Binding to antithrombin of heparin fractions with different molecular weights

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(Received 16 June 1980/Accepted 29 September 1980)

The interaction between bovine antithrombin, a plasma proteinase inhibitor, and heparin species of different molecular weights was studied. A commercial heparin preparation was divided by gel chromatography into a number of fractions with average molecular weights ranging from 6000 to 34 700. Each of these fractions was further fractionated by affinity chromatography on matrix-bound antithrombin. In the latter procedure, those heparin fractions that had molecular weights lower than about 14 000 were separated into three peaks. The material in the first of these was not adsorbed on the column, and the other two peaks corresponded to the low-affinity and high-affinity peaks described previously. In contrast, high-molecular-weight heparin samples gave only the low-affinity and high-affinity fractions. U.v. difference absorption studies showed that the non-adsorbed heparin fraction bound to antithrombin in solution with a binding constant at physiological ionic strength only slightly lower than that of low-affinity heparin. The division between the two fractions thus is arbitrary and only dependent on the conditions selected for the affinity-chromatography experiment. Stoichiometries and binding constants for the binding of several high-affinity heparin species to antithrombin were determined by fluorescence titrations. High-affinity heparin fractions of equal elution positions in the beginning of the peaks of the affinity chromatographies, but with different molecular weights, showed stoichiometries that were not experimentally distinguishable from 1:1 and also had no appreciable differences in binding constants. However, the anticoagulant activities, calculated on a molar basis, of these fractions increased markedly with molecular weight, a behaviour that thus cannot be explained by differences in the binding of the fractions to antithrombin. In contrast, high-affinity samples of similar molecular weights, which were eluted at increasing ionic strengths from matrix-linked antithrombin, were found to have an increasing proportion of chains with two binding sites for antithrombin and also to have progressively higher binding constants. These binding properties at least partly explain the increasing anticoagulant activities that were observed for these fractions.

The anticoagulant polysaccharide heparin greatly accelerates the rate by which the plasma proteinase inhibitor antithrombin inactivates the serine proteinases of the intrinsic coagulation system (for reviews see Rosenberg, 1977*a,b*; Barrowcliffe *et al.*, 1978). Most evidence suggests that binding of heparin to antithrombin is a necessary component of this accelerating effect (Rosenberg & Damus, 1973; Li *et al.*, 1976; Villanueva & Danishefsky, 1977; Einarsson & Andersson, 1977; Nordenman & Björk, 1978*a,b*; Nordenman *et al.*, 1978). Commercial heparin preparations are heterogeneous with respect to their biological activity, and can be separated into two functionally distinct fractions by

affinity chromatography on matrix-linked antithrombin or analogous methods. One of these fractions has high affinity for antithrombin and high anticoagulant activity, whereas the other has low affinity for the inhibitor and is almost inactive (Lam *et al.*, 1976; Höök *et al.*, 1976; Andersson *et al.*, 1976). Previous reports from our laboratory and by others have dealt with the binding of these two fractions to antithrombin in solution (Nordenman & Björk, 1978*a*; Nordenman *et al.*, 1978; Danielsson & Björk, 1978; Jordan *et al.*, 1979). However, these studies were carried out with specific, extensively purified, heparin fractions of restricted molecular weights. As some differences in the binding to

antithrombin may be expected between individual low-affinity or high-affinity heparin species, we have now extended our earlier investigations to other heparin fractions. The emphasis has been placed on characterizing the binding of high-affinity heparin species of different molecular weights and of different elution positions in affinity chromatography. Binding constants and stoichiometries for the binding of these fractions to antithrombin are presented and are correlated to the anticoagulant activities of the fractions.

Materials and methods

Antithrombin was prepared from bovine plasma as described by Miller-Andersson *et al.* (1974). A molecular weight for antithrombin of 56 000 (Nordenman *et al.*, 1977) was used in all calculations. Heparin from pig intestinal mucosa (stage 14; Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A.) was purified as described by Lindahl *et al.* (1965).

Molecular weights were determined by sedimentation equilibrium in a Beckman model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). All runs were conducted (after dialysis of the solutions to equilibrium against the buffered saline) in 0.1 M-Tris/HCl buffer, pH 7.4, containing 1.0 M-NaCl, with initial heparin concentrations of about 1 g/l. The rapid long-column meniscus-depletion method (Chervenka, 1970) was used. All calculations of molecular weights are based on a partial specific volume for heparin of 0.45 ml/g, which is an average of the two literature values reported for the polysaccharide (Laurent, 1961; Lasker & Stivala, 1966).

Titration of antithrombin with low-affinity heparin fractions were monitored by difference spectroscopy measurements of the heparin-induced u.v. absorbance change of antithrombin (Nordenman & Björk, 1978a). Similar titrations of antithrombin with high-affinity heparin fractions were monitored by fluorescence (Nordenman *et al.*, 1978). Stoichiometries (expressed as mol of heparin bound/mol of antithrombin) and binding constants were determined by computer-fitting the data from these titrations to a theoretical equation (Krause *et al.*, 1974; Nordenman & Björk, 1978a).

Anticoagulant activities were determined by a whole-blood clotting assay (*British Pharmacopoeia*, 1968), or by a spectrophotometric assay based on the ability of heparin to accelerate the reaction between purified bovine antithrombin and thrombin (Björk & Nordenman, 1976; Laurent *et al.*, 1978; Nordenman *et al.*, 1980). In the latter procedure the amounts of thrombin inactivated at several sample concentrations were related to the inactivation produced by the IIIRD International Heparin Stan-

dard under the same conditions. The results of both types of assay were expressed as units/mg or units/nmol of polysaccharide.

Analyses of glucosamine and galactosamine were performed with an amino acid analyser after hydrolysis with 6 M-HCl at 110°C for 24 h.

Protein concentrations were measured spectrophotometrically, by using a previously measured specific absorption coefficient ($A_{280}^{1\%,1\text{cm}}$) of 6.7 for bovine antithrombin (Nordenman *et al.*, 1977). Heparin solutions of known concentrations were prepared by dissolving weighed amounts of dry salt-free polysaccharide in the appropriate solvent. Heparin distributions in chromatograms were analysed by the carbazole method (Bitter & Muir, 1962).

Results and discussion

Fractionation of heparin

Heparin samples with decreased polydispersity and different average molecular weights were obtained by gel chromatography of purified commercial heparin (Fig. 1). The material in each of fractions I to V was used for further studies.

The different molecular-weight fractions of heparin were further fractionated according to their affinity for antithrombin on a column containing matrix-linked antithrombin (Höök *et al.*, 1976; Nordenman & Björk, 1978a; Laurent *et al.*, 1978). The fractions behaved differently on this column. When low-molecular-weight heparin (fraction V) was chromatographed, most of the material was not adsorbed on the antithrombin-Sepharose (Fig. 2, fraction V, peak NA). As the salt gradient was

Fig. 1. Gel chromatography of commercial heparin. A 1 g amount of purified commercial pig mucosal heparin was applied to a column (5 cm × 90 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 50 mM-Tris/HCl buffer, pH 8.0, containing 1.0 M-NaCl. The column was eluted at a rate of 70 ml/h, and 15 ml fractions were collected. The material was pooled as indicated by the vertical lines. The void (V_0) and total (V_t) volumes of the column are marked.

Fig. 2. Affinity chromatography on antithrombin–Sephacrose of heparin fractions with different molecular weights. A 50 mg amount of each of heparin fractions I–V (Fig. 1) was applied to a 2.6 cm $\times$ 15 cm column in 50 mM-Tris/HCl buffer, pH 7.4, containing 50 mM-NaCl. The column was eluted at a flow rate of 45 ml/h with a linear salt gradient (total volume 1000 ml) to the same Tris buffer containing 3 M-NaCl. The fraction volume was 8 ml. The start of the gradient is marked with an arrow (G) in each chromatogram, but the complete gradient is shown only for fraction V. The fractions were pooled as indicated by the vertical lines. Key to peaks: NA, non-adsorbed heparin; LA, low-affinity heparin; HA, high-affinity heparin.

started, a low-affinity peak (peak LA) emerged at a salt concentration of about 0.2 M, followed by the broad high-affinity peak (peak HA) at a salt concentration of about 0.8 M. As heparin fractions with increasingly higher molecular weights (fractions IV through to I) were applied to the column, the elution pattern changed gradually (Fig. 2, fractions IV–I). The amount of non-adsorbed heparin thus diminished with increasing molecular weight, and concomitantly the low-affinity and high-affinity

peaks increased. The high-affinity peak also became broader and skewed, with more material being eluted at high ionic strengths. The general pattern was broken by the high-molecular-weight end fraction (fraction I), which contained material not retained by the matrix-bound antithrombin. However, hexosamine analysis of this non-adsorbed peak showed that its glucosamine/galactosamine ratio was only 0.09, demonstrating that this peak consisted mainly of contaminating non-heparin molecules, presum-

ably dermatan sulphate. Selected fractions from the affinity-chromatography separations were pooled as indicated by the vertical lines in Fig. 2.

Molecular weights

The molecular weight of each high-affinity heparin fraction was determined by sedimentation equilibrium (Tables 1 and 2). All plots of the logarithm of fringe displacement versus the square of the distance from the centre of rotation were linear from fringe displacements of 75–100 μm to the cell bottoms. This indicates apparent molecular-size homogeneity of each of the samples during the conditions of the experiments (i.e. narrow molecular-weight distributions) and a negligible concentration-dependence of the apparent molecular weights within the

concentration range covered in the cells (about 0.1–1.5 g/l). The molecular weights obtained from these experiments may be 10–20% in error. This is due to the uncertainty associated with the partial specific volume of heparin (Laurent, 1961; Lasker & Stivala, 1966), to the slight residual polydispersity of the preparations, and to the influence on the determinations of the high negative charge of the polysaccharide (Tanford, 1961).

Comparison of non-adsorbed heparin and low-affinity heparin

As indicated above, a large portion of the low-molecular-weight heparin fractions was not retained by matrix-bound antithrombin under the usual separation conditions (Fig. 2, fractions V–IV,

Table 1. *Stoichiometries and binding constants for the binding to antithrombin, and anticoagulant activities, of high-affinity heparin fractions of different molecular weights but similar elution positions in affinity chromatography*

The high-affinity heparin fractions used were those designated peak HA in Fig. 2. The activities were determined by the B.P. whole-blood assay method and by a spectrophotometric antithrombin-activation assay method with purified reagents; for further details see the Materials and methods section. Most binding parameters were obtained by titrations, monitored by fluorescence measurements, at protein concentrations of 0.4 μM in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.26 M-NaCl (I0.3). For selected fractions, stoichiometries were also measured at protein concentrations of 1.7 μM in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl (I0.14). All titrations were performed at least in duplicate; average values are given.

Parent gel-chromatographic fraction	Mol.wt. of high-affinity heparin fraction	Anticoagulant activity						10 ⁻⁶ × Binding constant (M ⁻¹)
		B.P. assay		Antithrombin-activation assay		Stoichiometry of binding of heparin to antithrombin		
		(Units/mg)	(Units/nmol)	(Units/mg)	(Units/nmol)	(0.4 μM, I0.3)	(1.7 μM, I0.14)	
V	6000	168	1.01	131	0.79	1.0:1	—	8
IV	8400	221	1.86	269	2.26	1.0:1	1.0:1	10
III	13 200	207	2.73	271	3.58	1.2:1	—	8
II	21 500*	179	3.85	186	4.00	1.3:1	1.2:1	6
I	34 700	137	4.75	142	4.93	1.3:1	—	13

* Peak HA-1 material of fraction II (Fig. 2) was used.

Table 2. *Stoichiometries and binding constants for the binding to antithrombin, and anticoagulant activities, of high-affinity heparin fractions of similar molecular weights (about 22000) but different elution positions in affinity chromatography*

The high-affinity heparin fractions used were those indicated for fraction II in Fig. 2. For further details of the measurements, see Table 1.

High-affinity heparin fraction peak material	Mol.wt.	Anticoagulant activity						10 ⁻⁶ × Binding constant (M ⁻¹)
		B.P. assay		Antithrombin- activation assay		Stoicheiometry of binding of heparin to antithrombin		
		(Units/ mg)	(Units/ nmol)	(Units/ mg)	(Units/ nmol)	(0.4 μM, I0.3)	(1.7 μM, I0.14)	
HA-1	21 500	179	3.85	186	4.00	1.3 : 1	1.2 : 1	6
HA-2	21 300	195	4.15	287	6.11	0.9 : 1	—	10
HA-3	22 800	206	4.70	322	7.34	0.8 : 1	0.6 : 1	26

peak NA), an observation also made by Laurent *et al.* (1978). When rechromatographed, this non-adsorbed heparin fraction was eluted at its original position, which shows that its appearance was not due to overloading of the column. The binding to antithrombin of this material from one molecular-weight fraction (Fig. 2, fraction IV, peak NA) was compared with the binding to the protein of the low-affinity fraction (Fig. 2, fraction IV, peak LA) of the same molecular weight. The binding constants at physiological ionic strength, determined by difference spectroscopy titrations, were similar, i.e. $4 \times 10^4 \text{ M}^{-1}$ for the non-adsorbed fraction and $7 \times 10^4 \text{ M}^{-1}$ for the low-affinity fraction. In the calculations, the same molecular weight for these two fractions as for the corresponding high-affinity fraction (8400; Table 1) was assumed. Further similarity between the two fractions was established when a low-molecular-weight heparin fraction obtained by gel chromatography (Fig. 1, fraction IV) was applied to the antithrombin-Sepharose column in 50 mM-Tris/HCl buffer, pH 7.4, i.e. at a lower ionic strength than usual (0.04 instead of 0.09). A decrease of the amount of unretained material and a concomitant increase of the low-affinity fraction were apparent, although the size of the high-affinity peak did not change appreciably.

These results show that the non-adsorbed heparin fraction does not differ sharply from the low-affinity fraction, but, in fact, can be regarded as being part of the latter. Each heparin preparation thus apparently contains a number of molecules with a range of slightly different, but low, affinities for antithrombin. The separation of the non-adsorbed and low-affinity peaks in affinity chromatography is only an arbitrary result of the ionic strength of the chromatography, rather than of a distinct difference in the affinity of the two fractions for antithrombin. The absence of the non-adsorbed peak in the affinity chromatographies of the high-molecular-weight heparin samples indicates that the average affinity for antithrombin within the low-affinity heparin fraction increases with increasing heparin size.

Anticoagulant activities and binding of high-affinity heparin fractions to antithrombin

Stoichiometries and binding constants for the binding to antithrombin, as well as anticoagulant activities, were determined for two different sets of high-affinity heparin fractions. One of these consisted of a number of high-affinity heparins of different molecular weights with similar elution positions in the beginning of the peaks in affinity chromatography (Table 1). The other set consisted of three high-affinity fractions of the same, relatively high, molecular weight (about 22000; Fig. 2, fraction II), but with different elution positions in the skewed peak from the antithrombin-Sepharose

column (Table 2). The activities were determined both by a whole-blood assay and by a spectrophotometric antithrombin-activation assay with purified reagents. Because of the different principles of the two assay methods, their results should only be compared relative to each other. Binding-constant determinations were made at an ionic strength of 0.3 to improve precision (Nordenman *et al.*, 1978); the values thus are lower than those to be expected at physiological ionic strength (Nordenman & Björk, 1980). However, the stoichiometries obtained in these experiments were somewhat inaccurate. For selected fractions more reliable stoichiometries were therefore measured at a higher protein concentration and at physiological ionic strength. Both the stoichiometries and the binding constants are associated with rather large uncertainties, since they incorporate the experimental errors of two molecular-weight measurements and two concentration determinations.

High-affinity fractions with different molecular weights. The two methods used for the determination of anticoagulant activity gave similar trends for the set of high-affinity heparin samples that had different molecular weights (Table 1). The activity of these fractions, expressed as units per weight, showed a maximum at a molecular weight of 8000–13000, whereas the activity calculated on a molar basis increased monotonously over the whole molecular-weight range studied. Binding constants between $6 \times 10^6 \text{ M}^{-1}$ and $1.3 \times 10^7 \text{ M}^{-1}$ were measured for the five fractions (Table 1). However, since no trend was apparent, most of the variation probably is due to the experimental error of the fluorescence titrations (Nordenman *et al.*, 1978). The values obtained for the stoichiometry of binding also varied somewhat, but all values most likely reflect equimolar stoichiometries. Thus most of the molecules in the different fractions have one, identical or highly similar, binding site for antithrombin.

A similar molecular-weight-dependence of the anticoagulant activity of high-affinity heparins as that observed in the present work has been shown earlier (Laurent *et al.*, 1978; Andersson *et al.*, 1979). However, these investigators measured the average activity of the whole broad high-affinity peak from affinity chromatography and did not quantitatively characterize the binding of the fractions to antithrombin. Our results show that differences in the number of heparin molecules bound to antithrombin, and in the affinity of the binding, cannot be the main reasons for the molecular-weight-dependence of the anticoagulant activity of high-affinity heparins, since the activity of high-affinity molecules with essentially identical binding properties to antithrombin also increases with molecular weight. Other factors therefore are likely to be of primary importance. These may include the necessity for a

region of a high-affinity heparin molecule outside the antithrombin-binding site to bind either to thrombin, or weakly to an additional site on antithrombin, for the anticoagulant activity of the molecule to be fully expressed (Laurent *et al.*, 1978). Another possibility is that differences in the kinetics of the binding of the various fractions to antithrombin, or in their dissociation from the antithrombin-thrombin complex, may be responsible for the molecular-weight-dependence of the activity.

The five fractions analysed were all obtained from the beginning of the high-affinity heparin peaks. The fact that similar binding constants were measured for these fractions therefore suggests that the minimum affinity for high-affinity heparins of different molecular weights is about the same. This minimum affinity apparently is shown by most molecules in low-molecular-weight high-affinity fractions, since these fractions are eluted from the affinity column within a rather narrow range of ionic strengths, and the major parts of the fractions therefore were included in the material used in the binding studies. However, the proportion of high-affinity molecules with higher binding constants increases in heparin fractions with higher molecular weights (see below). A value at physiological ionic strength of slightly below 10^8 M^{-1} may be estimated for the minimum affinity from the binding constants determined at higher ionic strengths in the present work, in conjunction with the previously established dependence of the binding constant for high-affinity heparin on ionic strength (Nordenman & Björk, 1980). Essentially the same binding constant, i.e. $8 \times 10^7 \text{ M}^{-1}$, was also determined previously at physiological ionic strength for one selected molecular-weight fraction of high-affinity heparin that was eluted early from the affinity column (Nordenman *et al.*, 1978).

The observation that antithrombin bound equimolar amounts of the different high-affinity heparin fractions, regardless of the molecular weight of the polysaccharide, is in contrast with a proposal by Piepkorn *et al.* (1978). These authors suggested, on the basis of fluorescence titrations, that one molecule of antithrombin can bind two molecules of low-molecular-weight (about 8000) heparin but only one molecule of high-molecular-weight (about 14000) heparin. However, the heparin samples studied were not fractionated according to their affinity for antithrombin. Since the proportion of low-affinity heparin (including the non-adsorbed fraction) increases with decreasing heparin molecular weight (Laurent *et al.*, 1978; see also Fig. 2), the presence of a large amount of weakly binding low-affinity heparin in the low-molecular-weight fraction used by Piepkorn *et al.* (1978) most probably accounts for the observed apparent stoichiometry value of 2:1.

High-affinity fractions with different elution positions in affinity chromatography. The analyses of the other set of high-affinity heparin fractions, i.e. those that had similar, high, molecular weights but were eluted at different ionic strengths in a skewed peak in affinity chromatography, gave the results presented in Table 2. The anticoagulant activities of these fractions increased with increasing elution volume from the antithrombin-Sepharose column, when they were expressed both on a weight basis and on a molar basis. Moreover, and not surprisingly, high-affinity fractions that were eluted progressively later from the affinity column were found to have higher binding constants than the high-affinity heparin fraction that was eluted at the lowest ionic strength. More strikingly, the late-eluted high-affinity heparin fractions also gave markedly lower stoichiometries (expressed as mol of heparin/mol of antithrombin) than the high-affinity fraction that was most weakly bound to the column. Indeed, the stoichiometry of 0.6:1 obtained for the fraction that was eluted at the highest ionic strength indicates that a large proportion of the molecules in this fraction has two binding sites of similar affinity for antithrombin. This is in agreement with a study by Rosenberg *et al.* (1979), who used a gel-chromatographic method to demonstrate the existence of high-affinity heparin molecules with two binding sites for antithrombin.

There is thus an increasing occurrence in high-molecular-weight high-affinity heparin fractions of molecules with two binding sites for antithrombin, as well as of molecules with higher affinity for the protein than the minimum affinity discussed above, and possibly also of molecules with both these properties. This behaviour accounts for the observation that the high-affinity heparin peak is progressively more skewed in affinity chromatographies of heparin fractions with increasing molecular weights. The higher anticoagulant activities of high-affinity fractions eluted late from the affinity column may also at least partly be explained by the same behaviour. The binding constant determinations at an ionic strength of 0.3 indicate that the affinity at physiological ionic strength of the most tightly bound high-affinity fraction from the skewed peak investigated would be about $3 \times 10^8 \text{ M}^{-1}$, which is 4–5 times the binding constant for the most weakly bound fraction. However, the existence of a small number of high-molecular-weight heparin molecules with even somewhat higher binding constants is suggested by the shape of the affinity-chromatographic elution patterns. The observed range of affinities may be due to a variation of the structure within the antithrombin-binding site of the high-affinity heparin molecules (Lindhall *et al.*, 1979). However, a variation of the number of negatively charged sulphate groups outside the

binding area of the molecules could also result in different affinities for antithrombin owing to a general electrostatic effect.

This work was supported by grants from the Swedish Medical Research Council (Project no. 04212) and from the Swedish Society for Medical Research. We are grateful to Dr. B. Ajaxon (AB Vitrum, Stockholm, Sweden) for performing the anticoagulant assays, and to Ms. E. Raub for excellent technical assistance.

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