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THE INTERACTION OF HEPARIN WITH THROMBIN AND ANTITHROMBIN

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SUMMARY

We have monitored the binding of heparin to thrombin as well as antithrombin by fluorescence techniques. The interaction of mucopolysaccharide with thrombin is characterized by a stoichiometry of 2:1 with $K^{H1T}_{DISS} = K^{H2T}_{DISS} = 8 \times 10^{-7}$ M. The interaction of heparin with antithrombin is typified by a stoichiometry of 1:1 with $K^{H-A}_{DISS} = 5.7 \times 10^{-8}$ M. A plot of the initial velocity of the thrombin-antithrombin reaction versus mucopolysaccharide concentration exhibits an ascending limb, descending limb and final plateau. The ascending limb of the kinetic profile corresponds to the level of heparin-antithrombin complex. The descending limb of the initial velocity plot coincides with the formation of tertiary complexes between two molecules of heparin and one molecule of thrombin. Thus, our data demonstrates that the generation of heparin-antithrombin complex is responsible for the mucopolysaccharide dependent acceleration of enzyme neutralization. Furthermore, our results also suggest that interactions between heparin bound to inhibitor and free enzyme can account for only a small fraction of the total kinetic effect of the complex carbohydrate. Therefore, we conclude that the direct binding of heparin to antithrombin is probably responsible for the anticoagulant effect of the mucopolysaccharide.

INTRODUCTION

Heparin functions as an anticoagulant by dramatically accelerating the rate at which antithrombin complexes with the serine proteases of the hemostatic mechanism (1). In prior communications we have suggested that this augmentation in reaction velocity is a result of the binding of mucopolysaccharide to the protease inhibitor (2). However, in the past few years, numerous investigators have claimed that heparin-enzyme interactions may be of paramount importance to the biologic action of the mucopolysaccharide. On the one hand, it has been claimed that heparin binds preferentially to hemostatic system enzymes and that the resultant complex is subsequently neutralized by free antithrombin (3). On the other hand, it has been proposed that the mucopolysaccharide is initially bound to antithrombin but that the subsequent interaction of the complex carbohydrate with free enzyme ("approximation") is responsible for the

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anticoagulant properties of heparin(4).To date,no one has been able to estimate the relative kinetic significance of these three postulated mechanisms.

MATERIALS AND METHODS

Chemicals - Fluorescamine (Fluram) was purchased from Roche Diagnostics, Nutley, N.J. All other chemicals utilized were reagent grade or better.

Preparation of Proteins and Mucopolysaccharide - Human antithrombin and human thrombin were isolated in physically homogeneous form by methods previously reported from our laboratory (5). Porcine heparin of molecular weight ~6500 daltons was obtained by utilizing a fractionation technique based upon the mucopolysaccharide's affinity for antithrombin. The final product exhibited a specific anticoagulant activity of 373 units/mg, and was homogeneous with respect to its interactions with inhibitor (5).

Measurement of the Concentrations and Biologic Activities of Reactants - The concentrations and biologic activities of proteins and mucopolysaccharides were quantitated as previously described (5).

Labeling of Heparin - In order to introduce a fluorescent tag into the heparin molecule, we treated the mucopolysaccharide with fluorescamine (6). The fluorescent intensity of the heparin conjugate was quantitated by exciting this species at 380 nm and measuring the level of emission at 475 nm. Comparison with labeled components such as o-methyl serine suggest that approximately 0.3 groups of fluorescamine are incorporated per molecule of heparin.

Fluorescence Spectroscopy - Fluorescence measurements were performed with a Perkin-Elmer MPF-44A spectrofluorometer equipped with thermostated sample compartment and polarization accessory. The binding of unlabeled heparin to antithrombin was quantitated by measuring the enhancement in the intrinsic fluorescence of the protease inhibitor at 330 nm (ΔF) after excitation at 280 nm as described earlier (5).

The interactions of fluorescamine-heparin with thrombin or antithrombin were examined by monitoring the enhancement in polarization (ΔP) of labeled mucopolysaccharide as a function of protein concentration. The ΔP values were obtained by calculating the difference in polarization of fluorescamine-heparin in the presence and absence of various levels of either thrombin or antithrombin. All measurements were conducted at excitation and emission wavelengths of 390 nm and 500 nm, respectively in order to minimize contributions from high levels of protein. Furthermore, a 430 nm cutoff filter was employed in front of the emission monochrometer to suppress transmission of stray or scattered light. Polarization values were computed from the magnitudes of the vertically and horizontally polarized emission beams after excitation by vertically and horizontally polarized light according to a standard formula (7).

Estimates of Reaction Stoichiometry - The stoichiometries of the various processes were determined by admixing either varying concentrations of unlabeled mucopolysaccharide with a constant level of protein (intrinsic fluorescence method) or varying concentrations of protein with a constant level of labeled mucopolysaccharide (polarization fluorescence method). The concentrations of all reactants utilized were at least 10 fold above the dissociation constants of the respective interactions (see below). The enhancement in the intrinsic fluorescence of antithrombin or in polarization fluorescence of labeled mucopolysaccharide were quantitated and the various measurements were normalized to the maximal observed increase in the respective parameters. Subsequently, the $\Delta F/\Delta F_{max}$ or $\Delta P/\Delta P_{max}$ were plotted versus the respective molar ratios of reactants employed. Estimates of stoichiometry were obtained by constructing a line through the initial portions of the ascending limbs of the binding isotherms and determining their intersection with the horizontal asymptomates that signify virtually complete saturation of antithrombin with heparin or of labeled mucopolysaccharide with protein, respectively.

Estimates of Dissociation Constants and Levels of Heparin-Protein Complexes - The interaction of thrombin with labeled mucopolysaccharide exhibits a stoichiometry of 2:1 (see Results). To obtain estimates of the two dissociation constants, we added varying concentrations of the enzyme to a fixed level of labeled heparin. The reactants were employed at concentrations similar to the dissociation constants of this interaction. The enhancements in the fluorescence polarization of labeled mucopolysaccharide were determined and the data obtained was normalized to the maximal projected value of this parameter. In this fashion, a plot of $\Delta P/\Delta P_{max}$ versus thrombin concentration was generated. The heparin-thrombin interaction can be described by the following set of equations:

Equation I - $\Delta P/\Delta P_{max} = (H_1T + 2H_2T)/Ho$

Equation II - $H_2T = (Ho[H_1T] - [H_1T]^2)/(K_{DISS}^{H_2T} + 2[H_1T])$

Equation III - $[H_1T]^3 + \frac{\phi_2}{\phi_1}[H_1T]^2 + \phi_3/\phi_1 [H_1T] + \phi_4/\phi_1 = 0$

Where Ho and To represent the initial concentrations of heparin and thrombin, respectively $[H_1T]$ and $[H_2T]$ are the levels of the mucopolysaccharide enzyme complexes with stoichiometries of 1:1 and 2:1, respectively. $K_{DISS}^{H_1T}$ and $K_{DISS}^{H_2T}$ signify the dissociation constants of the two heparin-protein complexes and

$$\phi_1 = 4K_{DISS}^{H_1T} - K_{DISS}^{H_2T}$$

$$\phi_2 = 4K_{DISS}^{H_1T} K_{DISS}^{H_2T} + 2[To] K_{DISS}^{H_2T} - (K_{DISS}^{H_2T})^2$$

$$\phi_3 = [Ho](K_{DISS}^{H_2T})^2 + K_{DISS}^{H_2T} [Ho]^2 + [To](K_{DISS}^{H_2T})^2 - 2[Ho][To]K_{DISS}^{H_2T} + K_{DISS}^{H_1T}(K_{DISS}^{H_2T})^2$$

$$\phi_4 = [Ho] [To](K_{DISS}^{H_2T})^2$$

Theoretical profiles of $\Delta P/\Delta P_{max}$ versus enzyme concentration were obtained by numerical evaluation of the above equations utilizing multiple sets of assumed dissociation constants, and reactant concentrations identical to those employed in the experimental protocol. These theoretical plots were compared to the experimental results by appropriate statistical procedures in order to identify the $K_{DISS}^{H_1T}$ and $K_{DISS}^{H_2T}$ which best fit the polarization fluorescence determinations.

When kinetic behaviour was investigated, the concentrations of various heparin-thrombin complexes within a given reaction mixture were calculated by employing Equations II and III in conjunction with the optimal values of the dissociation constants. The details of these procedures will be described in a subsequent communication.

The interaction of heparin with antithrombin exhibits a stoichiometry of 1:1 (see Results). The dissociation constant of this interaction was estimated by nonlinear least squares fit of the data to equations analogous to those previously described (5). The concentrations of mucopolysaccharide-inhibitor complex within reaction mixtures were calculated as outlined in a prior communication (5).

Kinetic Evaluation of the Thrombin-Antithrombin Interaction - The kinetics of this interaction were examined by quantitating the residual levels of enzymatic activity as a function of the time of incubation of the serine protease with inhibitor and varying concentrations of mucopolysaccharide. The initial concentrations of thrombin and antithrombin were set at 5×10^{-9} M. The solvent environment was established at 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5. Studies of the neutralization process were initiated by admixing the enzyme with a solution containing specific levels of heparin or equimolar concentrations of antithrombin or both. The various mixtures were incubated at 37°C for nine separate time intervals that ranged from 0 to 17 sec. The reactions were quenched by the addition of protamine sulfate at a final concentration of 2 mg/ml and the residual levels of thrombin were measured. The values obtained were corrected to eliminate

losses due to the adsorption of enzyme on vessel surfaces. Subsequently, the initial velocity of the enzyme-inhibitor interaction at a given concentration of heparin was determined by fitting the initial level of thrombin (To) as well as all subsequent levels of residual enzyme (T) at time (t) to the equation

$$\frac{1}{T} - \frac{1}{To} = - k^1 t$$

The following equation provides the relationship between the initial velocity of enzyme neutralization, the concentration of mucopolysaccharide added, and the level of enzyme employed. This equation is valid for reactant concentrations and experimental conditions utilized in the studies cited below.

Equation IV

$$\text{initial velocity} = b(1-c)k_1To^2 + bc\ k_2\ To^2$$

Where To is the initial concentration of enzyme, k_1 is the bimolecular rate constant for the interaction of a given enzyme with the heparin-antithrombin complex, k_2 is the bimolecular rate constant for the interaction of a given heparin-enzyme complex with heparin-inhibitor complex, b is the fractional saturation of antithrombin with mucopolysaccharide, and c is the fractional saturation of enzyme with mucopolysaccharide. The latter two parameters can be directly calculated from the dissociation constants of the heparin-antithrombin and the heparin-thrombin interactions.

RESULTS AND DISCUSSION

Evaluation of Fluorescamine Heparin-Protein Interactions - We have studied the interaction of heparin with antithrombin by spectroscopic techniques. Initially, the binding of unlabeled mucopolysaccharide to the inhibitor was analyzed by fluorescence spectroscopy. Employing this approach, the above interaction is characterized by a molar stoichiometry of inhibitor to mucopolysaccharide of 1.08 and a dissociation constant of 5.74×10^{-8} M. Subsequently, the binding of fluorescamine-heparin to antithrombin was examined by polarization fluorescence spectroscopy. Utilizing this methodology, we obtained a molar stoichiometry of mucopolysaccharide to inhibitor of 0.96 and a dissociation constant of 1.06×10^{-7} M. On the basis of this data, we believe that the labeling of heparin with a fluorescent tag does not significantly alter the functional characteristics of this mucopolysaccharide. We have also analyzed the binding of heparin to thrombin with respect to the stoichiometry and avidity of this interaction. The stoichiometry of this process was determined by the polarization fluorescence technique as described in Methods. Experiments were initiated by adding varying concentrations of enzyme that ranged from 5×10^{-7} M to 3×10^{-5} M to a constant level of fluorescamine-heparin

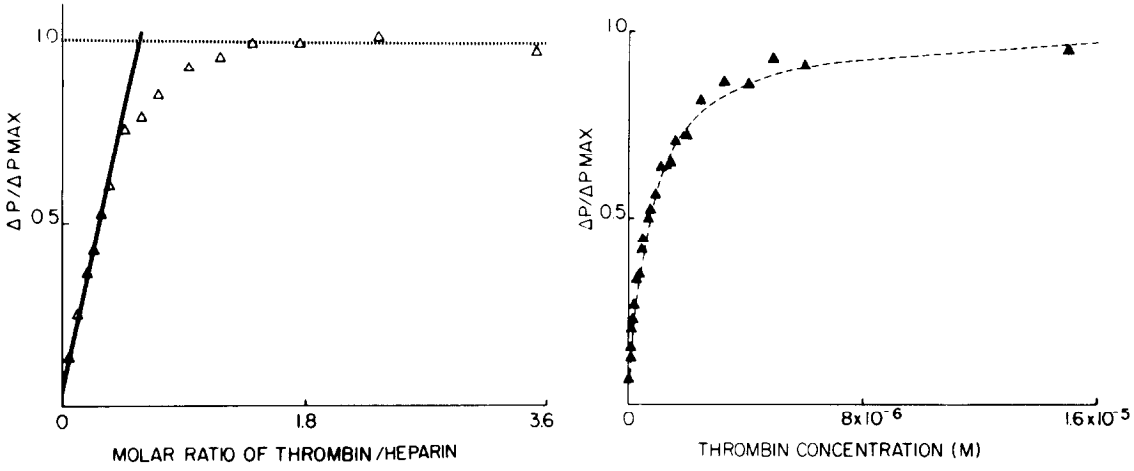

Fig. 1 The binding of fluorescamine-heparin to thrombin as monitored by fluorescence polarization spectroscopy. A, Estimate of reaction stoichiometry. The solid line (▬) represents a linear least squares fit of binding data contained within the ascending limb of the isotherm (r=0.99). B, Determination of $K_{DISS}^{H_1T}$ and $K_{DISS}^{H_2T}$. The dashed line (---) represents theoretical values calculated from the two binding site model that best fits our experimental data. These estimates were obtained by numerical evaluation of Equations II and III with $K_{DISS}^{H_1T}=K_{DISS}^{H_2T}=8\times10^{-7}$M.

maintained at 8.57×10^{-6} M. The environmental conditions were established at 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5 and 37°C. Fig 1A depicts a plot of the $\Delta P/\Delta P_{max}$ of the various mixtures versus the molar ratio of thrombin to fluorescamine-heparin added. Extrapolation of the linear portion of the binding isotherm indicates that the stoichiometry of this process is 1.8. This suggests that a single molecule of thrombin is capable of binding two molecules of mucopolysaccharide.

Subsequently, we examined the avidity of the heparin-thrombin interaction as outlined above. However, the concentration of labeled mucopolysaccharide was reduced to 8.57×10^{-7} M which is optimal for evaluating the dissociation constants of this process. Fig 1B shows a plot of the $\Delta P/\Delta P_{max}$ of the various mixtures versus the molar concentration of thrombin added. The best statistical fit of our data was obtained utilizing a 2-binding site model with $K_{DISS}^{H_1T} = K_{DISS}^{H_2T} = 8 \times 10^{-7}$ M.

The Kinetics of Interaction of Heparin, Antithrombin, and Thrombin - We have analyzed the ability of heparin to accelerate thrombin-antithrombin inter-

actions. To this end, the initial velocity of this process was experimentally determined as a function of mucopolysaccharide concentration. Subsequently, this kinetic profile was correlated with the levels of heparin-enzyme or heparin-antithrombin complexes present within the respective reaction mixture. The concentrations of the various mucopolysaccharide-protein interaction products were calculated from the initial levels of reactants utilized and the dissociation constants provided above. This approach allowed us to ascertain whether the binding of heparin to antithrombin or thrombin or both is critical for the mucopolysaccharide dependent acceleration of this interaction.

In Fig 2A, we compare the ascending limb of the initial velocity profile with the plots of heparin-antithrombin complex as well as various types of heparin-thrombin interaction products. These latter components include binary complexes of enzyme and mucopolysaccharide [TH_1], tertiary complexes of these species [TH_2] and a summation of both products [TH_1 + TH_2]. It is apparent that this area of the kinetic profile exhibits a higher degree of correspondence to the level of heparin-antithrombin complex than to the concentrations of any heparin-thrombin interaction product. Thus, our data indicates that the former species must be responsible for the mucopolysaccharide dependent acceleration of this inhibitory process.

In Fig 2B, we contrast the descending limb of the initial velocity plot with the concentrations of [To-TH_1], [To-TH_2], and [To-TH_1-TH_2]. It would appear that this region of the kinetic profile coincides best with the level of [To-TH_2]. Thus, our data suggests that the TH_2 complex rather than the TH_1 interaction product is neutralized at a significantly slower rate than free enzyme.

However, a small discrepancy is noted between the concentration of the heparin-antithrombin complex and the uppermost portion of the ascending limb of the initial velocity plot (Fig 2A). Furthermore, a more significant lack of concordance is observed between the level of [To-TH_2] and the descending

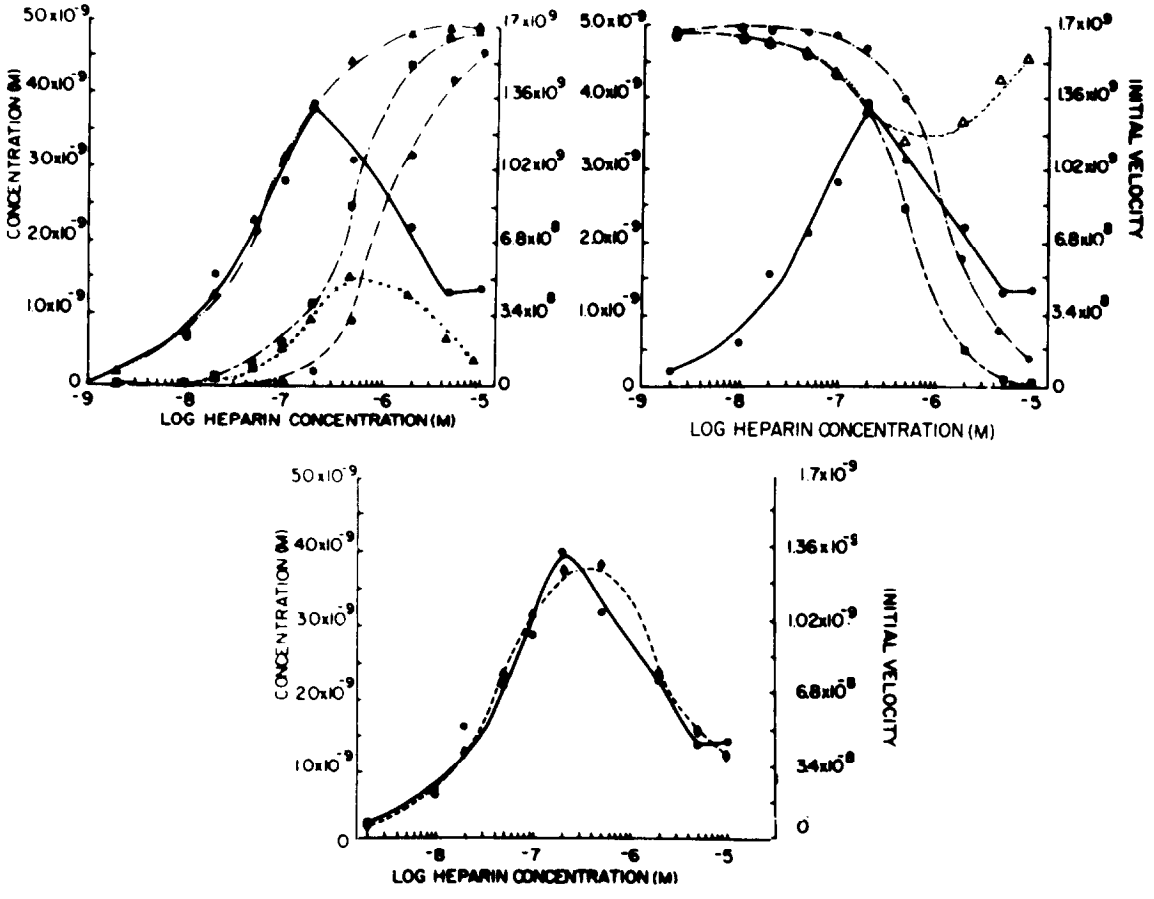

Fig. 2 Correlation between the binding of heparin to thrombin as well as antithrombin and the ability of the mucopolysaccharide to accelerate thrombin-antithrombin interactions. A, Comparisons of the levels of heparin-protein complexes with the kinetics of the thrombin-antithrombin interaction. (o—o) initial velocity, (▲—···—▲) concentration of heparin-antithrombin complex, (■---■) concentration of heparin-thrombin complexes (TH_1+TH_2), (●—·—●)concentration of tertiary complex of heparin and thrombin (TH_2), and (Δ·····Δ) concentration of binary complex between heparin and thrombin (TH_1). B, Comparison of various combinations of free thrombin and heparin-thrombin complexes with the kinetics of the thrombin-antithrombin interactions. (o——o) initial velocity, (■— - —■) concentration of free thrombin (T-TH_1-TH_2), (●—·—●) concentration of free thrombin and TH_1 (T-TH_2), and (Δ·····Δ) concentration of free thrombin and TH_2 (T-TH_1). C, Comparison of the observed (o---o) and theoretically predicted (◆---◆) kinetics of the thrombin-antithrombin interaction.

limb of the kinetic profile (Fig 2B). We suspected that these discrepancies were due to two independent phenomena. On the one hand, competition exists between thrombin and antithrombin for limiting quantities of mucopolysaccharide. On the other hand, the heparin-antithrombin complex interacts with the heparin-thrombin complex at a different rate than with free enzyme. For these reasons, Equation IV was employed to construct a theoretical plot of the initial velocity

profile. The experimentally determined values of the various dissociation constants were utilized to generate the theoretical estimate. We also assumed that TH_2 rather than TH_1 exhibited a reduced rate of interaction with the heparin-antithrombin complex. As shown in Fig 2C, this approach resulted in a theoretical kinetic plot that is virtually indistinguishable from the experimental profile when $k_1 = 1.7 \times 10^9\ M^{-1}min^{-1}$ (rate of interaction between thrombin and heparin-antithrombin complex) and $k_2 = 3.0 \times 10^{8-1}min^{-1}$ (rate of interaction between heparin-thrombin complex and heparin-antithrombin complex). We have previously demonstrated that the bimolecular rate constant (k) of this interaction in the absence of mucopolysaccharide is $4.25 \times 10^5 M^{-1}min^{-1}$ (5). Therefore, the normalized values of the above two parameters are $k_1' = \frac{k_1}{k} = 4000$ and $k_2' = \frac{k_2}{k} \simeq 700$.

The parameter k_1' represents the summation of all kinetic influences of the mucopolysaccharide on the above reaction. This would include contributions from the direct binding of heparin to antithrombin as well as those from the interaction between mucopolysaccharide bound to inhibitor and free thrombin. The parameter k_2' provides a minimal estimate of the kinetic results due to the direct binding of mucopolysaccharide to antithrombin. This interpretation is justified since "approximation" sites on the enzyme -if present- would be saturated with heparin and unable to function in a normal fashion. The kinetic importance of "approximation" phenomena can be calculated as either k_1'/k_2' or $k_1' - k_2'$, respectively. We favor the use of k_1'/k_2' based upon our belief that the "approximation" of thrombin via mucopolysaccharide bound to antithrombin and the assembly of enzyme-inhibitor complexes are likely to represent sequential events in the neutralization of this serine protease. If such is the case our data would indicate that "approximation" phenomena are responsible for no more than 1%-2% of the direct effect of heparin on antithrombin.

To extend our findings, we have analyzed in similar fashion the interactions of heparin and antithrombin with factor IXa, factor Xa, as well as plasmin (not shown). In each instance, the binding of complex carbohydrate to the protease inhibitor correlates with the mucopolysaccharide dependent acceleration of enzyme

neutralization. Our data also suggests that the "approximation" mechanism contributes minimally to the total kinetic effect of the mucopolysaccharide when factor IXa is inhibited by the heparin-antithrombin complex and is of no importance when factor Xa or plasmin are neutralized by the same interaction product. Therefore, we conclude that the direct binding of heparin to the antithrombin molecule is probably responsible for the anticoagulant effect of the mucopolysaccharide.

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