Molecular-size-dependent variations in the proportions of chains with high binding affinities for antithrombin in rat skin heparin proteoglycans

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Approximately half of all rat skin heparin proteoglycans have polysaccharide chains that have no sites with high binding affinity for antithrombin. The rest have chains with high-affinity antithrombin-binding-site densities ranging from zero to five sites per chain, with a high degree of variation. Proteoglycans vary in size because of diversity in the number of chains per molecule; the relationship between proteoglycan size and high-affinity antithrombin-binding-site density has not been studied previously. Polydisperse heparin proteoglycans from rat skin, labelled biosynthetically with ^{35}S , were fractionated by gel filtration on Bio-Gel A-150m and arbitrarily divided into five fractions of decreasing average molecular size. Fractionation of these products on antithrombin-agarose showed that the proportion of proteoglycans with high affinity for antithrombin decreased from 39% to 25% as molecular size decreased. However, as the molecular size of high-affinity proteoglycans decreased, the proportion of their chains that had high affinity increased from 29% to 59%. Therefore molecular size is a significant factor in determining the proportion of high-affinity chains in heparin proteoglycans. A model of heparin biosynthesis is proposed in which areas of specific enzyme activity that control the synthesis of the antithrombin-binding-site sequence are sparsely and non-randomly distributed on mast-cell Golgi membranes. It is postulated that the likelihood of a developing proteoglycan encountering one of these hypothetical areas is molecular-size-dependent.

INTRODUCTION

A rat skin heparin proteoglycan preparation contains proteoglycans with low binding affinity for antithrombin (LA-proteoglycans) in which all the heparin chains have low binding affinity for antithrombin (LA-chains), and proteoglycans with high binding affinity for antithrombin (HA-proteoglycans) in which some of the heparin chains have high binding affinity for antithrombin (HA-chains) (Horner & Young, 1982). HA-chains may have one to five binding sites with high binding affinity for antithrombin (HA binding sites) (Horner *et al.* 1988). HA-proteoglycans are highly variable in composition, there being an inverse relationship between the proportion of LA-chains and the mean HA binding-site density of the HA-chains (Horner, 1987).

Heparin proteoglycans are clearly polydisperse with respect to molecular size. Horner (1971) observed that the electrophoretic mobility in agarose gel of rat skin heparin proteoglycans eluted from a column of agarose-gel granules was inversely proportional to molecular size. Metcalfe *et al.* (1980) used gel filtration through agarose-gel granules to demonstrate the polydispersity of rat peritoneal mast-cell heparin proteoglycans and showed that this was due to variation in the number of chains per molecule rather than to variations in chain size.

In the earlier work on HA binding-site distribution cited above, the possible influence of proteoglycan size was not studied. This aspect was addressed in the present work, with five proteoglycan fractions obtained by gel filtration.

EXPERIMENTAL

Eight male Wistar rats (average body wt. 331 g) were each given 7.4 mCi of $\text{H}_2^{35}\text{SO}_4$ in 1 ml of 0.15 M-NaCl by intraperitoneal injection. Their food was then removed and they were killed 18 h later by decapitation. The methods for recovering [^{35}S]heparin proteoglycans from rat skin, for releasing chains from the proteoglycans with NaOH and for fractionating [^{35}S]heparins by gradient elution from antithrombin-agarose have been described in detail previously (Horner & Young, 1982; Horner, 1987). The human antithrombin used was generously given by Dr. Milton Mozen of Cutter Laboratories, Berkeley, CA, U.S.A.

To produce [^3H]heparin chains, unlabelled rat skin heparin proteoglycan (500 μg) was dissolved in 2.0 M-NaCl (100 μl) and mixed with NaB^3H_4 (25 mCi, 60 μmol ; ICN Radiochemicals) in 1.0 M-NaOH (100 μl) at 0 °C. After 72 h acetic acid was added to give pH 5 and the mixture was applied to a column (1.6 cm $\times$ 18 cm) of Bio-Gel P6-DG (Bio-Rad Laboratories) equilibrated with 1.2 M-NaCl/0.05 M-Tris/HCl buffer, pH 8.5. ^3H -containing material eluted in the void volume was recovered by precipitation with ethanol (Horner & Young, 1982), which was repeated three times, until the product contained 99% of the remaining radioactivity. This was fractionated on antithrombin-agarose (Horner, 1987) yielding an ^3H -end-labelled HA-heparin chain preparation that contained 12% of the total radioactivity.

For electrophoresis, commercially prepared 1%-agarose-gel films on plastic backings (Corning Electro-Trace Universal Electrophoresis Film) were used. These

Fig. 1. Final purification of total ^{35}S -labelled rat skin heparin proteoglycans by gel filtration on a column (1.0 cm $\times$ 36 cm) of Sepharose CL-4B)

The column was equilibrated with 1.2 M-NaCl adjusted to pH 2.5 with HCl. Fractions (1.0 ml) were collected at a flow rate of 5 ml/h into tubes containing 2 M-sodium acetate (100 μl). They were analysed for radioactivity, then recombined as shown. Products A, B and C were recovered by precipitation with ethanol. Samples (3 μg) of each were electrophoresed in agarose gel at pH 3.0 and stained with Toluidine Blue (see inset). Products A and B were combined to constitute a proteoglycan preparation free of depolymerization products (Horner, 1986).

were washed in distilled water, then in 0.87 M-acetic acid adjusted to pH 3.0 with LiOH (Horner, 1967). Samples (1 μl) were applied in pre-formed sample slots. After electrophoresis for 13 min at 300 V in a water-cooled Shandon thin-layer electrophoresis tank, gels were stained with Toluidine Blue (0.1% in water) and destained in water.

RESULTS AND DISCUSSION

Fig. 1 shows gel filtration of rat skin heparin proteoglycans on Sepharose CL-4B. This is an additional step, relative to earlier work. It was undertaken because of the possibility that the smallest proteoglycans might overlap with the largest 'depolymerization products' (Horner, 1986). The fractionation was therefore evaluated by agarose-gel electrophoresis.

In a total heparin preparation from rat skin, agarose-gel electrophoresis shows two components (Horner, 1971). The slower component, which has a characteristic irregular shape, is composed of proteoglycan molecules. The faster component, of regular shape, is composed of depolymerization products. Gel electrophoresis indicated some overlap in fraction C, which was therefore excluded from further study. Thus fractions A and B, which were combined to give a 'total proteoglycan' product, actually comprised 97% of the total proteoglycans.

Fig. 2. Gel filtration of ^{35}S -labelled rat skin heparin proteoglycans on a column (1.0 cm $\times$ 31 cm) of Bio-Gel A-150 m

The column was equilibrated with 1.2 M-NaCl/0.05 M-Tris/HCl buffer, pH 8.5. (a) $\blacksquare$, Fractionation of the total ^{35}S -labelled proteoglycans. The radioactivity in each fraction is expressed as a percentage of the total radioactivity applied to the column (see left-hand ordinate). Fractions were combined to give products I-V as indicated, heparin being recovered by precipitation with ethanol. Portions of products I-V were fractionated on a column of antithrombin-agarose (see Fig. 3) to determine their HA-proteoglycan contents, which are shown as vertical bars ($\blacksquare$) and expressed as percentages of the total proteoglycans in each product (see right-hand ordinate). Portions of the HA-proteoglycan components of products I-V were treated with NaOH to release their polysaccharide chains, which were fractionated on antithrombin-agarose (see Fig. 4) to determine their HA-chain contents, which are shown as vertical bars ($\square$) and expressed as percentages of the total chains in the corresponding HA-proteoglycans (see right-hand ordinate). (b) Re-chromatography of portions of the five proteoglycan products: $\circ$, I; $\bullet$, II; $\triangle$, III; $\blacktriangle$, IV; $\square$, V. The fraction volume was 0.76 ml. V_t indicates the total column volume, determined with $^3\text{H}_2\text{O}$. The flow rate was approx. 1 ml/h.

Fig. 2(a) illustrates gel filtration of the total proteoglycans on Bio-Gel A-150m and the arbitrary division of column fractions to give five products for further study. This gel, containing 1% agarose, gives a more extended elution pattern than observed previously with 2%-agarose gels (Horner, 1971) or 4%-agarose gels (Metcalf *et al.*, 1980; Horner, 1986); its use is only appropriate after removal of depolymerization products on gels with lower exclusion limits. Fig. 2(b) shows the re-chromatography of samples of the five products. This demonstrates proteoglycan polydispersity quite dramatically. There is considerable overlap, but the mean hydrodynamic volumes of the five products are clearly different, with very little overlap at the extremes represented by products I and V. The weight-average M_r of total rat skin heparin proteoglycan preparations has been estimated to be 1×10^6 by sedimentation-equi-

Fig. 3. Fractionations of [^{35}S]heparin proteoglycan products by gradient elution from a column (1.6 cm $\times$ 9 cm) of antithrombin-agarose

Portions of products I (●), III (○) and V (▲) obtained by gel filtration (see Fig. 2) were fractionated as described previously (Horner, 1987). Fractions (1.5 ml) were collected at a flow rate of 12 ml/h. The radioactivity in each fraction is expressed as a percentage of the total radioactivity in each column sample. Δ , NaCl concentration. The vertical lines through the elution patterns indicate the divisions made between the LA-proteoglycan and HA-proteoglycan products. Products II and IV were treated in the same way. The HA-proteoglycan contents of all five products, determined from these fractionations, are presented in Fig. 2(a).

Fig. 4. Fractionation of a mixture of ^{35}S -labelled and ^3H -labelled HA-heparin chains by gradient elution from a column (1.6 cm $\times$ 9 cm) of antithrombin-agarose

●, [^{35}S]Heparin chains released from the high-affinity component of proteoglycan product V by treatment with NaOH; ○, ^3H -labelled heparin HA-chains obtained from an unlabelled total rat skin heparin proteoglycan preparation by treatment with NaOH and NaB^3H_4 . Fractions (1.5 ml) were collected at a flow rate of 12 ml/h. The radioactivity in each fraction is expressed as a percentage of the total ^{35}S or ^3H radioactivity applied to the column. Δ , NaCl concentration. Portions of the high-affinity components of proteoglycan products I–V were treated in this way to determine their HA-chain contents (presented in Fig. 2a). The elution patterns for fractionations of three of these products, showing only the regions in which HA-chains were eluted, are presented in Fig. 5.

librium analysis (Horner, 1971; Robinson *et al.*, 1978). Attempts to determine the M_r values of products I–III by this technique failed: plots of $\log(\text{fringe displacement})$ against the square of the radius of rotation deviated markedly from linearity. The polydispersity of products I–V can therefore only be discussed here in relative terms. However, the degree of separation between products I and V achieved by gel filtration must surely represent, conservatively, a 2–3-fold molecular-size range.

Portions of products I, III and V were treated with NaOH, and the chains released were chromatographed on Sepharose CL-4B, as described previously (Horner, 1987). There were no significant differences between the elution profiles of the three chain preparations (results not shown). This confirmed earlier work by Metcalfe *et al.* (1980), indicating that size differences between heparin proteoglycans are due to variations in chain numbers, not in chain lengths.

The histograms in Fig. 2(a) show that the proportion of HA-proteoglycans fell as molecular size decreased, from 39% in product I to 25% in product V. Fractionations on antithrombin–agarose, from which these data were calculated, are shown in Fig. 3. (These elution patterns also suggest that, once released from the matrix, which was 2% agarose, the larger products were excluded from the gel granules. This effect was not seen when chains were fractionated on the same affinity gel.) Conversely, in the HA-proteoglycan products, the proportion of HA-chains increased from 29% to 59% as molecular size decreased. An example of the fractionation of chains from an HA-proteoglycan product, from which these data were calculated, is shown in Fig. 4.

When ^{35}S -labelled chains prepared from HA-proteoglycan products I–V were fractionated on antithrombin–agarose, they were co-chromatographed with ^3H -end-labelled HA-chains prepared from an unlabelled total proteoglycan product (see Figs. 4 and 5). This approach was taken for the following reason. In earlier work (Horner, 1987; Horner *et al.*, 1988) HA-proteoglycans that had not been fractionated on the basis of molecular size were shown to cover a wide range of degrees of affinity for antithrombin. They contained LA-chains and HA-chains with from one to five antithrombin-binding sites (mean value three sites per chain). At the low end of the range HA-proteoglycans had high proportions of LA-chains, and the HA-chains contained predominantly one to three antithrombin-binding sites. At the high end of the range, HA-proteoglycans had much lower proportions of LA-chains, and the HA-chains contained predominantly three to five antithrombin-binding sites. These differences were very clearly demonstrated by gradient elution from antithrombin–agarose (see Fig. 3 of Horner, 1987). Therefore, if the molecular size of a proteoglycan were a significant parameter in determining the highly asymmetric distribution of antithrombin-binding sites on HA-chains, the elution patterns for HA-chains from large and small HA-proteoglycans should differ markedly from the elution pattern for HA-chains from a total proteoglycan preparation. Fig. 5 shows that there is actually only a small molecular-size effect in this respect. In the case of HA-proteoglycan V, the proportion of chains at the high end of the HA-chains' affinity range is slightly greater than in an 'average' HA-chain preparation. There are no significant differences in the cases of HA-proteoglycans I and III. Therefore the major

Fig. 5. Gradient elution of ^{35}S -labelled HA-chains (●), co-chromatographed with ^3H -labelled HA-chains (○), from a column of antithrombin–agarose

The ^{35}S -labelled HA-chain products shown in panels (a), (b) and (c) were derived from the high-affinity components of proteoglycan products I, III and V respectively. The ^3H -labelled HA-chain product is the same in all three fractionations (see the legend to Fig. 4). The data presented here were derived from fractionations of the type presented in Fig. 4 by expressing radioactivity as a percentage of the total ^{35}S or ^3H radioactivity in fractions 38–58.

difference between large and small HA-proteoglycans lies in their proportions of LA-chains. [It also follows, from the earlier work described above (Horner 1987; Horner *et al.*, 1988), that the marked variations in both HA-chain proportions and antithrombin-binding-site densities in HA chains are independent of proteoglycan size.]

Lindahl *et al.* (1986) have presented an elegant hypothetical model of the post-polymerization reactions occurring during heparin proteoglycan biosynthesis in the mast-cell Golgi complex. Polysaccharide chains composed of an alternating sequence of glucuronic acid and *N*-acetylglucosamine units, linked to a poly(Ser-Gly) peptide core, pass by rows of membrane-bound enzyme 'stations', at which closely linked polymer-modification

reactions occur in a strict sequence. The marked structural heterogeneity of heparin chains can be explained in general terms by assuming that the stepwise biosynthetic reaction sequence frequently fails to go to completion. With this proviso, the model proposed by Lindahl *et al.* (1986) can be reconciled with all aspects of heparin proteoglycan biosynthesis except the highly asymmetric distribution of antithrombin-binding sites in the final products. At present we do not know if the spacing between these sites on HA-chains is regular or irregular; there is, however, no current evidence that would suggest that they are concentrated within circumscribed segments of these chains (Oscarsson *et al.*, 1989).

The antithrombin-binding site is a pentasaccharide sequence comprising monosaccharide units 2–6 of an HA octasaccharide sequence studied extensively by Lindahl and co-workers (see, e.g., Kusche *et al.*, 1988). The work of Kusche *et al.* (1988) strongly suggests (i) that 3-*O*-sulphation of the internal glucosamine unit (unit 4) concludes the biosynthesis of the antithrombin-binding site and (ii) that this final step occurs only in regions of a heparin chain where all other structural features essential for antithrombin binding are already expressed. These features comprise a recognition sequence for a 3-*O*-sulphotransferase that shows absolute specificity for glucosamine unit 4. Candidates for the enzyme catalysing the final step in the biosynthesis of the recognition sequence have been discussed by Kusche *et al.* (1988).

As antithrombin-binding sites are not randomly distributed in heparin proteoglycans, the distribution of putative recognition sequences for 3-*O*-sulphation must be equally non-random. If this were not the case, it would be necessary to conclude that some recognition sequences were not converted into antithrombin-binding sites because they failed to encounter 3-*O*-sulphation sites as they traversed the Golgi complex. In this scenario LA-chains in completed proteoglycans would contain 'unused' recognition sequences. This possibility is not supported by recent findings. Kusche *et al.* (1988) incubated a synthetic pentasaccharide, with all structural features of the antithrombin-binding region except the 3-*O*-sulphate group on the internal glucosamine unit, with adenosine 3'-phosphate 5'-phospho[³⁵S]sulphate in the presence of a microsomal fraction from mouse mastocytoma tissue. 3-*O*-[³⁵S]sulphation of the internal glucosamine unit occurred and high affinity for antithrombin was conferred on the product. More recently, ³H-end-labelled LA-chains from rat skin heparin proteoglycans have been incubated in the same system: their LA characteristics were retained and [³⁵S]sulphate was not incorporated (M. Kusche, U. Lindahl & A. A. Horner, unpublished work). This finding refutes the 'unused recognition sequence' hypothesis. It must therefore be assumed that recognition sequences for the 3-*O*-sulphotransferase are non-randomly distributed in developing heparin proteoglycans and that all such sequences are normally recognized and 3-*O*-sulphated.

The simplest concept that can be reconciled with the available data on the asymmetric distribution of antithrombin-binding sites is that one enzyme involved in biosynthesis of the recognition sequence for the 3-*O*-sulphotransferase is non-randomly distributed on Golgi membranes. This key enzyme for the synthesis of the recognition sequence must occur in areas that are so

circumscribed that they cannot interact with all the chains of even the smallest proteoglycans, for all HA-proteoglycans contain some LA-chains. Within these areas, sites of the key enzyme must be spaced much further apart than the enzymes involved in the earlier biosynthetic reactions. (Maximal antithrombin-binding-site density corresponds to 3-*O*-sulphation of, on average, every 25th glucosamine residue.) The number of recognition sequences synthesized per chain will depend on the degree of contact made between a chain and a recognition-sequence-synthesizing area. A maximal degree of contact would generate five recognition sequences on one chain.

Conceivably, the 3-*O*-sulphotransferase has the same non-random distribution as the key enzyme for the synthesis of the recognition sequence, at immediately adjacent membrane sites. The two enzymes would then constitute an antithrombin-binding-site-sequence completion site, their activities being closely linked. Such coupling would be efficient. The concept is similar to the coupling of *N*-deacetylation with *N*-sulphation and of C-5-epimerization with 2-*O*-sulphation, for which there is considerable experimental evidence (see, e.g., Lindahl & Kjellén, 1987).

The current findings can then be explained by assuming that the distance between adjacent areas containing 3-*O*-sulphotransferase recognition-sequence-synthesizing activity (or perhaps antithrombin-binding-site-sequence completion activity, as discussed above) is sufficiently wide to allow proteoglycans, of all sizes, to pass between them without making contact. Statistically, the smallest proteoglycans would be least likely to encounter these areas. However, if large and small proteoglycans did make contact with the area to the same degree (i.e. equal numbers of their chains overlapped the area to the same extent), the smaller proteoglycan would acquire a higher proportion of chains with antithrombin-binding sites.

The hypothetical biosynthetic assembly line would be much wider than the span of a proteoglycan molecule and would have numerous points of entry. Spatial relationships between the point of entry and the positioning on the assembly line of the specialized areas that control antithrombin-binding-site synthesis would determine whether proteoglycan-size-dependent chance encounters resulting in antithrombin-binding-site synthesis would occur.

The proposed modification to the concept introduced by Lindahl *et al.* (1986) is an attempt to explain some of the complexities of heparin biosynthesis in the simplest terms consistent with current knowledge. This essentially two-dimensional concept will have to be reconciled with three-dimensional reality when the topography of the biosynthetic enzyme sites in the Golgi complex is elucidated. In addition, changes in both the primary and secondary structures of developing proteoglycans undoubtedly influence their interactions with biosynthetic enzymes as they traverse Golgi membranes. Chains gain considerable flexibility when iduronic acid units are formed (Casu *et al.*, 1988). As subsequent sulphation steps increase the negative-charge densities of the chains dramatically, mutual repulsion effects, within and between chains, will give a more extended proteoglycan structure and cause a concomitant increase in hydrodynamic volume.

Clearly, any attempt to explain the current findings on the basis of our present knowledge involves a high degree

of speculation. However, the ultimate elucidation of the mechanisms involved will have to take the observed proteoglycan-size-dependent differences in proportions of HA-chains into account.

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