URIDINE DIPHOSPHO-N-ACETYLGALACTOSAMINE-4-SULFATE SULFOHYDROLASE ACTIVITY OF HUMAN ARYLSULFATASE B AND ITS DEFICIENCY IN THE MAROTEAUX-LAMY SYNDROME

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Summary: The hydrolysis of UDP-N-acetylgalactosamine-4-sulfate by human arylsulfatase B has been demonstrated with an enzyme preparation purified 200-fold from placenta. No hydrolysis was observed with arylsulfatase A. UDP-N-acetylgalactosamine-4-sulfate is the first fully characterized physiological compound shown to be a substrate for arylsulfatase B, confirming that arylsulfatase B is an N-acetylgalactosamine-4-sulfate sulfohydrolase. Cultured fibroblasts derived from patients with Maroteaux-Lamy syndrome were deficient in UDP-N-acetylgalactosamine-4-sulfate sulfohydrolase to the same extent that they were deficient in arylsulfatase B.

The Maroteaux-Lamy syndrome, mucopolysaccharidosis VI, is an autosomal recessive disorder characterized by severe skeletal deformities, gross corneal opacity, marked retardation of growth, and early death; but with normal intellect [1]. Patients with this disorder accumulate in tissues and excrete in urine excess amounts of dermatan sulfate. A deficiency of the lysosomal enzyme, arylsulfatase B, noted in tissues of patients with Maroteaux-Lamy syndrome by Stumpf et al. [2], has subsequently been observed in cultured fibroblasts [3-6], urine [3], and leukocytes [5]. Defective desulfation of dermatan sulfate [7] and chondroitin 4-sulfate [8] by Maroteaux-Lamy syndrome fibroblasts has implied that arylsulfatase B functions as an N-acetylgalactosamine-4-sulfate sulfohydrolase.

Tsuji et al. [9] recently reported the occurrence of a sulfatase in hen oviduct which acted on uridine diphospho-N-acetylgalactosamine-4-sulfate (UDP-GalNAc-4-S). This report indicated that a sulfatase with similar activity was present in rat tissue. Similarities between the UDP-GalNAc-4-S sulfatase and

arylsulfatase B on ion exchange chromatography coupled with structural analogies between the enzyme susceptible moiety of the sugar nucleotide and the region thought to be hydrolyzed in mucopolysaccharides prompted the present study. UDP-GalNAc-4-S was found to be an excellent substrate for human arylsulfatase B; moreover, Maroteaux-Lamy syndrome fibroblasts showed impaired ability to hydrolyze the sulfated sugar nucleotide.

MATERIALS AND METHODS

[^{35}S]UDP-GalNAc-4-S. [^{35}S]Labeled nucleotide sugar sulfate was prepared with tissue from the isthmus region of the hen oviduct as described by Suzuki and Strominger [10] and isolated by ion exchange chromatography as described by Strominger [11]. Briefly, the isthmus of four actively laying hens was sliced and incubated in Krebs-Ringer buffer containing 40 mCi carrier-free $H_2{}^{35}SO_4$ (New England Nuclear) for 90 min at 37°. The mixture was heated, homogenized, centrifuged, and applied to a column of Dowex 1-Cl; the UDP-GalNAc-4-S was isolated by a gradient elution scheme. The material was recovered in essentially the same fractions as the compound designated UDP-GalNAc-S by Strominger [11]. UDP-GalNAc-4-S concentrations were estimated spectrophotometrically using a molar extinction coefficient of 10,000 at 260 nm [11].

Placental arylsulfatase B. Fresh placenta was freed of adhering blood, passed through a meat grinder and converted to an acetone powder. The acetone powder was extracted with 100 mM Tris-HCl, pH 7.5; dialyzed overnight against 25 mM Tris-HCl, pH 7.5; and applied to a DEAE-cellulose column coupled in tandem to a CM-cellulose column. The enzyme was eluted from the CM-cellulose with a NaCl gradient and subjected to gel filtration on Sephadex G-200. The preparation was devoid of arylsulfatase A activity and about a 200-fold purification was achieved to a specific activity of 36 units per mg protein. A unit is defined as one μmole of 4-nitrocatechol sulfate hydrolyzed per hour.

Fibroblast extracts. Fibroblasts were cultured and harvested in the usual manner [4], but the preparation of extracts was modified. Cells were suspended in an equal volume of 25 mM Tris-HCl, pH 7.5, and lysed by six cycles of freezing and thawing. Cell debris was sedimented (1 min, 14,000 x g) and the supernatant fluid dialyzed (Spectrapor 2 dialysis tubing, molecular weight cut off 12 to 14,000, Spectrum Medical Industries) overnight against 1000 vol of 12.5 mM Tris-HCl, pH 7.5, followed by one hour against 1000 vol of water. Adequate dialysis was essential to remove inhibitory material and to decrease the buffering capacity of the extracts. Protein was determined by the procedure of Lowry et al. [12].

Enzyme assay. The sulfatase assay mixture contained the following in a total volume of 0.1 ml: 2.25 nmoles [^{35}S]UDP-GalNAc-4-S (~ 3000 cpm); 5 μmoles sodium acetate-acetic acid buffer, pH 3.5; 10 μg bovine serum albumin; and enzyme, either 0.01 units of the arylsulfatase B preparation or 5-10 μliters of dialyzed fibroblast extract. The mixture was incubated at 37° for 30 min and the reaction was stopped by the addition of 0.1 ml 1 N HCl and 2-3 mg activated charcoal (Mallinckrodt). After 10 min the mixture was filtered through Whatman 42 filter paper directly into a scintillation vial followed by 0.3 ml 0.5 N HCl wash. The combined filtrate-wash was dispersed in 10 ml of a toluene-methylcellosolve scintillant [13] and counted. Zero time blanks as well as controls without charcoal treatment were included in each assay series. Enzyme activity is expressed as nmoles sulfate released/h/mg protein.

RESULTS

The yield of [^{35}S]UDP-GalNAc-4-S was 10 μmoles from 18.5 g of isthmus

tissue at a specific activity of 1.5 Ci/mole. The product gave one discrete band which co-chromatographed with authentic UDP-GalNAc-4-S (kindly provided by Drs. Y. Nakanishi and S. Suzuki) on thin layer chromatography in four solvent systems.

UDP-GalNAc-4-S was hydrolyzed by the arylsulfatase B preparation at a rate within the range of commonly used synthetic substrates. The reaction was sensitive to ionic composition of the assay mixture, so the pH optimum was dependent on the type and concentration of buffer. In 50 mM acetate, the optimum was at pH 3.5 (Fig. 1) and the K_m was 13 μM (Fig. 2). The reaction was linear with time or protein concentration until about one-half of the substrate was consumed and essentially complete hydrolysis was achieved with either extended incubation or increased amounts of enzyme. Heating the arylsulfatase B preparation at 64° resulted in a loss of UDP-GalNAc-4-S sulfohydrolase activity at a rate identical to that of the 4-nitrocatechol sulfate sulfohydrolase activity (Fig. 3).

Optimal conditions for the UDP-GalNAc-4-S sulfatase assay of crude fibroblast extracts were essentially identical to those for the arylsulfatase B preparation provided the extracts were adequately dialyzed. The sulfatase activity was deficient in Maroteaux-Lamy fibroblasts, but was normal in fibroblasts of other types of mucopolysaccharidoses or metachromatic leukodystrophy (Table I). The deficiency of UDP-GalNAc-4-S sulfatase was comparable to the

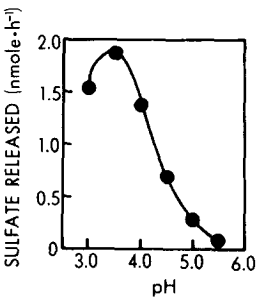

Fig. 1. Effect of pH on UDP-GalNAc-4-S sulfohydrolase activity. Enzyme assay was as described in the text except that sodium acetate buffers were adjusted to the indicated pH and 0.007 units of arylsulfatase B was used.

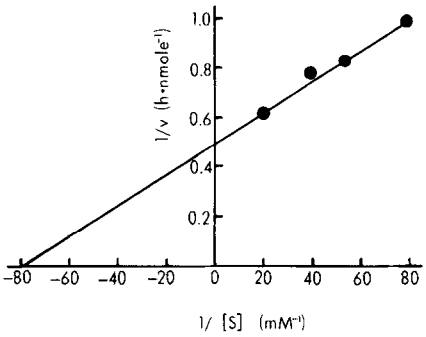

Fig. 2. Lineweaver-Burk reciprocal plot of UDP-GalNAc-4-S sulfohydrolase activity. The assay was as described in the text except that the concentration of UDP-GalNAc-4-S was varied.

deficiency of arylsulfatase B. Pure human arylsulfatase A failed to hydrolyze the nucleotide substrate, even at a 1000-fold excess.

DISCUSSION

UDP-GalNAc-4-S, a constituent of hen oviduct, has been observed to be hydrolyzed by a sulfatase from the same source [9]. We have shown that this sulfated sugar nucleotide is also hydrolyzed effectively by a partially purified preparation of human arylsulfatase B. This is the first fully characterized physiological compound to be demonstrated as a substrate for this enzyme. UDP-GalNAc-4-S appears to be highly specific for arylsulfatase B, so it should prove to be valuable in a variety of applications.

A convenient analytical procedure for the nucleotide sulfohydrolase reaction was developed using charcoal adsorption of the unreacted substrate and radioactive estimation of the unbound inorganic sulfate. This procedure does not differentiate between released inorganic sulfate and N-acetylgalactosamine-4-sulfate. The latter would arise from the action of phosphoesterases which were found in crude hen oviduct extracts [9]. To confirm that we were measuring a sulfohydrolase reaction and not a phosphoesterase reaction, parallel assays were analyzed by the charcoal adsorption procedure and by a barium sulfate precipitation protocol. Nearly identical results were obtained by both

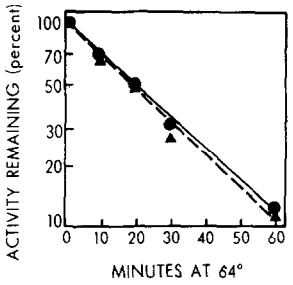

Fig. 3. Effect of heat treatment on UDP-GalNAc-4-S sulfohydrolase (●—●) and 4-nitrocatechol sulfate sulfohydrolase (▲- - -▲) activity. The enzyme was diluted to 0.5 units/ml in 10 mM Tris-HCl, pH 7.5, containing 1 mg/ml bovine serum albumin, incubated at 64° for indicated periods, chilled, and assayed.

methods indicating that only the sulfohydrolase reaction was occurring. This was also true with dialyzed fibroblasts extracts.

The pH 3.5 optimum for the hydrolysis of the nucleotide substrate was surprisingly low. In contrast, Tsuji et al. [9] observed an optimum of pH 5.0 for hen oviduct and rat kidney and liver enzymes. Synthetic substrates, such as 4-nitrocatechol and 4-methylumbelliferyl sulfates, are hydrolyzed optimally between pH 5 and 6 by arylsulfatase B. Hydrolysis of dermatan sulfate and chondroitin 4-sulfate by fibroblast extracts also occurred at pH's lower than that for synthetic substrates [7,8]. It thus appears that synthetic substrates are not appropriate for defining physiological reactions of arylsulfatase B.

While the arylsulfatase B preparation employed in these studies was completely free of arylsulfatase A and other anionic sulfatase components it was not purified to homogeneity. There was the possibility that UDP-GalNAc-4-S sulfatase and arylsulfatase B were not identical. The following, however, eliminates this possibility. Maroteaux-Lamy fibroblasts were deficient in UDP-GalNAc-4-S sulfatase activity to the same extent that they were deficient in arylsulfatase B activity. Arylsulfatase A was inactive toward the nucleotide, while metachromatic leukodystrophy fibroblasts, which are genetically deficient in arylsulfatase A, had normal UDP-GalNAc-4-S sulfatase activity. Fibroblasts derived from patients with genetic defects in other mucopolysaccharide

TABLE I

SULFOHYDROLASE ACTIVITIES OF FIBROBLAST EXTRACTS

Aliquots of the dialyzed extracts were assayed for UDP-GalNAc-4-S sulfohydrolase activity as described in the text and for 4-nitrocatechol sulfate (4-NCS-B) sulfohydrolase activity by the arylsulfatase B specific procedure of Baum et al. [14].

Cell Type	UDP-GalNAc-4-S	4-NCS-B
	nmole/h/mg protein	nmole/h/mg protein
Maroteaux-Lamy 1	2.9	190
Maroteaux-Lamy 2	3.7	90
Hurler	62	1,350
Hunter	26	1,530
Sanfilippo A	61	2,100
Morquio	115	1,260
Metachromatic Leukodystrophy	87	2,500
Normal (N = 6)	51(25-90)[a]	1,050(550-2,500)[a]

[a]Average and range.

sulfatases, i.e., Hunter, Sanfilippo A and Morquio syndromes, also had normal nucleotide sulfatase activity. Finally, heat treatment resulted in identical rates of inactivation of UDP-GalNAc-4-S and 4-nitrocatechol sulfate sulfohydrolase activities. These observations clearly establish that human arylsulfatase B, the sulfatase deficient in Maroteaux-Lamy syndrome, and the enzyme catalyzing the hydrolysis of UDP-GalNAc-4-S are one and the same, namely, N-acetylgalactosamine-4-sulfate sulfohydrolase.

Similar conclusions have been inferred by O'Brien et al. [7] who observed that Maroteaux-Lamy fibroblasts failed to cleave sulfate groups from dermatan sulfate accumulated by such fibroblasts, and by Matalon et al. [8] who

observed that Maroteaux-Lamy fibroblasts showed reduced release of sulfate from chondroitin 4-sulfate and a heptasaccharide derived from the latter. In both of these studies only the sulfate groups at or near the non-reducing terminus of the polysaccharides were susceptible to hydrolysis. Consequently, even after prolonged incubations (18 hours) only a small percentage of total sulfate was released by normal fibroblasts. The UDP-GalNAc-4-S system offers a number of advantages over the mucopolysaccharide substrates: It is readily prepared; its structure is fully characterized; all the sulfate groups are available for hydrolysis; and the assay is sensitive, simple and rapid. In addition, the nucleotide sulfatase assay can be carried out in the presence of arylsulfatase A without interference, unlike the situation with synthetic substrates. The fibroblast assay was equally applicable to leukocytes [15]. Thus, UDP-GalNAc-4-S offers the opportunity to investigate systematically the physiologically relevant parameters of the arylsulfatase B reaction, and at the same time it provides a valuable diagnostic tool for Maroteaux-Lamy syndrome.

The investigation of UDP-GalNAc-4-S as a substrate was undertaken to establish the structural specificity of arylsulfatase B in mucopolysaccharide catabolism. It is possible, however, that the UDP-GalNAc-4-S:arylsulfatase B system may be directly involved in connective tissue metabolism. The occurrence of nucleotide sugar sulfates in mammalian tissue has been known for some time. Administration of [^{35}S]sulfate to young rats resulted in a rapid incorporation of isotope into UDP-N-acetylgalactosamine sulfates in ossifiable cartilage and aorta [16]. Thus, UDP-GalNAc-4-S may prove to be more important for mucopolysaccharidoses pathology than simply being a convenient substrate for arylsulfatase B.

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