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INHIBITION OF THE HEPARIN-ANTITHROMBIN III/THROMBIN REACTION BY ACTIVE SITE BLOCKED-THROMBIN[1]

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SUMMARY

Active site blocked-thrombin, prepared by reacting thrombin with valyl-isoleucyl-prolyl-arginine chloromethyl ketone, inhibits the heparin enhanced-antithrombin III/thrombin reaction. Since active site blocked-thrombin does not interact with antithrombin III it was concluded that active site blocked-thrombin was competing for heparin in the reaction system. The heparin concentration dependence for maximum enhancement of the antithrombin III/thrombin reaction in the presence and absence of active site blocked-thrombin indicated that heparin was binding to thrombin to enhance the reaction rate. A dissociation constant value of 6.4×10^{-9}M was estimated for the heparin·thrombin complex which is similar to the value of 5.8×10^{-9}M previously reported (Griffith, M.J. (1979) J. Biol. Chem. in press). Antithrombin III·thrombin complexes were also found to bind heparin with an affinity equivalent to thrombin. The results were interpreted to indicate that heparin binds to thrombin as the first step in the mechanism of action of heparin in enhancing the antithrombin III/thrombin reaction.

Heparin has been shown to enhance the rate of inactivation of thrombin and other activated coagulation factors (1-6) by the plasma proteinase inactivator, antithrombin III (7-9). The interaction of heparin with thrombin and antithrombin III has received considerable attention as a model system for examining the anticoagulant mechanism of action of heparin. There are conflicting reports which suggest either the primary importance of the interaction of thrombin and heparin (10-20) or antithrombin III and heparin (1,21-25) while several groups have proposed the importance of the simultaneous binding of heparin to both proteins (26-28). Our recent studies involving the phosphopyridoxylation of thrombin indicate that heparin and thrombin bind very tightly, i.e. K_{diss} of less than 10^{-8}M (19). This is at least an order of magnitude lower than the dissociation constant determined for heparin and antithrombin III (24). We concluded from these studies that the first step in the mechanism of action of heparin involves the binding of heparin to thrombin.

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It has been demonstrated that active site blocked-thrombin does not interact with antithrombin III (4,26). In the present communication we have taken advantage of this observation to further study the binding of heparin to thrombin and antithrombin III. Our results indicate that active site blocked-thrombin and antithrombin III·thrombin complexes are effective inhibitors of the heparin enhanced-antithrombin III/thrombin reaction, presumably by competing with thrombin for heparin.

EXPERIMENTAL PROCEDURES

Materials. N-α-p-tosyl-L-glycyl-L-prolyl-L-arginine-p-nitroanilide (TosGlyProArgNaN) was purchased from Boerhinger-Mannheim. Prothrombin complex concentrates and antithrombin III were obtained from the American Red Cross Fractionation Center.[2] Polyethylene glycol (PEG 6000) was purchased from Baker Chemicals. Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) was purchased from Aldrich. Bovine serum albumin was purchased from Miles Laboratories. Valyl-isoleucyl-prolyl-arginine chloromethyl ketone (ValIleProArgCH_2Cl) was the gift of Drs. Kettner and Shaw, Brookhaven National Laboratories. Heparin (bovine lung, 183 USP units/mg, mol. wt. 14,000) was obtained from Drs. Mathews and Cifonelli, University of Chicago.[3]

Human alpha-thrombin (3,200 NIH clotting units/mg) was isolated from thromboplastin (Difco) activated prothrombin complex concentrates as described previously (29). Active site blocked-thrombin was prepared by adding ValIleProArgCH_2Cl to a solution containing 0.1 M triethanolamine (TEA; pH 8.0) and 1.0×10^{-5}M thrombin. The final ValIleProArgCH_2Cl concentration was 2.0×10^{-5}M. After 1 h at room temperature, greater than 99% of the thrombin amidase activity was lost. The modified enzyme was exhaustively dialyzed against 0.1 M TEA (pH 8.0) at 4°. Control enzyme was treated similarly except that ValIleProArgCH_2Cl was omitted.

Inactivation of thrombin by antithrombin III. Thrombin was added to an inactivation solution containing an equimolar concentration of heparin and a 15- to 20-fold molar excess of antithrombin III. Samples of 0.2 ml were removed at timed intervals and the residual enzyme activity determined by adding the sample to 2.0 ml of assay solution containing 0.1 M TEA (pH 8.0), 0.1% PEG 6000, 5×10^{-5}M TosGlyProArgNaN and 0.2 mg/ml of polybrene to complex the heparin. The assay reaction was terminated by adding 1.5 ml of 50% glacial acetic acid and the amount of product formed determined spectrophotometrically using a 5 cm path length cuvette in a Zeiss PM QII spectrophotometer ($E_{400} = 1.14 \times 10^4 M^{-1} cm^{-1}$; p-nitroaniline).

Inactivation of thrombin by antithrombin III in the presence of antithrombin III·thrombin complexes. Antithrombin III and thrombin were premixed at equimolar concentration then diluted into the inactivation solution described above. After 15 min there was no detectable thrombin amidase activity indicating complete formation of the antithrombin III·thrombin complex. At this time, thrombin was added to the inactivation solution as above and the rate of inactivation determined.

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[2]Antithrombin III and prothrombin complex concentrates used for the preparation of thrombin were provided by the American Red Cross Fractionation Center with the partial support of NIH grant HL-13881.

[3]National Institutes of Health (NIAMD) Contract No. NO1-AM-5-2205.

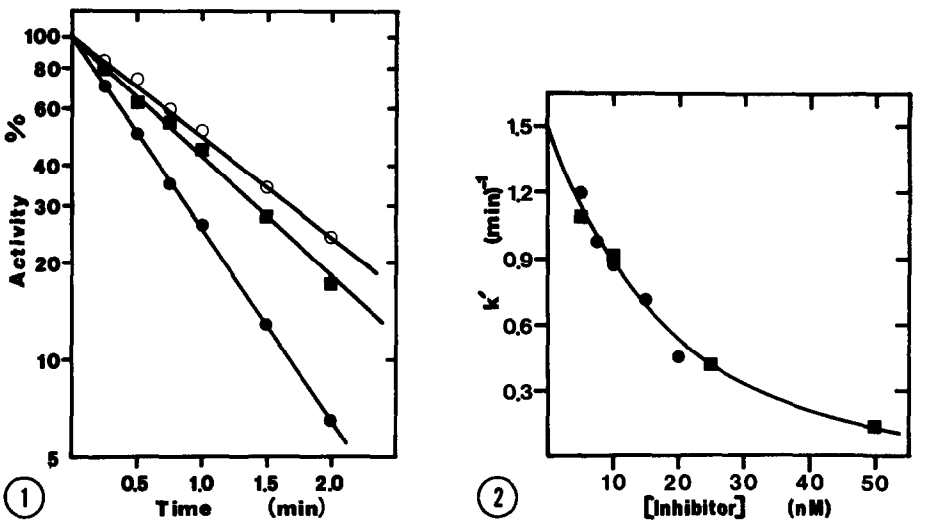

Figure 1. Inhibition of the heparin-antithrombin III/thrombin reaction. Thrombin was added to a solution containing 0.1 M TEA (pH 8.0), 0.1% PEG 6000, 7.7×10^{-8}M antithrombin III and 3.6×10^{-9}M heparin, ● . The final thrombin concentration was 4.7×10^{-9}M. At timed intervals samples were removed and the percentage of residual enzyme activity determined as described in Experimental Procedures. Active site blocked-thrombin, ■ , or antithrombin III·thrombin complexes, ○ , were also added to the inactivation solution. The final concentrations were 8.6×10^{-9}M and 9.4×10^{-9}M, respectively. The pseudo first order rate constant was determined from the slope of the lines (m = -k').

Figure 2. Concentration dependence for inhibition of the heparin-antithrombin III/thrombin reaction. Thrombin was inactivated in the presence of inhibitor; active site blocked-thrombin, ● , or antithrombin III·thrombin complexes, ■ , as described in the legend to Fig. 1. The final concentrations of thrombin, antithrombin III and heparin were 4.7×10^{-9}M, 7.7×10^{-8}M, and 5.0×10^{-9}M, respectively. The pseudo first order rate constant was determined as described in the legend to Fig. 1.

RESULTS

Inhibition of the heparin enhanced-antithrombin III/thrombin reaction. The heparin enhanced-antithrombin III/thrombin reaction rate was inhibited by the addition of either active site blocked-thrombin or antithrombin III·thrombin complexes to the reaction solution. These results are shown in Fig. 1. The pseudo first order rate constant value, k', for the inactivation reaction was 1.39 min^{-1} in the absence of inhibitor (active site blocked-thrombin or antithrombin III·thrombin complex).[4] In the presence

[4]The term inhibitor is reserved for active site blocked-thrombin or antithrombin III·thrombin complexes since these molecules inhibit the heparin enhanced-antithrombin III/thrombin reaction. Heparin is an effector in the antithrombin III/thrombin reaction. Antithrombin III is a thrombin inactivator, not an inhibitor.

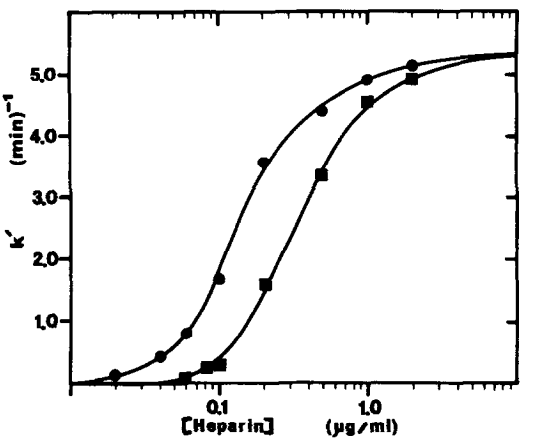

Figure 3. Heparin concentration dependence for enhancement of the antithrombin III/thrombin reaction. Thrombin (4.7×10^{-9}M) was inactivated in the presence, ■ , and absence, ● , of active site blocked-thrombin (2.6×10^{-8}M) as described in the legend to Fig. 1.

of active site blocked-thrombin, k' was equal to 0.85 min^{-1}. In the presence of antithrombin III·thrombin complexes, k' was equal to 0.70 min^{-1}. Neither inhibitor affected the antithrombin III/thrombin reaction rate in the absence of heparin, k' = 0.026 min^{-1} (not shown). Increasing the concentration of the inhibitor progressively decreased the antithrombin III/thrombin reaction rate, in a manner indicating a saturation phenomenon. These results are shown in Fig. 2. At a concentration of 1.2×10^{-8}M, which was approximately 15% of the concentration of the antithrombin III in the system, antithrombin III·thrombin complexes inhibited the inactivation rate 50%. At a concentration of 1.5×10^{-6}M bovine serum albumin decreased the reaction rate by less than 20%.

Effect of heparin on the antithrombin III/thrombin reaction rate.

The rate of the antithrombin III/thrombin reaction was greatly accelerated by increasing the concentration of heparin in the reaction solution. These results are shown in Fig. 3 (solid circles). The maximum k' value was approximately 5.2 min^{-1} or 200-fold greater than k' in the absence of heparin (0.026 min^{-1}). The concentration of heparin which half-maximally enhanced the reaction was 0.12 μg/ml or 8.7×10^{-9}M. Since this is 10-fold lower than the antithrombin III concentration it seems reasonable to conclude that heparin is binding to thrombin to enhance the antithrombin III/thrombin reaction. The dissociation constant for the heparin·thrombin complex can be estimated by assuming the following equilibrium.

$$E + H \underset{}{\overset{K_{diss}}{\rightleftharpoons}} E \cdot H$$

and

$$K_{diss} = \frac{[E][H]}{[EH]} = \frac{([E_t] - [EH])([H_t] - [EH])}{[EH]} \quad (1)$$

E_t is the total enzyme concentration in the reaction system and H_t is the total heparin concentration. K_{diss} is the dissociation constant for the enzyme·heparin complex, EH. If it is presumed that the half-maximal reaction rate is obtained when half of the thrombin is bound to heparin, i.e. $E_t/2 = EH$, then,

$$K_{diss} = ([H_t]^* - [E_t]/2) \quad (2)$$

where $[H_t]^*$ is the concentration of heparin (8.7×10^{-9}M) when this occurs. In the present experiment, the total concentration of enzyme was 4.7×10^{-7}M and therefore, $K_{diss} = 6.4 \times 10^{-9}$M. In the presence of active site blocked-thrombin the heparin saturation curve was shifted to the right (solid squares, Fig. 3), but attained the same maximum rate of reaction. The concentration of heparin which half-maximally enhanced the reaction rate was 2.2×10^{-8}M. Since the total thrombin concentration in the system (native plus active site blocked) was 3.1×10^{-8}M the dissociation constant for heparin and thrombin was 6.5×10^{-9}M, indicating that active site blocked- and native thrombin have nearly identical affinities for heparin. Since antithrombin III·thrombin complexes are as effective as active site blocked-thrombin in inhibiting the heparin enhanced-inactivation reaction (Fig. 2) it was concluded that native thrombin and the antithrombin III·thrombin complex also have nearly identical affinities for heparin.

DISCUSSION

The results of the present study demonstrate that heparin binds to thrombin as the first step in the mechanism of action of heparin in enhancing the antithrombin III/thrombin reaction. The binding of heparin to thrombin is reversible as is the binding of heparin to the antithrombin III·thrombin complex. The data indicate that heparin has approximately the same affinity for the antithrombin III·thrombin complex as it does for thrombin, in contrast with previous results (30) which indicated a lower affinity. The following scheme can be proposed to describe the inactivation process in the presence of heparin.

$$\begin{array}{ccc} E & + \text{AtIII} \xrightarrow{k_2'} & E \cdot \text{AtIII} \\ + & & + \\ H & & H \\ K_H \downarrow\uparrow & & \uparrow\downarrow K_H \\ E \cdot H & + \text{AtIII} \xrightarrow{\alpha k_2'} & E \cdot H \cdot \text{AtIII} \end{array}$$

This scheme suggests that the formation of the antithrombin III·thrombin complex is essentially irreversible. Under pseudo first order reaction conditions we have found that the rate of thrombin inactivation increased proportionately with an increase in the antithrombin III concentration with no evidence for the formation of a dissociable antithrombin III·thrombin complex prior to the formation of a covalently bound complex.[5] This observation suggests that the binding of heparin to thrombin induces or stabilizes a conformation of the enzyme which is more reactive, by a factor of α, with antithrombin III. This may be a subtle change in the orientation of reactive residues at the active center or a general conformational change involving the entire enzyme. Dramatic fluorescence changes in thrombin have been observed when heparin is present (31). In either case, heparin would have the same affinity for the antithrombin III·thrombin complex as it does for thrombin as was observed in the present study.

We are presently investigating the effect of active site blocked thrombin on the heparin enhanced inactivation of Factor X_a and Factor IX_a by antithrombin III. The results of these studies may be useful in determining the relative importance of the heparin-antithrombin III/-thrombin, Factor X_a and Factor IX_a reactions in regulating blood coagulation *in vivo*.

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[5] Unpublished observation.

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