Determination of the range in binding-site densities of rat skin heparin chains with high binding affinities for antithrombin

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Rat skin heparin proteoglycans vary markedly in the proportions of their constituent polysaccharide chains that have high binding affinity for antithrombin. As the proportion of such chains in a proteoglycan rises, their degree of affinity for antithrombin also increases [Horner (1987) *Biochem. J.* **244**, 693–698]. The antithrombin-binding-site densities of such chains have now been determined, by measuring heparin-induced enhancement of the intrinsic fluorescence of antithrombin and by chemical analysis for the disaccharide sequence glucuronosyl-*N*-sulphoglucosaminyl (3,6-di-*O*-sulphate), which is unique to this site in heparin [Lindahl, Bäckström, Thunberg & Leder (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6551–6555]. Antithrombin-binding-site density ranged from one to five sites per chain.

INTRODUCTION

It has been demonstrated (Horner, 1987) that heparin chains with high affinity for antithrombin (HA-chains), which are components of HA-heparin proteoglycans from rat skin (Horner & Young, 1982), are very heterogeneous, with specific anticoagulant activities covering an approximately 10-fold range. It was postulated, by deductions that involved comparisons with published data obtained with commercially derived HA-heparins of much lower M_r , that the upper limit for the number of antithrombin-binding sites per chain in rat skin heparin proteoglycans was probably five or six.

In order to determine the upper limit in binding-site density and to establish that the variation in antithrombin affinity of HA-chains is correlated with the number of discrete antithrombin-binding sites on the rat skin proteoglycan chains, chemical and physicochemical analyses were used to determine the number of antithrombin-binding sites on the HA-chain fractions previously isolated and assayed for anticoagulant activity by Horner (1987).

MATERIALS AND METHODS

The methods of isolating HA-chain fractions from ^{35}S -labelled rat skin heparin proteoglycans have been described previously in detail (Horner, 1987). HA-chains were eluted from antithrombin-agarose with an NaCl concentration gradient, recovered by precipitation with ethanol and quantified by metachromasia with Azure A. Their specific anticoagulant activities (with respect to thrombin neutralization) were determined with a chromogenic assay. The fractions analysed in the current work are these same materials. To facilitate comparison with the earlier work, the heparin fraction numbers assigned previously (see the text and Fig. 5 of Horner, 1987) have been retained for this presentation.

HA-chain fractions were analysed by measuring the heparin-induced enhancement in the intrinsic fluorescence of human antithrombin. The initial fluorescence of a solution of antithrombin ($0.17\ \mu\text{M}$, $10\ \mu\text{g/ml}$, $1.7\ \text{ml}$ volume in $0.01\ \text{M}$ -sodium phosphate buffer, pH 7.4) was determined. Small portions of a solution of a heparin fraction in the same buffer were then added successively to this solution. After each addition fluorescence intensity was measured, with correction mathematically for dilution by the heparin titrant. Each heparin fraction was added to antithrombin to a final concentration of at least $7\ \mu\text{M}$, assuming an M_r of 80000 (Horner, 1987). Fluorescence was measured on an SLM model SPF-500C spectrofluorimeter, with an excitation wavelength of 280 nm with a 1 nm slit width and an emission wavelength of 314 nm with a 20 nm slit width. Stoichiometries were calculated from the fluorescence data, by using a computerized non-linear least-squares fitting routine, as described by Peterson & Blackburn (1985).

Antithrombin-binding regions were also determined by analysis of the unique disaccharide marker sequence glucuronosyl-*N*-sulphoglucosaminyl (3,6-di-*O*-sulphate), which is confined exclusively to these regions in HA-heparins (Lindahl *et al.*, 1980; Thunberg *et al.*, 1982; Lindahl *et al.*, 1984). The method has been fully described by Jacobsson *et al.* (1986). HA-chain fractions were deaminated with nitrous acid at pH 1.5 followed by reduction with NaB^3H_4 . The ^3H -labelled disaccharides were then fractionated by high-performance ion-exchange chromatography on a column of Partisil-10SAX (Bienkowski & Conrad, 1985). Glucuronosyl-[1- ^3H]anhydromannitol (3,6-di-*O*-sulphate) was obtained as a minor but well-resolved component of the total disaccharides. The number of binding sites per chain was calculated by expressing this marker as a percentage of the total disaccharides and assuming that the intact chains and their constituent disaccharides had average M_r values of 80000 and 600 respectively.

Abbreviations used: HA, high affinity for antithrombin; LA, low affinity for antithrombin.

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Fig. 1. Enhancement of the intrinsic fluorescence of human antithrombin by HA-heparin chain fractions

Experimental details are given in the Materials and methods section. Fractions 28, 29, 30, 31, 32, 33 and 35 are represented by the symbols ◆, ○, ●, Δ, ▲, □ and ■ respectively. The plot for fraction 34, which lay midway between the plots of fractions 33 and 35, has been omitted. In calculating antithrombin-binding-site densities from these data an M_r value of 80000 has been assumed. This value, previously used by Jacobsson *et al.* (1986) and Horner (1987), is based on the ultracentrifugal analyses made by Robinson *et al.* (1978).

RESULTS AND DISCUSSION

Rat skin heparin proteoglycan was treated with alkali, and the released polysaccharide chains were fractionated by affinity chromatography on antithrombin-agarose (see the Materials and methods section and Horner, 1987). Individual fractions from the high-affinity portion of the chromatogram (Fig. 2a) were tested for their ability to enhance the fluorescence of human antithrombin, an effect demonstrated by Einarsson & Andersson (1977) and first used to determine the stoichiometry of the interaction between antithrombin and a high-affinity mucosal heparin preparation by Nordenman *et al.* (1978). This readily measurable change in fluorescence was used to generate saturation curves as the protein was titrated with HA-heparin chains. The change in protein fluorescence induced by the available amounts of fractions 26 and 27 was too weak to generate reliable data. Fig. 1 shows the fluorescence-enhancement data for fractions 28–35. The antithrombin-binding site densities calculated from these measurements are presented as histograms in Fig. 2(b). They range from 1.0 site per chain for fraction 28 to 4.8 sites per chain for fractions 34 and 35. The mean binding-site density for fractions 28–35 was 3.1 sites per chain.

The binding-site densities determined by direct chemical analysis of fractions 28, 30, 32, 34 and 36 also are presented as histograms in Fig. 2(b). They range from 0.4 site per chain for fraction 28 to 3.6 sites per chain for fraction 36, with a mean value of 2.0 sites per chain. The marker disaccharide could not be detected in fractions 26 and 27. If one estimates the binding-site densities of the odd-numbered fractions, which were not analysed, by making linear interpolations between the values for the even-numbered fractions, the mean binding-site density for fractions 28–36 is also 2.0 sites per chain. This value

is appreciably lower than the value of 2.8 sites per chain reported by Jacobsson *et al.* (1986) for an HA-chain preparation. A feasible explanation of the disparity is that the latter value was for a selectively fractionated preparation from the upper end of the range in affinity of the total HA-proteoglycans, representing approx. 63% of the HA-chains derived from the total proteoglycan starting material.

Antithrombin-binding-site densities calculated from the physicochemical analyses were consistently higher than those obtained by chemical analysis. Although there is no obvious explanation for this discrepancy, it may be due to selective loss of the marker disaccharide, which was a minor proportion (0.3–2.7%) of the total ^3H -labelled disaccharides, during the degradation procedures and multiple steps involved in the chemical analyses. However, it is clear that the proportions of the ^3H -labelled marker disaccharide essentially paralleled the antithrombin-binding-site densities determined by fluorescence enhancement.

It is reasonable to accept that the non-degradative physicochemical analyses give quantitative data, since a known amount of antithrombin is used in the fluorescence titration with known amounts of HA-chains. Antithrombin binds in a 1:1 stoichiometric fashion with antithrombin-binding sites in heparin (Jordan *et al.*, 1982; Nesheim *et al.*, 1986), so that titration curves such as those in Fig. 1, which are linear at low concentrations of titrant and for which a saturating level of fluorescence is measurable, can be easily extrapolated to determine the molar concentration of antithrombin-binding sites on a given heparin sample. The low-ionic-strength buffer used in the fluorescence measurements ensured tight binding of the ligand to the protein (K_d 10^{-8} M), so that the titration curves shown were easily fitted by a non-linear least-squares routine to determine the density of antithrombin-binding sites for each HA-chain fraction. These chains were found to contain from one to five antithrombin-binding sites, with the predominant type (fractions 30 and 31, Fig. 2a) having three sites per chain. Fig. 2(b) also shows that the relationship between antithrombin-binding-site density and anticoagulant specific activity is approximately linear.

The analytical data confirm and extend the findings and proposals made on the basis of anticoagulant assays of the same HA-chain preparations by Horner (1987). In the case of the fluorescence-enhancement data, an interesting comparison can be made with results obtained by the same procedure with HA-heparin fractions prepared from commercial pig mucosal heparin by Nesheim *et al.* (1986). They obtained six fractions with M_r values ranging from 10000 to 30000 and observed a linear increase in the number of antithrombin-binding sites per molecule from 1.0 to 2.1. If their data are extrapolated linearly, heparin with an M_r of 80000, comparable with that of the HA-chains used in the present study, would have 4.9 antithrombin-binding sites per molecule. It thus appears that the upper limit of binding-site density is essentially the same in heparins from rat skin and pig intestinal mucosa.

Establishing the upper limit of antithrombin-binding-site density allows us to elaborate on the findings of Horner (1987) concerning the heterogeneity of HA-proteoglycans and HA-chains. By fractionating HA-proteoglycans and their constituent chains, it was shown that the ratio of LA-chains to HA-chains in

Fig. 2. (a) Elution of HA-heparin chains from a column of antithrombin-agarose with an NaCl concentration gradient, and (b) antithrombin-binding-site densities and anticoagulant specific activities of HA-heparin chain fractions 28–36

(a) Elution of HA-heparin chains from a column of antithrombin-agarose with an NaCl concentration gradient. ●, Heparin mass, expressed as a percentage of the total heparin in fractions 28–36, determined by metachromasia with Azure A after recovering the heparin in each fraction by precipitation with ethanol; ○, NaCl concentration, determined by measuring conductivity. The data, including the fraction numbers, are taken from Fig. 5 in the paper by Horner (1987). (b) Antithrombin-binding-site densities and anticoagulant specific activities of HA-heparin chain fractions 28–36. □, Antithrombin-binding sites per chain, determined from single chemical analyses for the disaccharide sequence glucuronosyl-*N*-sulphoglucosaminyl (3,6-di-*O*-sulphate) (Jacobsson *et al.*, 1986). ▨, Antithrombin-binding sites per chain, determined by fluorescence enhancement; $n = 1$ for fraction 28; other values are means $\pm$ s.d., $n = 2$ for fraction 29, $n = 3$ for fractions 31, 33, 34 and 35 and $n = 4$ for fractions 30 and 32. ■, Anti-(Factor IIa) activity, determined by amidolytic assay (Horner, 1987); values are means $\pm$ s.d., $n = 4$.

Fig. 3. Hypothetical heparin proteoglycan molecules, each comprised of 12 heparin chains attached to a polypeptide core, corresponding to average M_r values of 1×10^6 for rat skin heparin proteoglycans (Horner, 1971) and 8×10^4 for the chains (Robinson *et al.*, 1978)

The black circles represent antithrombin-binding sites, introduced sequentially into an LA-proteoglycan (A) to give HA-proteoglycans containing chains with increasing antithrombin-binding-site densities, labelled B to F. (Intermediate species with odd numbers of antithrombin-binding sites per proteoglycan molecule have been omitted.) This progressive sequence is consistent with earlier data (Horner, 1987).

HA-proteoglycans ranged from approx. 4:1 to approx. 1:4 in six HA-proteoglycan subfractions. Furthermore, as the proportions of HA-chains increased, so did their degree of affinity for antithrombin, i.e. their binding-site densities (see Table 1 and Figs. 3 and 4 in Horner, 1987).

These findings can be rationalized into the hypothetical biosynthetic scheme presented in Fig. 3. The incorporation of the 3-*O*-sulphate group concludes the formation of the antithrombin-binding site (Riesenfeld *et al.*, 1983; Kusche *et al.*, 1987). The scheme thus illustrates how these sites are generated by progressive 3-*O*-sulphation of glucosamine residues in a precursor proteoglycan (model A). Limited interaction with the 3-*O*-sulphotransferase would give products at the low end of the HA-proteoglycan spectrum such as model B. Extensive 3-*O*-sulphation would produce proteoglycans in which some chains achieved maximal binding-site density (model F). Introduction of antithrombin-binding sites has been stopped at this stage, as even proteoglycans of the highest affinity contain some LA-chains (Horner, 1987). The orderly distribution of sites is entirely speculative.

There are two major possible explanations, not necessarily mutually exclusive, for the asymmetric distribution of antithrombin-binding sites in heparin proteoglycans. The first envisages limited access of polysaccharide chains to 3-*O*-sulphotransferase molecules. Lindahl *et al.* (1986) presented a model of heparin biosynthesis in mast cells in which the proteoglycan traverses distinct groups of membrane-bound enzyme complexes in the Golgi apparatus in an order that reflects the sequence of post-polymerization modification reactions. The authors suggested that a restricted distribution within the membrane complex of the glucosaminyl 3-*O*-sulphotransferase required for synthesis of antithrombin-binding sites might explain the non-random distribution of these sites in the final products. According to this model, the results obtained by Jacobsson *et al.* (1986), by Horner (1987) and in the present study would imply that some heparin chains passing through the Golgi encounter more 3-*O*-sulphotransferase enzyme 'stations' than others, and furthermore that a majority of chains, including in many cases all the chains comprising a complete proteoglycan molecule, do not encounter the 3-*O*-sulphotransferase at all. If this proposal is valid, variations in the number of glucosaminyl 3-*O*-sulphotransferase molecules at station III in different biosynthetic units (see Lindahl *et al.*, 1986) could correlate with variations in antithrombin-binding-site density.

The model as presented would produce HA-chains that were uniform with respect to antithrombin-binding-site density, in the proteoglycans formed by any one biosynthetic unit, rather than products of the general type shown in Fig. 3. However, for the sake of simplicity, this model presented proteoglycans undergoing polymer modification as being two-dimensional, which is not necessarily the case. If the array of enzyme stations in a biosynthetic unit in the Golgi apparatus is in fact a flat bed of membrane-bound enzymes, traversed by a three-dimensional proteoglycan molecule, it is easier to envisage how different polysaccharide chains could interact to differing degrees with glucosaminyl 3-*O*-sulphotransferase molecules. Clearly all chains do interact with the other sulphotransferases, which must be much more abundant.

The other factor that may control antithrombin-binding-site synthesis is the distribution along the polysaccharide chains of recognition sites for the 3-*O*-sulphotransferase. The studies cited above, showing that 3-*O*-sulphation concludes the formation of the antithrombin-binding region, also point to a stringent substrate specificity for the 3-*O*-sulphotransferase: only pentasaccharide sequences that express all the structural features required for antithrombin binding except the 3-*O*-sulphate group are recognized by the enzyme. Conceivably, the key to the distribution of antithrombin-binding sites lies in the distribution of these recognition sites, synthesized during earlier stages of polymer modification. Polysaccharide chains predestined to remain LA-chains would lack these recognition sites and would therefore not be attacked by the 3-*O*-sulphotransferase.

The elucidation of the precise regulatory mechanisms involved in the synthesis of antithrombin-binding sites is a challenge, considering the marked diversity of products containing these sites. The current work and earlier studies with rat skin heparin proteoglycans (Horner & Young, 1982; Jacobsson *et al.*, 1986; Horner, 1987) illustrate the diversity of antithrombin-binding-site densities in products from a single source. In such a case structural differences may be related, for example, to variations in degree of maturation of mast cells (Yong *et al.*, 1975). The extent of antithrombin-binding-site synthesis also varies markedly in heparins in different tissues of a single species (Horner, 1986), and in heparins from different species (Hovingh *et al.*, 1986). Heparan sulphates also differ markedly in antithrombin-binding-site density (Marcum *et al.*, 1986; Hovingh *et al.*, 1986; Pejler *et al.*, 1987). Addition of *n*-butyrate to cell cultures stimulated the synthesis of binding sites in mastocytoma-cell heparin (Jacobsson *et al.*, 1985) and in endothelial-cell heparan sulphate (Marcum *et al.*, 1986). Thus variations in antithrombin-binding-site density occur naturally and can be induced pharmacologically. Such changes are likely to regulate the expression of anticoagulant activity in endogenous heparins and heparan sulphates. Their control therefore has important physiological and pathological implications.

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