The heparin-catalysed inhibition of human Factor XIa by antithrombin III is dependent on the heparin type

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The effect of various well-characterized heparin preparations on the inactivation of human Factor XIa by human antithrombin III was studied. The heparin preparations used were unfractionated heparin and four heparin fractions obtained after anion-exchange chromatography. Inactivation of Factor XIa was monitored with S2366 as chromogenic substrate and followed pseudo-first-order reaction kinetics under all reaction conditions tested. Enhancement of the rate of inhibition of Factor XIa in the presence of unfractionated heparin correlated to the binding of antithrombin III to heparin. From the kinetic data a binding constant of $0.1 \mu\text{M}$ was inferred. The maximum rate enhancement, achieved at saturating heparin concentrations, was 30-fold. The rate enhancement achieved in the presence of each of the heparin fractions could also be correlated to the binding of antithrombin III to the heparin. The binding constant inferred from the kinetic data varied from 0.10 to $0.28 \mu\text{M}$ and the number of binding sites for antithrombin III varied from 0.06 to 0.74 site per heparin molecule. The maximum rate enhancements, achieved at saturating heparin concentrations, were strongly dependent on the type of heparin used and varied from 7-fold for fraction A to 41-fold for fraction D. Therefore, although the stimulation of Factor XIa inactivation by antithrombin III could be quantitatively correlated to the binding of antithrombin III to heparin, the heparin-catalysed inhibition of Factor XIa is dependent not only upon the degree of binding of antithrombin III to heparin but also upon the type of heparin to which antithrombin III is bound.

INTRODUCTION

Human Factor XIa is the proteinase that links the contact phase of blood coagulation to intrinsic Factor X activation. It is a dimeric molecule, which is composed of two heavy chains (M_r 50000) and two light chains (M_r 33000) held together by disulphide bonds (Bouma & Griffin, 1977). It is the only known dimeric enzyme participating in blood coagulation, and the light chain of each monomer contains an active site (Fujikawa *et al.*, 1986).

Antithrombin III is one of the four plasma proteinase inhibitors that have been reported to inactivate Factor XIa (Scott *et al.*, 1982). Both active sites in Factor XIa interact with antithrombin III (Kurachi & Davie, 1977), and we have shown that the two sites are inhibited independently of each other both in the absence and in the presence of heparin (Soons *et al.*, 1987). Heparin is a mixture of mucopolysaccharides that is heterogeneous with respect to M_r , charge density and affinity to antithrombin III. In a previous study we have shown that the rate of inactivation of Factor XIa by antithrombin III is a direct measure for the binding of antithrombin III to heparin (Soons *et al.*, 1987). The mechanism via which the heparin-induced rate enhancement for bound antithrombin III is brought about remains, however, to be established. Holmer *et al.* (1981) reported that the rate enhancement of Factor XIa inhibition by antithrombin III at a single heparin concentration is dependent upon the M_r of the heparin. However, more recently Beeler *et al.* (1986) reported equal reaction rates for the inactivation of Factor XIa by antithrombin III in the presence of saturating amounts of heparin preparations with M_r values of 7000 and 15000 respectively.

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The present study concerns a detailed kinetic analysis of the inactivation of Factor XIa by antithrombin III in the presence of different well-characterized heparin preparations. The data show that the rate enhancement by heparin is not solely caused by binding of antithrombin III to heparin as such, but that the final magnitude of the reaction rate at saturating heparin concentrations also depends upon the type of heparin used.

MATERIALS AND METHODS

Materials

Chromogenic substrates pyro-Glu-Pro-Arg *p*-nitroanilide (S2366) and D-Pro-Phe-Arg *p*-nitroanilide (S2302) were purchased from AB Kabi Diagnostica, Stockholm, Sweden. Pig mucosal sodium heparin was fractionated by ion-exchange chromatography on DEAE-Sephacel with increasing salt molarities. The four fractions obtained, corresponding to the fractions A, B, C and D described by Sache *et al.* (1982), as well as the unfractionated heparin, were kindly given by the Choay Institute, Paris, France. The physicochemical properties of the different heparin preparations were reported by Sache *et al.* (1982) (M_r as estimated by h.p.l.c. and amount of antithrombin III-binding material as estimated by the percentage of material adhering to antithrombin III-Sepharose) and are listed in Table 1. Heparin concentrations were

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determined with Azure A (Nesheim, 1983), with unfractionated heparin as a standard. The molar concentrations were calculated by using the average M_r as indicated in Table 1.

Proteins

Human Factor XI was purified by the method of Bouma *et al.* (1983). Human Factor XII was isolated as described by Griffin & Cochrane (1976). Human antithrombin III was purified as described by Thaler & Schmer (1975). β -Factor XIIa was prepared from purified Factor XII as described by Fujikawa & McMullen (1983). Factor XI, Factor XII and antithrombin III preparations were homogeneous and pure as determined by gel electrophoresis in the presence of SDS on 10% polyacrylamide gels according to the procedure of Laemmli (1970). The specific activities determined in a clotting assay for Factors XI and XII were 211 and 74 units/mg respectively, assuming 1 unit to be present per ml of normal human plasma. All proteins were stored at -70°C after dialysis against 175 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.9, for human antithrombin III and 150 mM-NaCl/0.5 mM-EDTA/4 mM-sodium citrate/2 mM-acetic acid buffer, pH 5.0, for human Factors XI and XII.

Preparation of human Factor XIa

Preparation of human Factor XIa from human Factor XI by using human β -Factor XIIa was performed as described previously (Soons *et al.*, 1986). Factor XIa was separated from β -Factor XIIa on a DEAE-Sephadex column (1.5 $\times$ 11.5 cm) at 4°C in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0.

Amidolytic activity of human Factor XIa was measured with the chromogenic substrate S2366 in 175 mM-NaCl/20 mM-EDTA/50 mM-Tris/HCl buffer, pH 7.9, containing 0.5 mg of human serum albumin/ml. The kinetic parameters of the hydrolysis of the chromogenic substrate S2366 by human Factor XIa were $K_m = 0.42$ mM and $k_{cat} = 758$ s $^{-1}$.

Protein concentration

Protein concentrations were routinely determined by the method of Bradford (1976). Factor XIa concentrations were expressed as 80000- M_r subunits (van der Graaf *et al.*, 1983). Antithrombin III concentration was measured by employing an $A_{1\text{cm}, 280}^{1\%}$ value of 5.7 (Kurachi & Davie, 1977) and by a titration against a known concentration of human thrombin.

Kinetic data analysis

In a previous study we showed that the inactivation of Factor XIa by antithrombin III, both in the absence and in the presence of heparin, can be fully described according to a mechanism in which both active sites in Factor XIa are inhibited independently of each other with the same rate constant of inhibition. Furthermore, it was shown that the stimulation of the reaction rate in the presence of heparin can be satisfactorily explained by the binding of antithrombin III to heparin (Soons *et al.*, 1987). Therefore in a first approximation the data obtained in the present study with different heparin preparations were also analysed by assuming that the stimulation of the reaction rate in the presence of heparin

was due to antithrombin III binding. In that case the reaction can be written as:

in which XIa is the 80000- M_r subunit of Factor XIa and in which AT-III $_f$ and AT-III $_b$ are free antithrombin III and antithrombin III bound to heparin respectively. k_1 and k_2 are the rate constants of inactivation by free antithrombin III and by the antithrombin III-heparin complex. The rate equation can be written as:

$$d[\text{XIa}]/dt = -(k_1 \cdot [\text{AT-III}_f] + k_2 \cdot [\text{AT-III}_b]) \cdot [\text{XIa}] \quad (2)$$

and the slope of a pseudo-first-order plot will equal $-(k_1 \cdot [\text{AT-III}_f] + k_2 \cdot [\text{AT-III}_b])$. k_1 and k_2 can be independently determined in the absence of heparin (all antithrombin III is free) and in the presence of saturating amounts of heparin (all antithrombin III is bound). Since also the total amount of antithrombin III is known, the amounts of bound antithrombin III and of free antithrombin III can be calculated.

It should be stressed that in the above it is tacitly assumed that the equilibrium of binding of antithrombin III to heparin is not influenced by Factor XIa or by products formed during the reaction. Since the inactivation of Factor XIa remained pseudo-first-order throughout and since the binding data obtained were independent of the amounts of Factor XIa present over the whole range of Factor XIa concentrations tested (0.1–200 nM), this assumption appears to hold.

Binding of antithrombin III to heparin

The parameters (K_d and amounts of binding sites) of antithrombin III binding to heparin were obtained by determination of the rate of inactivation of Factor XIa by antithrombin III in the presence of heparin at various concentrations of heparin and antithrombin III. From the slopes of the pseudo-first-order plots the amounts of free antithrombin III and of antithrombin III bound to heparin were calculated as described above by using the independently determined k_1 and k_2 . The binding constant K_d is given as:

$$K_d = \frac{[\text{AT-III}_f] \cdot [S_f]}{[\text{AT-III}_b]} \quad (3a)$$

in which $[S_f]$ is the concentration of free binding sites available in which $[\text{AT-III}_f]$ and $[\text{AT-III}_b]$ equal the concentrations of free and bound antithrombin III respectively. Eqn. (3a) can be rearranged to a linear form with bound and free antithrombin III as the variables:

$$1/[\text{AT-III}_b] = (K_d/[S_{tot.}]) (1/[\text{AT-III}_f]) + 1/[S_{tot.}] \quad (3b)$$

or with free and occupied binding sites present on heparin as the variables:

$$1/[S_f] = (K_d/[\text{AT-III}_{tot.}]) (1/[S_f]) + 1/[\text{AT-III}_{tot.}] \quad (3c)$$

Thus the binding constant K_d and the total amount of binding sites ($[S_{tot.}]$) present at a given heparin concentration can be determined from a plot of $1/[\text{AT-III}_b]$ versus $1/[\text{AT-III}_f]$. Subsequently from a plot of $1/[S_b]$ versus $1/[S_f]$ at a given constant antithrombin III concentration the binding constant K_d and the total amount of antithrombin III used in the experiment are obtained (e.g. eqn. 3c). This enables an internal check for the

Fig. 1. Heparin-catalysed inactivation of Factor XIa by antithrombin III measured at various concentrations of antithrombin III and unfractionated heparin

Inactivation of Factor XIa was monitored by measurement of the disappearance of amidolytic activity with time. From the data obtained pseudo-first-order plots were constructed, and the rate constant, k , was obtained from the slope of these plots by linear regression of the data, using at least six time points for each determination. The standard error in the rate constants thus obtained was less than 1.5%. Panel (a) shows the changes in the pseudo-first-order reaction rate constant, k , when Factor XIa (8 nM) and antithrombin III (0.16 μM) were titrated with unfractionated heparin (range 0.06–11.6 μM) (● symbols). The rate constant in the absence of heparin is given by a ▲ symbol. Panel (b) shows the changes in the pseudo-first-order rate reaction constant, k , when Factor XIa (8 nM) and unfractionated heparin (0.28 μM) were titrated with antithrombin III (range 0.05–1.0 μM) (●). The same experiment was also performed in the absence of heparin (▲).

kinetic data analysis, because both experiments should give the same K_d for a given heparin preparation, and the calculated total antithrombin III concentration should of course be equal to the amount of antithrombin III added to the reaction mixture.

RESULTS AND DISCUSSION

The inhibition of human Factor XIa by human antithrombin III was studied in the presence of different heparins by measurement of the disappearance of Factor XIa amidolytic activity towards the chromogenic substrate S2366. The experiments presented here were performed several times on separate days with essentially the same results, and representative experiments are shown. Under all conditions tested the inactivation of Factor XIa was pseudo-first-order in Factor XIa since semi-logarithmic plots of the residual activity versus time yielded straight lines. From such plots the apparent first-order reaction rate constants (k) were obtained. Fig. 1(a) shows a saturation curve obtained when the apparent first-order rate constant of inhibition of Factor XIa (8 nM) by antithrombin III (0.16 μM) was determined as a function of the heparin concentration (0.06–11.6 μM). Fig. 1(b) shows the pseudo-first-order reaction rate constants obtained when at constant concentrations of Factor XIa (8 nM) and heparin (0.28 μM) the concentration of antithrombin III was varied (0.05–1.0 μM). In the latter case the rate constant did not reach a plateau (● symbols), because the rate of Factor XIa inhibition in the absence of heparin was not negligible (▲ symbols).

The data of Fig. 1 can be analysed according to a mechanism in which the stimulation by heparin is

assumed to be due to binding of antithrombin III to heparin (see the Materials and methods section). For this it is necessary that the rate constants of inactivation of Factor XIa by free antithrombin III (k_1) and by antithrombin III bound to heparin (k_2) are known. k_1 can be determined from the rate of Factor XIa inhibition in the absence of heparin (▲ symbols in Fig. 1b) and k_2 is obtained from the rate of Factor XIa inhibition at saturating heparin concentrations (plateau in Fig. 1a). From the data shown in Fig. 1 k_1 was determined to be $1.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_2 was $29.6 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$.

By using these two rate constants the binding of antithrombin III was calculated at each heparin and antithrombin III concentration and plotted as described in the Materials and methods section to obtain the parameters of antithrombin III binding to heparin. Fig. 2 shows the result obtained. A plot of $1/[\text{AT-III}]_f$ versus $1/[\text{AT-III}]_t$ at a given heparin concentration (data from Fig. 1b) yielded a straight line from which a K_d value of 101 nM was determined (Fig. 2, ○ symbols). This is in good agreement with the values reported in the literature (Jordan *et al.*, 1979; Griffith, 1982). The amount of antithrombin III-binding sites was calculated to be 0.276 mol of antithrombin III/mol of heparin assuming an M_r of 17300. From the rate constants obtained at various heparin concentrations (Fig. 1a) again a straight line was obtained (Fig. 2, ● symbols). As can be seen the same K_d was obtained, and from the intercept at the ordinate the total antithrombin III concentration present in the reaction mixture was calculated to be 167 nM (160 nM was added to the reaction mixture). Thus analysis of the data presented in Figs. 1(a) and 1(b) yields a set of internally consistent data that support the assumption

Fig. 2. Replot of data from Fig. 1 according the presented model described in eqn. (1)

For each total antithrombin III concentration (Fig. 1b) the concentrations bound and free antithrombin III were calculated as described in the Materials and methods section, and the reciprocals of them are plotted against each other (○). From the intercept at the abscissa a K_d of 101 nM was obtained, and from the intercept at the ordinate the amount of binding sites present on heparin was calculated to be 0.276 mol of sites/mol of heparin (see the Materials and methods section, eqn. 3b). Each total heparin concentration shown in Fig. 1(a) was expressed in antithrombin III-binding sites by using the value of 0.276 mol of sites/mol of heparin. The concentrations of occupied and free antithrombin III-binding sites were calculated as described in the Materials and methods section and the reciprocals of them are plotted against each other (●).

Table 1. Characteristics of heparin fractions obtained by ion-exchange chromatography on DEAE-Sephacel

Data are taken from Sache *et al.* (1982).

Compound	Elution position (M-NaCl)	M_r	Charge density* (Z^2)	High-affinity sites for antithrombin III (%)
Unfractionated heparin	—	17300	10.96	52
Fraction A	0.55	7300	6.81	23
Fraction B	0.65	13200	9.67	43
Fraction C	0.75	17300	10.56	86
Fraction D	0.85	20300	11.02	100

* Calculated by using the data of Sache *et al.* (1982) according to the procedure of Hurst *et al.* (1979). $Z = 1 + \text{sulphate content/uronic acid content}$.

that stimulation of the inactivation of Factor XIa by antithrombin III in the presence of heparin is due to the binding of antithrombin III to heparin.

Standard heparin is a heterogeneous mixture of polysaccharide chains with different M_r , charge density and affinity for antithrombin III. To gain insight in the mode

of action by which binding of antithrombin III to heparin stimulated the inactivation of Factor XIa, the reaction was also analysed with four different well-characterized heparin fractions. These fractions were obtained by ion-exchange chromatography, and the physicochemical properties of them are listed in Table 1. They differ in M_r , charge density and their affinity for antithrombin III. Fig. 3(a) shows the saturation curves obtained when Factor XIa (8 nM) and antithrombin III (0.16 μM) were titrated with the four heparin fractions. Fig. 3(b) shows that when the antithrombin III concentration was varied (0.05–1.0 μM) the pseudo-first-order reaction rate constant increased more in the presence of the heparin fractions (●, ▲, ■ and ★ symbols) than in the absence of heparin (○ symbols). The amounts of heparin chosen for the different fractions in the experiment presented in Fig. 3(b) was chosen such that the amounts of antithrombin III-binding sites were approximately equal. Thus, although the amounts of heparin present in each antithrombin III titration experiment was different, on a molar basis the amounts of antithrombin III-binding sites present were the same in each titration experiment presented in Fig. 3(b). In all cases the stimulating effect of heparin increased in the order of fraction A < fraction B < fraction C < fraction D.

From the data shown in Fig. 3(a) the rate constants of inhibition at saturating heparin concentration (k_2) were obtained, and these are summarized in Table 2. The binding of antithrombin III to fraction A caused a 7-fold increase in the rate constant compared with the rate constant obtained in the absence of heparin. Binding of antithrombin III to fraction D caused an acceleration of 41.2-fold.

From the data from experiments in which the antithrombin III concentrations were varied at a given heparin concentration (Fig. 3b) the parameters of binding of antithrombin III to heparin were determined as described in the Materials and methods section. The results are also summarized in Table 2. The calculated K_d values showed a 3-fold variation (280 nM–96 nM) whereas the number of antithrombin III-binding sites varied from 0.058 to 0.74 site per molecule of heparin. These data are in agreement with the data of Sache *et al.* (1982), who also described an increase in antithrombin III-binding material from fraction A to fraction D. However, comparison of the data in Tables 1 and 2 shows that on a quantitative basis our fractions contained fewer antithrombin III-binding sites than expected on basis of the data originally reported by Sache *et al.* (1982). This likely reflects some variability in the preparation of these fractions as well as some uncertainty in the M_r used to calculate the number of sites present per molecule of heparin for each fraction. By using the total amounts of sites as determined above the data in Fig. 3(a) were also replotted as described in the Materials and methods section to obtain the binding parameters. From such plots the same K_d values were obtained (results not shown). Therefore, as described for the unfractionated heparin, both titration experiments yielded a set of internally consistent data. We therefore conclude that the inactivation of Factor XIa by antithrombin III in the presence of heparin can be satisfactorily explained in terms of the binding of antithrombin III to heparin.

However, the final magnitude of the rate constant obtained at saturating heparin concentration was dependent upon the type of heparin used (cf. Fig. 3a and

Fig. 3. Heparin-catalysed Factor XIa inactivation by antithrombin III in the presence of four different heparin fractions

The pseudo-first-order reaction rate constants, k , were determined at various heparin concentrations (panel *a*) and at various antithrombin III concentrations (panel *b*) as described in the Materials and methods section for each of the heparin fractions. The symbols *, ■, ▲ and ● in the Figure represent data obtained for the heparin fractions A, B, C and D respectively. ○ symbols show the inhibition of Factor XIa in the absence of heparin. Panel (*a*) shows the changes in the pseudo-first-order reaction rate constant, k , when Factor XIa (8 nM) and antithrombin III (0.16 μM) were titrated with each heparin fraction. The concentration ranges used were: fraction A, 1.4–13.7 μM; fraction B, 0.2–11.4 μM; fraction C, 0.15–11.6 μM; fraction D, 0.06–9.9 μM. Panel (*b*) shows the changes in the pseudo-first-order reaction rate constant, k , when Factor XIa (8 nM) and one of the heparin fractions were titrated with antithrombin III (range 0.05–1.0 μM). The concentrations of fractions A, B, C and D were 1.49, 0.44, 0.17 and 0.12 μM respectively. Expressed as concentrations antithrombin III-binding sites present in the experiment this corresponded to 86, 105, 85 and 89 nM sites for fractions A, B, C and D respectively.

Table 2. Kinetic data of heparin fractions in Factor XIa inhibition by antithrombin III

The kinetic data given in this Table were calculated from the data presented in Figs. 1 and 3 according to the analysis described in the Materials and methods section. The second-order reaction rate constant, k_2 , was calculated by using the data given in Fig. 1(*a*) for the unfractionated heparin and the data given in Fig. 3(*a*) for the heparin fractions. The antithrombin III-heparin binding constants, K_d , and the number of antithrombin III binding sites per molecule of heparin were derived from the data given in Fig. 1(*b*) for the unfractionated heparin and from the data given in Fig. 3(*b*) for the heparin fractions.

Compound	$10^{-3} \times k_2$ ($M^{-1} \cdot s^{-1}$)	K_d (nM)	Antithrombin III-binding sites/heparin (mol/mol)
Unfractionated heparin	29.6	101	0.276
Fraction A	7.2	280	0.058
Fraction B	15.3	243	0.239
Fraction C	26.7	205	0.502
Fraction D	41.2	96	0.740

Table 2). With respect to this it may be argued that interpretation of the data is hampered by the fact that the different heparin preparations used contain varied amounts of antithrombin III-binding material. Thus fractions D, C, B and A contain increasing amounts of material that will not adhere to an antithrombin III-Sepharose column (Sache *et al.*, 1982; cf. Table 1). We therefore repeated the results obtained with fragment A with high-affinity material obtained by passing fragment A over an antithrombin III-Sepharose column. The same rates of Factor XIa inhibition were obtained with the high-affinity material as with the unfractionated fraction A, and addition of the non-adhering material had no influence on the reaction rates (results not shown). It appears, therefore, that the stimulation of the inactivation of Factor XIa by antithrombin III is exclusively due to the high-affinity heparin in the preparations and that the reaction rates are not influenced by the other material present. This is of course consistent with the finding that the stimulation of Factor XIa inhibition can be explained in terms of the binding of antithrombin III to heparin. However, since the final magnitude of the rate constant shows a large variation of saturating heparin concentrations, our data also clearly show that besides the binding of antithrombin III as such the type

of heparin is also important. Which physicochemical parameter of the heparin (e.g. size or charge density) is responsible for these observed differences can at this moment not be precisely established. However, since the M_r of the fractions increases from fraction A to D our data may with some caution be regarded as to lend support to the data of Holmer *et al.* (1981), who reported an increase in the stimulation of Factor XIa-antithrombin III inhibition with heparins of increasing M_r .

At this moment at least two possibilities remain to explain the observations reported in this paper. First, it is possible that the inactivation of Factor XIa is solely dependent upon the binding of antithrombin III and that no interactions between heparin and Factor XIa contribute to the observed reaction rate. This so-called allosteric reaction model of activation of antithrombin III by heparin has been proposed (Jordan *et al.*, 1979). However, in such a model our data can only be accommodated if one assumes that the change in antithrombin III upon binding to heparin differs for each different type of heparin. A second explanation is that Factor XIa-heparin interactions influence the rate of inactivation of Factor XIa by antithrombin III bound to heparin. Such a possibility is supported by the observation that Factor XIa has affinity for heparin-Sephadex (Østerud & Rapaport, 1977). However, direct binding experiments of Factor XIa binding to the different types of heparins will be needed to gain more insight into this question.

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