was also evidenced by SDS–polyacrylamide gel electrophoresis (not shown). The catalytic efficiency of methylated factor Xa is 0.7 of that of the native enzyme, which reflects the difference in K_m for the synthetic substrate Spectrozyme FXa (Table I). The clotting activity of the methylated factor Xa preparation is 0.005 of that of the native enzyme (Table I).

Heparin binding was assayed by gel chromatography, which distinguishes between free factor Xa and factor Xa bound to heparin (Figure 4). Crude heparin (median molecular weight 16000) was used in all experiments so as not to exclude a

Table I: Properties of Methylated Factor Xa

	factor Xa	methylated factor Xa
activation rate (X-CP)[a] (M^{-1} s^{-1})	k'' = 40 000	k'' = 1300
one stage clotting time = 40 s[b]	[Xa] = 0.01 nM	[m-Xa] = 2.0 nM
K_m[c] (M)	2×10^{-4}	4×10^{-4}
k_{cat}[c] (s^{-1})	50	70

[a] Half-life ($t_{1/2}$) was determined from the data in Figure 3; k'_{obs} was derived by dividing 0.7 by $t_{1/2}$, and k''_{obs} was derived by dividing the k'_{obs} by the concentration of the activator, X-CP. [b] Factor Xa or methyl-factor Xa in 25 mM $CaCl_2$ (100 μL) was assayed by the time required to clot 100 μL of bovine plasma to which 100 μL of phosphatidylcholine/phosphatidylserine vesicles (final concentration = 30 μg/mL) had been added. [c] K_m and k_{cat} were determined from double-reciprocal plots of initial substrate hydrolysis rates and substrate concentrations.

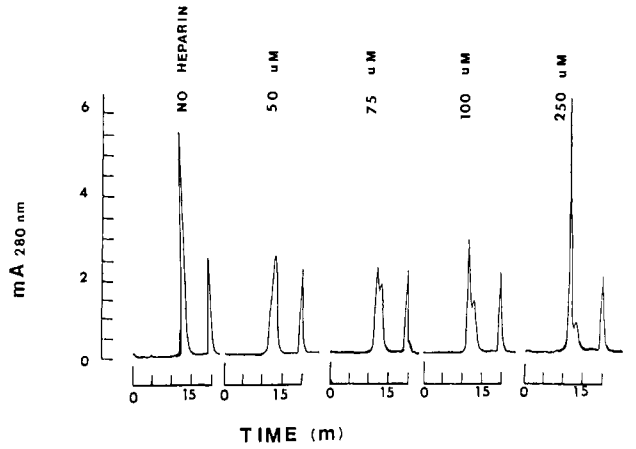

FIGURE 4: Gel chromatography of factor Xa and heparin. Samples contained 4 μM factor Xa, heparin at the concentrations indicated, and vitamin B_{12}, the internal marker peak eluted at 20 min.

population of heparin that might have a higher affinity for factor Xa. Heparin with an average molecular weight of 16 000 is eluted at a position comparable to that a 50 000–60 000 molecular weight protein. Consequently, a complex between heparin and factor Xa is eluted at a position corresponding to twice the molecular weight of free factor Xa. At λ = 280 nm, heparin is undetected by the spectrophotometer, so the size of the peak is proportional to the amount of protein. The concentration of heparin required to drive half the protein into complex is between 50 and 100 μM. A dissociation constant was not determined from these measurements since the actual concentration of heparin, protein, and heparin–protein complexes is changing throughout chromatography. The sharpness of the peak corresponding to complex implies that most of the bound heparin falls within a narrow oligosaccharide chain length and the retention time is that obtained in separate experiments with heparin fractions of M_r 16 000.

Methylated factor Xa requires at least a 5–10-fold higher heparin concentration to form complex (Figure 5). At concentrations of heparin between 100 and 250 μM, complex formation can just be detected as a shoulder on the leading edge of the protein peak.

Confirmation that the material eluted ahead of the free protein comprised protein–heparin complex was obtained by low-speed sedimentation equilibrium analysis (Figure 6). Both aPMS–factor Xa and aPMS–methyl-factor Xa appeared as homogeneous solutes having a molecular weight of 46 000 (curve 1, Figure 6A,B). Addition of heparin to the solutions in concentrations from 5 to 50 μM raised the molecular weight of factor Xa to 62 000 (Figure 6A, curve 2) but had no discernible effect on methyl-factor Xa (Figure 6B, curve 2).

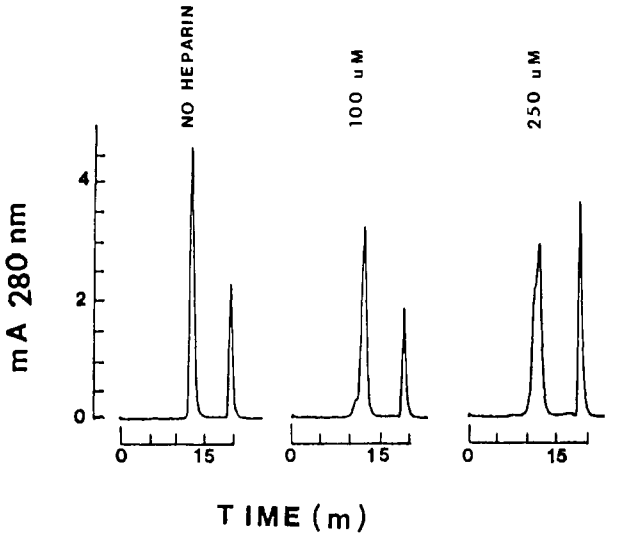

FIGURE 5: Gel chromatography of methylated factor Xa and heparin. Samples contained 4 μM methyl-factor Xa, heparin at the concentrations indicated, and vitamin B_{12}, the peak eluted at 20 min.

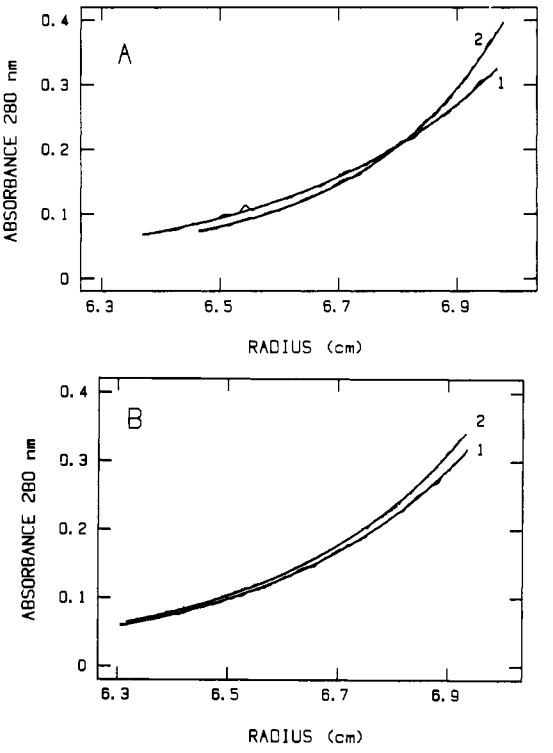

FIGURE 6: Sedimentation equilibrium of methylated factor Xa and heparin. Each curve represents data as acquired directly from the scanner, overlaid with a fit to the equation for a noninteracting monomer as described under Methods. (A) Data for 5 μM aPMS–factor Xa without (curve 1) and with (curve 2) 12 μM heparin. (B) Data for 5 μM aPMS–methyl-factor Xa without (curve 1) and with (curve 2) 12 μM heparin. The data are representative of scans of two preparations of each protein, each analyzed in duplicate, that yielded calculated molecular weights of 46 000 ± 2000 for factor Xa and methyl-factor Xa, 48 000 ± 3000 for methyl-factor Xa with 12 μM heparin, and 62 000 ± 3000 for factor Xa with 12 μM heparin.

Antithrombin III inhibits methylated factor Xa at one-tenth the rate of unmodified factor Xa (Figure 7). Kinetic measurements were made under pseudo-first-order reaction conditions where the decay of factor Xa activity was first order; the half-life of 34 min, determined graphically, was used to calculate the observed first- and second-order rate constants of inhibition, which were 3.4×10^{-4} s^{-1} and 340 M^{-1} s^{-1}, respectively. These values can be compared to the native

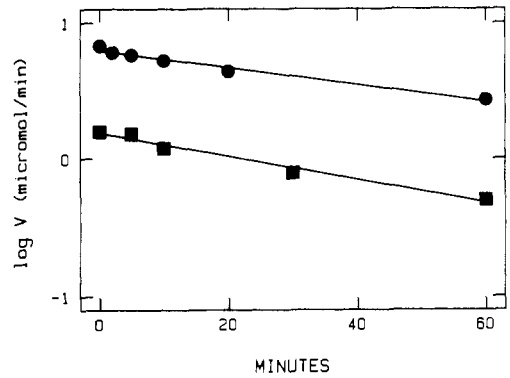

FIGURE 7: Inhibition by antithrombin III of factor Xa and methyl-factor Xa. Reaction mixtures contained 1 nM factor Xa and 100 nM antithrombin III (●) or 1 nM methyl-factor Xa and 1 μM antithrombin III (■).

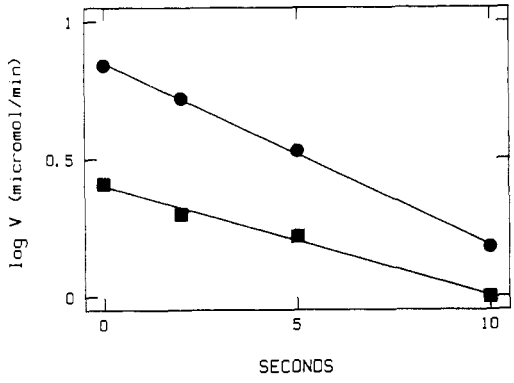

FIGURE 8: Inhibition by antithrombin III and heparin of factor Xa or methyl-factor Xa. Reaction mixtures contained 100 nM antithrombin III and either 1.0 nM factor Xa with 3 nM heparin (●) or 1.0 nM methyl-factor Xa with 30 nM heparin (■).

enzyme which is inhibited by antithrombin III with an observed second-order rate constant of 3000 M^{-1} s^{-1}.

When the heparin is saturated with antithrombin III, the observed second-order rate constant[2] for the inhibition of unmodified factor Xa is 4×10^7 M^{-1} s^{-1}. Methylated factor Xa is inhibited by antithrombin–heparin with an observed second-order rate constant of 3×10^6 M^{-1} s^{-1}, one-tenth the rate of the native enzyme (Figure 8).

The effect of heparin concentration on the rate of inhibition of native and modified factor Xa by antithrombin–heparin is shown in Figure 9. The ascending limb of the curve reflects binding of heparin to antithrombin, where the increase in inhibition rate constant is proportional to the heparin–antithrombin concentration. The descending limb of the curve reflects the decrease in the rate of inhibition by antithrombin–heparin of factor Xa bound to heparin. In the case of methylated factor Xa, the descending limb is shifted to the right to reflect the lower affinity of methylated factor Xa for heparin. Factor Xa and methylated factor Xa were assayed in parallel at all heparin concentrations to determine the effect of high concentrations of heparin on the rate of factor Xa hydrolysis of Spectrozyme FXa. At a heparin concentration of 1 mM, a 30% increase of factor Xa hydrolysis of Spectrozyme FXa was observed for methylated factor Xa, but not for native factor Xa (not shown), and at a heparin concentration of 2.5 mM, the substrate hydrolysis rate of modified

[2] Previously reported values for the observed second-order rate constants for inhibition of factor Xa by thrombin–heparin range between 7×10^5 and 7×10^6 M^{-1} s^{-1} (Craig et al., 1989; Jordan et al., 1980; Ceustermans et al., 1982).

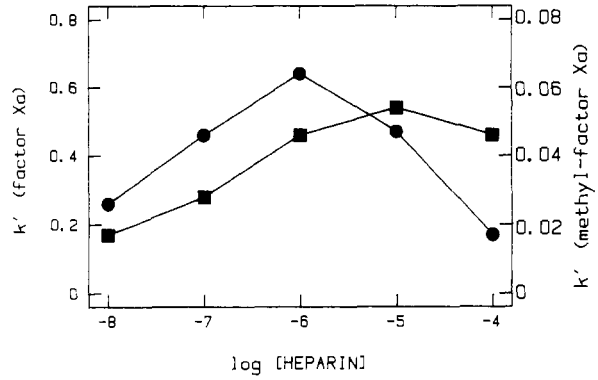

FIGURE 9: Effect of heparin on inhibition of factor Xa and methyl-factor Xa. Pseudo-first-order rate constants (k') were calculated as $0.7/t_{1/2}$ from reactions of 1 nM factor Xa (●) or 2 nM methyl-factor Xa (■) with 30 nM antithrombin III.

factor Xa approached that of the unmodified enzyme.

Discussion

The mechanism by which antithrombin III inhibits target enzymes has been a subject of investigation for nearly 2 decades. Of particular interest is the capacity of heparin to enhance the inhibition rate of the coagulation factors IXa, Xa, XIa, and thrombin by several orders of magnitude. Determining the relative importance of either heparin–antithrombin or heparin–protease interactions has proved difficult because either could cause the same effect, a change in the rate of inhibition. The role in rate enhancement of binding of heparin to the inhibitor was determined from experiments with fractions of heparin that do not bind antithrombin III and derivatives of antithrombin III that do not bind heparin (Lam et al., 1976; Rosenberg & Damus, 1973). Heparin fractions with low affinity for antithrombin III lack the ability to enhance the rate of inhibition. This evidence was later substantiated with the characterization of genetically variant antithrombins with impaired ability to bind heparin (Borg et al., 1988; Koide et al., 1984; Change & Tran, 1986; Brennan et al., 1988). Loss of heparin binding and impaired antithrombin III inhibition have also been associated with selective chemical modifications (Chang, 1989; Peterson et al., 1987; Jorgensen et al., 1985; Blackburn et al., 1981).

The contribution that a heparin–protease interaction makes to the overall rate of inhibition appears to depend on the protease involved. A heparin–thrombin interaction is required for the enhanced inhibition rate of this protease by antithrombin III and heparin. Indirect evidence has suggested that this is not the case for activated factor X. Foremost, the size of the heparin required for factor Xa inhibition can be as small as a pentasaccharide, which has been found to increase the inhibitory capacity of antithrombin III against factor Xa nearly as efficiently as heparin of 12–18 monosaccharides (Choay et al., 1983; Danielsson et al., 1986). Interestingly, heparins longer than 20 monosaccharides have been found further to increase the rate of inhibition of factor Xa by antithrombin–heparin; the basis of this, however, is unknown (Danielsson et al., 1986). Relative insensitivity to ionic strength of the inhibition of factor Xa by antithrombin–heparin has also been used as an argument that a heparin–factor Xa interaction is not obligatory for effective rate enhancement (Craig et al., 1989).

Kinetic measurements of factor Xa inactivation by antithrombin III and heparin (Jordan et al., 1980) are complicated by two factors. First, the weak nature of the heparin–factor Xa interaction exacts the use of very high heparin concen-

trations to achieve saturation of factor Xa. Second, the limiting rate constant for the reaction of factor Xa with antithrombin–heparin of 200 s^{-1} (compared to 5 s^{-1} for the reaction with thrombin) effects inactivation rates that become difficult to measure (Craig et al., 1989; Olson & Shore, 1986b).

Modification of factor Xa by reductive methylation provides an enzyme that effectively does not bind heparin. Although modified factor Xa is inhibited by antithrombin III at one-tenth the rate of the native enzyme, the finding that the rate increase upon the addition of heparin is the same, 10 000-fold, provides direct evidence that a heparin–factor Xa interaction makes no contribution to the enhanced rate of inhibition by antithrombin–heparin. We find also that, in a manner analogous to thrombin and factor IXa, heparin–factor Xa complexes are inhibited more slowly than free factor Xa by antithrombin–heparin. The significance of the differences in mechanism by which antithrombin–heparin inhibits factor Xa and thrombin remains unknown.

Registry No. Blood coagulation factor Xa, 9002-05-5; heparin, 9005-49-6; spectrozyme F-Xa, 95752-07-1.

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