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MINOR ANIONIC ARYLSULFATASES IN CULTURED HUMAN FIBROBLASTS

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SUMMARY

A group of minor anionic arylsulfatases (EC 3.1.6.1) was found in extracts of cultured human fibroblasts. These arylsulfatases, hitherto undescribed in fibroblasts, were separated from arylsulfatase A and arylsulfatase B by DEAE-cellulose chromatography. The optimal condition for separation was 25 mM Tris–HCl buffer (pH 7.5) with a NaCl gradient. Arylsulfatase B was not retained by the ion-exchanger; the minor arylsulfatases were eluted as a single peak at about 0.15 M NaCl; and arylsulfatase A was eluted at about 0.3 M NaCl. The minor arylsulfatases were further separated into several bands of activity on polyacrylamide-gel electrophoresis with mobilities intermediate between the A and B forms of the enzyme. The minor arylsulfatase fraction was similar to arylsulfatase B, not arylsulfatase A, in its response to the differential 4-nitrocatechol sulfate assays and in sensitivity to Ag^+. This enzyme fraction failed to hydrolyze cerebroside sulfate, the physiological substrate for arylsulfatase A, and it was present in normal levels in fibroblast extracts derived from a patient with metachromatic leukodystrophy (deficiency of arylsulfatase A). The minor anionic arylsulfatases appear to be totally unrelated to arylsulfatase A. They are kinetically indistinguishable from arylsulfatase B but differ from the latter by their overall net charge.

INTRODUCTION

Cultured human fibroblasts contain soluble arylsulfatases A and B [1] which can be distinguished in crude extracts by the differential 4-nitrocatechol sulfate assays [2]. The accurate assessment of their activities is, however, difficult in fibroblasts of arylsulfatase deficiency disorders where there can be low levels of either of these enzymes. While examining conditions for separating arylsulfatases A and B by ion-exchange chromatography for reliable determination of low enzyme activity levels, a new group of arylsulfatases, hitherto undescribed in fibroblasts, was fractionated from the A and B enzymes. This new group of arylsulfatases was distinct from what are normally designated as arylsulfatases A and B by their elution pattern from DEAE-cellulose and by their behavior on polyacrylamide-gel electrophoresis.

MATERIALS AND METHODS

Materials

4-Nitrocatechol sulfate and *p*-nitrophenyl sulfate were purchased as the potassium salts from Sigma Chemical Co. 4-Methylumbelliferyl sulfate was synthesized by the procedure of Sherman and Stanfield [3] and purified according to Rinderknecht et al. [4]. Whatman DEAE-cellulose (microgranular DE-32) was purchased from Reeve Angel. Polyacrylamide electrophoresis reagents were obtained from Calbiochem.

Enzyme assays

Arylsulfatase A and arylsulfatase B activities were determined by hydrolysis of 4-nitrocatechol sulfate with the A and B specific assays of Baum et al. [2]. Fractions from the DEAE-cellulose chromatography were analyzed by hydrolysis of 4-methylumbelliferyl sulfate by a procedure similar to that of Harinath and Robbins [5]. Aliquots of fractions (50 μl) were added to 100 μl of substrate solution (10 mM 4-methylumbelliferyl sulfate, 100 mM sodium acetate (pH 5.5), 100 mM NaCl, and 0.2 mg/ml bovine serum albumin). The mixtures were incubated at 37 °C for 1 h and the reactions terminated by addition of 1 ml of 0.4 M glycine–NaOH buffer (pH 10.5). The fluorescence was determined in an Aminco–Bowman spectrophotofluorometer at an excitation wavelength of 365 nm and emission wavelength of 450 nm. The variable NaCl and Tris–HCl buffer concentration in fractions from the DEAE-cellulose chromatography did not affect enzyme activities.

Hydrolysis of cerebroside [^{35}S]sulfate was determined by release of [^{35}S]sulfate [6]. Determinations of *p*-nitrophenyl sulfate hydrolysis were carried out at pH 5.5 and 7.5 according to Dodgson et al. [7].

Fibroblast cell extracts

Human fibroblast cultures were initiated from skin biopsies and maintained as previously described [1]. The cells were cultured on Medium 199 with CO_2–bicarbonate buffering, but identical results were obtained when they were cultured on minimum essential medium with HEPES buffer. They were harvested by trypsinization and extracted by repeated cycles of freezing and thawing in the presence of one volume of 25 mM Tris–HCl buffer (pH 7.5). The extracts were then dialyzed overnight against Tris–HCl buffer. The concentration of the buffer was adjusted to that at which the DEAE-cellulose chromatography was to be performed (cf. below).

DEAE-cellulose chromatography

DEAE-cellulose was allowed to swell overnight in 10 mM Tris–HCl (pH 7.5). The chromatographies were performed with 1, 10, 25, 50 and 100 mM Tris–HCl buffer, so the cellulose was washed several times with buffer of the appropriate concentration, deaerated, and poured into 8 mm $\times$ 17 cm columns. The columns at room temperature were charged with dialyzed fibroblast extracts and about 7 ml of buffer pumped through before the gradient was applied. A linear salt gradient (26 ml) between 0 and 0.6 M NaCl in Tris–HCl buffer was pumped to each column from a common mixing chamber at 12 ml/h and 1.2-ml fractions were collected from the

time the columns were charged. Enzyme-containing fractions were concentrated by ultrafiltration (Amicon Minicon B cell with a 15 000 molecular weight cutoff).

Gel electrophoresis

Discontinuous polyacrylamide-gel electrophoresis was performed with 7.5% acrylamide gels in the buffer system of Orr et al. [8]. A Canalco Model 12 unit with air cooling was employed. The cathode buffer was pH 6.3, the resolving gel pH 6.8, and the anode buffer pH 5.8. The electrophoresis was carried out at 4 °C for 2.5 h at 3 mA/tube with a constant rate power supply. Enzyme bands were located by placing gels in 4-methylumbelliferyl sulfate (10 mM in 500 mM sodium acetate (pH 5.2)) at 37 °C and photographing the fluorescent product under a long wavelength ultraviolet lamp, usually after a 30-min reaction period. When there was limited or low activity material, the gels were immersed in 1 M NaOH to intensify the enzyme bands, but this resulted in rapid diffusion of the product.

RESULTS

DEAE-cellulose chromatography

DEAE-cellulose chromatography of fibroblast extracts in 25 mM Tris–HCl buffer (pH 7.5), with a NaCl gradient yielded three fractions containing arylsulfatase activity (Fig. 1). They were designated: I, unadsorbed fraction, the material in the void volume; II, low-salt fraction, the material eluted around 0.15 M NaCl; and III, high-salt fraction, the material eluted around 0.3 M NaCl.

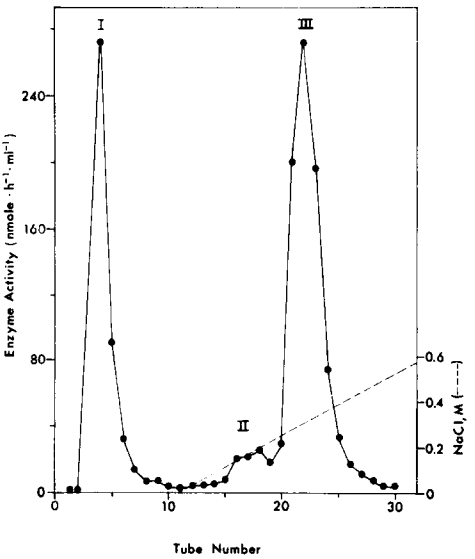

Fig. 1. The fractionation pattern of fibroblast arylsulfatases on DEAE-cellulose with 25 mM Tris-HCl buffer (pH 7.5). The column was charged with 7.8 mg protein of fibroblast extract. Enzyme activity was determined on an aliquot of each tube by hydrolysis of 4-methylumbelliferyl sulfate (see Materials and Methods). The dashed line indicates the calculated concentration of NaCl in the eluate. The activity peaks are designated: I, unadsorbed; II, low-salt; and III, high-salt fractions.

It was noted that when fibroblast extracts were dialyzed against buffer of low ionic strength, the proportion of sulfatases in the low-salt fraction was increased. This prompted the examination of the chromatography with Tris buffer from 1 to 100 mM (Fig. 2). At 1 mM Tris–HCl buffer the amount of arylsulfatase in the

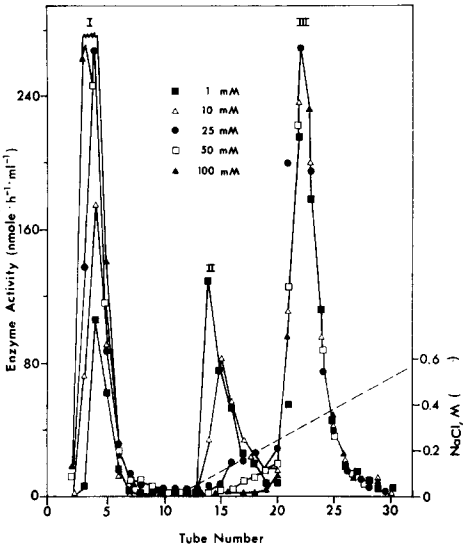

Fig. 2. The fractionation pattern of fibroblast arylsulfatases on DEAE-cellulose with Tris–HCl buffer (pH 7.5), at different concentrations. Each column was charged with 7.8 mg protein of fibroblast extract and chromatographed with buffer at the indicated concentration. Enzyme activity and the concentration of the NaCl gradient were determined as in Fig. 1. The activity peaks are designated: I, unadsorbed; II, low-salt; and III, high-salt fractions.

unadsorbed fraction was greatly reduced and was surpassed by that in the low-salt fraction. As the buffer concentration was increased, the enzyme activity in the unadsorbed fraction increased while the amount in the low-salt fraction decreased and at 100 mM Tris–HCl buffer there was no discernable activity in the low-salt fraction. In addition to the decrease in the amount in the low-salt fraction, there was a shift of the enzyme peak to higher NaCl concentrations with increasing ionic strength of the buffer. In contrast, the enzyme in the high-salt fraction was unaffected, either in quantity or position, by buffer concentration.

Characterization of chromatographic fractions

The three fractions derived from the 25 mM Tris chromatography were examined by polyacrylamide-gel electrophoresis (Fig. 3A). The bulk of the unadsorbed fraction (I) failed to penetrate the gel; the high-salt fraction (III) migrated with an R_F of about 0.4; and the low-salt fraction (II) yielded two or three diffuse activity bands with lower mobilities than the component in the high-salt fraction.

Electrophoretic examination of the unadsorbed fractions from the 1–100 mM Tris chromatography series showed quite clearly that at 1 mM there was only material that did not penetrate the gel (Fig. 3B). At 25 mM Tris–HCl buffer only a small portion of the intermediate migrating arylsulfatases was evident in the unadsorbed

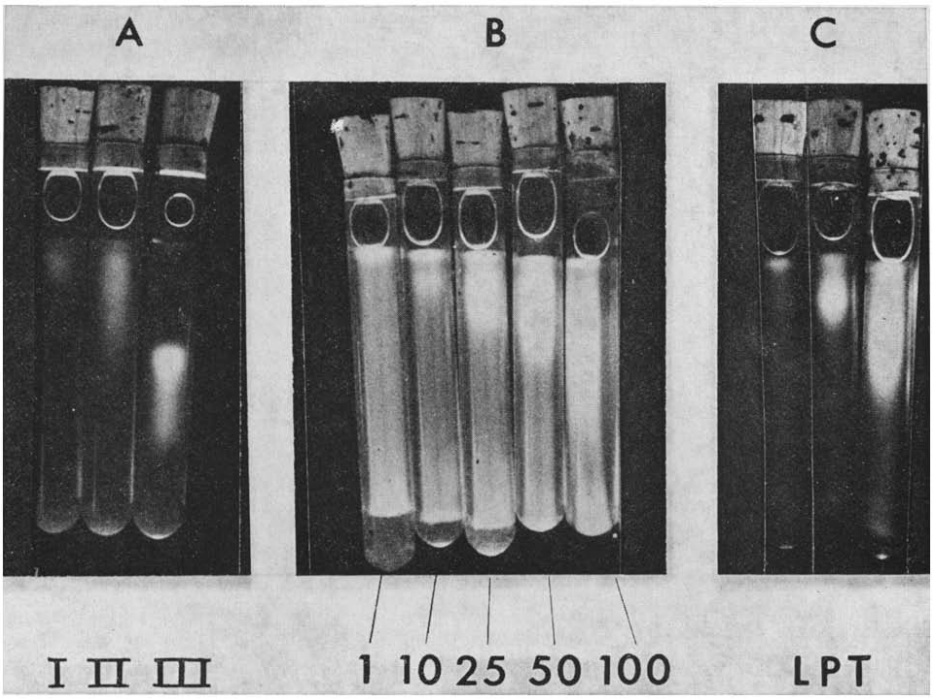

Fig. 3. Polyacrylamide-gel electrophoretograms of arylsulfatase fractions derived from DEAE-cellulose chromatography. (A) Fractions from 25 mM Tris–HCl buffer (pH 7.5) chromatography: I, unadsorbed fraction; II, low-salt fraction; III, high-salt fraction. (B) Unadsorbed fractions from chromatography at the indicated (mM) Tris–HCl buffer concentrations. (C) Low-salt fraction from the 10 mM Tris–HCl buffer chromatography: L, leading shoulder (Tube 14); P, peak (Tube 16); T, tailing shoulder (Tube 18). See Materials and Methods for details of electrophoresis and enzyme staining procedure. Gels from A were photographed after a 30-min reaction period with 4-methylumbelliferyl sulfate. Gels from B and C were immersed in 1 M NaOH after reaction with substrate and photographed within 1 min.

fraction. When the buffer concentration was increased to 100 mM the unadsorbed fraction contained all of the intermediate migrating enzymes. A similar examination of subfractions of the low-salt fraction of the 10 mM Tris chromatography indicated that the leading shoulder (Gel L, Fig. 3C) was mainly material which did not penetrate the gel; the tailing shoulder (Gel T, Fig. 3C) was identical with the low-salt fraction of the 25 mM Tris chromatography (cf. Gel II, Fig. 3A); and the peak (Gel P, Fig. 3C) contained only the slower migrating components of Gel T, Fig. 3C.

The enzymatic properties of the three fractions from the 25 mM Tris chromatography were compared. The K_m (12 mM) and pH optima (5.5) were similar for enzyme from all three fractions toward 4-methylumbelliferyl sulfate. In the presence of 0.2 mM Ag^+, the activities of the unadsorbed and low-salt fractions were slightly stimulated, while that of the high-salt fraction was strongly inhibited. Enzyme from all three fractions showed complete inhibition by 2.5 mM phosphate and 50% inhibition by 10 mM KCN. Both the unadsorbed and low-salt fractions showed activity in the arylsulfatase B specific assay, but not in the arylsulfatase A specific assay nor in the cerebroside sulfate sulfohydrolase assay. The high-salt fraction responded in

the arylsulfatase A specific assay and the cerebroside sulfate sulfohydrolase assay. With *p*-nitrophenyl sulfate as the substrate, enzyme from all three fractions showed greater activity at pH 5.5 than at 7.5.

The results of chromatography of extracts from metachromatic leukodystrophy fibroblasts, which are deficient in arylsulfatase A [1], are shown in Fig. 4 to illustrate

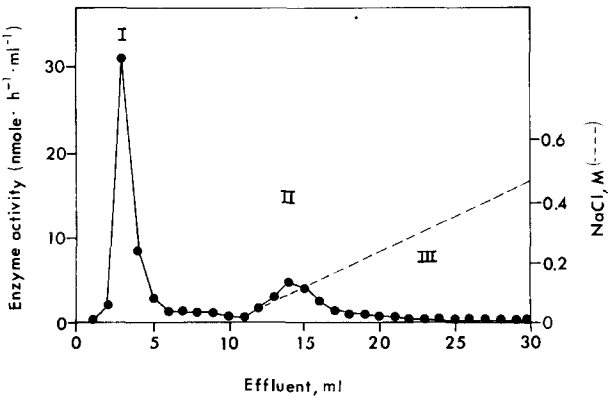

Fig. 4. The fractionation pattern of arylsulfatases from an adult metachromatic leukodystrophy fibroblast extract on DEAE-cellulose with 25 mM Tris–HCl buffer (pH 7.5). The column was charged with 2.0 mg protein of fibroblast extract. Enzyme activity and the concentration of the NaCl gradient were determined as in Fig. 1. The enzyme peaks are designated: I, unadsorbed; II, low salt fraction; and III, the position normally occupied by the high-salt fraction.

the unequivocal presence of the low-salt fraction and as additional evidence that it is generically unrelated to arylsulfatase A (cf. Discussion).

DISCUSSION

The soluble arylsulfatases of fibroblast extracts have been separated into three types by DEAE-cellulose chromatography. One is cationic at neutral pH and constitutes 40–50% of the total 4-methylumbelliferyl sulfate sulfatase activity of the extracts. This is the enzyme normally called arylsulfatase B. In spite of its cationic nature, this enzyme was partially retarded by DEAE-cellulose at very low ionic strengths. Since charge binding is unlikely, a non-specific adsorption to the cellulose matrix or gross filtration of aggregated enzyme, which arylsulfatase B forms at low ionic strength [9], may occur.

The second type of arylsulfatase, designated high-salt fraction, is arylsulfatase A. This was the only fraction which showed cerebroside sulfate sulfohydrolase activity and also comprised 40–50% of the total arylsulfatase activity of fibroblast extracts. Arylsulfatase A, being highly anionic, was bound strongly to DEAE-cellulose and was eluted only by a relatively high concentration of NaCl.

The third type of arylsulfatase comprised 5–10% of the total enzyme. This material was also anionic at neutral pH and was adsorbed by DEAE-cellulose in 25 mM Tris–HCl buffer, but was eluted at a lower NaCl concentration than arylsulfatase A. The material in this fraction was polydisperse on gel electrophoresis and

appeared to contain several arylsulfatases with mobilities lower than arylsulfatase A. Provisionally we will refer to this group of enzymes as "minor anionic arylsulfatases".

It is quite clear that the minor anionic arylsulfatases are neither identical with nor related to arylsulfatase A by the following criteria. They showed no activity in the arylsulfatase A specific assay with 4-nitrocatechol sulfate or in the cerebroside sulfate sulfohydrolase assay; they were stimulated by Ag^+, whereas the A enzyme was inhibited by this metal ion; and they were present in normal amounts in metachromatic leukodystrophy fibroblasts. The possibility that the minor arylsulfatases may be derived from adventitious solubilization of arylsulfatase C has been precluded by their greater activity toward *p*-nitrophenyl sulfate at pH 5.5 than at pH 7.5, by their complete inhibition with 2.5 mM phosphate, and by only partial inhibition with 10 mM KCN. The minor anionic arylsulfatases were enzymatically indistinguishable from arylsulfatase B, but because the physiological function of arylsulfatase B is undetermined, comparisons were made only with synthetic substrates. The only difference between the minor anionic arylsulfatases and arylsulfatase B was in the net charge. In this respect they appear to be related to the minor B-like enzymes reported by other investigators, i.e., arylsulfatases a, b and c [10], B_1, B_2 and B_3 [11], B_α and B_β [9], α, β and γ [12], B_1 and B_2 [13], and $B_{1\alpha}$, $B_{1\beta}$, B_x, $B_{2\alpha}$, $B_{2\beta}$, $B_{2\gamma}$ and B_3 [14]. However tempting it may be to speculate whether the minor enzymes are isomeric or polymeric forms of arylsulfatase B, definitive characterizations cannot be established without physiological substrates. Thus, it may be prudent to retain a noncommittal designation such as "minor anionic arylsulfatases" for this group of enzymes rather than to associate them arbitrarily to arylsulfatase B on the basis of activity toward highly nonspecific synthetic substrates.

The term minor, as applied to the anionic arylsulfatases in this report, is not to be interpreted as meaning insignificant. It is used descriptively to distinguish this enzyme group from arylsulfatase A, the major anionic arylsulfatase fraction. The minor anionic arylsulfatases do form a real and significant portion of the total arylsulfatases present in extracts of human fibroblast cultures. This enzyme fraction may contain activity levels that are one-fifth as great as those of either arylsulfatase A or B. Minor anionic arylsulfatase levels become quite relevant when it is desirable to measure extracts of fibroblasts derived from patients with metachromatic leukodystrophy where levels of arylsulfatase A are less than 5% of normals [1]. In the analysis of arylsulfatase deficiency disorders care must be taken to avoid grouping this enzyme fraction with those containing the A and B enzymes. Most ion exchange procedures easily separate arylsulfatases A and B, however, gross separation techniques cannot be applied to the fractionation of minor arylsulfatases from arylsulfatases A and B. Our present results demonstrate that these three groups of arylsulfatases can be well separated by using the stated conditions for DEAE-cellulose chromatography.

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