Heparin-catalyzed inhibitor/protease reactions: Kinetic evidence for a common mechanism of action of heparin

(antithrombin III/heparin cofactor II/thrombin/factor Xa)

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ABSTRACT Three different heparin-catalyzed inhibitor/protease reactions were studied: antithrombin III/thrombin, heparin cofactor II/thrombin, antithrombin III/factor Xa. The three reactions were saturable with respect to both inhibitor and protease. The initial reaction velocity, for each reaction, could be described by the general rate equation for a random-order bireactant enzyme-catalyzed reaction. The kinetic parameters for the heparin-catalyzed antithrombin III/thrombin and antithrombin III/factor Xa reactions differed in terms of apparent maximum velocity (V_{max}) and apparent heparin-protease dissociation constant values. The apparent heparin-antithrombin III dissociation constant values were the same for both reactions. The kinetic parameters for the heparin-catalyzed antithrombin III/thrombin and heparin cofactor II/thrombin reactions differed in terms of apparent V_{max} and apparent heparin-inhibitor dissociation constant values. The apparent heparin-thrombin dissociation constant values were the same for both reactions. The results are consistent with a general mechanism of action of heparin for the three reactions that, in its simplest form, requires only that both protease and inhibitor bind to heparin for catalysis to occur.

Heparin increases the rate of thrombin inhibition by antithrombin III (1–3). In a recent paper, it was reported that the heparin-catalyzed antithrombin III/thrombin reaction is saturable with respect to both thrombin and antithrombin III (4). The initial reaction velocity, under any set of experimental conditions, can be described by the general rate equation for a random-order bireactant enzyme-catalyzed reaction, which is mathematically identical to the “template” model for the mechanism of action of heparin (5, 6). In principle, the template model for the mechanism of action of heparin implies that the binding of both thrombin and antithrombin III to heparin is required for the catalytic activity of heparin (7–10).

Heparin also accelerates other antithrombin III/protease reactions (11). By varying the heparin concentration over a relatively large range, it has been shown that the kinetics of factor IXa, factor Xa, plasmin, and thrombin inhibition by antithrombin III appear to differ significantly (12). It has been suggested (13) that “heparin possesses multiple functional domains that modulate different functions of antithrombin.” An alternative explanation for the apparent differences in the kinetics of the various antithrombin III/protease reactions is that different proteases bind to heparin with different affinities.

Thrombin is also inhibited at an increased rate in the presence of heparin by heparin cofactor II (14–18). Although it has been shown that higher heparin concentrations are required to observe a significant acceleration of the heparin cofactor II/thrombin reaction as compared with the antithrombin III/thrombin reaction (16–18), a detailed analysis of the kinetics of

the heparin-catalyzed heparin cofactor II/thrombin reaction has not been reported.

The purpose of the present study was to evaluate the kinetics of different heparin-catalyzed reactions to determine whether or not a common underlying mechanism of action of heparin exists. It has been found that the apparent catalytic activity of heparin is related to the apparent binding affinity of the heparin molecule for both inhibitor and protease. Since the initial reaction velocities in each reaction system can be described by the general rate equation for a random-order bireactant enzyme-catalyzed reaction (4, 19), it is concluded that the general mechanism of action of heparin in the reactions reported in the present paper is similar.

MATERIALS AND METHODS

N^{α} -*p*-Tosyl-L-glycyl-L-prolyl-L-arginine-*p*-nitroanilide (Tos-GlyProArgNaN) was purchased from Boehringer Mannheim. 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene) was purchased from Aldrich. Porcine mucosal heparin (165 USP units/mg), essentially devoid of protein, was generously provided by G. van Dedem and E. Coyne, Dionsynth B.V. (Oss, The Netherlands). Concanavalin A-agarose was purchased from Sigma. Human α -thrombin (3,600 NIH units/mg) and human antithrombin III were prepared as described (5). Heparin cofactor II was purified to homogeneity, as judged by NaDodSO₄/polyacrylamide gel electrophoresis, essentially as described by Tollefsen and co-workers (17). Human factor Xa was prepared as described (20).

Heparin Fractionation. Heparin was fractionated by gel filtration and affinity chromatography. Approximately 250 mg of heparin was applied to a Sephadex G-200 column (2.5 cm $\times$ 95 cm) previously equilibrated with 0.05 M Tris-HCl, pH 7.4/0.15 M NaCl. Material with an apparent molecular weight of 10,000–13,000 was pooled and dialyzed against 0.02 M Tris-HCl, pH 7.0/0.05 M NaCl. Ten milligrams of dialyzed heparin was applied to a 1.1 cm $\times$ 4.0 cm column of concanavalin A-agarose to which approximately 6 mg of heparin cofactor II or antithrombin III had been noncovalently bound. The columns, which were equilibrated with 0.02 M Tris-HCl, pH 7.0/0.05 M NaCl, were washed with 25 ml of this buffer, and then bound heparin was eluted with a 60 ml $\times$ 60 ml 0.05–0.5 M NaCl gradient in 0.02 M Tris-HCl (pH 7.0). Heparin activity was evaluated by determining the rate of thrombin (2.0 nM) inhibition by antithrombin III (20 nM) or heparin cofactor II (20 nM) in the presence of heparin at 5.0 ng/ml. Heparin concentration (wt/vol) was determined by uronic acid assay as described (21). To obtain the relative specific activity of a given heparin fraction, the reaction velocity obtained in the presence of fractionated

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Abbreviations: TosGlyProArgNaN, N^{α} -*p*-tosyl-L-glycyl-L-prolyl-L-arginine-*p*-nitroanilide.

FIG. 1. Kinetics of the heparin-catalyzed antithrombin III/thrombin reaction. The initial rate of thrombin inhibition by antithrombin III, in the presence of heparin at 5.0 ng/ml, was determined. (A) Initial thrombin concentration = 2.0 nM. Data are plotted according to Eq. 5. (B) Initial antithrombin III concentration = 500 nM. Data are plotted according to Eq. 3.

heparin was divided by the reaction velocity obtained in the presence of unfractionated heparin (UF-heparin). Heparin with the highest relative specific activity, which eluted from both affinity columns between 0.3 M and 0.5 M NaCl, was pooled. Heparin obtained from the heparin cofactor II/concanavalin A-agarose column (HC-heparin) had a relative specific activity of approximately 1.3 in both the antithrombin III/thrombin reaction and the heparin cofactor II/thrombin reaction. Heparin obtained from the antithrombin III/concanavalin A-agarose column (AT-heparin) had a relative specific activity of approximately 1.3 in the heparin cofactor II/thrombin reaction and of approximately 3.0 in the antithrombin III/thrombin reaction. The recovery of heparin, as determined by uronic acid and activity assays, from both affinity columns was essentially 100% ($\pm 4\%$). By weight, HC-heparin and AT-heparin constituted approximately 5% of the total heparin applied to the respective columns. HC-heparin (1.0 $\mu\text{g}/\text{ml}$) and AT-heparin (1.0 $\mu\text{g}/\text{ml}$) were incubated separately with thrombin (5 nM) for 30 min. There was no detectable loss of thrombin activity under these conditions, indicating that the fractionated heparins were not contaminated by either heparin cofactor II or antithrombin III.

Inhibitor/Protease Reaction Velocity Determination. Inhibitor/protease reaction measurements were made essentially as described (4). All reactions were carried out in 0.1 M triethanolamine, pH 8.0/0.1 M NaCl/0.1% polyethylene glycol (M_r , 6,000–7,500). The final protein and heparin concentrations were varied. Reactions were initiated by adding the protease to a solution containing inhibitor and heparin. At timed intervals after the addition of protease, samples were removed and added to 0.1 M triethanolamine, pH 8.0/0.1 M NaCl/0.1% polyethylene glycol containing Polybrene at 0.5 mg/ml and TosGlyProArgNaN. The final TosGlyProArgNaN concentrations were 0.15 mM and 0.3 mM in experiments measuring thrombin and factor Xa inhibition, respectively. The hydrolysis of TosGlyProArgNaN was terminated by addition of acetic acid. The amount of TosGlyProArgNaN hydrolyzed, determined by absorbance at 400 nm, was proportional to the thrombin or factor Xa concentration. In all experiments, the initial inhibitor concentration was significantly greater than the initial protease concentration, such that the heparin-catalyzed antithrombin III/thrombin reaction followed apparent pseudo-first-order kinet-

ics over the time course studied (4). The initial reaction velocity (v_i) was calculated by multiplying the observed pseudo-first-order rate constant by the initial protease concentration.

General Inhibitor/Protease Reaction Velocity Equations. In the present study, the heparin-catalyzed inhibitor/protease reactions are considered to be analogous to a random-order bi-reactant enzyme-catalyzed reaction (4, 19). The system is described by the equilibria shown below.

In this system, protease (P) and inhibitor (I) bind randomly to heparin (H). K_P and K_I are the heparin-protease and heparin-inhibitor dissociation constants, respectively, and k is the apparent first-order rate constant for the rate-limiting step in product formation. K_C is the dissociation constant for heparin and the product of the reaction, the stable inhibitor-protease complex (I-P). Assuming rapid equilibrium, the initial reaction velocity, v_i , is described by the following rate equation (19):

$$v_i = k[\text{H}] \times \frac{[\text{P}]}{K_P + [\text{P}]} \times \frac{[\text{I}]}{K_I + [\text{I}]} \quad [1]$$

When the protease concentration is varied in the presence of

Table 1. Kinetic parameters for the heparin-catalyzed antithrombin III/thrombin reaction

	K_T , nM	V_{max}^T , nM/min	V_{max}^* , nM/min	K_{AT} , nM	V_{max}^{AT} , nM/min	V_{max}^* , nM/min
UF-heparin	7.0	6.3	6.7	35	1.42	6.4
HC-heparin	7.0	6.9	7.4	35	1.70	7.7
AT-heparin	7.0	18.0	19.3	35	4.24	19.1

Kinetic parameters were determined from the data in Fig. 1.

FIG. 2. Kinetics of the heparin-catalyzed heparin cofactor II/thrombin reaction. The initial rate of thrombin inhibition by heparin cofactor II, in the presence of heparin at 10 ng/ml, was determined. (A) Initial thrombin concentration = 50 nM. Data are plotted according to Eq. 5. (B) Initial heparin cofactor II concentration = 375 nM. Data are plotted according to Eq. 3.

a fixed inhibitor concentration, the rate equation becomes

$$v_i = V_{\max}^P \times \frac{[P]}{K_P + [P]} \quad [2]$$

and

$$\frac{[P]}{v_i} = \left(\frac{1}{V_{\max}^P} \times [P] \right) + \frac{[P]}{V_{\max}^P}, \quad [3]$$

where $V_{\max}^P$ is the apparent maximum reaction velocity at saturation with respect to the protease. Similarly, when the inhibitor concentration is varied in the presence of a fixed protease concentration,

$$v_i = V_{\max}^I \times \frac{[I]}{K_I + [I]} \quad [4]$$

and

$$\frac{[I]}{v_i} = \left(\frac{1}{V_{\max}^I} \times [I] \right) + \frac{[I]}{V_{\max}^I}, \quad [5]$$

where $V_{\max}^I$ is the apparent maximum reaction velocity at saturation with respect to inhibitor. The apparent maximum reaction velocities, $V_{\max}^P$ and $V_{\max}^I$ are related to each other according to the following:

$$V_{\max}^* = k[H] = V_{\max}^I \times \frac{K_P + [P]}{[P]} = V_{\max}^P \times \frac{K_I + [I]}{[I]}, \quad [6]$$

where $V_{\max}^*$ is the maximum reaction velocity at saturation with

respect to both inhibitor and protease. $V_{\max}^*$ values calculated from $V_{\max}^I$ and $V_{\max}^P$ values should be equivalent if Eq. 1 is a valid description of the reaction system.

RESULTS

The heparin-catalyzed antithrombin III/thrombin reaction velocity was determined as a function of antithrombin III (Fig. 1A) and thrombin (Fig. 1B) concentrations. Apparent saturation kinetics were obtained with the three heparin preparations studied. The kinetic parameters, summarized in Table 1, indicate that the three heparin preparations do not differ measurably with respect to apparent heparin-thrombin and heparin-antithrombin III binding affinities. The relative activities, as indicated by the $V_{\max}^*$ values, did, however, differ. AT-heparin had approximately three times the activity of UF-heparin. HC-heparin activity was only slightly (1.3-fold) greater than the activity of UF-heparin.

The heparin-catalyzed heparin cofactor II/thrombin reaction velocity was determined as a function of heparin cofactor II (Fig. 2A) and thrombin (Fig. 2B) concentrations. Apparent saturation kinetics were obtained with the three heparin preparations studied. The kinetic parameters, summarized in Table 2, indicate that the apparent heparin-thrombin and heparin-heparin cofactor II binding affinities are similar for the three heparin preparations studied. The relative specific activities of AT-heparin and HC-heparin were also similar. Both heparins were approximately 1.3-fold more active than UF-heparin.

The heparin-catalyzed antithrombin III/factor Xa reaction velocity was determined as a function of factor Xa (Fig. 3) and antithrombin III (data not shown) concentrations. Apparent saturation kinetics were obtained. The kinetic parameters for the three reactions studied are summarized in Table 3, where the $V_{\max}^*$ values are expressed in terms of maximum reaction velocity per nanogram of AT-heparin.

DISCUSSION

Three different heparin-catalyzed inhibitor/protease reactions were studied. In each reaction, the initial reaction velocity can be described by the general rate equation for a random-order

Table 2. Kinetic parameters for the heparin-catalyzed heparin cofactor II/thrombin reaction

	K_T , nM	$V_{\max}^T$, nM/min	$V_{\max}^*$, nM/min	K_{HC} , nM	$V_{\max}^{HC}$, nM/min	$V_{\max}^*$, nM/min
UF-heparin	7.5	4.6	6.4	150	5.8	6.7
HC-heparin	7.5	6.0	8.4	150	7.4	8.5
AT-heparin	7.5	6.5	9.1	150	7.8	9.1

Kinetic parameters were determined from the data in Fig. 2.

FIG. 3. Kinetics of the heparin-catalyzed antithrombin III/factor Xa reaction. The initial rate of factor Xa inhibition by antithrombin III, in the presence of AT-heparin at 15 ng/ml, was determined. The initial antithrombin III concentration was 500 nM. Data are plotted according to Eq. 3.

bireactant enzyme-catalyzed reaction (4, 19).

The kinetic parameters for the three reactions studied were dependent on the components of the reaction mixture (Table 3). The apparent binding affinity of antithrombin III for heparin was, however, independent of the protease in the reaction system. Similarly, the apparent binding affinity of thrombin for heparin was independent of the inhibitor in the reaction system. These results are consistent with the random-order binding of reactants to heparin. The apparent binding affinity of antithrombin III for heparin differed significantly from the apparent binding affinity of heparin for heparin cofactor II. These results are compatible with the relative affinities of antithrombin III and heparin cofactor II for heparin-agarose (17, 18). The apparent binding affinity of thrombin for heparin differed significantly from the apparent binding affinity of factor Xa for heparin. These results are also compatible with the relative affinities of thrombin and factor Xa for heparin-agarose.

Heparin fractionation by affinity for antithrombin III had a significant effect on the catalytic properties of heparin. The kinetic parameters for the antithrombin III/thrombin reaction catalyzed by AT-heparin and UF-heparin differed, however, only in terms of the $V_{\max}^*$ values (Table 1). This result is consistent with the expectation that AT-heparin is relatively free of "inactive" heparin and should have a higher specific activity

than UF-heparin. The heparin-antithrombin III dissociation constant (K_{AT}) values observed for AT-heparin and UF-heparin would not be expected to differ since the kinetically determined K_{AT} values reflect antithrombin III binding to "active" heparin—i.e., heparin that can catalyze the antithrombin III/thrombin reaction.

Heparin fractionation by affinity for heparin cofactor II had a slight effect on the catalytic properties of heparin. The $V_{\max}^*$ values for the antithrombin III/thrombin and heparin cofactor II/thrombin reactions catalyzed by HC-heparin were only about 30% higher than the $V_{\max}^*$ values for the UF-heparin-catalyzed reactions (Tables 1 and 2). Interestingly, if antithrombin III and heparin cofactor II bind to the same oligosaccharide sequence within the heparin molecule, AT-heparin, which has a 3-fold higher specific activity ($V_{\max}^*$ value) than UF-heparin with respect to the antithrombin III/thrombin reaction, should also have a 3-fold higher specific activity than UF-heparin with respect to the heparin cofactor II/thrombin reaction. The $V_{\max}^*$ values for the heparin cofactor II/thrombin reaction catalyzed by AT-heparin and HC-heparin were, however, similar (Table 2). This observation is taken as indirect evidence that the binding sites in the heparin molecule for antithrombin III and heparin cofactor II are not identical.[†]

The apparent $V_{\max}^*$ value was higher for the AT-heparin-catalyzed antithrombin III/factor Xa reaction than for the AT-heparin-catalyzed antithrombin III/thrombin reaction (Table 3). The lower heparin-catalyzed antithrombin III/factor Xa reaction velocity, relative to the heparin-catalyzed antithrombin III/thrombin reaction velocity, which was observed with nonsaturating factor Xa concentrations (12), can be attributed, in part, to the large difference between K_{Xa} and K_T values (Table 3). Differences in the heparin preparations used in the present and previous (12) studies could also, in part, explain the observed difference in reaction velocities.

The present study has shown that the kinetically determined mechanism of action of heparin is similar for three different heparin-catalyzed inhibitor/protease reactions.[‡] Thrombin and factor Xa were specifically chosen for study because several reports have suggested that the mechanism of action of heparin in catalyzing their reaction with antithrombin III is different (12, 13). Although the present study explains this difference in terms of simple heparin binding affinity differences, the binding orientation of the protease on the heparin molecule may also be important in determining the reaction velocity. In this regard, it is important to note that kinetics does not prove chemical mechanisms. The working model described here for the mechanism of action of heparin in catalyzing protease/inhibitor reactions appears to describe the kinetics of these reactions. The apparent antithrombin III-heparin and protease-heparin binding affinities, determined kinetically, may not be equivalent to the corresponding true dissociation constants. Therefore, at present, it can be concluded that the binding of both inhibitor and protease to heparin is one important aspect of the catalytic mechanism of action of heparin, but more subtle events are also likely to be important in determining the overall reaction velocity.

Table 3. Summary of kinetic parameters for the heparin-catalyzed inhibitor/protease reactions

	Reaction		
	Antithrombin III/thrombin	Heparin cofactor II/thrombin	Antithrombin III/factor Xa
Apparent K_m for inhibitor, nM	35	150	35
Apparent K_m for protease, nM	7.0	7.5	160
Apparent $V_{\max}^*$ (nM/min)/ng	3.8	0.9	6.4

Kinetic parameters were determined for inhibitor/protease reactions catalyzed by AT-heparin. $V_{\max}^*$ values are normalized to AT-heparin at 1.0 ng/ml.

[†] Heparin has been fractionated to obtain material that does not bind to antithrombin III-agarose. This material was essentially devoid of activity in terms of catalyzing the antithrombin III/thrombin reaction but catalyzed the heparin cofactor II/thrombin reaction as effectively as heparin, which bound tightly to antithrombin III-agarose (22).

[‡] It has been found that the dermatan sulfate-catalyzed heparin cofactor II/thrombin reaction is saturable with respect to both heparin cofactor II and thrombin. The reaction velocity can be described by Eq. 1 (unpublished data).

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