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THE ENZYMIC DEFECT IN MORQUIO'S DISEASE: THE SPECIFICITY OF N-ACETYLHEXOSAMINE SULFATASES

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SUMMARY

Extracts of Morquio fibroblasts lack N-acetylgalactosamine 6-sulfate sulfatase activity, but exhibit normal levels of N-acetylglucosamine 6-sulfate sulfatase activity. Thus, the enzyme defective in Morquio's disease is a sulfatase specific for the 6-sulfate linked to sugars with the galactose configuration. Hydrolysis of ester sulfate by this enzyme is limited to 6-sulfate groups occurring at the non-reducing terminal.

The mucopolysaccharidoses result from lack of enzymic activity involved in cleavage of linkages present in glycosaminoglycans (1). Extracts of Maroteaux-Lamy fibroblasts show decreased hydrolysis of oligosaccharides containing GalNAc 4-SO_4 linkages (2). Since Morquio patients excrete keratan sulfate as well as chondroitin sulfate (3) and since keratan sulfate contains both GlcNAc 6-SO_4 and galactose 6-SO_4 linkages, it is not clear whether the deficient enzyme is specific for the galactose configuration or hexosamine 6-SO_4. The present study demonstrates that extracts of Morquio fibroblasts remove sulfate from a keratan sulfate fragment containing N-acetylglucosamine 6-sulfate. Additionally, these studies indicate that 6-sulfate cleavage occurs only on non-reducing residues.

MATERIALS AND METHODS

Chondroitin 6-sulfate oligosaccharides were obtained by testicular hyaluronidase digestion followed by Sephadex G-50 chromatography of [$^{35}SO_4$]chondroitin sulfate produced in differentiated limb bud cultures (4). Analysis of chondroitinase

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ABC digestion products (5) of the hexasaccharide demonstrated that 88% of the non-reducing terminal disaccharide was labeled in the 6 position. The tetrasaccharide was subjected to chromatography on Whatmann 3MM paper in butanol-acetic acid-1N NH_4OH (2:3:1) for 72 hr to resolve the three sulfated isomers. The slowest migrating material was found to contain sulfate only in the 6 position of both N-acetylgalactosamines. Penta- and trisaccharides were prepared by β-glucuronidase digestions of the hexa- and tetrasaccharide. Keratan sulfate was obtained by the labeling of 13-day embryonic chick epiphyses with $^{35}SO_4$ in Krebs-Ringer-bicarbonate buffer. The material resistant to papain digestion was degraded by nitrous acid and chondroitinase ABC. The resistant material was digested by partially purified keratan sulfate β-endogalactosidase (6) (gift of Dr. N. Kitamikado, Kyushu University, Japan). The resultant [$^{35}SO_4$]-disacchride, isolated by Sephadex G-50 chromatography, had paper chromatographic and electrophoretic mobilities identical with a disaccharide isolated from skeletal keratan sulfate (gift of Dr. M. B. Mathews) (7).

Skin fibroblasts were cultured as previously described (8). Cells deficient in β-glucuronidase, as measured by digestions of p-nitrophenylglucuronide (10% of normal activity), or 4-methylumbelliferyl glucuronide (less than 5% of normal), were obtained from a patient with clinical and urinary glycosaminoglycan findings similar to that described by Sly et al. (9). Cells were sonicated in 0.05M sodium acetate, 0.1M NaCl, pH 5.5 and centrifuged at 600xg for 10 min. The supernatants, utilized for enzymic activities using the chondroitin sulfate oligosaccharides, did not show significant sulfatase activity utilizing the keratan sulfate disaccharide until dialyzed (0.03M sodium acetate, pH 5.5) and centrifuged 10,000xg, 10 min.

Sulfate release from chondroitin 6-sulfate oligosaccharides was measured in 0.05M sodium acetate, pH 4.5, with 100 to 200 µg of enzyme protein in a final volume of 0.1 ml. Following incubation at 37° for 6 hr, 5 µl of 0.1M Na_2SO_4 was added prior to paper electrophoresis. The areas corresponding to free sulfate were eluted and counted. Incubation with the keratan sulfate disaccharide was carried out in 0.05M sodium acetate, pH 6.5, with 200 to 300 µg of protein in a total volume of 0.1 ml at 37° for 18 hr.

Radioactivity on paper strips was localized with a Packard strip scanner. Radioactivity was measured by dissolving 1 ml of aqueous sample in 10 ml aquasol (New England Nuclear) and counted in a Packard scintillation counter.

RESULTS

Release of sulfate from chondroitin 6-sulfate pentasaccharides was linear with time up to 8 hr and with amounts of enzyme up to 300 µg. Sulfate release from the pentasaccharide by Morquio fibroblasts was 10-12% of that found in extracts of normal fibroblasts, a percentage similar to the proportion of 4-sulfate on the non-reducing N-acetylgalactosamine of this substrate. This activity may be due to the 4-sulfatase (aryl-

sulfatase B) which is present in Morquio cells. Table 1 demonstrates that with a pure 6-O-sulfated substrate, extracts of Morquio fibroblasts show less than 2% of normal activity. Extracts of fibroblasts from other sulfatase deficient mucopolysaccharidoses (Table 1) show normal levels of GalNAc 6-SO_4 sulfatase activity.

Release of sulfate from the keratan sulfate disaccharide proceeded at normal rates in extracts of Morquio fibroblasts as well as those of other mucopolysaccharidoses (Table 1).

Fig. 1 indicates that the pH optimum for activity on the chondroitin 6-sulfate pentasaccharide is 4.5, while that for sulfate release from the keratan sulfate disacchride is 6.5. The later activity was stable from pH 5.5 to 7.5, but activity was lost upon storage at pH 4.5 at 4^{o} for more than 24 hr.

Table 2 indicates that removal of the non-reducing terminal glucuronic acid was necessary for release of the 6-sulfate from chondroitin sulfate oligosaccharides. Incubation of normal fibroblast extracts with a hexasaccharide (having non-reducing terminal glucuronic acid) gave the same rate of release of sulfate as from the pentasaccharide. Analysis of the digestion products of this reaction by chondroitinase degradation and chromatography revealed that the non-reducing glucuronic acid was removed by endogenous β-glucuronidase activity as evidenced by complete absence of the saturated disaccharide. When the β-glucuronidase inhibitor, saccharo-1,4-lactone (10) was included, there was almost no release of sulfate from hexasaccharide although release from pentasaccharide was unchanged. Likewise, a lack of endogenous glucuronidase activity in extracts of cells with glucuronidase deficiency correlated with a defect of sulfate release from a hexasaccharide, but not from the pentasaccharide.

TABLE 1

Substrate	GalNAc-GlcUA-GalNAc 6 6 SO_4 SO_4	GlcNAc-Gal 6 SO_4
	cpm x 10^{-3}/mg	cpm x 10^{-3}/mg
Normal	9.55	52.0
Normal	7.21	71.5
Morquio	0.20	41.3
Morquio	0.17	31.0
Sanfilippo A	3.52	81.9
Hunter	4.50	30.6
Maroteaux-Lamy	7.19	38.6
Boiled normal	0.10	0.3

Incubations each contained 150 to 200 µg of protein. Incubation with 140 x 10^3cpm of $^{35}SO_4$ trisaccharide was in a final volume of 100 µl of 0.05 M sodium acetate for 6 hr. The disaccharide, 120 x 10^3cpm, was incubated in 100 µl of 0.05 M sodium acetate, pH 6.5 for 18 hrs.

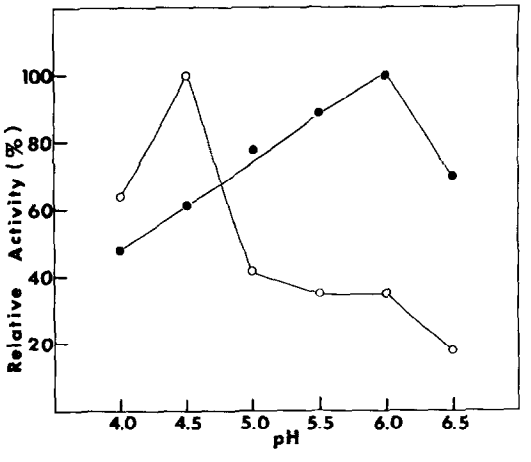

Figure 1. Effect of pH on sulfatase activities. Incubations with chondroitin 6-sulfate pentasaccharide were carried out as in Table 2 and with the keratan sulfate disaccharide as described in Table 1, except the acetate buffer were at the indicated pH. The percent activity is relative to that at the maximum pH. o-----o , rate of sulfate release from chondroitin 6-sulfate pentasaccharide; ●-----● , rate of sulfate release from the keratan sulfate disaccharide.

TABLE 2

Substrate	$(GlcUA\text{-}GalNAc)_3$ (6-SO_4)	$GalNAc\text{-}(GlcUA\text{-}GalNAc)_2$ (6-SO_4)
	cpm/mg x 10^{-3}	cpm/mg x 10^{-3}
Normal	15.60	14.7
Normal + Saccharolactone	1.12	14.1
β-Glucuronidase deficiency	3.51	13.2
β-Glucuronidase deficiency + Saccharolactone	1.23	13.0
Morquio	1.62	1.57
Morquio + Saccharolactone	1.24	1.43

Incubations each contained 150 to 200 µg of protein. Incubation with 3 x 10^5cpm of hexasaccharide was in a final volume of 110 µl of 0.05 M sodium acetate, pH 4.5, for 6 hr. Saccharo-1,4 lactone was present at a concentration of 1.5 mM. Radioactive sulfate released was isolated by electrophoresis.

Further evidence for the specificity of sulfate hydrolysis was obtained by examination of the oligosaccharide substrate after sulfate release. As shown in Fig. 2, chondroitinase digestion and chromatography of untreated chondroitin 6-sulfate trisaccharide demonstrates GalNAc 6-SO_4 derived from the non-reducing terminal and unsaturated 6-sulfated disaccharide derived from the reducing terminal. Following incubation with fibroblast extract, there is a reduction of GalNAc 6-SO_4 and a corresponding appearance of inorganic sulfate with no detectable change in the amount of unsaturated disaccharide. Thus, release was predominantly from the non-reducing terminal.

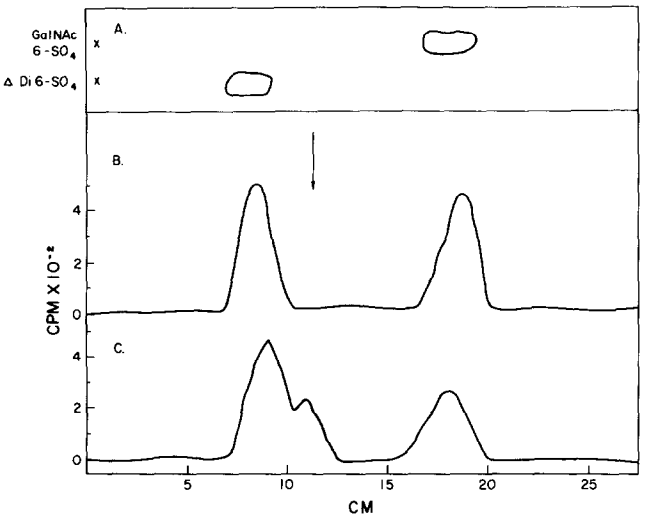

Figure 2. Analysis of digestion products of N-acetylgalactosamine 6-sulfate sulfatase. The $^{35}SO_4$-labeled chondroitin 6-sulfate trisaccharide was incubated as described in Table 1 for 24 hr. Following incubation, digestion was carried out with chondroitinase ABC followed by paper chromatography. Radioactivity was determined by a strip scanner. The standards were obtained by paper chromatography of the chondroitinase digestion products of a chondroitin 6-sulfate pentasaccharide. A: location of GalNAc 6-SO_4 and unsaturated 6-sulfated chondroitin sulfate disaccharide. B: Chondroitinase digest of untreated trisaccharide. The arrow represents location of radioactive sulfate run parallel to the sample. C: Chondroitinase digest following incubation with active enzyme.

DISCUSSION

The enzymic deficiency in Morquio's disease can now be considered an N-acetylgalactosamine 6-sulfate sulfatase specific for the galactose configuration. Results obtained using a pure 6-sulfated substrate indicated an essentially complete lack of this sulfatase in the Morquio fibroblasts. The small amounts of sulfate release (8-10% of normal) previously described (11) were probably due to small amounts of 4-sulfate released. The sulfate hydrolysis utilizing the keratan sulfate disaccharide containing GlcNAc 6-SO_4 implies that the defect in keratan sulfate degradation must be in activity on galactose 6-sulfate. The separate activities for the glucosamine and galactosamine

sulfate isomer is consistent with the findings by Ginsberg et al. (12) of a patient with mucopolysaccharidurıa whose fibroblasts extracts are able to release sulfate from a chondroitin 6-sulfate oligosaccharide, but not from synthetic GlcNAc 6-SO_4. Extracts of Morquio's disease hydrolyzed this latter substrate. The preferential hydrolysis of sulfate from the non-reducing terminal explains why the excreted glycosaminoglycans in Morquio's disease appear to be normally sulfated.

The exact cause of lack of enzymic activity in Morquio's disease will require further efforts to purify this enzyme and to detect abnormal enzyme protein.

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