Heterogeneous distribution of antithrombin-binding sites in rat brain heparan sulphate proteoglycans

Alan A. HORNER

Department of Physiology, Medical Sciences Building, University of Toronto, Toronto, Ont. M5S 1A8, Canada

Heparan sulphates with high binding affinity for antithrombin (HA-HS), labelled *in vivo* with [³⁵S]sulphate, were extracted from rat brains and purified by chromatography on DEAE-cellulose and on antithrombin-agarose. HA-HS proteoglycans (HA-HSPG) were then separated from HA-HS chains on Sepharose CL-6B. The total HA-HSPG product was rechromatographed on antithrombin-agarose. Six HA-HSPG subfractions with differing degrees of affinity for antithrombin were recovered and treated with NaOH to release their chains. Rechromatography of these six ³⁵S-labelled HS chain preparations on antithrombin-agarose showed that their proportions of chains with no affinity for antithrombin (NA-HS chains) ranged from 36 to 71 %. There was a reciprocal relationship between the proportion of NA-HS chains in each HA-HSPG subfraction and the degree of affinity for antithrombin of the rest of its chains (assessed relative to ³H-labelled HA-heparin chains with which they were co-chromatographed). Similar characteristics of antithrombin-binding-site distribution apply to HA-heparin proteoglycans from rat skin studied previously [Horner (1987) *Biochem. J.* 244, 693–698]. The data suggest that the sites at which 3-*O*-sulphation of some glucosamine *N*-sulphate residues occurs in the Golgi complex of brain cells (probably endothelial cells) which synthesize HA-HSPGs (as in mast cells, which synthesize HA-heparin PGs) are distributed sparsely but not randomly.

INTRODUCTION

The heparan sulphates (HS) are a family of glycosaminoglycans with many subtle variations in the composition of their polysaccharide chains and in the ways in which the chains are arranged on different core proteins in heparan sulphate proteoglycans (HSPGs) (see the review by Fransson, 1989).

HS chains and HSPGs with anticoagulant activity, due to high binding affinity for antithrombin (HA-HS chains and HA-HSPGs), have been isolated from various mammalian tissues and cultured cells (see the review by Marcum & Rosenberg, 1989a). An HA-HS chain must contain at least one antithrombin-binding site, the affinity for antithrombin being conferred by a pentasaccharide sequence which includes a unique 3-*O*-sulphated glucosamine *N*-sulphate residue, first discovered in heparins with high affinity for antithrombin (HA-heparins) by Lindahl *et al.* (1980).

Pejler *et al.* (1987) and Marcum & Rosenberg (1987) have described HA-HSPGs in which almost all the chains are HA-HS chains. This is in marked contrast with rat HA-heparin PGs, which are extremely heterogeneous. The proportions of HA-chains in rat skin HA-heparin PGs range from 17 to 76 % (Horner, 1987) and the number of antithrombin-binding sites per HA-heparin chain ranges from one to five (Horner *et al.*, 1988). Both these parameters are also proteoglycan-size-dependent (Horner, 1989).

From the above discussion, it might appear that HA-HSPG synthesis is controlled as an essentially all-or-none phenomenon, a simpler situation than is the case with HA-heparin PGs. A survey of various Pronase-digested rat tissues for their HS contents (Horner, 1990) showed that whole brain contained the highest proportion of HA-HS chains (21.5 % of total HS). This study also demonstrated the marked heterogeneity of rat HA-HS chains. The current study was therefore undertaken to determine the pattern of distribution of antithrombin-binding sites in rat brain HA-HSPGs, for comparison with the known distribution of antithrombin-binding sites in rat skin HA-heparin PGs.

EXPERIMENTAL

Animals

Each of six male Wistar rats (average weight 313 g) received an intraperitoneal injection of H₂³⁵SO₄ (8.6 mCi) in 1 ml of 0.15 M-NaCl and was killed by decapitation 18 h later.

Preparation of brain extract

The brains (weight 11.7 g) were removed, rinsed in ice-cold 0.15 M-NaCl and homogenized (Polytron homogenizer with PTA 20TS generator) in 70 ml of ice-cold 0.15 M-NaCl/0.2 % sodium deoxycholate/0.05 M-Tris/HCl, pH 8.25 (Klinger *et al.*, 1985) containing the following proteinase inhibitors; 0.005 M-benzamidinium hydrochloride, 0.01 M-disodium EDTA and 0.1 M-6-aminohexanoic acid. (This mixture of proteinase inhibitors was also added to several other solutions; see below.) The homogenate was stirred magnetically at 4 °C for 2 h and centrifuged for 1 h at 12000 *g* at 0 °C (Sorvall SS34 rotor). The cloudy supernatant was transferred to Quickseal tubes (Beckman) and centrifuged for 1.5 h at 200000 *g* at 0 °C (Beckman 50 Ti rotor).

DEAE-cellulose chromatography (see Fig. 1)

The clear supernatant from the ultracentrifugation was pumped into a column (1.6 cm × 18 cm) of DEAE-cellulose DE-23 (Whatman; in the chloride form), equilibrated with 0.15 M-NaCl/0.05 M-Tris/HCl, pH 8.5, containing proteinase inhibitors and Triton X-100 (0.5 %, v/v), followed by the same solution (160 ml), at a flow rate of approximately 30 ml/h. Fractions (5.2 ml) were collected by timed flow. A linear NaCl concentration gradient, from 0.15 M to 1.20 M, total volume 320 ml, in the above buffer system was then pumped through the column, and a portion (50 µl) of each fraction was taken to determine its ³⁵S radioactivity by liquid-scintillation counting in PCS (Amersham Searle). Fractions comprising a peak of radioactive material eluted in the gradient were combined, adjusted to 1.0 M-NaCl, mixed with ethanol (3 vol.) and left at 4 °C overnight.

Abbreviations used: CSA, chondroitin sulphate A; HS, heparan sulphate; NA, LA, HA, no affinity, low affinity and high affinity for antithrombin respectively; PG, proteoglycan.

Insoluble products were recovered by centrifuging (4 h at 3000 *g* at 0 °C, Sorvall HS-4 rotor) and air-dried at 4 °C for 2 h.

First fractionation on antithrombin-agarose (see Fig. 2)

The product from the DEAE-cellulose column was dissolved in 0.14 M-NaCl/0.003 M-CaCl₂/0.1 M-Hepes/NaOH, pH 7.3, containing proteinase inhibitors and Triton X-100 (0.5%, v/v) and applied to a column of antithrombin-agarose (1.6 cm × 9 cm) equilibrated with the same solution. Fractions (1.5 ml) were collected at a rate of 15 ml/h. A sample (50 µl) of each fraction was taken for liquid-scintillation counting. After an initial elution under isocratic conditions, a discontinuous NaCl concentration gradient was applied: a relatively shallow linear gradient to approx. 0.8 M-NaCl followed by a steeper linear gradient to 3.0 M-NaCl. Fractions containing ³⁵S-labelled material eluted by increasing the NaCl concentration were combined and mixed with ethanol, as described earlier, after the addition of 2 mg of chondroitin sulphate A (CSA) from shark cartilage (Sigma) as a carrier. The precipitate which formed was recovered by centrifuging (2 h at 5800 *g* at 0 °C, Sorvall HB-4 rotor) and air-dried at 4 °C for 2 h.

Fractionation on Sepharose CL-6B (see Fig. 3)

The material recovered from the first antithrombin-agarose column was dissolved in 1.2 M-NaCl/0.05 M-Tris/HCl, pH 8.5, containing proteinase inhibitors and Triton X-100 (0.1%, v/v) and applied to a column (1.6 cm × 23 cm) of Sepharose CL-6B equilibrated with the same solution. Fractions (1.2 ml) were collected at a flow rate of 5 ml/h. A portion (25 µl) of each fraction was taken for liquid-scintillation counting. ³⁵S-containing fractions comprising the first, major, peak eluted were designated to contain proteoglycans. These fractions were combined, and carrier CSA (1 mg) was added before the addition of ethanol. Material precipitated by ethanol was recovered as described earlier. A portion (5%) of this product was treated with NaOH (Horner & Young, 1982) and rechromatographed on the same column. Another portion (5%) was treated with HNO₂ (see below). The rest was fractionated on antithrombin-agarose.

Treatment with HNO₂ (see Fig. 4)

Samples (approx. 5000 d.p.m.) of the total ³⁵S-labelled HSPG product recovered from the Sepharose CL-6B column and of ³⁵S-labelled heparin proteoglycans prepared (Horner & Young, 1982) from the skins of the same rats were treated with HNO₂ at pH 1.5 (Shively & Conrad, 1976). The products were fractionated on a column (1.0 cm × 25 cm) of Sephadex G-25 (F) equilibrated with 1.2 M-NaCl/0.05 M-Tris/HCl, pH 8.5. Fractions (0.68 ml) were collected at a flow rate of 7 ml/h. The whole of each fraction was used to determine radioactivity.

Second fractionation on antithrombin-agarose (see Fig. 5)

The proteoglycans recovered from the Sepharose CL-6B column were dissolved in 0.3 M-NaCl/0.05 M-Tris/HCl, pH 8.5, containing proteinase inhibitors and Triton X-100 (0.1%, v/v) and applied to a column (1.0 cm × 8 cm) of antithrombin-agarose equilibrated with the same solution. Fractions (1.2 ml) were collected at a rate of 5 ml/h. After elution under isocratic conditions, which yielded an initial peak (A), an extended linear NaCl concentration gradient, from 0.3 M-NaCl to 2.8 M-NaCl, was applied. A portion (150 µl) of each fraction was taken for liquid-scintillation counting. Column fractions comprising a broad peak of material eluted by the gradient were arbitrarily assigned to six HA-HSPG subfractions labelled B–G. All carrier CSA from the previous step was eluted with product A. CSA

(1 mg) was added to each of the six subfractions. Products B–G were then recovered by precipitation with ethanol.

Fractionations of HS chains, released from HA-HSPG subfractions B–G, on antithrombin-agarose (see Fig. 6)

HA-HSPG subfractions B–G were each treated with NaOH to release their chains, as described for heparin proteoglycans (Horner & Young, 1982). A portion (10000 d.p.m.) of a preparation of ³H-end-labelled HA-heparin chains from rat skin heparin proteoglycans (Horner, 1989) was added to each ³⁵S-labelled HS chain product. Each mixture, in 0.14 M-NaCl/0.003 M-CaCl₂/0.01 M-Hepes/NaOH, pH 7.3, was applied to a column (1 cm × 9 cm) of antithrombin-agarose equilibrated with the same solution. Fractions (1.5 ml) were collected at a flow rate of 5 ml/h. After an initial elution under isocratic conditions, a discontinuous NaCl concentration gradient was applied, as for the first fractionation on antithrombin-agarose. The whole of each fraction was used to determine ³⁵S and ³H radioactivities by liquid-scintillation counting.

RESULTS AND DISCUSSION

The material initially retained on DEAE-cellulose (Fig. 1) and subsequently applied to antithrombin-agarose comprised 53% of the radioactivity extracted from the brains. Only 16% of the radioactivity applied to the first antithrombin-agarose column was initially retained on the column (Fig. 2). This high-affinity material was subsequently fractionated by gel filtration on Sepharose CL-6B into two overlapping peaks (Fig. 3). The first and major peak was comprised of HA-proteoglycans, for treatment with NaOH gave a product which was markedly retarded on Sepharose CL-6B, being only slightly larger than commercial pig mucosal heparin (*M_r* approx. 13000; Laurent *et al.*, 1978). Susceptibility to HNO₂ at pH 1.5 indicated that the HA-proteoglycan product contained only HS chains (Fig. 4). Relative to HNO₂-degraded heparin, it yielded a higher proportion of larger degradation products, consistent with a higher proportion of *N*-acetylated glucosamine residues. The extent of degradation was rather high for a heparan sulphate; however, previous work (Horner, 1990) established the absence of heparin from rat brain. The material in the second smaller peak eluted from the Sepharose CL-6B column was not degraded by NaOH, but was degraded by HNO₂ to the same extent as the proteoglycan fraction (results

Fig. 1. Fractionation of solubilized components of rat brain by elution from a column of DEAE-cellulose with a gradient of NaCl concentration at pH 8.5

●, ³⁵S radioactivity; △, NaCl concentration. Material in the fractions indicated by the horizontal bracket was recovered by precipitation with ethanol.

Fig. 2. Fractionation of material recovered after DEAE-cellulose chromatography by gradient elution from a column of antithrombin-agarose

●, ^{35}S radioactivity; △, NaCl concentration. Material in the fractions indicated by the horizontal bracket was recovered by precipitation with ethanol.

Fig. 3. Fractionations on a column of Sepharose CL-6B

●, ^{35}S radioactivity of material recovered after antithrombin-agarose chromatography (see Fig. 2). (The ordinate refers only to the elution curve for this product. The other elution curve data are expressed in arbitrary units, chosen to facilitate visual comparisons.) The fractions labelled PG comprised the HA-HSPG product recovered for further fractionation. ○, ^{35}S radioactivity of chains released from the PG fraction by treatment with NaOH. ▲, Commercial pig mucosal heparin (1 mg), quantified colorimetrically with 1,9-dimethyl Methylene Blue (Farndale *et al.*, 1986). V_0 , void volume, determined with rat skin ^{35}S -labelled heparin PG. HC (heparin chains) marks the peak of the elution curve for chains released from rat skin ^{35}S -labelled heparin PG by NaOH. V_1 , total volume, determined with $^3\text{H}_2\text{O}$.

not shown), indicating it to be comprised of HA-HS chains and/or chain fragments.

The HA-HSPGs were smaller than the rat skin heparin proteoglycans (M_r approx. 1×10^6) used to determine the void volume of the Sepharose CL-6B column, but slightly larger, on average, than rat skin heparin chains (M_r approx. 80000; Robinson *et al.*, 1978).

HA-HSPG subfractions B–G had increasing degrees of affinity for antithrombin, proportional to the NaCl concentrations required to dissociate them from antithrombin-agarose (Fig. 5).

In earlier work in this laboratory, working definitions of NA-, LA- and HA-heparins were established, based on fractionation

Fig. 4. Fractionation of products treated with HNO_2 at pH 1.5 on a column of Sephadex G-25.

●, ^{35}S -labelled HSPG recovered from the Sepharose CL-6B column; ○, ^{35}S -labelled heparin PG from rat skin.

Fig. 5. Fractionation of the PG product recovered after Sepharose CL-6B chromatography by gradient elution from a column of antithrombin-agarose

●, ^{35}S radioactivity; △, NaCl concentration. Column fractions were assigned to subfractions A–G as indicated.

on antithrombin-agarose with a discontinuous two-step NaCl concentration gradient from 0.14 M-NaCl to 3.0 M-NaCl (Horner & Young, 1982). NA-heparins (found only in commercial heparins and rat skin heparin chain fragments) did not bind to the gel. LA-heparins bound, but were displaced by relatively small increases in NaCl concentration. HA-heparins, displaced at higher NaCl concentrations, were the only heparins with antithrombin-binding sites (Horner, 1987; Horner *et al.*, 1988).

In recent work with the total ^{35}S -labelled HS chains recovered from Pronase-digested rat brain (Horner, 1990) a high proportion (approx. 35%) of these chains was eluted from antithrombin-agarose as a distinct peak of LA material, as previously defined for heparins. LA chains were only minor components of the HA-HSPGs extracted from rat brain in the current study.

The six HA-HSPG subfractions contained NA-chains in proportions which covered a twofold range: from 71% in subfraction B to 36% in subfraction G (Fig. 6). (The NA-chain content of the complete HA-HSPG product, calculated from the data for the subfractions, was 54%.) The separation of the HS chains that initially bound to antithrombin-agarose into two distinct peaks was less clear-cut than in the earlier work cited above. Most of these chains from subfractions B and C were

Fig. 6. Fractionations of ^{35}S -labelled chains from HA-HSPG subfractions B-G, by gradient elution from a column of antithrombin-agarose

●, ^{35}S radioactivity; ○, radioactivity of HA- ^3H -labelled heparin chains from rat skin heparin PG, co-chromatographed with each ^{35}S -labelled HS chain preparation. △, NaCl concentration. The same NaCl concentration gradient shown in the lowest panel was

eluted in the LA region, i.e. ahead of the HA-heparin chains with which they were co-chromatographed. Subfractions D-G contained increasing proportions of HA-HS chains, which were eluted coincident with or later than the HA-heparin chains.

Previous work on the heterogeneity of HA-heparin PGs from rat skin has shown them to be comprised of various proportions of LA-chains and HA-chains (Horner & Young, 1982; Jacobsson *et al.*, 1986; Horner, 1987, 1989; Horner *et al.*, 1988). LA-chains predominate in heparin PGs at the low end of the HA-heparin PG spectrum and their HA-chains have relatively low affinity for antithrombin. HA-chains predominate in heparin PGs at the high end of the HA-heparin PG spectrum and these chains have much higher affinity for antithrombin. The degree of affinity of HA-heparin chains for matrix-bound antithrombin was shown, first, to be directly related to their specific anticoagulant activities (Horner, 1987) and, later, to be directly proportional to their antithrombin-binding-site densities, which ranged from one to five sites per chain (Horner *et al.*, 1988).

Thus in HA-heparin PGs, there is an inverse relationship between the proportion of LA chains and the degree of affinity of the HA chains. In HA-HSPGs, there is a similar inverse relationship between the proportion of NA chains and the degree of affinity of the HA chains. In other words, in both heparin PGs and HSPGs, as the proportion of HA chains increases, the degree of affinity for antithrombin of these chains also increases. The varying degrees of affinity of HA-HS chains correlate directly with their specific anticoagulant activities (Horner, 1990). Their antithrombin-binding-site densities have not been determined. The highest specific anticoagulant activity of an HA-HS chain (Horner, 1990) is approximately one-third of the corresponding value for an HA-heparin chain (Horner, 1987).

The present study demonstrates similarities in the patterns of variable non-random distribution of antithrombin-binding sites in HA-HSPGs and HA-heparin PGs in the rat. Many of the complexities of heparin and heparan sulphate biosynthesis have been elucidated (Lindahl, 1989). The factors controlling the 3-*O*-sulphation of only a limited proportion of ostensibly legitimate acceptor sites on HS chains and on heparin chains, thereby completing antithrombin-binding-site synthesis, are still not fully understood (Kusche *et al.*, 1990). The mechanisms involved in the asymmetric and variable distribution of antithrombin-binding sites in HA-heparin PGs and HA-HSPGs are still conjectural. However, structural features of rat organ products, both HSPGs in the current study and heparin PGs in earlier work cited above, support the concept that 3-*O*-sulphation sites are sparsely and non-randomly distributed on Golgi membranes (Horner, 1989).

There is no theme common to the current study and the work of others. Detailed comparisons are precluded by differences in experimental approach. However, it is clear that there is great source-dependent diversity in the proportions of total HSPGs which are HA-HSPGs. Pejler *et al.* (1987) isolated a Pronase-resistant high-density HSPG preparation from cultures of murine Reichert's membrane. The intact HSPGs were not affinity-fractionated but approx. 80% of their chains were clearly HA-HS chains, covering a wide range of antithrombin-binding-site densities. This is unequivocal, as they were co-chromatographed with a heparin marker on antithrombin-Sepharose and eluted with an extended NaCl concentration gradient.

The proportions of HA-HS chains isolated from enriched endothelial cell preparations from calf aorta and calf cerebral

obtained reproducibly in each fractionation, using Pharmacia equipment: a P-3 pump and a GP-250 + gradient programmer coupled with a PSV-100 solenoid valve.

microvessels and from cultured endothelial cells from calf aorta and rat microvessels (from epididymal fat pads) ranged from 0.3 % to 10 % of the total HS chains from these sources, with the microvessels yielding the highest proportions (see the review by Marcum & Rosenberg, 1989a). Hydrophobic papain-degradable HA-HSPGs isolated from cloned bovine aortic endothelial cells by Marcum *et al.* (1986) comprised < 1 % of the total HSPGs. These were step-eluted (0.15 M-NaCl to 1.0 M-NaCl) from antithrombin-Affi-Gel 15, so that heterogeneity of antithrombin-binding site density at the proteoglycan level would not have been observed.

In several review articles published since 1986, Marcum and collaborators have reported that cloned rat microvascular endothelial cells produce up to 10 % HA-HSPGs, in which virtually all of the chains are HA-HS chains (Marcum *et al.*, 1987; Marcum & Rosenberg, 1987, 1989a,b). The possible heterogeneity of these HA-HSPGs, due to variations in chain antithrombin-binding-site density, cannot be dismissed.

Thus there are reports in the literature of a proteinase-resistant HA-HSPG which is the major, and possibly the only, type of HSPG produced by Reichert's membrane in culture and of proteinase-degradable HA-HSPGs which constitute very minor proportions of the total HSPGs produced by endothelial cells. In both these types of HA-HSPG it appears that almost all of the HS chains are HA-HS chains. In the Reichert's membrane product, antithrombin-binding-site density is clearly variable.

The stimulation by butyrate of HA-heparin synthesis in cultured mastocytoma cells (Jacobsson *et al.*, 1985) and of HA-HS synthesis by cultured endothelial cells (Marcum *et al.*, 1986) indicates that the degree of expression of the 3-*O*-sulphotransferase can be altered *in vitro*. The great variability in antithrombin-binding-site distribution in HA-heparin PGs and HA-HSPGs in the rat raises questions concerning the expression of 3-*O*-sulphation activity *in vivo*, questions that cannot be answered by analysing products from whole tissues or organs. Do individual cells produce uniform products? For example, were HA-HSPG subfractions B-G produced by different populations of brain endothelial cells in which 3-*O*-sulphation was expressed to increasing degrees? Alternatively, to take the other extreme, do brain endothelial cells uniformly synthesize the whole range of HA-HSPGs, as well as HSPGs with no antithrombin-binding sites?

To elucidate the distribution of antithrombin-binding sites in HA-proteoglycans, the distribution of the 3-*O*-sulphotransferase on Golgi membranes must be determined (Lindahl & Kjellén, 1987). It is also important, particularly from a pathophysiological standpoint, to determine the factors (e.g. cytokines) that control the marked variability in the expression of 3-*O*-sulphotransferase

activity and consequent variations in the anticoagulant activities of HS and heparin chains.

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