Biosynthesis and Maturation of Arylsulfatase B in Normal and Mutant Cultured Human Fibroblasts*

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The biosynthesis of arylsulfatase B in normal and mutant human skin fibroblasts was studied by metabolic labeling with radioactive amino acids, monosaccharides, or 32P, and by specific immunoprecipitation followed by polyacrylamide gel electrophoresis and fluorography. Three major polypeptides with apparent molecular weights of 47,000, 40,000, and 31,000 were found intracellularly and one of 64,000 in the medium. Pulse-chase labeling and uptake experiments showed that arylsulfatase B synthesized and secreted as a 64,000 precursor was intracellularly processed within less than 24 h via short lived intermediates to two different forms. Form I (chains of 47,000 and 11,500) was labeled earlier and was about twice as stable as form II (chains of 40,000 and 31,000). The secreted 64,000 precursor and the 40,000 chain of form II contained oligosaccharides resistant to endo-β-N-acetylglucosaminidase H. In the other chains mainly cleavable and phosphorylated oligosaccharides were found. Arylsulfatase B activity was associated with the 64,000 precursor and with form I, but not with form II. Fibroblasts of four patients with the severe form of mucopolysaccharidosis type VI, which were deficient in arylsulfatase B activity, synthesized and secreted the 64,000 precursor at a normal rate. This precursor, however, had little if any catalytic activity and one of its mature forms (I) was rapidly degraded.

Arylsulfatase B (EC 3.1.6.1) is a lysosomal hydrolase cleaving the sulfate ester bond in N-acetylgalactosamine 4-sulfate residues of dermatan sulfate (1, 2). Deficiency in arylsulfatase B causes intralysosomal accumulation of partially degraded dermatan sulfate and is observed in several genetic disorders. Profound deficiency of arylsulfatase B causes the Maroteaux-Lamy syndrome (mucopolysaccharidosis type VI) (1–3). In multiple sulfatase deficiency, arylsulfatase B is one of several sulfatases missing (4). In this disorder all known lysosomal and nonsulfosomal sulfatases, whether coded for by autosomal chromosomes or by the X chromosome, are deficient. The primary defect in this disease is unknown. Furthermore, arylsulfatase B is one of a number of lysosomal enzymes that are secreted rather than transported into lysosomes by fibroblasts from patients with 1-cell disease (mucolipidosis II) or pseudo-Hurler polydystrophy (mucolipidosis III) (5). The transport defect in these two diseases is due to the absence of mannose 6-phosphate recognition markers on lysosomal enzymes caused by the deficiency of N-acetylgalactosamine 1-phosphotransferase (6–8).

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In the present study we have examined the synthesis and maturation as well as some structural properties of arylsulfatase B in normal and mutant skin fibroblasts. Previous studies have established that a large number of lysosomal enzymes are synthesized as larger precursors and subsequently processed to mature forms by a limited proteolysis (for review see Refs. 9 and 10). We extended these findings to arylsulfatase B and report on the biosynthesis and processing of mutant arylsulfatase B in Maroteaux-Lamy fibroblasts.

MATERIALS AND METHODS

[1-35S]Methionine (specific activity 950–1200 Ci/mmol) and 32P-methylated standards were from New England Nuclear. [6-3H]-Galactose (9.3 Ci/mmol), [2-3H]mannose (22.3 Ci/mmol), and carrier-free 32P were from Amersham, p-Nitrocatechol sulfate was from Sigma, endo-β-N-acetylgalactosaminidase H from Seikagaku. Cell Culture—Human diploid fibroblasts were maintained at 37 °C in 5% CO2 in Eagle’s minimal essential medium supplemented with antibiotics, nonessential amino acids, and 7.5% fetal calf serum (Boehringer Mannheim) as described (11). Normal and mutant fibroblasts were obtained from biopsies submitted to our laboratory for diagnosis. The four Maroteaux-Lamy fibroblast lines were derived from patients affected with the severe form of the disease (12).

Labeling of Arylsulfatase B—Radioactive labeling of cells was performed as described by Hasilik and Neufeld (13, 14) with minor modifications (14–16). Cells in 25- or 75-cm2 flasks were labeled with 0.05–0.15 mCi of [35S]methionine, 0.4 mCi of [2-3H]mannose, 0.5 mCi of [6-3H]galactose, or 0.5 mCi of 32P. Arylsulfatase B was adjusted in extracts of cells and medium to 0.3 μg/ml with partially purified arylsulfatase B from human placenta. Antiserum was used at a final dilution of 1:25.

Endocytosis of Labeled Arylsulfatase B—Confluent cultures in 75-cm2 flasks were incubated for 24–30 h with 5 ml of labeling medium supplemented with 10 mM NH4Cl. The media were concentrated to 0.3 pg/ml with partially purified arylsulfatase B from human placenta. Antiserum was used at a final dilution of 1:25.

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Treatment with Endo-β-N-acetylgalactosaminidase H—Extracts of cells and medium were incubated 36 h at 37 °C with 1 unit/ml of endo-β-N-acetylgalactosaminidase H prior to immunoprecipitation as described (16).

Gel Electrophoresis and Fluorography—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) with or without dithiothreitol was performed according to Laemmli (17) and fluorography according to Bonner and Laskey (18).

Enzymatic Activity of Arylsulfatase B Precursor—Confluent cultures in 75-cm2 flasks were incubated for 72 h with 15 ml of labeling medium supplemented with 10 mM NH4Cl. The media were concentrated 50-fold by ultracentrifugation and mixed with either arylsulfatase B antiserum or preimmune serum. After incubation for 30 min at

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room temperature and 20 h at 4 °C and centrifugation at 10,000 x g for 10 min, arylsulfatase B activity was determined in the supernatant. Arylsulfatase B activity was defined as the activity precipitable with the antiserum and was calculated from the decrease in the enzyme activity in the soluble fraction.

In Vitro Phosphorylation of Arylsulfatase B—Purified arylsulfatase B (2 μg) was incubated with UDP-N-acetylgalactosaminyltransferase from rat liver Golgi membranes and [γ-32P]UDP-N-acetylgalactosamine (19). After incubation for 2 h at 37 °C arylsulfatase B was isolated by immunoprecipitation.

Enzyme Assay—In cell extracts and secretions, arylsulfatase B activity was measured with the aid of differential precipitation as described above. During the purification the activity was determined in untreated aliquots. Samples of 30 μl were incubated with 120 μl of 12.5 mM p-nitrophenyl phosphate in 0.5 M sodium acetate, pH 5.5 (20), for 30 min at 37 °C. The reaction was stopped by adding 300 μl of 0.6 N NaOH and absorbance was measured at 410 nm.

One unit is the amount of enzyme catalyzing the formation of 1 μmol of p-nitrophenol/min. Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as standard.

Purification of Arylsulfatase B—In tissue extraction, frozen placenta, 9.6 kg, was passed through a meat grinder and 1 kg portions were homogenized in 13 volumes of ice-cold 50 mM Tris/HCl, pH 7.5 (dissolved in 0.5 M NaCl) with an Ultra-Turrax. The homogenate was centrifuged for 30 min at 3000 x g, and the pellet re-extracted with 1 volume of the buffer. At this and the following stages, the protein was concentrated by precipitation with (NH₄)₂SO₄, 500 g/liter, collected by centrifugation, and equilibrated with a proper buffer by dialysis. The protein in buffer was further treated with DE52 cellulose (0.98 ml/mg of protein).

The unbound protein was concentrated and applied to CM52-cellulose in 25 mM sodium acetate at pH 5.1. The enzyme was eluted with a terminal buffer, the elution was effected with 10% methyl-α-D-mannoside.

The following "C-methylated standards were used: phospho-

Results

Synthesis and Maturation in Human Fibroblasts—Human skin fibroblasts labeled for 23 h with [35S]methionine contained three major and several minor radioactive polypeptides precipitable with antiserum against human placental arylsulfatase B (Fig. 1). The apparent molecular weights of the major polypeptides were 47K, 40K, and 31K, and those of the minor polypeptides 64K, 62 K, and 53K (Fig. 1A). Of the minor polypeptides only the 64K and 62K were regularly observed. In the medium, a single polypeptide with an apparent molecular weight of 64K was precipitable. In the presence of 10 mM NH₄Cl, which is known to induce the secretion of newly synthesized lysosomal enzymes (13, 22, 23), 70−75% of total labeled arylsulfatase B was secreted. Further, the 64K polypeptide was the most prominent among the intracellular forms. In fibroblasts from a patient with I-cell disease, more than 70% of the newly synthesized arylsulfatase B was secreted.

Table I

| Purification step | Protein (mg) | Activity (units/mg) | Specific activity (units/mg) | Purification fold |
|-------------------|-------------|---------------------|-----------------------------|------------------|
| Homogenate (3,000 x g supernatant) | 462,900 | 1,652 |
| 70% (NH₄)₂SO₄ | 207,000 | 929 |
| DE-52 cellulose I (pH 7.5) | 96,000 | 380 | 0.004 |
| CM-52 cellulose I | 4,910 | 162 | 0.033 |
| CM-52 cellulose II | 1,440 | 243 | 0.169 |
| Concanavalin A-Sepharose 4B | 45 | 85 | 1.89 |
| Sephadex G-100 | 4.4 | 68 | 15.5 |
| Sephadex G-75 | 3.3 | 24.9 | 12.1 |
| DE-52 cellulose II (pH 8.3) | 0.5 | 24.8 | 46.2 |

Arylsulfatase B polypeptides in human skin fibroblasts and human placenta. Fibroblasts of controls and patients affected with I-cell disease were incubated for 23 h in the presence of [35S]methionine and, where indicated, of 10 mM NH₄Cl. Arylsulfatase B (ASB) was immunoprecipitated from cells and media. The immunoprecipitates were solubilized under reducing (A) or nonreducing (B) conditions. After polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, labeled polypeptides were visualized by fluorography. Arylsulfatase B purified from human placenta and used for immunization was subjected to gel electrophoresis directly after in vitro phosphorylation (see "Materials and Methods") and visualized by staining with Coomassie blue or by fluorography. The positions of arylsulfatase B polypeptides obtained under reducing conditions (A) and nonreducing conditions (B) are indicated by arrows. The following "C-methylated standards were used: phosphorylase B, 92.5K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; and cytochrome c, 12.3K.

Fig. 1. Arylsulfatase B polypeptides in human skin fibroblasts and human placenta. Fibroblasts of controls and patients affected with I-cell disease were incubated for 23 h in the presence of [35S]methionine and, where indicated, of 10 mM NH₄Cl. Arylsulfatase B (ASB) was immunoprecipitated from cells and media. The immunoprecipitates were solubilized under reducing (A) or nonreducing (B) conditions. After polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, labeled polypeptides were visualized by fluorography. Arylsulfatase B purified from human placenta and used for immunization was subjected to gel electrophoresis directly after in vitro phosphorylation (see "Materials and Methods") and visualized by staining with Coomassie blue or by fluorography. The positions of arylsulfatase B polypeptides obtained under reducing conditions (A) and nonreducing conditions (B) are indicated by arrows. The following "C-methylated standards were used: phosphorylase B, 92.5K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; and cytochrome c, 12.3K.
gel stained with Coomassie blue. The smaller polypeptides in placental arylsulfatase B were linked via disulfide bonds to the 47K polypeptide. Under nonreducing conditions they appeared as a single molecule with an apparent Mₐ of 57K (Fig. 1B). Under nonreducing conditions, the arylsulfatase B from normal fibroblasts migrated as two incompletely separated polypeptides of apparent Mₐ = 58K and 56.5K and the arylsulfatase B secreted into the medium as a rather diffuse 60K polypeptide (Fig. 1B).

To establish the relationship between the polypeptides precipitable with antiserum against arylsulfatase B, fibroblasts were labeled for up to 3 h and then subjected to a chase of up to 24 h (Fig. 2). During the first 2 h only the 64K polypeptide was detectable intracellularly. After a 3-h pulse, in addition to the 64K polypeptide the 47K polypeptide became visible. During a following chase for 3 h, the 40K and 31K polypeptides and a processing intermediate of 62K appeared. The polypeptide (Fig. 1B).

During a following chase for 3 h, the 40K and 31K polypeptides were visible. After incubation for 6 and 24 h and a chase for up to 24 h the 47K, 40K and 31K polypeptides were visible. After incubation for 3 and 7 days in cultivation medium, 45 and 15% of the endocytosed 47K polypeptides were recovered.

Oligosaccharides in Arylsulfatase B—The 64K precursors and the mature intracellular polypeptides of arylsulfatase B were all labeled with [2-¹⁴C]mannose, [6-¹⁴C]galactose, and [³²P]Pi, for 16-24 h. Extracts of cells and media were prepared, split into equal parts, and incubated with or without 1 unit/ml of endo-β-N-acetylgalcosaminidase H. The 11.5K polypeptide seen after labeling with [¹⁴C]mannose or [³²P]Pi is indicated by an asterisk. The additional polypeptide (smaller by 2K than the 31K polypeptide and its 29K product) seen in cell extracts labeled with [³⁵S]methionine is supposed to result from limited proteolysis. The immunoprecipitate obtained from the [³²P]-labeled secretions contains a high molecular weight contamination. For standards see Fig. 1.

and the mature intracellular polypeptides of arylsulfatase B were all labeled with [2-¹⁴C]mannose, [6-¹⁴C]galactose, and [³²P]Pi, for 16-24 h. Extracts of cells and media were prepared, split into equal parts, and incubated with or without 1 unit/ml of endo-β-N-acetylgalcosaminidase H. This endoglycosidase is specific for oligosaccharides of the high mannos and hybrid type. It affected the size and the radioactivity in the polypeptides labeled with [³⁵S]methionine as follows. The secreted 64K precursor was largely converted into a 62K form presumably due to the loss of one sensitive oligosaccharide. The 62K product was still labeled with [¹⁴C]mannose indicating the presence of resistant (complex) oligosaccharides. The presence of complex oligosaccharides in the secreted 64K precursor was further indicated by its labeling with [¹⁴C]galactose. The 47K polypeptide was converted into two products smaller in size by 4K and 6K suggesting the loss of two or three sensitive oligosaccharides. Part of the 40K polypeptide was converted into a 38K product. Both the 40K forms resistant to the endoglycosidase and the 38K product were labeled with [¹⁴C]mannose suggesting that the 40K polypeptide carries at least two oligosaccharides, of which only one may be cleavable. The 31K form is converted into two products smaller in size by 2K and 3K (in the experiment shown in Fig. 4, the products are incompletely separated). The assignment of the products to the parent polypeptides was facilitated by similar experiments with Maroteaux-Lamy fibroblasts which are deficient in the 47K polypeptide (see below).

After labeling with [¹⁴C]mannose and [³²P]Pi, a polypeptide with an apparent molecular weight of 11.5K was immunoprecipitable which was not detected in immunoprecipitates of cells labeled with [³⁵S]methionine or [¹⁴C]leucine (not shown). We assume that this fragment is related to the smaller phosphorylatable fragments in placental arylsulfatase B (see Fig. 1, A and B).

Synthesis and Maturation of Arylsulfatase B in Maroteaux-Lamy Fibroblasts—In fibroblasts of four patients with the

FIG. 2 (left). Arylsulfatase B in pulse-chase labeled fibroblasts. Normal fibroblasts were labeled with [³⁵S]methionine for 3 h and subjected to a chase as indicated. Arylsulfatase B was immunoprecipitated from extracts of cells. The immunoprecipitates were solubilized under reducing conditions and subjected to gel electrophoresis and fluorography. The positions of arylsulfatase B polypeptides (arrows) and standards (see Fig. 1) are indicated.

FIG. 3 (right). Endocytosis of arylsulfatase B. Secretions were prepared from fibroblasts labeled with [³⁵S]methionine in the presence of 10 mM NH₄Cl. The secretions containing labeled arylsulfatase B precursor were added to confluent cultures of fibroblasts. After incubation for 6 and 24 h and a chase for up to 7 days, cells were harvested and arylsulfatase B immunoprecipitated from the cell extracts. The medium released after incubation for 24 h with the cells contained only the precursor and the cells contained only processed forms of arylsulfatase B. For standards see Fig. 1.
severe form of Maroteaux-Lamy syndrome incubated for 36 h with [35S]methionine. Arylsulfatase B was immunoprecipitated from extracts of cells (C) and medium (M). The immunoprecipitates were solubilized under reducing (A) or nonreducing (B) conditions and subjected to gel electrophoresis and fluorography. The positions of arylsulfatase B polypeptides are indicated by arrows. For standards see Fig. 1.

![Synthesis of arylsulfatase B](image)

**Fig. 5. Synthesis of arylsulfatase B in Maroteaux-Lamy fibroblasts.** Fibroblasts of four unrelated patients affected with the severe form of Maroteaux-Lamy syndrome were labeled for 36 h with [35S]methionine. Arylsulfatase B was immunoprecipitated from extracts of cells (C) and medium (M). The immunoprecipitates were solubilized under reducing (A) or nonreducing (B) conditions and subjected to gel electrophoresis and fluorography. The positions of arylsulfatase B polypeptides are indicated by arrows. For standards see Fig. 1.

**Fig. 6 (left).** Endocytosis of normal and mutant arylsulfatase B. Secretions were prepared from normal and Maroteaux-Lamy fibroblasts (cell line A) labeled with [35S]methionine in the presence of 10 mM NH4Cl. The secretions were added to confluent cultures of Maroteaux-Lamy fibroblasts (cell line G). After incubation for 24 h and a chase for 3 days, cells were harvested and arylsulfatase B (polypeptides indicated by arrows) immunoprecipitated.

**Fig. 7 (right).** Arylsulfatase B in pulse-chase labeled Maroteaux-Lamy fibroblasts. Maroteaux-Lamy fibroblasts (cell line G) were labeled for 0 h with [35S]methionine and subjected to a chase of up to 24 h. The positions of arylsulfatase B polypeptides (arrows) and standards (see Fig. 1) are indicated.

Thiol proteinases participate in the processing of lysosomal enzymes (24, 25) and may be responsible for the degradation of the mutant 47K polypeptide. Therefore, uptake experiments were performed in the presence of leupeptin, an inhibitor of cathepsin B-like thiol proteinases. Leupeptin caused the accumulation of the 64K precursor and several polypeptides not seen in untreated cells. The amount of 47K polypeptide and its 50K processing intermediate formed from mutant arylsulfatase B was less than 10% of normal (not shown). Thus, leupeptin has little protective effect on the mutant 47K polypeptide.

The catalytic activity of normal and mutant arylsulfatase B precursor secreted in the presence of 10 mM NH4Cl was...
assayed using p-nitrocatechol sulfate as substrate. Secreted arylsulfatase B activity was defined as the activity precipitable with antiserum against arylsulfatase B. Controls secreted 0.56 milliunit of arylsulfatase B/mg of cell protein daily. The arylsulfatase B activity in four Maroteaux-Lamy cell lines was below the limit of detection (0.02 milliunit/mg).

**DISCUSSION**

In human fibroblasts, arylsulfatase B is synthesized as a larger precursor. At least one-third of the precursor is secreted into the medium. The secreted precursor is amenable to uptake in fibroblasts. Within a few hours of synthesis or endocytosis, the precursor matures to smaller polypeptides. Without reduction the precursor and the products behave as molecules of 60K and 56.5-58K, respectively. After reduction, the precursor has an apparent molecular weight of 64K and the mature products fragment to polypeptides of 47K, 40K, 31K, and 11.5K. The shortest polypeptide can conveniently be labeled with 32P or [3H]mannose, but not with [3H]leucine or [35S]methionine. We suggest that in fibroblasts, two different products are formed from the 64K precursor, one product (form I) containing the 47K and 11.5K polypeptides and the other (form II) containing the 40K and 31K polypeptides. The assignment of the polypeptides to the two processing products is based on the following observations. The mutation in Maroteaux-Lamy fibroblasts, which are deficient in arylsulfatase B activity, causes a nearly complete absence of form I, whereas it elevates the amount of form II by about 50%. Arylsulfatase B purified from human placenta resembles form I. It contains three polypeptides of 47K, 14.5K, and 11.5K. The two smaller polypeptides can be detected only after subjecting the enzyme to enzymic phosphorylation and to reduction of disulfide bridges. Probably the 47K polypeptide is linked to either of the two smaller polypeptides in a ratio of 1:1. In fibroblasts, form I can be detected 2-3 h after synthesis of the precursor. The production of form II starts 3-6 h after synthesis of the precursor. In endocytosis experiments an even greater temporal displacement in the appearance of two forms was observed (Fig. 3). The delayed production of form II might suggest form I to be an intermediate in the processing of the precursor to form II. Such relationship would require cleavage of the 47K polypeptide of form I into the 40K and 31K polypeptides of form II. This relation is unlikely because the 40K polypeptide contains largely noncleavable [3H]mannose-labeled oligosaccharides, whereas in the 47K polypeptide all are cleavable. We propose, therefore, that form I and form II are products of alternative processing of the precursors. This finding indicates that the 64K precursors while sharing antigenic determinants with arylsulfatase B are heterogeneous at least in respect to their carbohydrate side chains. It should be of interest to find out whether the carbohydrate or another signal is responsible for the specific processing of the precursor to either form I or II products.

The enzymic activity of arylsulfatase B is apparently associated with form I. This is based on the following observations. The polypeptides found in form I are similar to those in the purified placental enzyme. Cells deficient in arylsulfatase B activity contain little or no form I polypeptides but normal amounts of form II. This was observed in all four cell lines from Maroteaux-Lamy patients examined in this study as well as in several multiple sulfatidosis fibroblasts. Whereas the precursor made in Maroteaux-Lamy fibroblasts is deficient in catalytic activity, multiple sulfatase-deficient fibroblasts synthesize catalytically active precursor. As in Maroteaux-Lamy fibroblasts, the form I polypeptides are rapidly degraded in multiple sulfatase-deficient fibroblasts, whereas normal amounts of form II polypeptides accumulate.2 The precursors secreted in control fibroblasts are endowed with arylsulfatase B activity. It is not known, however, whether the activity is associated with all precursor molecules. If so, processing of precursors to form II of arylsulfatase B would be associated with loss of catalytic activity.

The mutation in the severe form of Maroteaux-Lamy syndrome has pleiotropic effects on arylsulfatase B. The mutant enzyme is inactive and the mutant form I is unstable. Our results are in agreement with an earlier report on the presence of immunologically cross-reactive material in Maroteaux-Lamy fibroblasts (26).

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