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Dependence of the Rate of Thrombin/Antithrombin III Reaction upon the Turnover Rate of a Catalytic Amount of Heparin

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Commercially available heparin preparations slightly enhanced the rate of thrombin/antithrombin (AT) III reaction at pH 6.05 in the absence of NaCl. However, this accelerative activity was significantly lower than that induced by heparin with high affinity for AT III (HA-heparin), probably due to the formation of the binary complexes of HA-heparin–AT III as well as that composed of thrombin and heparin with low affinity for AT III (LA-heparin). The HA-heparin-catalyzed thrombin/AT III reaction was faster in the presence of 0.1 M NaCl at pH 6.05 than that in the absence of the salt.

LA-heparin and dextran sulfate (DS) were also found to accelerate the thrombin/AT III reaction rate, but neither substance catalyzed the formation of the complex in the presence of 0.1 M NaCl at pH 7.4. LA-heparin was also confirmed to compete with HA-heparin for enhancement of the thrombin/AT III reaction. Thus, it appears that AT III tends to form a ternary complex with the thrombin–DS or thrombin–LA-heparin complex, even in the presence of 0.1 M NaCl, whereas factor X_a reacts with the AT III–DS or AT III–LA-heparin complex.

These results indicate that HA-heparin is the only substance having the ability to catalyze the thrombin/AT III reaction, and that its turnover rate is markedly elevated in the presence of strongly electropositive and electronegative ions because of the decreased affinity of the enzyme for heparin under such conditions.

Keywords thrombin; antithrombin; factor X_a; heparin; dextran sulfate; blood coagulation

The potent anticoagulant activity of heparin largely depends upon its interaction with the circulating protease inhibitor antithrombin III (AT III) (also called heparin cofactor), which has the ability to inhibit some, if not all, of the circulating serine proteases under *in vitro* assay conditions.[1] There is general agreement that heparin enhances the slow-rate anticoagulant reaction, in which AT III normally neutralizes serine proteases of the coagulation cascade. However, the majority of investigators have studied the mechanism of catalysis only from the standpoint of the reaction beween AT III and thrombin, partly because of the difficulty in studying heparin's mechanism of action.

Since it is commonly thought that the formation of a ternary complex of heparin, AT III and thrombin is essential to the acceleration of the enzyme/inhibitor reaction,[2] the formation of binary heparin–AT III and heparin–thrombin complexes at a high concentration of heparin is inhibitory to the reaction.[2c,3] Some investigators have pointed out that there exist optimal salt concentrations for the heparin-enhanced thrombin/AT III reaction.[4] It has also been noted that although thrombin tightly binds to heparin or dextran sulfate (DS) in the absence of NaCl,[4a,5] this association largely depends upon the concentration of salt.[4a,b,5d–f] On the other hand, the interaction between HA-heparin (heparin with high affinities for AT III) and AT III seemed to be affected less by salt concentration.[4b,6] Therefore, it can be said that a decrease in thrombin/AT III reaction rate at low salt concentration is due to the formation of inhibitory binary heparin–thrombin and heparin–AT III complexes, and that DS abolishes the heparin-enhanced enzyme/inhibitor reaction through the formation of binary heparin–AT III and DS–thrombin complexes.[4,5c]

Whereas the binding of HA-heparin to AT III has been found to be stoichiometric in the enhancement of the thrombin/AT III reaction by heparin, the binding of heparin to thrombin has also been determined to be stoichiometric in the absence of NaCl.[3a,4a,5a] Although heparin is a quasi catalyst of the thrombin/AT III reaction, under limited conditions,[7] it fails to act as such unless its affinity for the inactive enzyme–inhibitor complex is reduced.[5a] The author previously reported that the interaction between thrombin and sulfated polysaccharides depends upon the charge density of the polysaccharides and is strong at pH 6.05.[5f,g] Thus, in this study, the author undertook to investigate the heparin-enhanced thrombin/AT III reaction at pH 6.05, in order to ensure a tight association of thrombin with heparin in the face of the above-mentioned paradoxical actions of heparin.

Materials and Methods

Materials Porcine intestinal mucosal heparin (168 USP units/mg) and DS (8 kDa) were purchased from Sigma Chemical Co., St. Louis, MO; bovine factor X_a was purchased from Boehringer Mannheim-Yamanouchi, Ltd., Tokyo; Boc (*tert*-butoxycarbonyl)–Val–Pro–Arg–MCA (methylcoumarylamide) and Boc–Ile–Glu–Gly–Arg–MCA were purchased from the Protein Research Foundation, Minoh, Osaka; polybrene was purchased from Aldrich Chemical Co. Inc., Milwaukee, WIS.

Bovine AT III was prepared as previously reported; bovine α-thrombin was prepared, activated and purified using methods previously described.[8] The purified proteins appeared to be homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis. These protein preparations were stored at −80 °C until use. Concentrations of the preparations were determined on weight and molar bases as previously stated;[5c] those of bovine factor X_a were estimated from its specific activity at a 55 kDa molecular weight.[9]

Heparin preparations [HA- and LA-heparins (high and low affinities for AT III) at 227 and 9 USP units/mg, respectively], purified *via* a method previously described,[10] were a kind gift from Prof. K. Nagasawa of our university.

Measurement of Activities The amidolytic activity of thrombin was fluorometrically measured using the continuous method previously reported;[5c] that of factor X_a was estimated in the same way at 460 nm with excitation at 380 nm, at 37 °C.

Tris–acetate solutions were prepared following a method previously reported.[5g]

The rate of reaction between thrombin or factor X_a and AT III was measured in the presence of substrate by mixing the enzyme with a solution containing AT III, the substrate and heparin, following the method originally introduced by Griffith.[11] The author selected this

method because it takes advantage of the competition between a substrate and an inhibitor for the active site of the enzyme. Since only a fraction of the enzyme is unprotected and is, therefore, free to react with the inhibitor in the presence of substrate, the reaction rate is slowed down. When substrate and AT III are used at suitable concentrations, the reaction can be monitored as a pseudo-first-order reaction using an ordinary spectrofluorometer operating in the derivative mode (dF/dt). For thrombin neutralization, a reaction mixture containing 1 nM thrombin, 10 nM AT III, 40 μM Boc–Val–Pro–Arg–MCA and an indicated amount of heparin in 50 mM Tris–acetate (pH 6.05) with or without 0.1 M NaCl was used, unless otherwise mentioned. The reaction mixture used for the factor X_a neutralization contained 5 nM factor X_a, 165 nM AT III, 40 μM Boc–Ile–Glu–Gly–Arg–MCA and an indicated amount of heparin in 0.1 M NaCl and 50 mM Tris–acetate (pH 7.4), unless otherwise mentioned.

In another measurement of thrombin neutralization, 25 μl of AT III (final concentration, 20 nM) and 1 μl of the enzyme were added to 949 μl of a heparin solution containing 40 mM NaCl and 0.1 M Tris–HCl (pH 7.4) in a quartz cell. The mixture was incubated at 37 °C for various periods of time, then 25 μl of 4 mM substrate containing polybrene (final concentration, 0.4 mg) was added to the thrombin residue and the residual activity was measured. Because polybrene seemed to be nonspecifically adsorbed on the surface of every cell, the cell was neutralized once with a commercially-available heparin solution (0.1 mg/ml) after the removal of each reaction mixture, and then washed thoroughly with detergent and water before reutilization.

Although NaCl or sodium ions were not removed from the protein or the sulfated polysaccharides, the concentration of sodium introduced into the reaction mixture during the process of preparation was less than 0.5 mM.

Pseudo-first-order rate constant, k', values were determined using the least-squares analysis of initial reaction rates. Second-order rate constant, k'', values were calculated using a plot of ln [AT III/(AT III $-i$)] *vs.* time, as described by Downing *et al.*[12]

Results and Discussion

Interaction of Heparin with AT III and Thrombin at pH 6.05 Since the direct interaction between thrombin and heparin is stronger at pH 6 than at higher pH values,[5g] the author carried out the experiments described herein at pH 6.05. The inactivation of thrombin in a dilute solution was significantly enhanced by commercially available heparin at pH 6.05, even in the presence of synthetic substrate (Fig. 1A). It has been confirmed that the rate of substrate hydrolysis is linearly correlated to time in the presence of an excess amount of substrate, even though heparin exerts an instantaneous activating or inhibiting action on thrombin, depending upon the conditions used; therefore, the hydrolysis rate decreases over time as a result of thrombin inactivation when an excess amount of substrate is present. In this study, the degree of thrombin inactivation increased with increasing heparin concentration, up to 100 nM in the case of commercially available heparin. In the presence of substrate, the neutralization of thrombin by AT III was also enhanced by a commercially available heparin (Fig. 1B). However, the heparin-enhanced thrombin/AT III reaction did not display pseudo-first-order kinetics under the conditions used. Since AT III in a dilute solution was irreversibly inactivated time-dependently within 4 min at pH 6.05 at 37 °C (data not shown), as noted by other investigators,[13] the deviation of the heparin-enhanced thrombin/inhibitor reaction from the pseudo-first-order kinetics was thought to result from fast inactivation of AT III.

In the presence of HA-heparin, the thrombin/AT III reaction was markedly enhanced; namely, within 2 min, more than 90% of the initial thrombin was neutralized by AT III in the presence of HA-heparin at an amount of more than 1 nM, while only about 13% was neutralized by AT III alone, within the same time. In the presence of 0.1 nM HA-heparin, more than 60% of thrombin was inactivated by AT III (Fig. 2A). In the presence of 0.1 M NaCl, HA-heparin alone protected thrombin against inactivation (Fig. 2B). Since it has been reported that DS protects thrombin against inactivation in the presence of NaCl, even though DS stimulated thrombin inactivation in a dilute solution in the absence of NaCl,[5d–f] the protecting effect against thrombin inactivation in the presence of 0.1 M NaCl can be said to be commom to highly sulfated polysaccharides. Paradoxically, thrombin was neutralized by AT III in the presence of HA-heparin at a very fast rate when 0.1 M NaCl and 50 mM Tris–acetate were present at pH 6.05. HA-heparin (0.1 nM) enhanced the neutralization of more than

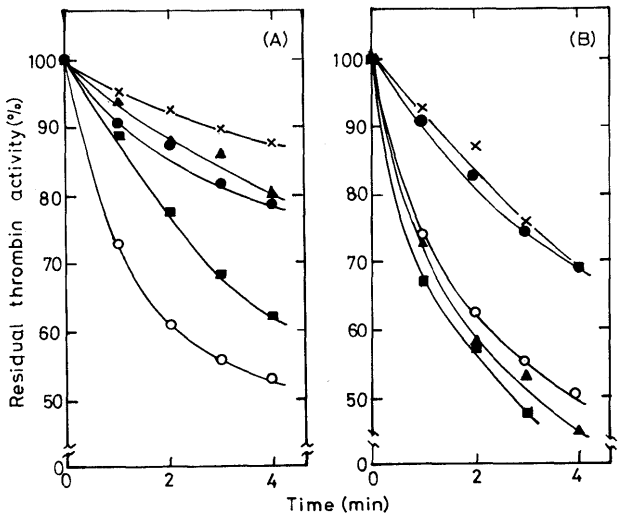

Fig. 1. Effect of Commercially Available Heparin on Thrombin Inactivation (A) and on the Thrombin/AT III Reaction (B) in the Presence of Synthetic Substrate

(A) Residual thrombin activity was measured at the indicated times through continuous monitoring of fluorescence intensity in a reaction mixture containing 40 μM Boc–Val–Pro–Arg–MCA and 1 nM thrombin with or without (×) an indicated amount of commercially available heparin in 50 mM Tris–acetate (pH 6.05). (B) Residual thrombin activity in a reaction mixture containing 40 μM substrate, 1 nM thrombin and 10 nM AT III with or without (×) an indicated amount of commercially available heparin was measured using the same procedure as described in (A). ●, 0.1 nM; ▲, 1 nM; ■, 10 nM; ○, 100 nM.

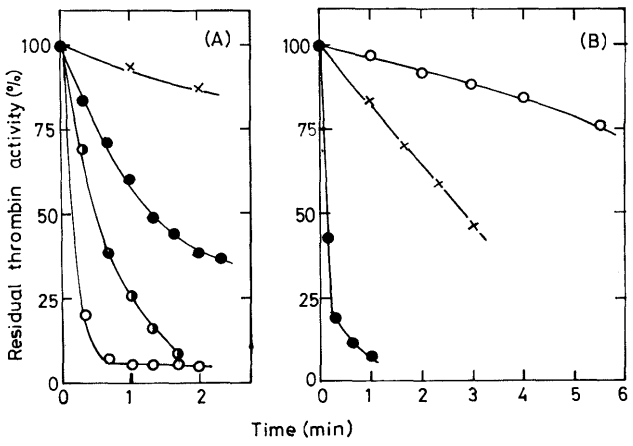

Fig. 2. Effect of HA-Heparin on the Thrombin/AT III Reaction in the Absence (A) and Presence (B) of 0.1 M NaCl

(A) The reaction mixture contained 40 μM substrate, 1 nM thrombin and 10 nM AT III alone (×) with 0.1 M (●), 1 nM (◑) and 10 nM (○) HA-heparin in 50 mM Tris–acetate (pH 6.05). (B) The reaction mixture contained 40 μM substrate and 1 nM thrombin alone (×), and 0.1 nM HA-heparin with (●) or without (○) 10 nM AT III in 0.1 M NaCl and 50 mM Tris–acetate (pH 6.05).

80% of initial 1 nM thrombin by AT III. Thrombin and heparin form 1 : 2[14] and 2 : 1[5a] complexes at molar excess of heparin and the enzyme, respectively. Moreover, Griffith reported enhancement by 0.3 nM heparin of the neutralization of more than 500 nM thrombin in the presence of 40 μM AT III.[15] Thus, it appears that HA-heparin has a catalytic action on the thrombin/AT III reaction in the presence of 0.1 M NaCl.

Compared with the rate of the thrombin/AT III reaction in the absence of NaCl, 0.1 nM HA-heparin significantly accelerated the rate in the presence of 0.1 M NaCl. This indicates that HA-heparin can catalyze the thrombin/AT III reaction at a faster rate in the presence of NaCl than in the absence of NaCl.

Since no reliable assay is available for the thrombin/AT III reaction with marked deviation from pseudo-first-order kinetics at pH 6.05, as described above, no further study of the reaction at this pH was attempted.

Interaction of Heparin with Proteins at pH 7.4 Next, the thrombin/AT III reaction was monitored in the absence of substrate. It has been considered that optimal salt concentrations shift with absolute concentrations of proteins or with their molar ratios. The author found that the HA-heparin-enhanced thrombin/AT III reaction was maximized at 0.04 M NaCl in the presence of 0.1 M Tris–HCl (pH 7.4) under the conditions used (data not shown). Under the same conditions, the rate of LA-heparin-enhanced thrombin/AT III reaction increased with increasing LA-heparin concentration (Fig. 3). The second-order rate constant of thrombin/AT III reaction in the presence of 1 nM HA-heparin without LA-heparin was calculated to be $1.47 \times 10^6 \cdot M^{-1} \cdot s^{-1}$. Provided that HA-heparin cooperates with LA-heparin in enhancing the reaction, the constant must increase with increasing LA-heparin concentration in the presence of HA-heparin at a fixed concentration. However, the constant decreased with increasing LA-heparin concentration. For example, the constant in the presence of both HA-heparin and LA-heparin at a 10-molar ratio was determined to be almost the same as that in the presence of 10 nM LA-heparin alone. As Fig. 4 shows, the thrombin/AT III reaction was somewhat enhanced by LA-heparin at a concentration of lower than 1 μM in the presence of substrate and 0.1 M NaCl, but was inhibited by 10 μM LA-heparin.

In the absence of heparin, second-order rate constants of the thrombin/AT III reaction shown in Figs. 3 and 4 were calculated to be 1.7 and $5.4 \times 10^{-5} \cdot M^{-1} \cdot s^{-1}$, respectively. Although these values were about three orders of magnitude higher than those reported by other investigators,[12,16] this difference could be due to the fact that the rate of thrombin/AT III reaction was determined by molar ratios of the proteins rather than their absolute concentrations for some unknown reason.[8b,17] Thus, second-order rate constant values of thrombin/AT III reaction seemed to have

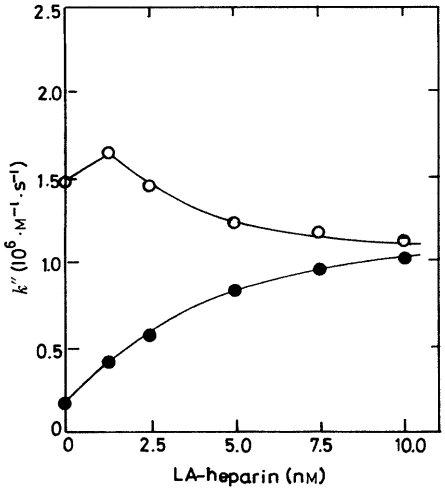

Fig. 3. Competition between HA-Heparin and LA-Heparin for Enhancement of the Thrombin/AT III Reaction

A reaction mixture containing 1 nM thrombin, 20 nM AT III and an indicated amount of LA-heparin with (○) or without (●) HA-heparin was incubated in 975 μl of 40 mM NaCl, 0.1 M Tris–HCl (pH 7.4) for various periods of time at 37°C. Residual thrombin activity was measured by adding 25 μl of 4 mM Boc–Val–Pro–Arg–MCA with polybrene to the mixture.

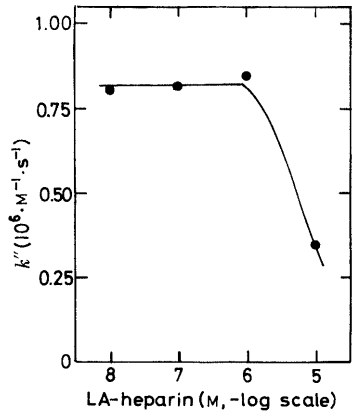

Fig. 4. Slight Enhancement of the Thrombin/AT III Reaction by LA-Heparin, even in the Presence of 0.1 M NaCl

Residual thrombin activity in a reaction mixture containing 0.1 nM thrombin, 10 nM AT III, 40 μM Boc–Val–Pro–Arg–MCA and an indicated amount of LA-heparin in 0.1 M NaCl, 50 mM Tris–acetate (pH 7.4) was measured using the method described in Fig. 1. The second-order rate constant k'' without LA-heparin was $5.4 \times 10^5 \cdot M^{-1} \cdot s^{-1}$; values appearing in the figure were obtained for free enzyme without correction for substrate binding.

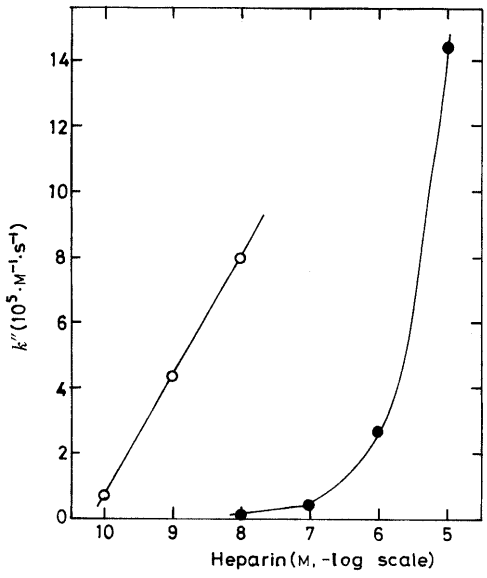

Fig. 5. Enhancement of the Factor X_a/AT III Reaction by HA- or LA-Heparin in the Presence of Synthetic Substrate

Residual factor X_a activity in a reaction mixture containing 40 μM Boc–Val–Pro–Arg–MCA, 5 nM factor X_a, 165 nM AT III and an indicated amount of HA- (○) or LA-heparin (●) was measured using the procedure described in Fig. 1. The k'' value without heparin was calculated to be $8.4 \times 10^3 \cdot M^{-1} \cdot s^{-1}$ without correction for substrate binding.

only little experimental significance for the evaluation of the reaction.

Although there was a possibility that the enhancement of thrombin/AT III reaction by LA-heparin might actually be induced by active HA-heparin contaminating the LA-heparin preparation, LA-heparin at higher than 10 nM significantly enhanced the factor X_a/AT III reaction while HA-heparin enhanced the reaction to a large extent when present at concentrations higher than 0.1 nM (Fig. 5). Based on the results shown in Fig. 5, it can be said that if LA-heparin preparation is contaminated with active HA-heparin, such a contaminated preparation can only yield a constant value of one-thousandth less than that seen in the presence of HA-heparin. Since LA-heparin interacts weakly with AT III,[10,18] it seemed reasonable to say that LA-heparin by itself enhanced the factor X_a/AT III reaction, but that contaminating HA-heparin did not. Thus, there is a strong possibility that AT III associates with LA-heparin at a concentration of higher than 0.1 μM in the presence of 0.1 M NaCl and 50 mM Tris–acetate (pH 7.4). With this in mind, the author hypothesized that slight enhancement of the thrombin/AT III reaction by LA-heparin, as shown in Figs. 3 and 4, resulted from the formation of the ternary complex essential to the neutralization of thrombin through the association of AT III with the thrombin–LA-heparin complex, and that the action of 10 μM LA-heparin against the reaction was due to the formation of inhibitory binary LA-heparin–thrombin and LA-heparin–AT III complexes, even in the presence of NaCl. This hypothesis agrees with previous observations; namely, that the enhancement of thrombin/AT III reaction by LA-heparin results from association of the inhibitor with the LA-heparin–thrombin complex in the absence of NaCl.[4a,8a] Griffith has also revealed that thrombin primarily binds to heparin as well as to DS.[3a]

Although factor X_a can bind to heparin,[14,19] it has been confirmed through several experiments that there is no necessity for ternary complex formation of heparin,[2b,g,3b,d,20] AT III and factor X_a for the enhancement of the factor X_a/AT III reaction. In addition, the results obtained in this study showed no simultaneous formation of inhibitory binary LA-heparin–factor X_a and LA-heparin–AT III complexes, even when LA-heparin was present at 10 μM. Since the HA-heparin–AT III complex is the sole essential component for enhancement of factor X_a neutralization, the enhancement of factor X_a/AT III reaction by HA-heparin at 0.1 nM, as observed in this study, is suggestive of the preferential association of HA-heparin with AT III. Therefore, it was thought that the enhancement of the thrombin/AT III reaction by 0.1 nM HA-heparin at pH 6.05 in the absence of NaCl was also due to primary binding of HA-heparin to AT III. The thrombin/AT III reaction rate in the presence of a commercially available heparin preparation was significantly low in comparison with that in the presence of HA-heparin, as shown in Fig. 1B. Since commercially-available heparin preparations contain both HA- and LA-heparins,[10,18,21] such a weak enhancement of the thrombin/AT III reaction by a commercially available heparin preparation was probably due to the formation of inhibitory binary HA-heparin–AT III and LA-heparin–thrombin complexes.

Interaction of DS with Proteins The author also investigated the effect of DS, a highly sulfated glycan, on the factor X_a/AT III reaction, in order to determine whether or not enhancement of the factor X_a/AT III reaction was specific to heparin. As Fig. 6 clearly illustrates, the factor X_a/AT III reaction was significantly enhanced by DS at relatively high concentrations. The binding constant of AT III with DS has been reported to be somewhere between that with HA-heparin and that with LA-heparin.[18a,22] The effective concentration of DS in enhancing the factor X_a/AT III reaction was found to be about 10-fold that of LA-heparin. The second-order rate constant value of the DS-enhanced factor X_a/AT III reaction was almost the same in the presence and absence of 0.1 M NaCl, suggesting that the association of DS with AT III was little affected by salt concentration up to 0.1 M NaCl. These results seemed to indicate that the increased rate of the HA-heparin-enhanced thrombin/AT III reaction in the presence of 0.1 M NaCl, as shown in Fig. 2B, was not due to the dissociation of HA-heparin from AT III, but was due to that of HA-heparin from thrombin. It was, therefore, thought that the fast neutralization by HA-heparin in a catalytic amount and 0.1 M NaCl was probably due to an increase in the turnover rate of HA-heparin serving as a catalyst. Since

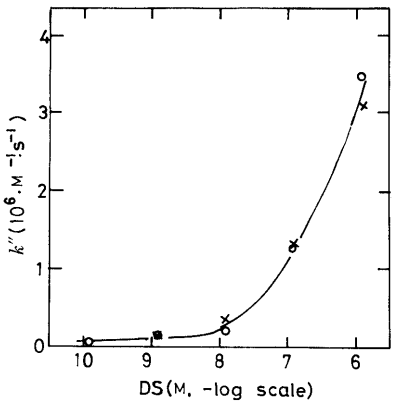

Fig. 6. Enhancement of the Factor X_a/AT III Reaction by DS

A reaction mixture contained 40 μM substrate, 1 nM factor X_a, 10 nM AT III and an indicated amount of DS in 50 mM Tris–acetate (pH 7.4) with (○) or without (×) 0.1 M NaCl. The k'' values were calculated to be 3.3 and $7.9 \times 10^4 \cdot M^{-1} \cdot s^{-1}$ with and without 0.1 M NaCl, respectively, without correction for substrate binding.

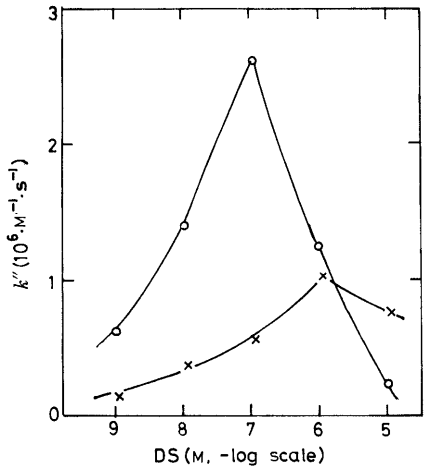

Fig. 7. Enhancement of the Thrombin/AT III Reaction by DS

A reaction mixture contained 40 μM substrate, 0.1 nM thrombin, 10 nM AT III and an indicated amount of DS in 50 mM Tris–acetate (pH 7.4) with (○) or without (×) 0.1 M NaCl. The k'' values were calculated to be 5.4 and $2.6 \times 10^5 \cdot M^{-1} \cdot s^{-1}$ with and without 0.1 M NaCl, respectively, without correction for substrate binding.

affinities of an enzyme for a number of substrates are generally expressed in terms of k_{cat}/K_m for convenience,[23] there should be no problem if the affinities of heparin for plasma serine proteases in neutralization by AT III as well as the static binding constants of the proteases to heparin are expressed using the same term.

The accelerative effect of DS on the thrombin/AT III reaction in the presence of 0.1 M NaCl was then investigated (Fig. 7). The degree of enhancement of the reaction by DS was determined to be greater in the presence of 0.1 M NaCl than that in the absence of 0.1 M NaCl, except for that induced by DS at an extremely high concentration (10 μM). An optimal concentration of DS for the enhancement in the presence of NaCl was found to be 0.1 μM. Under the conditions described in the legend to Fig. 6, AT III could only bind to DS when present at a concentration of more than 0.1 μM. Since it has been noted that thrombin is desorbed from the Sepharose 6B gel matrix through the influence of DS,[5d,g] it is clear that thrombin interacts with DS to some extent in the presence of 0.1 M NaCl. Thus, the ascending and descending parts of the curve, shown in Fig. 7, representing the enhancement of the thrombin/AT III reaction in the presence of 0.1 M NaCl, were thought to result from the increase in complexed thrombin and DS concentrations, and from the formation of inhibitory binary DS–thrombin and DS–AT III complexes.

Whereas the enhancement of the factor X_a/AT III reaction by DS was independent of salt concentration, as Fig. 6 shows, that of the thrombin/AT III reaction was significantly inhibited in the presence of NaCl. Some investigators have found that prior binding of heparin with thrombin markedly reduced the rate of the thrombin/AT III reaction, as determined by the stopped flow method.[24] The interaction of thrombin with DS has also been found to be so strong in the absence of NaCl as to induce conformational perturbation in thrombin molecules,[5e] suggesting that the prevention of DS enhancement of the thrombin/AT III reaction in the absence of NaCl was probably due to interaction between the enzyme and DS, which was too strong to allow cooperation with the thrombin/AT III reaction. Taking these results into account, there appears to be no way to satisfactorily explain the rate of thrombin/AT III reaction in the presence of highly sulfated polysaccharides in terms of a statical concentration of their complex, estimated based on binding constants of the enzyme and the polysaccharides.

Recently, Olson reported that the amount of AT III modified by thrombin in the heparin-enhanced enzyme/inhibitor reaction varied with change in NaCl concentration.[25] He examined the stoichiometry at equilibrium, while the author measured the overall velocity. This is the reason for the differences obtained in rates between forward reaction (neutralization) and pseudoreverse reaction.[25] He examined the stoichiometry at equilibrium, be directly compared with those of Olson, because he measured the amounts of the end product in the pseudoreverse reaction.

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