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A SECOND FORM OF ARYLSULFATASE A IN HUMAN URINE

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SUMMARY

Two forms of arylsulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1) have been isolated from human urine by $(NH_4)_2SO_4$ reverse gradient solubilization chromatography. The normal or predominant form is here designated arylsulfatase A_α and the new or second form designated arylsulfatase A_β. Both had similar activity toward the substrates 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and cerebroside sulfate. Rabbit anti-human arylsulfatase A precipitated both enzyme forms equally well, and each had the same migration behavior with polyacrylamide gel electrophoresis or gel isoelectric focusing. The rate of heat inactivation at two different temperatures did not reveal any differences between arylsulfatase A_α and A_β. Treatment with neuraminidase did not affect electrophoretic or catalytic properties of either enzyme. The A_β enzyme is apparently derived from the A_α form *in vitro* and there is partial reversion of isolated β-enzyme to the α-enzyme on storage. Neither ascorbic acid, dithiothreitol, nor urea appeared to enhance the interconversion between arylsulfatase A_α and A_β.

INTRODUCTION

There is considerable interest in the lysosomal enzyme, arylsulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1), because it is deficient in the neurological disorder metachromatic leukodystrophy[1]. This enzyme has been isolated from human brain[2], liver[3], placenta[4], kidney[5], and urine[6]. We have undertaken its purification from human urine for *in vitro* enzyme replacement studies.

While purifying arylsulfatase A from pooled human urine, a minor component with enzyme activity was observed on preparative isoelectric focusing. The possibility of genetic polymorphism prompted the examination of unpooled urine. In a fortuitous application of $(NH_4)_2SO_4$ reverse gradient solubilization chromatography[7] as a preparative step, resolution of a second form of arylsulfatase A was achieved. The second form of arylsulfatase A is here designated arylsulfatase A_β and the commonly

encountered form is designated arylsulfatase A_α. Procedures for the separation of the α- and β-forms of the enzyme and some of their properties are presented.

METHODS

The procedure described for the recovery of arylsulfatase A has been applied to 11-l batches of freshly collected pooled urine or to urine collected over a period of time from a single individual and stored frozen. The latter was thawed at room temperature, and in both cases $(NH_4)_2SO_4$ was added with stirring to 2.8 M (approximately 68% saturation). All subsequent procedures were carried out at 4 °C. After standing overnight, the bulk of the supernatant fluid was removed by aspiration, and the remaining 2 l or so were centrifuged at 500 × *g* for 10 min. To remove lipid[6] the sedimented material was slurried with 4 vol. of acetone, filtered, and washed on the filter with 2 vol. of acetone–diethyl ether (1:1, v/v) and 2 vol. diethyl ether. This acetone powder was extracted 3 times with 20 mM Tris–HCl (pH 7.5) at a ratio of 3–4 ml buffer to 1 g powder. The bulk of arylsulfatase A activity usually appeared in the second extract. The enzyme-containing extracts were diluted to 200 ml with buffer (protein about 1 mg/ml), slurried with 18 g analytical grade Celite (Johns-Manville) and adjusted to 3.28 M $(NH_4)_2SO_4$ (112 g) with stirring. After 1 h the mixture was poured into a 2.5-cm diameter column giving a packed height of about 10 cm. The column was eluted with a linear gradient (decreasing from 80 to 0% saturation in $(NH_4)_2SO_4$, see ref. 7) formed with 285 ml 20 mM Tris–HCl buffer (pH 7.5) containing 3.28 M $(NH_4)_2SO_4$ in the mixing chamber and 315 ml of the buffer alone in the reservoir. The flow rate was 36 ml/h. Fractions of 8 ml were collected, and enzyme activity was assessed on 10-μl aliquots with the 4-nitrocatechol sulfate sulfatase assay of Baum *et al.*[8]. An arylsulfatase A enzyme unit is defined as one μmole 4-nitrocatechol sulfate hydrolyzed per h at 37 °C.

Effective resolution by the reverse gradient procedure was also obtained on a smaller scale. Reproducible gradients were achieved by developing the gradient on a large scale, pumping from the mixing chamber through a multi-channel pump, and utilizing only as many lines as required. The procedure as described was effective for as little as 2 units of enzyme in solutions containing 0.2–1.5 mg protein per ml. 1 g of Celite and 2.86 g $(NH_4)_2SO_4$ were added with stirring to 5 ml of enzyme solution. After 1 h the mixture was poured into a 0.8-cm diameter column giving a packed height of about 6 cm. The eluting gradient was formed between 180 ml of buffer and 150 ml of $(NH_4)_2SO_4$-containing buffer. The gradient solution was drawn from the mixing chamber at a rate of 105 ml/h through a multi-channel pump. Each column was connected to a line delivering the gradient solution at 9 ml/h. The gradient solution from unused lines was discarded. Column effluent was collected in 1-ml fractions and enzyme activity was estimated on 25-μl aliquots. Enzyme activities are underestimated because of the presence of SO_4^{2-}, but this factor was usually ignored when making comparative evaluations. Under the assay conditions 1.4 M $(NH_4)_2SO_4$, typical of the arylsulfatase A_α peak, resulted in 62% inhibition, while 2.18 M $(NH_4)_2SO_4$, typical of the arylsulfatase A_β peak, resulted in 75% inhibition. When such a decrease in detection sensitivity could not be tolerated because of limited enzyme, fractions were dialyzed before analysis.

RESULTS AND DISCUSSION

We had established previously with pooled urine that on reverse gradient chromatography the bulk of arylsulfatase A is solubilized and eluted between 1.20 and 1.60 M $(NH_4)_2SO_4$ (peak at 1.4 M). When this procedure was applied to urine from individuals, the arylsulfatase A from one subject (S.D.) was unexpectedly resolved into two discrete fractions of approximately equal activity (Fig. 1). The new second form of the enzyme, arylsulfatase A_β, was solubilized between 1.96 and 2.40 M $(NH_4)_2SO_4$ (peak at 2.18 M), and was well separated from the α-form (peak at 1.4 M $(NH_4)_2SO_4$). Urine from two other subjects also contained the β-form, but it consti-

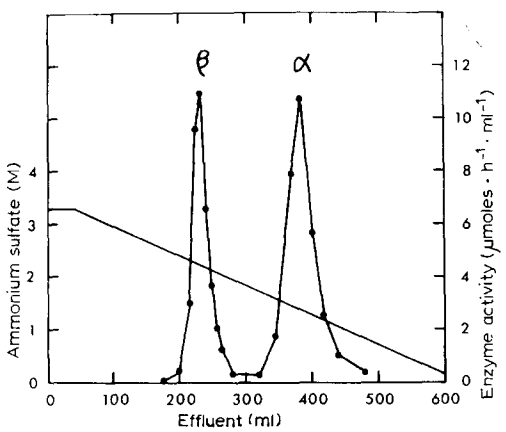

Fig. 1. Arylsulfatase A_α and A_β resolved by $(NH_4)_2SO_4$ reverse gradient chromatography. See text for details of the chromatography. The enzyme activity was measured by the hydrolysis of 4-nitrocatechol sulfate.

tuted less than 20% of the total activity. Urine from three additional subjects had no β-form.

Fractions from the chromatograph shown in Fig. 1 representing about 85% of the activity of each peak were chromatographed separately on a standardized Sephadex G-200 column[9] (100 mM NaCl in 10 mM Tris–HCl, pH 7.5). Each form of the enzyme was eluted as a single peak in an elution volume corresponding to a mol. wt of 125 000.

Arylsulfatase A_α and A_β from this Sephadex G-200 column showed indistinguishable substrate specificities; the relative activities toward two synthetic substrates, 4-nitrocatechol sulfate and 4-methylumbelliferyl sulfate, and the natural substrate, cerebroside sulfate, were similar (Table I). The ratio of activities for both forms of the enzyme toward the three substrates was also identical to that shown by an arylsulfatase A preparation from pooled human urine which had been subjected to preparative polyacrylamide gel electrophoresis (Prepn II-5A). The specific activities obtained with both enzyme forms of S.D. (Table I) were higher than the corresponding enzymes from other individuals or pooled urine, but the enzymes from all sources behaved similarly. Further studies with the 4-methylumbelliferyl sulfate as substrate showed that the K_m values for the 2 enzyme forms were nearly identical (10 $\pm$ 2 mM)

TABLE I

SPECIFIC ACTIVITIES OF ARYLSULFATASE A FORMS TOWARD DIFFERENT SUBSTRATES

Specific activities are expressed as μmoles substrate/h per mg protein. Hydrolysis of 4-nitrocatechol sulfate was determined by the assay of Baum *et al.*[8]. Hydrolysis of 4-methylumbelliferyl sulfate was determined by a procedure (Kihara, H. and Fluharty, A. L., unpublished) similar to that of Harinath and Robins[10]. Hydrolysis of cerebroside sulfate was determined as previously described[11].

	4-Nitrocatechol sulfate	*4-Methylumbelliferyl sulfate*	*Cerebroside sulfate*
Arylsulfatase A_α	127	6.5	6.0
Arylsulfatase A_β	18	1.1	1.0
Arylsulfatase A (Prepn II-5A)	183	10.6	9.0

and each had a wide pH optima between pH 5.7 and 6.0. Heat treatment of arylsulfatase A_α and A_β failed to reveal any differences between the 2 enzyme forms. At 55 °C and 60 °C first order inactivation curves were obtained with half-lives of 25 and 5 min, respectively, for both enzyme forms. Arylsulfatase A_α and A_β were both precipitated with apparent equal affinity by rabbit antibody produced against human urinary arylsulfatase A.

On analytical disc gel electrophoresis at alkaline pH[12], both arylsulfatase A_α and A_β had R_F values of 0.4 and could not be distinguished. On isoelectric focusing in polyacrylamide gel[13] both the α- and β-enzyme forms exhibited pI values near 4.7. In initial experiments it appeared that the α- and β-enzymes could be separated by this technique. However, upon closer examination utilizing fluorescein–hemoglobin[14] as a visual and fluorescent pH marker, it appeared that A_α and A_β migrated to the same area on the pH gradient and reliable resolution was not possible. In both disc gel electrophoresis and isoelectric focusing the enzyme band was located by placing the gel in 4-methylumbelliferyl sulfate (10 mM in 500 mM sodium acetate, pH 5.2) and visualizing the fluorescent product under ultraviolet light (long wavelength).

In order to establish that the appearance of arylsulfatase A_β was not due to adventitious generation during reverse gradient chromatography, the α- and β-forms were rechromatographed by this procedure. The arylsulfatase A_α fraction was eluted as a single peak at about the same $(NH_4)_2SO_4$ concentration as it had previously. Rechromatography of the arylsulfatase A_β fraction yielded a mixture of the α- and β-enzymes. However, between the initial chromatography and the rechromatography, the enzyme preparations had been subjected to considerable manipulation including dialysis, ultrafiltration, Sephadex G-200 chromatography, and repeated freezing and thawing. An immediate second rechromatography of the β-enzyme produced a single peak at the β-position with little or no α-enzyme. It thus appears that arylsulfatase A_α and A_β are unique molecular entities. It was of interest to note that the first rechromatography of arylsulfatase A_β resulted in two fractions with 40% of the activity eluting at about the original 2.18 M and 60% at about 1.4 M $(NH_4)_2SO_4$, the A_α position. The specific activity of the β-form from this column was not changed, but the specific activity of the α-form was 5 times as great as the β-form. Recovery of total enzyme activity from this column was nearly complete. While these results were obtained with A_β from a single individual (S.D.), similar behavior was observed with A_β prepared from pooled urine samples. From these experiments it must be assumed that

arylsulfatase A_α was generated from arylsulfatase A_β either during storage or the various manipulations. Interconversion of the α- and β-forms would provide an unique and effective step in the purification of arylsulfatase A if this process could be controlled.

The ratios of the two forms of the enzyme from a given individual were not constant. A subsequent urine sample from S.D. who previously had nearly equal quantities of the α- and β-enzymes (*cf.* Fig. 1) showed only 10% of the total arylsulfatase A in the β-form. A second test on another subject, who had about 20% of the enzyme in the β-form, showed no β-form. The variability of the ratios of α- and β-forms of arylsulfatase A in different urine samples from the same individual precludes genetic polymorphism as the explanation for the second enzyme form. The apparent interconversion of the two forms is also consistent with this conclusion.

Individual urines for the first examination included that collected at home and portions of it remained at room temperature up to 24 h before it was frozen. To test if the β-form might have been produced by such delays in freezing, a 1-l batch of urine from S.D. was left at room temperature for 48 h and only 2% of the arylsulfatase A was present in the β-form. Samples of urine left under the same conditions with antibacterial agents also contained low amounts of A_β. Thus, the high percentage of the

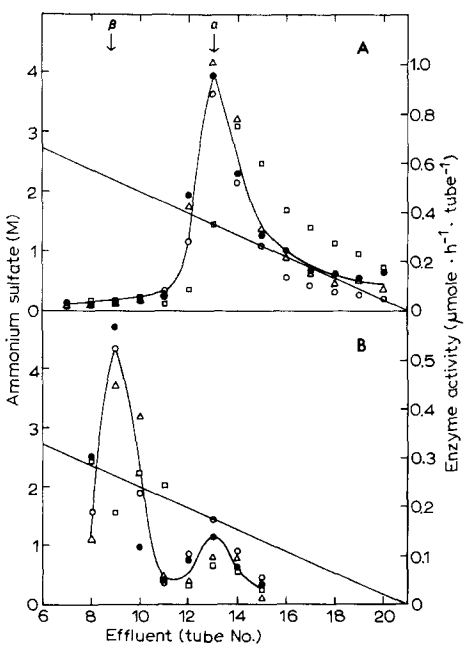

Fig. 2. $(NH_4)_2SO_4$ reverse gradient chromatography of arylsulfatase A_α and A_β subjected to various treatment. (A) Arylsulfatase A_α, 5 ml, 2.4 mg protein, 10 enzyme units. (B) Arylsulfatase A_β, 10 ml, 15 mg protein, 2.8 enzyme units. The enzyme solutions were incubated for 16 h at room temperature in 10 mM Tris–HCl (pH 7.5) containing 100 mM NaCl with: ●—●, no addition; ○—○, 1 mM ascorbic acid; □—□, 1 mM dithiothreitol; and △—△, 100 mM urea. To each of the 5 or 10 ml of enzyme solution was added 1 g Celite and solid $(NH_4)_2SO_4$ to 80% saturation. The 8 columns were poured and run simultaneously as described in the Methods section. In the lower chromatograph (B) each fraction was dialyzed before the enzyme was assayed to eliminate inhibitory SO_4^{2-}. The enzyme activity was measured by the hydrolysis of 4-nitrocatechol sulfate.

enzyme as A_β in the first urine sample from S.D. could not be attributed to failure to freeze the specimen immediately.

In an attempt to define conditions which may enhance the interconversion of these two enzyme forms, arylsulfatase A_α and A_β from pooled urine in 10 mM Tris–HCl (pH 7.5) were kept at room temperature for 16 h with (a) no additions, (b) urea, 100 mM, (c) dithiothreitol, 1 mM, or (d) ascorbate, 1 mM. The α-enzyme remained unchanged under all test conditions (Fig. 2A). No enzyme was detected in the β-position even after removal of SO_4^{2-} by dialysis. In the series originating with the β-enzyme (Fig. 2B) the various treatments did not enhance the formation of the α-enzyme when compared to the control (80% A_β, 20% A_α). In a separate series, arylsulfatase A_α and A_β were kept at room temperature for 10 h with 100 mM NaCl in (a) 10 mM Tris–HCl (pH 9.5); (b) 10 mM Tris–HCl (pH 7.5); and (c) 10 mM acetate (pH 4.5). At pH 9.5, both forms were completely inactivated. At pH 7.5 and 4.5, the α-enzyme remained unchanged while approximately 20% of the β-enzyme was again converted to the α-form irrespective of test conditions. The β to α conversion has also been noted in samples stored 2–4 weeks at −20 °C in the pH 7.5 Tris buffer. Thus, with partially-purified enzyme preparations the conversion of A_α to A_β under controlled conditions could not be achieved; storage of the partially purified β-enzyme resulted in significant production of A_α, but this process could not be accelerated by changes in temperature, pH, or chemical environment.

Altered forms of arylsulfatase A have been observed by others. Goldstone *et al.*[15] reported that arylsulfatase A was converted to an electrophoretically different form by treatment with a bacterial neuraminidase. Arylsulfatase A_α and A_β (from the Sephadex G-200 columns) treated with neuraminidase under conditions identical to these authors, showed no change in activities toward 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and cerebroside sulfate, nor were there alterations in migration properties on gel electrophoresis or isoelectric focusing. Purified ox liver arylsulfatase A has been shown to have two kinetically distinguishable forms[16]. An inactive modified form of arylsulfatase A was produced by incubation with 4-nitrocatechol sulfate. If A_β represented this inactive modified form, it would not have been detected under our assay conditions. The procedure of Baum *et al.*[8] would probably activate the modified form. When the α- and β-enzymes were assayed in the absence of pyrophosphate and Cl^-, providing a mixture similar to that employed by Nicholls and Roy[16], their activities did not differ. Arylsulfatase A purified to homogeneity from ox liver exhibits various degrees of polymerization dependent on pH and ionic strength[17], but in the present instance this possibility is precluded by essentially identical molecular weight behavior on Sephadex G-200 gel filtration by the α- and β-forms. Thus, arylsulfatase A_β does not appear to be any of the previously described modified forms of arylsulfatase A. The present data suggests that catalytically both enzyme forms are identical, although a rigorous kinetic analysis might prove otherwise.

Several facts concerning arylsulfatase A_β appear clear: it is of human origin; it does not represent genetic polymorphism; it cannot be arylsulfatase B because of its acidic pI; its catalytic properties are identical to those of arylsulfatase A_α; its pI value and molecular weight are similar to that of the α-enzyme. The conversion of the β- to α-form during experimental manipulation and differing ratios of the two forms in different urine specimens from the same individual strongly suggest that the β-form

arises from the α-enzyme and that the process is reversible. However, tests to date have offered no clues on the chemical or physical circumstances surrounding the interconversion.

The recognition of a second form of arylsulfatase A formed under as yet undetermined conditions provides an explanation for anomalous fractionation patterns occasionally observed during purification of this enzyme from pooled human urine. Stepwise $(NH_4)_2SO_4$ precipitation is expected to yield arylsulfatase B free of arylsulfatase A. On occasion significant amounts of arylsulfatase A has been found in such fractions from human urine. Arylsulfatase A_β being soluble in higher $(NH_4)_2SO_4$ concentrations would contaminate these fractions.

We have no evidence that arylsulfatase A_β is present in extracts of human tissue and, therefore, tentatively conclude that it is derived from A_α *in vitro*. Nevertheless, the mechanism of interconversion of the α- and β-enzymes is important in understanding more about arylsulfatase A *per se*. A controlled interconversion of the two forms should facilitate the purification of arylsulfatase A.

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