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A GLUCOSAMINE O,N-DISULFATE O-SULFOHYDROLASE WITH A PROBABLE ROLE IN MAMMALIAN CATABOLISM OF HEPARAN SULFATE

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SUMMARY: A trisaccharide of sequence: (glucosamine O,N-disulfate)-(iduronic acid O-sulfate)-(^{3}H-anhydromannitol O-sulfate) was prepared from degradation products of heparin and was used as a substrate to demonstrate, in rat and bovine tissues, a novel O,N-disulfoglucosamine O-sulfatase. The enzyme, purified 720-fold from extracts of beef kidney, has optimal activity at pH 4.1. It is distinct from arylsulfatases A or B, N-acetylglucosamine 6-sulfate sulfatase, and urinary 3,N-disulfoglucosamine 3-O-sulfatase. Data are given on the substrate specificities of the presently described O-sulfatase, of heparin sulfamidase, and of α-L-iduronidase.

INTRODUCTION

Catabolism of heparan sulfates in mammalian tissues involves sequential action of exoglycosidases and specific sulfatases at the nonreducing end of the carbohydrate chain (1,2). Although participation in this scheme of an O,N-disulfoglucosamine O-sulfatase has been postulated (2), a hydrolase of this specificity has not so far been detected in tissues. We now report our preliminary findings on demonstration, partial purification, and enzymic characterization of such a hydrolase with a trisaccharide substrate derived from heparin.

MATERIALS AND METHODS

The heparin di- and tetrasaccharides, as well as most of the methods, are described elsewhere (3,4). Electropherograms on "Baker-Flex" cellulose-coated film were dipped in scintillator and exposed to x-ray film. Enzymic digestion of oligosaccharide substrates was generally performed in 15 µl of sodium formate buffer, pH 4.1, of ionic strength 0.03, supplemented with 0.07 M sodium chloride and bovine serum albumin (50 µg/ml). After incubation at 37°, digests were heated for 2 min at 100°. Following electrophoresis of 5 µl aliquots, the species separated were located, using markers in adjacent lanes when necessary. The radioactivity of each excised spot was calculated as a fraction of total radioactivity in its lane. Quantitative results were expressed in enzyme units of µmol/min, which were also used for all of the enzymes listed following. Purified α-L-iduronidase (250 mU/mg; 4-methylumbelliferyl iduronide substrate; ref. 5) was prepared from beef liver by a modification of a published method for

Abbreviations: Gn, glucosamine; Gns, glucosamine N-sulfate; AcGn, N-acetylglucosamine; Ia, iduronic acid; aM, 2,5-anhydromannitol; GnS, GnsS, AcGnS, IaS, and aMS are the corresponding O-sulfates.

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human kidney (6); hydroxylapatite chromatography was omitted for a second, cruder preparation (12 mU/mg). Methyl-2-sulfamino2-deoxy-α-D-glucopyranoside 3- and 6-sulfates, gifts from Dr. Irwin Leder, were used to assay for 3- and 6-O-sulfatase activity (7); where specified, sodium formate of pH 4.1 was substituted as the assay buffer. Arylsulfatases A and B were assayed with p-nitrocatechol sulfate (8). Heparin sulfamidase was assayed with N-$^{35}SO_4$-heparin from Amersham-Searle (9). All enzyme preparations were exhaustively dialyzed against 0.05 M sodium chloride prior to tests and diluted with bovine serum albumin (150 μg/ml) as required.

RESULTS AND DISCUSSION

Preparation of trisaccharide substrates. Partial degradative deamination of beef lung heparin and reduction of the fragments with NaB^3H_4 gave, as one product, a tetrasaccharide tri-O-sulfate preparation that was largely Ia-GnS-IaS-aMS (4). By additional chromatography, the content of this species was increased to 85-90%. The resulting preparation was apparently identical with a less accessible similar preparation isolated after acid hydrolysis of IaS-GnsS-IaS-aMS (4). α-L-Iduronidase purified from beef liver had no effect on these preparations. When the free amino group in either preparation was chemically re-N-sulfated with aqueous trimethylamine-sulfur trioxide, the resulting N-sulfated tetrasaccharide (Ia-GnsS-IaS-aMS) was attacked by purified α-L-iduronidase, as illustrated in Fig. 1 (lanes B and C). The mobilities of the product at pH 1.7 and pH 5.3, relative to the mobilities of standards of known charge and size, were consistent with those expected for the species, GnsS-IaS-aMS (trisaccharide 1), anticipated from the structure of the substrate and the specificity of α-L-iduronidase.

For preparative experiments, cruder α-L-iduronidase was used, with the results exemplified in lanes D and E of Fig. 1. In addition to the fast spot for trisaccharide 1 a new slow spot was now seen, whose mobility at pH 1.7 and pH 5.3 corresponded closely to that predicted for the species GnS-IaS-aMS. This could arise from action of heparin sulfamidase (9) on trisaccharide 1 (GnsS-IaS-aMS, the iduronidase product), but not on Ia-GnsS-IaS-aMS because, as seen, Ia-GnS-IaS-aMS was not a substrate for iduronidase. In agreement with this interpretation, the crude iduronidase showed heavy contamination by sulfamidase. Moreover, 5 mM Na_2SO_3, a sulfamidase inhibitor (9), suppressed formation of the slow product (Fig. 1). For additional confirmation, both trisaccharides were isolated from crude iduronidase digests by chromatography on DEAE-Sephadex. As expected, chemical N-sulfation of GnS-IaS-aMS gave material having the electrophoretic mobility of trisaccharide 1, as shown in lanes G, H, and I of Fig. 1. (Trisaccharide 1 so prepared by re-N-sulfation was used in many of the experiments described following).

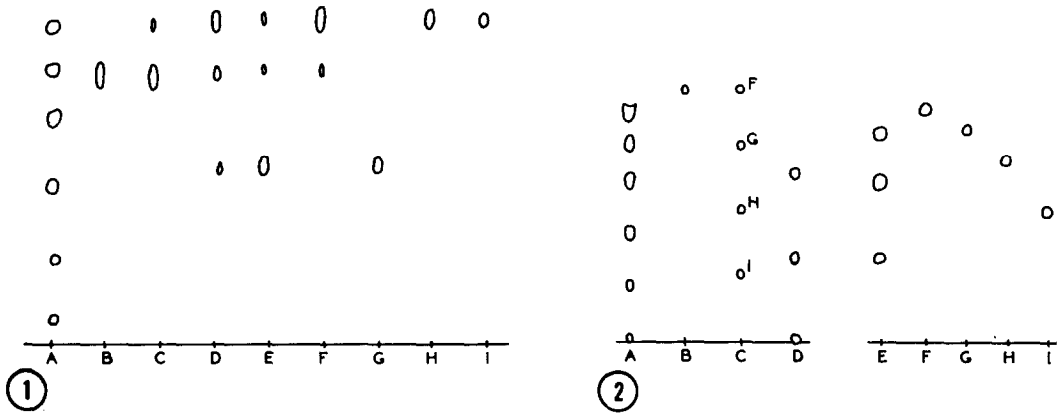


Figure 1. Preparation of heparin trisaccharides from tetrasaccharide. Thin film electrophoresis at pH 1.7. A: Tetrasaccharide markers (species of charge 0 to -5 at pH 1.7; ref. 4). B and C: Action of pure α-L-iduronidase on Ia-GnsS-IaS-aMS, control (BSA only) and digest. D and E: Crude α-L-iduronidase with Ia-GnsS-IaS-aMS, 14 mU/ml and 110 mU/ml. F: Digest as in E, supplemented with 5 mM Na_2SO_3. G: GnS-IaS-aMS isolated from digests. H: Trisaccharide 1 from chemical N-sulfation of material in G. I: Trisaccharide 1 isolated from digests.

Figure 2. Action of dialyzed rat spleen homogenate on trisaccharide 1. Electrophoresis at pH 1.7 (A-D) and 5.3 (E-I). A: Tetrasaccharide standards as in Fig. 1. B: Trisaccharide 1 marker. C: Digest of trisaccharide 1 with spleen homogenate. D: Disaccharide markers (from origin): Ia-aM, Ia-aMS, IaS-aMS. E: Disaccharide markers. F-I: Substances represented in C, isolated from digest and subjected to electrophoresis at pH 5.3. (The lane labels correspond to the letters identifying spots in lane C).

Occurrence of the O-sulfatase. Trisaccharide 1 was now used as a substrate to test for occurrence of the hypothetical O-sulfatase in mammalian tissues. The results with a dialyzed water homogenate of rat spleen (incubation for 2 days at 25° in sodium formate buffer of pH 4 and ionic strength 0.05 supplemented with 0.10 M NaCl) are illustrated in Fig. 2. The substrate and the three products resolved by electrophoresis were isolated by chromatography on ECTEOLA-cellulose for examination. Excellent correspondence was seen of their mobilities in electrophoresis at pH 1.7 and pH 5.3 (Fig. 2) with those predicted for trisaccharide species as follows: F, GnsS-IaS-aMS (trisaccharide 1, the substrate); G, Gns-IaS-aMS (the O-sulfatase product); H, GnS-IaS-aMS (the sulfamidase product already encountered in crude iduronidase digests); and I, Gn-IaS-aMS (probably a product of sequential O-sulfatase and sulfamidase action). Deamination of the four trisaccharide preparations, isolated from digests, gave IaS-aMS as the sole radioactive product in each case, in agreement with the sequences assigned.

TABLE I

SPECIFIC SULFATASE ACTIVITY WITH VARIOUS SUBSTRATES
(mU per mg of protein)

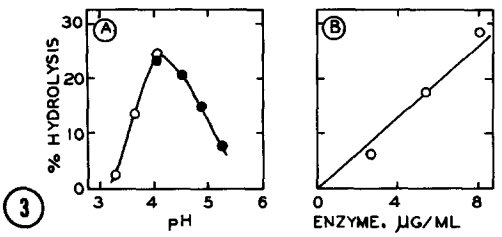
Enzyme	Trisac. 1 O-sulf.	Trisac. 1 N-sulf.	N-Ac-trisac.* O-sulf.	Arylsulfatases A	Arylsulfatases B
Kidney Extract	0.04	0.01			
Purified Prepn. (S-II)	28.5	nil	3.1	1.50	4.5
Fracn. B (45-60% A.S.)	0.09	0.03	0.12	0.63	0.48

*AcGnS-IaS-aMS

By separately summing the radioactivity of the O-sulfatase and the sulfamidase products, these activities in crude extracts could be roughly evaluated. For dialyzed water homogenates of rat spleen, liver, and kidney, incubation with 0.4 mM trisaccharide 1 (25°, 24 h, in sodium formate buffer of pH 4 and ionic strength 0.05 supplemented with 0.10 M NaCl) gave 0.4, 1.0, and 1.9 μmol per g moist tissue, respectively, of O-sulfatase products and 0.4, 1.0, and 1.1 μmol per g moist tissue of sulfamidase products.

Partial purification. A centrifuged water homogenate of frozen fresh beef kidney was treated with ammonium sulfate. Fractions collected at 45-60% satn. (Fraction B) and 60-70% satn. (Fraction C) were dialyzed. Fraction C was chromatographed on CM-cellulose and the most active O-sulfatase fractions were subjected to gel chromatography on Sephacryl S-200. The pooled peak fractions were concentrated by ammonium sulfate precipitation, dialyzed, and stored at -18° as Preparation S-II. As seen in Table I, substantial purification of the O-sulfatase activity (trisaccharide 1 as substrate) in the kidney extract was effected and the sulfamidase activity was eliminated.

Enzymic properties. As illustrated in Fig. 3, O-sulfatase action of Preparation S-II on trisaccharide 1 was optimal at pH 4.1 and product formed was roughly proportional to enzyme added to at least 25% hydrolysis of substrate. Preliminary measurements of K_m gave a value of about 0.5 mM. Decrease of the NaCl content of digests from 0.07 M to 0.01 M had little effect, but increase to 0.16 M caused some 50% inhibition. Tested at concentration of 0.2 mM, sulfite ion caused 97% inhibition; sulfate, 43%; phosphate, 22%.



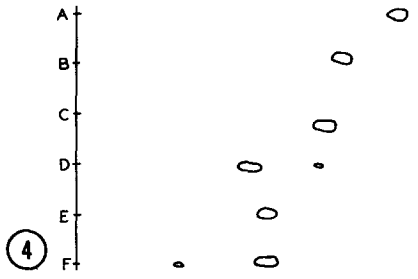


Figure 3. O-Sulfatase assays with trisaccharide 1 (0.1 mM, 2 hr., 37°, electrophoresis at pH 1.7). A: Enzymic hydrolysis as a function of pH, using Na formate buffers (o) and Na acetate (•) buffers. B: Enzymic hydrolysis at pH 4.1 as a function of enzyme added.

Figure 4. Action of the purified O-sulfatase preparation S-II (195 µg/ml) on oligosaccharide substrates. Electrophoresis at pH 1.7. A and B: Trisaccharide 1, control and digest; C and D: AcGnS-IaS-aMS, control and digest; E and F: GnS-IaS-aMS, control and digest. Under the conditions of this experiment hydrolysis of trisaccharide 1 was complete, hydrolysis of AcGnS-IaS-aMS was 88% complete, and hydrolysis of GnS-IaS-aMS was 13% complete.

Hydrolysis of methyl 2-sulfamino-2-deoxy-α-D-glucopyranoside 3-O-sulfate by Preparation S-II (0.23 mg/ml, pH 6.3 or pH 4.1) was undetectable (less than 5 pmol/min/mg), showing the O-sulfatase under examination to differ from urinary glucosamine 3,N-disulfate 3-O-sulfatase (7). For comparisons with N-acetylglucosamine 6-sulfate sulfatase (10), the potential substrate: AcGnS-IaS-aMS was prepared by reaction of GnS-IaS-aMS with aqueous acetic anhydride. At concentrations of Preparation S-II sufficient to effect complete hydrolysis of trisaccharide 1, hydrolysis of AcGnS-IaS-aMS was substantial in 2 h, as seen in Fig. 4. However, little, or perhaps none, of this N-acetylglucosamine 6-sulfate sulfatase activity resided in the enzyme that attacked trisaccharide 1, since, as shown in Table I, the ratio of activity with AcGnS-IaS-aMS to that with trisaccharide 1 for Preparation S-II (8µg/ml) was small when compared with this ratio for crude Fraction B. Significant arylsulfatase A and B activities were measured both in Preparation S-II and in crude Fraction B. Calculations from the values of Table I show, however, that less than 1% and 3% of the arylsulfatase A and B activities in Preparation S-II could have been associated with the O,N-disulfoglucosamine O-sulfatase. Small but significant O-sulfatase activity in Preparation S-II was displayed with the trisaccharide having an unsubstituted amino group, GnS-IaS-aMS (0.56 mU/mg; see also Fig. 4), and with methyl 2-sulfamino-2-deoxy-α-D-glucopyranoside 6-O-sulfate (0.019 mU/mg at pH 6.3, 0.056 mU/mg at pH 4.1).

Removal of the N-sulfo group thus greatly diminishes (or abolishes) the glucosamine O-sulfatase activity here described. A like effect of N-

sulfate substitution in action of α-L-iduronidase on tetrasaccharide substrates has already been noted. Possibly, in one or both cases these poor substrate properties of the free amines are attributable to presence of positive charge on the nitrogen at low pH values. Heparin sulfamidase, as seen, is apparently indifferent to the presence or absence of sulfate ester substitution (but not iduronosyl substitution) on the N-sulfoglucosamine residues subject to its N-sulfatase action. We hope to elaborate on these observations, which may well be relevant to details of mucopolysaccharide catabolism.

Only additional enzyme purification can show whether the weak O-sulfatase activities of Preparation S-II with GnS-IaS-aMS and the methyl glycoside 6-O-sulfate are inherent properties of the enzyme that hydrolyzes trisaccharide 1. This is also true for those small portions of the N-acetylglucosamine 6-sulfate sulfatase and arylsulfatase activities not otherwise accounted for in the experiments. It is in any case clear that the O-sulfatase now described differs from related enzymes reported previously. Since the sulfate ester groups on glucosamine residues in heparin (and, by extension, in trisaccharide 1) are generally considered (11) to occupy the 6-position, the new O-sulfatase is provisionally categorized as a 6,N-disulfoglucosamine 6-O-sulfohydrolase. Although trisaccharide substrates from heparin were used in this work, we consider it extremely likely that this enzyme plays a role in mammalian catabolism of heparan sulfate.

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REFERENCES

1. Neufeld, E. F., Lim, T. W., and Shapiro, L. J. (1975) Annu. Rev. Biochem. 44, 357-376.
2. Dorfman, A. and Matalon, R. (1976) Proc. Natl. Acad. Sci. USA 73, 630-637.
3. Weissmann, B., Chao, H., and Chow, P. (1981) Carbohydr. Res., in press.
4. Weissmann, B., and Chao, H. (1981) Carbohydr. Res., in press.
5. Weissmann, B. (1978) Methods Enzymol. 50, 141-150.
6. Rome, L. H., Garvin, A. J., and Neufeld, E. F. (1978) Arch. Biochem. Biophys. 189, 344-353.
7. Leder, I. (1980) Biochem. Biophys. Res. Comm. 94, 1183-1189.

8. Baum, H., Dodgson, K. S., and Spencer, B. (1959) Clin. Chim. Acta 4, 453-455.
9. Friedman, Y. and Arsenis, C. (1974) Biochem. J. 139, 699-708.
10. Basner, R., Kresse, H., and von Figura, K. (1979) J. Biol. Chem. 254, 1151-1158.
11. Lindahl, U. and Hook, M. (1978) Annu. Rev. Biochem. 47, 385-417.