Heterogeneity of rat skin heparin chains with high affinity for antithrombin

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Subfractions of ^{35}S -labelled rat skin heparin proteoglycans with various degrees of high affinity for antithrombin were obtained by gradient elution from a column of antithrombin–agarose. Heparin chains released from the proteoglycan preparations by β -elimination with alkali were re-fractionated on the same column. Proportions of chains with high affinity for antithrombin (HA-chains) ranged from 17% to 76%. These separations also revealed three overlapping subfractions of HA-chains. Their proportions varied in a manner consistent with a stepwise increase in the degree of affinity of HA-chains for antithrombin, this presumably being due to the biosynthesis of increasing numbers of antithrombin-binding sites per chain. The anticoagulant activity, with respect to thrombin neutralization, ranged from 32 units/mg to 287 units/mg. It is suggested that HA-chains may have from one to five or six antithrombin-binding sites. Thus the asymmetric distribution of these sites in rat skin heparin proteoglycans is much more marked than was realized from the earlier work of Horner & Young [(1982) *J. Biol. Chem.* 257, 8749–8754].

INTRODUCTION

In earlier work in this laboratory with ^{35}S -labelled rat skin heparins, heparin proteoglycans and heparin chains (released from proteoglycans by β -elimination) were separated into fractions with low affinity and high affinity for antithrombin by gradient elution from a column of antithrombin–agarose (Horner & Young, 1982). The high-affinity proteoglycan and high-affinity chain fractions were eluted with quite a steep gradient of increasing NaCl concentration, to facilitate recovery in a relatively small volume. This procedure was adopted after observing in pilot experiments that a shallow linear NaCl concentration gradient (necessary for the initial separation of low-affinity heparins) eluted high-affinity heparins over a wide concentration range. These observations, suggesting considerable heterogeneity in high-affinity proteoglycan and chain preparations, stimulated the present study.

In the previously published work it was found that four out of every ten chains in high-affinity proteoglycans had high affinity for antithrombin. This could well have been an average, rather than a constant, proportion. Therefore a batch of ^{35}S -labelled high-affinity proteoglycans was prepared by the published procedure and re-fractionated on antithrombin–agarose with an extended linear NaCl concentration gradient. This experimental modification facilitated the demonstration of extensive heterogeneity among high-affinity proteoglycans, which varied not only in their proportions of high-affinity chains but also in relative proportions of distinct subclasses of high-affinity chains that had not been demonstrated previously.

MATERIALS AND METHODS

Highly purified human antithrombin III was generously supplied by Dr. Robert Jordan of Cutter

Laboratories, Berkeley, CA, U.S.A. Bio-Gel A-50m (100–200 mesh) was purchased from Bio-Rad Laboratories, and Sepharose CL-4B from Pharmacia.

Biosynthetically labelled [^{35}S]heparin proteoglycans were prepared from rat skin, and [^{35}S]heparin chains were released from proteoglycans by β -elimination with alkali, as described by Horner & Young (1982). Antithrombin was covalently bound to Bio-Gel A-50m (Horner, 1986), and columns containing this gel were used to fractionate the heparin preparations. The buffers used for affinity chromatography contained 0.003 M-CaCl₂, 0.01 M-Hepes and various concentrations of NaCl, as described below, and were adjusted to pH 7.3 at ambient temperature with NaOH. NaCl concentration gradients were formed with an LKB Ultrograd coupled with a Pharmacia P-3 pump. [^{35}S]Heparins were recovered by precipitation with ethanol and quantified, relative to commercial pig mucosal heparin, by metachromasia with Azure A as described previously (Horner & Young, 1982).

The initial fractionation of [^{35}S]heparin proteoglycans (3.0 mg) on antithrombin–agarose into a low-affinity fraction (LA-proteoglycans), eluted with a shallow NaCl concentration gradient (0.10 M/10 ml of eluent) from 0.14 M-NaCl to 0.56 M-NaCl, and a high-affinity fraction (HA-proteoglycans), eluted with a steeper gradient (0.90 M/10 ml of eluent) from 0.56 M-NaCl to 3.0 M-NaCl, was performed essentially as described previously (Horner, 1986), on a column with a bed volume of 20 ml.

The HA-proteoglycans were re-fractionated on the same column with a single extended linear gradient (0.22 M/10 ml of eluent) from 0.30 M-NaCl to 2.2 M-NaCl. [^{35}S]Heparin-containing column fractions were arbitrarily assigned to proteoglycan subfractions A–G (see Fig. 2). These were recovered by precipitation with ethanol and treated with NaOH to release heparin chains.

The chain preparations from proteoglycan subfractions B-G were again fractionated on the same column with a two-gradient elution system (0.09 M/10 ml of eluent from 0.14 M-NaCl to 0.52 M-NaCl; 0.63 M/10 ml of eluent from 0.52 M-NaCl to 3.0 M-NaCl).

These fractionations demonstrated the marked heterogeneity of HA-proteoglycans and HA-chains with the use of only 3 mg of [35 S]proteoglycans. The next experiment stockpiled enough fractionated chains to determine specific anticoagulant activities across the entire range of LA-chains and HA-chains found in rat skin heparin proteoglycans. Another batch of [35 S]heparin proteoglycans (2.0 mg) was converted into [35 S]heparin chains and mixed with unlabelled chains (8.0 mg). Four 2.4 mg portions of this mixture were fractionated under identical conditions on a larger column of antithrombin-agarose (bed volume 46 ml). A two-gradient elution system was used: 0.05 M/10 ml of eluent from 0.14 M-NaCl to 0.59 M-NaCl; 0.34 M/10 ml of eluent from 0.59 M-NaCl to 3.0 M-NaCl. A sample of each fraction was taken for scintillation counting: the elution patterns of the four fractionations were essentially identical. Corresponding fractions were then combined. Heparin was precipitated with ethanol and quantified by metachromasia.

The relative molecular sizes of different preparations of chains were compared by gel filtration. Samples were applied to a column (bed volume 25 ml) of Sepharose CL-4B equilibrated with 1.2 M-NaCl/0.05 M-Tris adjusted to pH 8.5 with HCl.

Anticoagulant activity was determined with a modification of the amidolytic assay method of Teien *et al.* (1977), which measures the influence of heparin on the inactivation of thrombin by purified antithrombin. The reagents used were from a commercial assay kit (Chromostate-Heparin; General Diagnostics). The concentrations of antithrombin and thrombin were halved and incubation times doubled relative to the manufacturer's recommended conditions. The concentrations of the rat heparins tested were adjusted to give activities in the range 0.06–0.08 U.S.P. unit/ml, relative to a commercial pig mucosal heparin standard.

RESULTS AND DISCUSSION

Fig. 1 shows the initial separation of HA-proteoglycans from LA-proteoglycans. The former comprised 32% of the total radioactivity. Fig. 2 shows the re-fractionation of HA-proteoglycans. Subfraction A, presumably LA-proteoglycan, comprising 3.2% of the total radioactivity applied to the column, was not studied further.

The re-fractionations of chains prepared from HA-proteoglycan subfractions B-G with a two-gradient elution system are shown in Fig. 3. The proportion of LA-chains decreased from subfraction B to subfraction G. Fig. 3 shows not only the concomitant increases in the proportions of HA-chains, but also considerable heterogeneity among these chains. The elution patterns show three overlapping components in the HA region, designated I, II and III on the Figure. Their relative proportions are presented in Table 1. Type I was predominant in subfraction B, type II in subfractions C, D and E, and type III in subfractions F and G. The data support the previous observation (Horner & Young, 1982) that the ratio of LA-chains to HA-chains in HA-proteoglycans is approximately 3:2. However, this

Fig. 1. Fractionation of the total [35 S]heparin proteoglycans from rat skin by gradient elution from a column (1.6 cm $\times$ 10 cm) of antithrombin-agarose

○, 35 S radioactivity; ●, NaCl concentration, determined by measuring conductivity. Fractions (1.5 ml) were collected at a flow rate of 12 ml/h. The vertical line through the elution pattern indicates the division between the LA-proteoglycan and HA-proteoglycan fractions. The ordinate indicates percentages of the total radioactivity applied to the column.

Fig. 2. Further fractionation of the HA-proteoglycan fraction by gradient elution from a column (1.6 cm $\times$ 10 cm) of antithrombin-agarose

○, 35 S radioactivity; ●, NaCl concentration. Fractions (1.5 ml) were collected at a flow rate of 12 ml/h. Vertical lines through the elution pattern indicate the divisions made between a minor LA-proteoglycan subfraction (A) and HA-proteoglycan subfractions B-G.

ratio ranged from approximately 4:1 to approximately 1:4 in the six HA-proteoglycan subfractions obtained in the current work. Also, the degree of affinity of HA-chains for antithrombin varied, three types being demonstrated. The relative proportions of HA-chains types I, II and III in the six subfractions of HA-proteoglycans are presented graphically in Fig. 4. The data suggest a biosynthetic sequence, type I being converted into type II, which in turn is converted into type III. (The data would fit the reverse order equally well, but current concepts of heparin biosynthesis do not lend support to such an idea.) Overall, the conversion of type I into type II occurs to a greater extent than the conversion of type II into type III, so that type II is the

Fig. 3. Fractionations of [^{35}S]heparin chains prepared from HA-proteoglycan subfractions B-G by gradient elution from a column (1.6 cm $\times$ 10 cm) of antithrombin-agarose

●, ^{35}S radioactivity; ○, NaCl concentration. Fractions (1.5 ml) were collected at a flow rate of 12 ml/h. Vertical lines through the elution pattern indicate the divisions made between the LA-chain peaks and components I, II and III of the HA-chain peaks. The same NaCl concentration gradient shown in the lowest panel was obtained reproducibly in each fractionation, with the use of an LKB Ultrograd.

predominant type of HA-chain in the total HA-proteoglycan preparation.

The simplest interpretation of these observations would be that types I, II and III have one, two and three antithrombin-binding sites per chain respectively. If this were the case, the average number of sites per chain in the total HA-chains fraction, calculated from the data in

Table 1, would be 2.2. However, recent work (Jacobsson *et al.*, 1986; discussed more fully below in this section) indicates that the average value is approximately 3 sites per chain.

The relationship between the degree of affinity for antithrombin and specific anticoagulant activity of heparin chains is shown in Fig. 5. The specific activities of LA-chains ranged from 4 units/mg to 11 units/mg. HA-chains were eluted from fraction 26 to fraction 36 and their specific anticoagulant activities ranged from 18 units/mg to 287 units/mg. Fraction 26 may have been contaminated with LA-chains. Such contamination is probably trivial in fraction 27 (32 units/mg). Therefore the specific anticoagulant activities of HA-chains cover an approximately 10-fold range. The anticoagulant activity of the total HA-chain preparation (calculated from the activities of the individual fractions) was 142 units/mg. Horner & Young (1982) reported a value of 152 units/mg, determined by the Ca^{2+} /thrombin time test.

The differences in specific anticoagulant activities found in the current work are not related to differences in relative molecular mass. The relative sizes of LA-chains and HA-chains types I, II and III were determined by gel filtration on Sepharose CL-4B (see Fig. 6a). The M_r of purified chains determined by ultracentrifugation (Robinson *et al.*, 1978) was 81 000. It can be seen from Fig. 6(b) that this is a mean value, the chain preparations being polydisperse. A value of 80 000 is assumed here. Clearly there are only small differences among the average sizes and overall size ranges of LA-chains and the three types of HA-chains. Although LA-chains lack 3-*O*-sulphated glucosamine residues (Jacobsson *et al.*, 1986), they probably contain sequences of monosaccharide residues that are identical with the high-affinity binding site for antithrombin in all other aspects. Such structural similarity may account for their loose binding to antithrombin at physiological ionic strength, but the interaction is apparently too weak to induce appreciable conformational changes in the proteinase inhibitor.

Recently an HA-chains preparation from rat skin, with an anticoagulant activity of 122 units/mg (determined by the Ca^{2+} /thrombin time test), has been shown to contain, on the average, approximately three antithrombin-binding sites per molecule (Jacobsson *et al.*, 1986). This work involved quantitative analysis of the sequence glucuronosyl-*N*-sulphoglucosaminyl (3,6-di-*O*-sulphate), which is found only in heparins with high affinity for antithrombin.

The current experiments demonstrate the existence of HA-chains with specific anticoagulant activities twice that of the total HA-chains fraction. These most active chains presumably have more than three antithrombin-binding sites per molecule. The tempting assumption of an upper limit of six such sites per molecule may be too simplistic, for Nesheim *et al.* (1986), using fractions of commercial pig mucosal heparin, found that molar specific anticoagulant activity was directly related to the product of the number of antithrombin-binding sites and the number of thrombin-binding sites. However, the number of antithrombin-binding sites must be the dominant factor determining specific anticoagulant activity, for heparins with high affinity and low affinity for antithrombin, with high and low specific anticoagulant activities, have the same binding affinity for thrombin (Nordenman & Björk, 1978).

Table 1. Summary of results of fractionations of chains produced from HA-proteoglycan subfractions B–G on antithrombin–agarose (see Fig. 3)

For experimental details see the Materials and methods section. Values for the total HA-proteoglycan preparation were calculated from the data for the individual fractions.

Proteoglycan subfractions from which chains were produced	Percentage of total radioactivity in each high affinity proteoglycan subfraction	Percentage of total radioactivity in each chain preparation in the following fractions:				
		LA-chains	HA-chains			
			Type I	Type II	Type III	Total
B	12.9	83.0	10.2	5.6	1.2	17.0
C	32.2	76.3	7.0	12.4	4.2	23.6
D	25.4	65.6	3.0	22.8	8.6	34.4
E	16.6	51.3	4.5	22.6	21.7	48.8
F	9.2	32.5	5.5	19.4	42.6	67.5
G	3.7	23.9	5.7	14.5	55.8	76.0
Total	100	64.3	5.8	16.6	13.3	35.7

Fig. 4. Graphical presentation of the relative proportions of HA-chains types I (○), II (●) and III (△) in the total HA-chains obtained from HA-proteoglycan subfractions B–G

The ordinate indicates percentage of the total radioactivity in each total HA-chains peak obtained in the fractionations shown in Fig. 3.

Earlier work on the number of antithrombin-binding sites in HA-heparins has utilized fractions prepared from commercial pig mucosal heparin. Jordan *et al.* (1982) isolated a fraction (M_r approx. 7000) with one site per molecule and a fraction (M_r approx. 20000) with two sites per molecule. Nesheim *et al.* (1986) 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, heparin with an M_r of 80000 would have 4.9 antithrombin-binding sites per molecule,

whereas extrapolation of the data of Jordan *et al.* (1982) gives a value of 6.5 sites per molecule. Thus an upper limit of five or six sites per HA-chain seems likely, if comparisons between heparins from different species are valid.

Another interesting comparison can be made with earlier work. The heparin fraction with one antithrombin-binding site per molecule, of M_r approx. 7000, isolated by Jordan *et al.* (1982) had a specific anticoagulant activity, with respect to thrombin inactivation, of 350 units/mg. If the relative molecular mass of this heparin were increased to 80000 without any increase in its intrinsic anticoagulant activity, its specific activity would be 31 units/mg, a very similar value to that of fraction 27, at the low end of the range of specific activities found in HA-chains in the current work.

Thus the possibility that HA-chains contain from one to five or six antithrombin-binding sites per molecule seems reasonable, though it must obviously be established directly in future work. If this is the case, the affinity chromatography used in these experiments achieved only partial resolution of HA-chains into the three overlapping components designated types I, II and III. Even this degree of resolution was only achieved if HA-proteoglycans were initially fractionated and chains subsequently prepared from proteoglycan subfractions (compare Fig. 3 with Fig. 5). However, such incomplete resolution does not invalidate the concept that the biosynthesis of antithrombin-binding sites occurs in a stepwise manner, with only a minor proportion of HA-chains gaining a full complement of these sites.

The present work has not addressed the additional parameter of thrombin-binding sites in HA-chains. The wide range of specific anticoagulant activities of these chains probably reflects, not only different numbers of antithrombin-binding sites per chain, but also variations in the relative proportions of thrombin-binding sites. In the HA-heparin fractions analysed by Nesheim *et al.* (1986) the ratio of thrombin-binding sites to antithrombin-binding sites was approximately 3 (range 2.6–3.2). However, this ratio may be lower in some HA-heparins, for the fractions analysed did not constitute the total HA-heparins in the starting material. The bio-

Fig. 5. Fractionation of heparin chains prepared from the total heparin proteoglycans from rat skin by gradient elution from a column (2.2 cm $\times$ 12 cm) of antithrombin-agarose

Fractions (6.0 ml) were collected at a flow rate of 21 ml/h. ●, Heparin mass, determined by metachromatic assay; ○, specific anticoagulant activity, means $\pm$ S.E.M. ($n = 4$); ---, NaCl concentration. The left-hand ordinate indicates heparin recoveries from four identical fractionations. Corresponding fractions were combined and heparin was recovered before the assays for metachromasia and anticoagulant activity.

Fig. 6. Gel filtration of [35 S]heparin chains on a column (1.0 cm $\times$ 32 cm) of Sepharose CL-4B

(a) ○, LA-chains (the total LA-chains recovered from HA-proteoglycan subfractions B-G); ●, HA-chains type I (from HA-proteoglycan subfraction B); △, HA-chains type II (from HA-proteoglycan subfraction D); ▲, HA-chains type III (from HA-proteoglycan subfraction G). Mean values for three samples of each chain preparation have been plotted. (b) Single portions of individual fractions from the gel filtration of LA-chains illustrated in (a) were run through the same column: ○, fraction 21; ●, fraction 28; △, fraction 35. The arrow labelled PMH marks the elution peak for commercial pig mucosal heparin on this column. V_0 was determined with [35 S]heparin proteoglycan, V_t with $^3\text{H}_2\text{O}$. Each sample was applied in a volume of 0.20 ml. Fractions (0.60 ml) were collected at a flow rate of 13 ml/h.

syntheses of binding sites for thrombin and antithrombin almost certainly proceed independently of each other (see, e.g., Nordenman & Björk, 1978). Thus the number of thrombin-binding sites per antithrombin-binding site in HA-chains could theoretically range from zero to three (assuming similarities between pig heparins and rat heparins). If the number of antithrombin-binding sites per HA-chain ranges from one to six, as suggested above in this discussion, one can postulate 24 possible variants of HA-chains with different specific anticoagulant activities per mole. As all chain preparations are polydisperse with respect to relative molecular mass, the number of possible specific anticoagulant activities per unit mass could be much higher than 24. This hypothesis will probably need to be refined quantitatively in the light of future work, but the principles invoked would explain the current experimental findings.

Lindahl *et al.* (1986) have recently reviewed the intriguing topic of heparin biosynthesis by mast cells, presenting an elegant speculative model in which the proteoglycan traverses several 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 suggest that a restricted distribution within the membrane complex of the 3-*O*-sulphotransferase required for synthesis of the antithrombin-binding site may explain the non-random distribution of these sites in the final products. The different enzyme 'stations' in this model are sufficiently widely spaced to allow the formation of distinct intermediary proteoglycan species and so arranged that a proteoglycan substrate will interact with only one station at a time. If this model is correct, the distribution and spacing of the 3-*O*-sulphotransferase is obviously not ideal for the efficient synthesis of antithrombin-binding sites on all the polysaccharide chains passing through the Golgi apparatus. In the case

of rat skin mast cells, up to 60% of heparin proteoglycans contain no HA-chains (Horner & Young, 1982). The current work shows that the proportion of HA-chains in HA-proteoglycans varies from 17% to 76% and suggests that the number of antithrombin-binding sites per chain may range from one to five or six.

Jacobsson *et al.* (1985) found that the proportion of HA-chains produced by mouse mastocytoma cells in culture increased from 17% to 54% if the cells were preincubated with n-butyrate. This agent may act by lowering the rate at which proteoglycans pass by the membrane-bound biosynthetic enzymes, allowing the 3-O-sulphotransferase a longer time frame in which to act at appropriate sites on the polysaccharide chains. [The suggestion by Jacobsson *et al.* (1985) that increased N-acetylation of mouse heparins formed in the presence of n-butyrate is linked to antithrombin-binding-site synthesis is not supported by the rather unexpected finding (Jacobsson *et al.*, 1986) that HA rat skin heparins, unlike those from pig intestinal mucosa and mouse mastocytoma, contain virtually no N-acetyl groups.]

Clearly, control of the biosynthesis of antithrombin-binding sites is poorly understood. There is no uniformity, with respect to degree of affinity for antithrombin, in heparins isolated from different rat tissues (Horner, 1986). Within a single tissue, different populations of mast cells, varying in degrees of maturation for example (Yong *et al.*, 1975), may produce proteoglycans with differing proportions of antithrombin-binding sites. Even the possibility that different Golgi stacks within a single mast cell produce different heparins cannot currently be discounted.

An interesting new aspect of this problem arises from the demonstration by Rosenberg and co-workers that cells other than mast cells synthesize antithrombin-binding sites. These sites, apparently identical in structure with those found in heparins, are found in a very small proportion of the total polysaccharide chains of heparan sulphate proteoglycans in endothelial cells. The density of distribution of these sites is normally higher in anticoagulant-active heparan sulphate chains from microvascular endothelial cells (Marcum & Rosenberg, 1985) than in those from macrovascular endothelial cells (Marcum *et al.*, 1986). However, in the latter case more anticoagulant activity is produced by cells cultured in the presence of n-butyrate (Marcum *et al.*, 1986). The fact that the biosynthesis of antithrombin-binding sites by both mast cells and endothelial cells can be modified by external agents may have important physiological and pathological implications.

There is also an increasing body of literature on the variable affinities of heparan sulphate proteoglycans for ligands other than antithrombin. Subfractions from rat brain have different affinities for lipoprotein lipase

(Klinger *et al.*, 1985), and subfractions from cultured human neuroblastoma cells have varied degrees of affinity for platelet factor 4 (Maresh *et al.*, 1984).

The biosynthesis of ligand-specific binding sites on heparan sulphate chains can be modulated quite dramatically. Subfractions from cultured fibroblast substratum adhesion sites have differing affinities for platelet factor 4 and fibronectin. The proportions of binding sites for these ligands differ markedly, and independently, in newly formed and long-term culture-generated adhesion sites (Lark & Culp, 1984).

Microheterogeneity of glycosaminoglycan chain structure, due to variations in the extent of post-polymerization modifications, may well be the rule rather than the exception. As such reactions profoundly influence biological activity, elucidation of the control mechanisms involved presents interesting and important challenges.

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