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# The relative molecular mass dependence of the anti-factor Xa properties of heparin

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The effect of heparin fractions of various $M_r$, with high affinity for antithrombin III, on the kinetics of the reaction between factor Xa and antithrombin III have been studied using purified human proteins. Each of the heparin fractions, which varied between pentasaccharide and $M_r$ 32000, accelerated the inhibition of factor Xa although an increasing rate of inhibition was observed with increasing $M_r$. The chemically synthesized pentasaccharide preparation ($M_r$ 1714) gave a maximum inhibition rate constant of $1.2 \times 10^7$ M$^{-1}$·min$^{-1}$, compared with $6.3 \times 10^4$ M$^{-1}$·min$^{-1}$ in the absence of heparin, and this rose progressively to $4.2 \times 10^8$ M$^{-1}$·min$^{-1}$ with the two fractions of highest $M_r$ (22500 and 32000). The 35-fold difference in inhibition rates observed with the high-affinity fractions was virtually abolished by the presence of 0.3 M-NaCl. The disparity in these rates of inhibition was shown to be due to a change in the $K_m$ for factor Xa when a two-substrate model of heparin catalysis was used. The $K_m$ for factor Xa rose from 28 nM for the fraction of $M_r$ 32000 to 770 nM for the pentasaccharide, whilst 0.3 M-NaCl also caused an increase in $K_m$ with the high-$M_r$ fraction. These data suggest that the increased rates of inhibition observed with heparins of higher $M_r$ may be due to an involvement of heparin binding to factor Xa as well as to antithrombin III.

## INTRODUCTION

Heparin is a glycosaminoglycan which is heterogenous with respect to both its chemical structure and $M_r$. It is a straight-chain molecule composed of disaccharide units which contain glucosamine and uronic acid residues with varying degrees of *N*- and *O*-sulphation. Commercial heparin preparations typically contain molecules with $M_r$ values ranging from 5000 to 30000, with an average $M_r$ of 10000–15000 (Johnson & Mulloy, 1976).

Heparin acts as an anticoagulant by accelerating the rate at which antithrombin III inactivates the serine proteinases of the blood coagulation system (Damus *et al.*, 1973). The anticoagulant properties of heparin are markedly affected by the polysaccharide chain length, an effect which is primarily due to a differential acceleration of the rates of inactivation of thrombin and factor Xa (Andersson *et al.*, 1979; Thunberg *et al.*, 1979). Various studies using heparin oligosaccharides in the low-$M_r$ range, with high affinity for antithrombin III, have shown that very-low-$M_r$ heparins potentiate factor Xa inhibition but are unable to potentiate the inhibition of thrombin (Holmer *et al.*, 1980; Oosta *et al.*, 1981), with this function requiring heparin molecules of a size greater than octadecasaccharide (Lane *et al.*, 1984). The minimum heparin fragment which will support factor Xa inhibition has been shown to be a specific pentasaccharide sequence, which is also the smallest heparin fragment which binds to antithrombin III with high affinity (Choay *et al.*, 1983). Other studies have shown that the specific anti-factor Xa activity is relatively unaffected by heparin $M_r$ (Holmer *et al.*, 1981; Hoylaerts *et al.*, 1983). These differences in the heparin acceleration of the inhibition of the two proteinases have suggested that the binding of heparin to antithrombin III is sufficient to accelerate the inhibition of factor Xa, but that to accelerate the inhibition of thrombin, binding of heparin to both proteinase and inhibitor is required (Jordan *et al.*, 1980; Holmer *et al.*, 1981).

In the present paper we present evidence which demonstrates that acceleration of the antithrombin III inhibition of factor Xa is quite significantly dependent upon heparin $M_r$ and that an interaction between heparin and factor Xa may be responsible for this effect.

## MATERIALS AND METHODS

Human factor Xa and human antithrombin III were prepared and characterized as previously described (Ellis *et al.*, 1982; Scully & Kakkar, 1984*a*).

### Heparin fractionation

Porcine mucosal heparin (300 mg) (Leo Laboratories, Princes Risborough, Bucks., U.K.) was fractionated by gel chromatography on a 2.6 cm × 90 cm column of Ultrogel AcA44 (LKB) equilibrated with 0.05 M-Tris/HCl (pH 7.4)/1.0 M-NaCl and at a flow rate of 30 ml/h. Fractions were assayed for heparin by metachromatic assay using Azure A (Lam *et al.*, 1976) and calibration curves prepared from dilutions of heparin. The mean $M_r$ values of the pooled fractions obtained were determined by calibration of the column with heparin standards of known $M_r$ (measured by ultracentrifugation) as previously described (Lane *et al.*, 1978). Five pools were obtained with mean $M_r$ values of 32000, 22500, 16500, 9000 and 6200. These heparin pools were further fractionated for high affinity to antithrombin III by elution with 2 M-NaCl from antithrombin III immobilized on concanavalin A–Sepharose (Pharmacia) according to Denton *et al.* (1981).

CY216, a heparin of $M_r$ 4200 with high affinity for

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antithrombin III, was obtained from Choay Laboratories, Paris, France. A heparin oligosaccharide (primarily 8–10 monosaccharide units) prepared by nitrous acid depolymerization and affinity chromatography (Thunberg *et al.*, 1982) was generously provided by Dr. D. A. Lane, Charing Cross Medical School, London, U.K. A chemically synthesized antithrombin III-binding pentasaccharide, $M_r$ 1714 (Choay *et al.*, 1983) was the kind gift of Dr. J. Choay, Choay Laboratories, Paris, France.

### Kinetic measurements

The inhibition of factor Xa by antithrombin III in the presence of varying concentrations of heparin fraction was measured under pseudo-first-order reaction conditions. Factor Xa (3.6 nM) was incubated with 30 nM-antithrombin III and 0–10 $\mu$M heparin fractions in 0.05 M-Tris/HCl (pH 7.4)/0.1 M-NaCl/5 mM-$CaCl_2$/0.1% poly(ethylene glycol) 6000 at 37 °C. The rate of hydrolysis of the chromogenic substrate, benzoyl-Ile-Glu(piperidyl)-Gly-Arg *p*-nitroanilide (S2337; Flow Laboratories, Uxbridge, Middx., U.K.), present at a concentration of 0.5 mM, was measured as change in absorbance at timed intervals with an Abbott bichromatic analyser equipped with a 380/450 nm filter. The apparent first-order rate constants ($k'_{app.}$) and second-order rate constants were calculated as previously described (Ellis *et al.*, 1986; Scully & Kakkar, 1984*b*). In experiments performed with varying factor Xa concentrations the initial velocity of factor Xa inactivation was determined as the product of $k'_{app.}$ and the initial factor Xa concentration.

## RESULTS

### Effect of heparin $M_r$ on the acceleration of the antithrombin III inhibition of factor Xa

Fig. 1 shows the data obtained for the acceleration of the inhibition of factor Xa by antithrombin III for each of the eight high-affinity heparin fractions studied. The second-order rate constant for the inhibition of factor Xa is plotted against heparin concentration on a double-logarithmic scale. Each of the heparin fractions gave a concentration-dependent acceleration of factor Xa inhibition, which reached a plateau within the range of heparin concentrations studied. The two fractions of highest $M_r$ gave identical inhibition rates at all concentrations of heparin. The maximum rate constant observed for these fractions was $4.2 \times 10^8$ $M^{-1} \cdot min^{-1}$, at oligosaccharide concentrations of above 500 nM. This represents a 6700-fold acceleration over the rate constant for the inhibition of factor Xa in the absence of heparin, which has previously been shown to be $6.3 \times 10^4$ $M^{-1} \cdot min^{-1}$ (Ellis *et al.*, 1983). With the fraction of $M_r$ 16500 a decreased rate of factor Xa inhibition was observed at all polysaccharide concentrations. This effect of decreasing inhibition rate with fractions of decreasing $M_r$ was maintained throughout the range of heparin fractions. Comparison of the maximum inhibition rates with each polysaccharide (Table 1) revealed a 35-fold difference in the acceleration of factor Xa inhibition catalysed by the pentasaccharide and the fractions of $M_r$ 22500 and 32000.

The relationship between the acceleration of factor Xa inhibition and heparin $M_r$ is shown in Fig. 2. A linear relationship was observed up to an $M_r$ of approx. 9000.

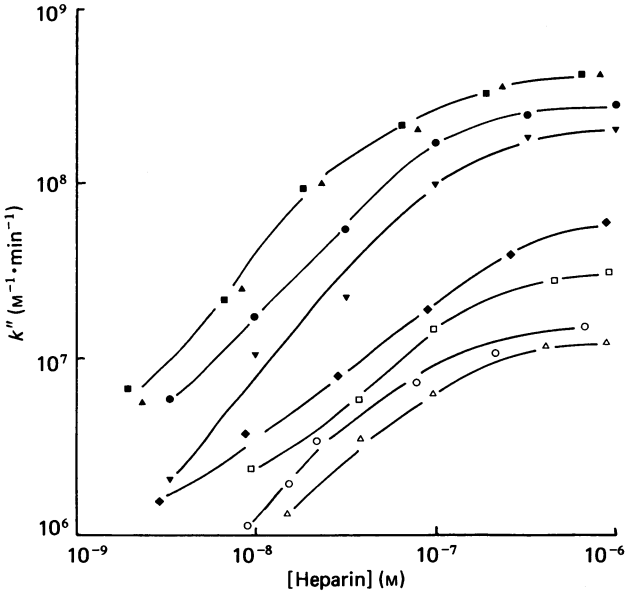


**Fig. 1. Effect of high-affinity heparin fractions of various $M_r$ on the rate constant for the inhibition of factor Xa by antithrombin III**

The second-order rate constant for the inhibition of factor Xa is plotted against molar concentration of heparin fraction on a double-logarithmic scale. The second-order rate constant was calculated from the rate of inhibition of 3.6 nM-factor Xa by 30 nM-antithrombin III in 0.05 M-Tris/HCl (pH 7.4)/0.1 M-NaCl/5 mM-$CaCl_2$/0.1% poly(ethylene glycol) 6000 at 37 °C. The $M_r$ values of the heparin fractions were: △, 1700; ○, 2700; □ 4200; ◆, 6200; ▼, 9000; ●, 16500; ▲, 22500; ■, 32000.

**Table 1. Maximum acceleration of factor Xa inhibition by antithrombin III in the presence of high-affinity heparin of various $M_r$**

The inhibition rate constants shown were obtained at oligosaccharide concentrations of approx. 2 $\mu$M, and in all cases are maximum rate constants which were unaltered at oligosaccharide concentrations of up to 10 $\mu$M. Acceleration of inhibition is with respect to the rate of factor Xa inhibition in the absence of heparin, which was $6.3 \times 10^4$ $M^{-1} \cdot min^{-1}$ under these conditions (Ellis *et al.*, 1983).

| Heparin $M_r$ | $k''$ ($M^{-1} \cdot min^{-1}$) | Acceleration of inhibition (-fold) |
|---|---|---|
| 32000 | $4.2 \times 10^8$ | 6700 |
| 22500 | $4.2 \times 10^8$ | 6700 |
| 16500 | $3.0 \times 10^8$ | 4800 |
| 9000 | $2.6 \times 10^8$ | 4100 |
| 6200 | $7.7 \times 10^7$ | 1200 |
| 4200 | $3.4 \times 10^7$ | 540 |
| 2700 | $1.7 \times 10^7$ | 270 |
| 1714 | $1.2 \times 10^7$ | 190 |

Above this $M_r$ only a small further gradual increase in the rate of factor Xa inhibition was observed, and no further increase in acceleration occurred with heparins of $M_r$ above approx. 22000.

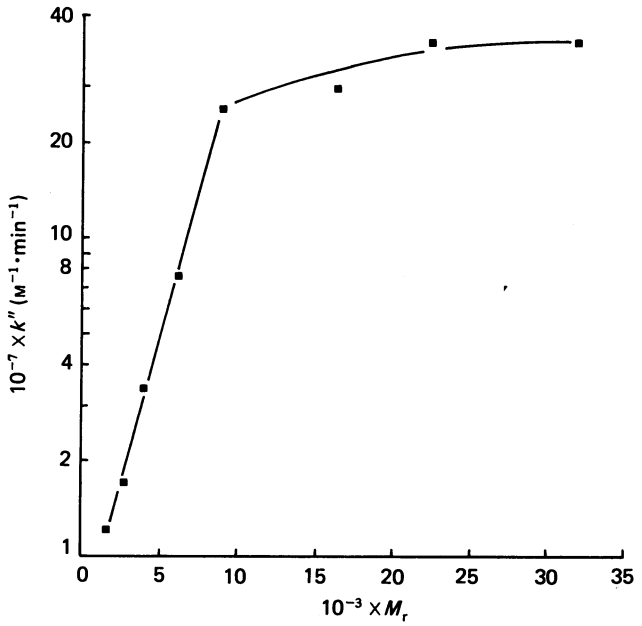


**Fig. 2. Relationship between the maximum rate constant for the inhibition of factor Xa by antithrombin III and heparin $M_r$**

The maximum rate constants obtained at saturating concentrations of heparin (see Table 1) are plotted against $M_r$ of the heparin fractions on a semilogarithmic scale.

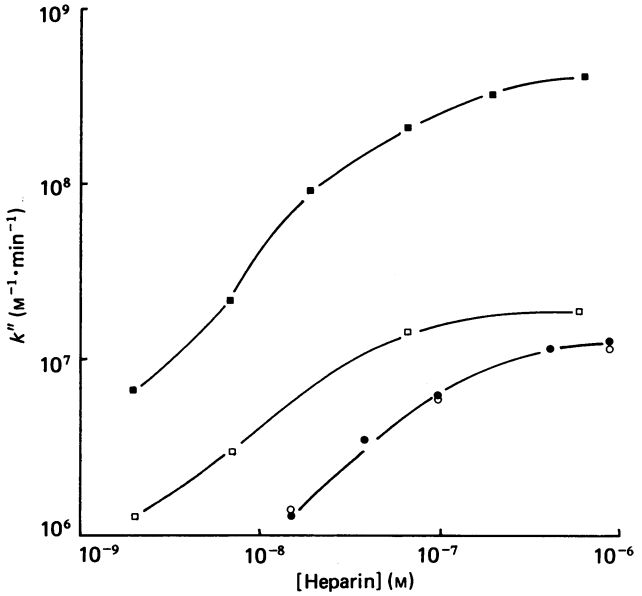


**Fig. 3. Effect of [NaCl] on the heparin-accelerated inhibition of factor Xa**

The second-order rate constant for the inhibition of factor Xa is plotted against molar concentrations of high-affinity heparin fraction on a double-logarithmic scale. The data for the fractions of $M_r$ 32000 (■) and 1714 (●) from Fig. 1 obtained at an [NaCl] of 0.1 M are compared with the data obtained for the same two heparin fractions in the presence of 0.3 M-NaCl (□ and ○).

### Influence of [NaCl] on the heparin-accelerated inhibition of factor Xa

The acceleration of factor Xa by heparin was also studied at an NaCl concentration of 0.3 M. The maximum rate constant obtained in the presence of the pentasaccharide fraction was $1.1 \times 10^7$ $M^{-1} \cdot min^{-1}$, identical with that previously obtained with 0.1 M-NaCl and consistent with the acceleration of factor Xa inhibition being due to high-affinity binding of heparin to antithrombin III. However, the maximum rate constant obtained with the high-affinity fraction of highest $M_r$ was $2.0 \times 10^7$ $M^{-1} \cdot min^{-1}$ (Fig. 3), which was 21-fold lower than that observed in the presence of 0.1 M-NaCl and less than 2-fold above that obtained with the pentasaccharide. Therefore the differential acceleration of factor Xa inhibition by heparins of various $M_r$ is virtually abolished in the presence of 0.3 M-NaCl.

### Effect of heparin $M_r$ on the $K_m$ for factor Xa in the heparin-catalysed inhibition of factor Xa by antithrombin III

The heparin-catalysed inhibition of proteinases by antithrombin III can be modelled as a two-substrate reaction (with heparin being treated as the enzyme) of either random (Griffith, 1982) or ordered (Pletcher & Nelsestuen, 1983) sequence, and therefore a value for the $K_m$ for factor Xa can be calculated under the appropriate conditions. Fig. 4 shows the Lineweaver–Burk plot used to calculate this parameter for the reaction catalysed by the two high-affinity heparin fractions of highest and lowest $M_r$. The values obtained were 28 nM for the $M_r$ 32000 fraction and 770 nM for the pentasaccharide. These constants can be used to predict the expected difference in the rate of inhibition of factor Xa with these two heparin fractions under the conditions employed in the earlier experiments. Substitution of the constants into the Michaelis–Menten equation reveals a 25-fold difference in $V_{max.}$, which correlates reasonably well with the 35-fold difference in the rate constants observed. In the presence of 0.3 M-NaCl the $K_m$ for factor Xa with the pentasaccharide was unaltered, but the $K_m$ with the $M_r$ 32000 fraction rose to 500 nM (Fig. 4), which is consistent with the effect of [NaCl] on the rate constants observed previously (Fig. 3). These data demonstrate that the increasing rates of inhibition of factor Xa observed with heparin fractions of increasing $M_r$ can be explained in terms of an increased affinity between factor Xa and the heparin–antithrombin III complex.

## DISCUSSION

The data presented in this paper demonstrate that the heparin-catalysed acceleration of factor Xa inactivation by antithrombin III is dependent upon heparin $M_r$. The inhibition rates with heparin fractions of $M_r$ above 22000 were 35-fold higher than those obtained with the pentasaccharide. Previous studies by other investigators have failed to detect such a dependency. The reasons for this are two-fold. Firstly, these studies have compared heparin fractions by their specific activities on a weight basis. This masks the effects observed here, and recalculation of such data (Holmer *et al.*, 1981) reveals an approx. 5-fold difference in inhibition rates over the $M_r$ range 3400–17000. Reconsideration of another study (Lane *et al.*, 1984) shows an approx. 3-fold difference in inhibition rates between a heparin octasaccharide and

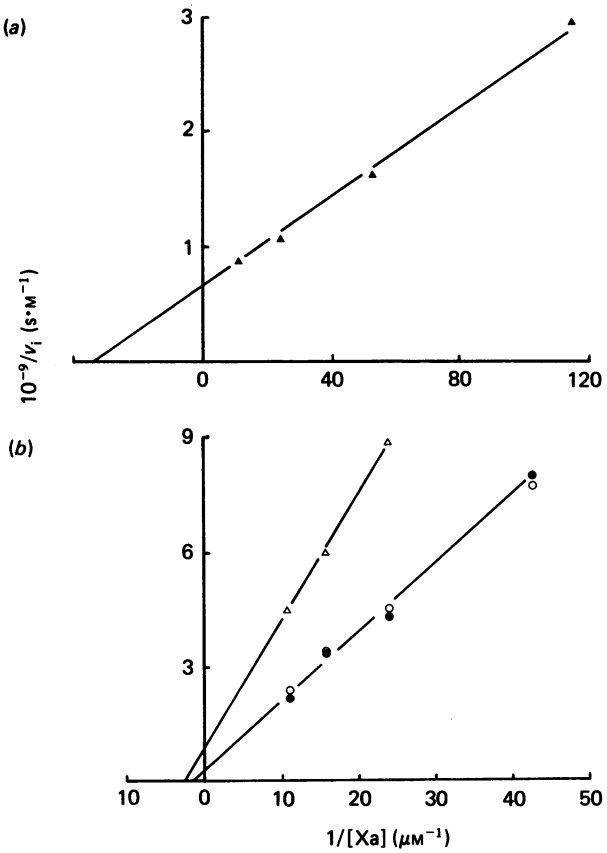


**Fig. 4. Effect of heparin $M_r$ and [NaCl] on the $K_m$ for factor Xa in the heparin-catalysed inhibition of factor Xa by antithrombin III**

Initial velocity of factor Xa inactivation ($v_i$) by 500 nM-antithrombin III in the presence of (*a*) 1 nM high-affinity $M_r$ 32000 fraction (▲) or (*b*) 1 nM pentasaccharide (●) at an [NaCl] of 0.1 M were measured and plotted against factor Xa concentration (9–90 nM) on a Lineweaver–Burk plot. △ and ○ in (*b*) show the data obtained for both fractions in the presence of 0.3 M-NaCl.

heparin of $M_r$ 13000. Secondly, these studies were all performed using factor Xa of bovine origin, rather than the human enzyme studied here. The acceleration of bovine factor Xa inhibition appears to be less dependent upon heparin $M_r$, and in preliminary experiments only a 5-fold difference was observed between the rates with pentasaccharide and heparin of $M_r$ 32000 (V. Ellis, M. F. Scully & V. V. Kakkar, unpublished work). In addition, measurements of the anti-factor Xa activity of heparin made in plasma are further complicated by the presence of lipoproteins which act as antagonists of high-$M_r$ heparin species (MacGregor *et al.*, 1979).

The anti-factor Xa properties of heparin have previously been supposed to be determined solely by the presence of a high-affinity antithrombin III-binding pentasaccharide sequence on the heparin molecule (Lindahl *et al.*, 1980, 1983; Casu *et al.*, 1981; Bjork & Lindahl, 1982; Thunberg *et al.*, 1982). However, the dependence of the anti-factor Xa properties of heparin upon its $M_r$ demonstrated here suggests that interactions other than this are taking place (Ellis & Scully, 1985). Two possible interactions may be envisaged with the higher $M_r$ heparins. Firstly, factor Xa may be interacting with the antithrombin III-bound heparin to cause the increased affinity observed. Secondly, there may be secondary interactions between the heparin molecule and antithrombin III which result in an antithrombin III molecule with a higher affinity for factor Xa. The first possibility is similar to the mechanism proposed for the potentiation of the thrombin–antithrombin III interaction by heparin (Machovich & Aranyi, 1978; Holmer *et al.*, 1980; Jordan *et al.*, 1980; Hoylaerts *et al.*, 1984), and evidence for possible secondary interactions between heparin and antithrombin III has been suggested by Stone *et al.* (1982) from observations of perturbations in the chiral absorption spectrum of antithrombin III upon binding of heparins of various $M_r$.

On the basis of the data presented here it is not possible to exclude either of these possible mechanisms for the observed dependence of heparin's anti-factor Xa activity upon its $M_r$. However, evidence for the mechanism involving heparin binding to factor Xa is provided by our previous observations on the heparin-accelerated inhibition of platelet prothrombinase complex by antithrombin III (Ellis *et al.*, 1986). In these experiments the rates of platelet-bound factor Xa inhibition in the presence of heparins of low $M_r$ were only slightly lower than those observed with free factor Xa. When heparin molecules of higher $M_r$ were studied the disparity in inhibition rates increased to approx. 7-fold. These observations are consistent with the hypothesis that binding of factor Xa to factor Va and phospholipids at the platelet surface masks a heparin-binding region of the factor Xa molecule, and therefore reduces the $M_r$ dependency of the heparin's anti-factor Xa activity. The difference in the heparin $M_r$ dependencies of factor Xa inhibition noted between the human and bovine enzymes is also suggestive of a mechanism involving a heparin–factor Xa interaction, rather than an additional interaction between heparin and antithrombin III.

The importance of these differences in the rates of inhibition of factor Xa with heparins of various $M_r$ is highlighted when the heparin concentration profiles for the reactions are studied. Fig. 1 shows that each of the heparin $M_r$ fractions reaches its maximum catalytic effect at a similar concentration, which is approx. $5 \times 10^{-7}$ M. However, due to the disparity in the maximum rates of inhibition, it can be seen that the rate obtained at this concentration of pentasaccharide is equivalent to the rate obtained with the highest $M_r$ fractions at a concentration of approx. $3.5 \times 10^{-9}$ M, a 140-fold lower concentration. A similar, although less marked, effect has also been observed in studies on the inhibition of thrombin by heparins of high $M_r$ (M. F. Scully, V. Ellis & V. V. Kakkar, unpublished work).

Heparin fractions of low $M_r$ are presently of much clinical interest due to their very low activities in the inactivation of thrombin. The data presented here, demonstrating that they also display a reduced activity in the inhibition of factor Xa, suggests that a re-evaluation of the clinical potency of these low-$M_r$ heparins may be necessary, in order to determine their exact mechanism of action *in vivo*.

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