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THE ACTIVITY OF ARYLSULFATASE A AND B ON TYROSINE *O*-SULFATES

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Summary

L-Tyrosine *O*-sulfate was hydrolyzed by pure human arylsulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1). The rate of hydrolysis was 1/20 of the rate with nitrocatechol sulfate, but was comparable to the rate with cerebroside sulfate. The reaction was optimal at pH 5.3—5.5 and displayed zero order kinetics with time and enzyme concentration. The K_m was about 35 mM.

The enzyme showed no stereospecificity and hydrolyzed D-tyrosine *O*-sulfate with K_m and V similar to those for the L-isomer. Arylsulfatase B was less than 5% as effective as arylsulfatase A in catalyzing the hydrolysis of the tyrosine sulfates. The daily urinary excretion of tyrosine sulfate by a patient with metachromatic leukodystrophy (arylsulfatase A deficiency) was comparable to the excretion by control subjects. The biological relevance of the tyrosine sulfatase activity of arylsulfatase A remains uncertain.

Introduction

Mammalian sulfatases which hydrolyze synthetic phenolic sulfate esters have been subjected to extensive investigation as arylsulfatases (aryl-sulfate sulfohydrolase, EC 3.1.6.1) [1]. Studies of human genetic disorders have indicated that the putative physiological substrates for the soluble sulfatases, arylsulfatase A and arylsulfatase B, are carbohydrate sulfates [2,3]. No naturally occurring phenolic sulfates have been identified as substrates for these enzymes, so their arylsulfatase activities are regarded as convenient artifacts.

One phenolic sulfate present in mammals is L-tyrosine *O*-sulfate. It is excreted in the urine in significant amount [4,5] and is believed to be a breakdown product of proteins which have undergone sulfation of selected tyrosine residues. The hydrolysis of this phenolic sulfate by partially purified human arylsulfatases was examined by Dodgson et al. [6]. They found no hydrolysis

by arylsulfatase C, but arylsulfatase A did catalyze some hydrolysis. However, the rate was only about one-sixth of the rate at which arylsulfatase A hydrolyzed the usual assay substrate nitrocatechol sulfate, so the reaction was considered too feeble to be of physiological significance.

Subsequently, it has been established that cerebroside sulfate is the physiological substrate of arylsulfatase A [2] and its rate of hydrolysis is considerably lower than the rate for nitrocatechol sulfate [7]. Therefore, it appeared important to reexamine the reaction of arylsulfatases with tyrosine sulfate. Toward this end, L-tyrosine and D-tyrosine sulfates were prepared and tested with human arylsulfatase A and arylsulfatase B. The excretion of tyrosine sulfate by a patient with metachromatic leukodystrophy was also examined.

Materials and Methods

Materials

L-Tyrosine and D-tyrosine were obtained from Calbiochem (La Jolla, CA), nitrocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate) from Sigma Chemical Co. (St. Louis, MO), Dowex-1 (AG1-X2) from Bio-Rad Laboratories (Richmond, CA) and Folin-Ciocalteu phenol reagent from Hareleco (Philadelphia, PA). Pure arylsulfatase A was isolated from human urine [7]. Arylsulfatase B was purified 200-fold from human placenta and was devoid of arylsulfatase A activity [8]. A unit of arylsulfatase activity is defined as the amount of enzyme required to hydrolyze 1 μmol nitrocatechol sulfate/h by the methods of Baum et al. [9] as modified in this laboratory for arylsulfatase A [7] and arylsulfatase B [10].

Synthesis of tyrosine sulfates

L-Tyrosine *O*-sulfate and D-tyrosine *O*-sulfate were synthesized and isolated by the method of Tallan et al. [4] as modified by Dodgson et al. [6]. Briefly, tyrosine was reacted with H_2SO_4 for 1 h at −12 to −20°C. The excess sulfate was removed as the Ba^{2+} salt. Tyrosine sulfate was absorbed on Dowex-1 in 0.01 N HCl and the unreacted tyrosine was washed through. The product was recovered by applying a 0.01—0.1 N HCl gradient to the resin. The yield of L-tyrosine *O*-sulfate from 2 g L-tyrosine was 0.97 g and of D-tyrosine *O*-sulfate from 1 g D-tyrosine was 0.12 g. The identification and purity of the sulfated products were established by absorbance ratios at 260 and 310 nm, high voltage paper electrophoresis (0.1 M sodium acetate, pH 4.6), thin-layer chromatography (ethyl acetate/acetic acid/0.1 mM KCl; 2 : 1 : 1, v/v), reactivity with ninhydrin and recovery of tyrosine and inorganic sulfate after acid hydrolysis.

Determination of enzyme activity

The hydrolysis of tyrosine sulfate was monitored by the increase in absorbance at 295 nm caused by the appearance of free phenolic groups [6]. The usual reaction mixture contained 5 μl enzyme (0.4 units) and 50 μl 40 mM tyrosine sulfate in 0.5 M sodium acetate (pH 5.4). The reaction was incubated at 37°C for 1 h, terminated by the addition of 0.5 ml 0.2 M NaOH and the absorbance measured at 295 nm. In early experiments an alternative method

for determining hydrolysis was used. The increase in product was monitored by reduction of a phosphomolybdate-phosphotungstate 'phenol' reagent. The 55 μl reaction mixture, after the usual 60 min incubation period, was added to 400 μl 2% Na_2CO_3 in 0.1 N NaOH. Then 40 μl of a 1 : 1 diluted Folin-Ciocalteu phenol reagent was added and the mixture incubated at room temperature for 10 min before being read at 500 nm.

Determination of urinary tyrosine sulfate

2.5-ml aliquots of 24-h urine specimens were prepared for chromatography by acidification to pH 2—3 with 1 N HCl, addition of 0.75 ml 62.5% sucrose, 0.2 ml 2.5 mM norleucine as internal standard, and water to 3.75 ml and stored at −20°C. The samples were analyzed on a Technicon Amino Acid Autoanalyzer with a 120 × 0.6 cm type B chromobead column. 1.5-ml samples were injected and the columns were developed with a gradient of sodium citrate buffer between pH 2.875 and 5.0 [11] for 140 min at 45°C then at 60°C for the remainder of the elution. Tyrosine sulfate was eluted as a sharp symmetrical peak at about 60 min after the gradient was applied. It was clearly separated from urea which peaked at about 90 min. Although the complete 20 h elution was not required for tyrosine *O*-sulfate, all runs were carried through the entire gradient to monitor excretion of all amino acids.

Results

Activity by arylsulfatase A

L-Tyrosine *O*-sulfate was hydrolyzed by arylsulfatase A, with a maximum rate about 5% of that with nitrocatechol sulfate. Variation of substrate concentration between 3 and 100 mM generated a K_m of 35 mM and a V of 400 μmol/h per mg from double reciprocal plots. The relative standard error of the reciprocals was ±0.4. An energy of activation of 12.5 cal/mol was estimated from an Arrhenius plot of V at 5 temperatures between 18 and 45°C. The K_m did not change appreciably over this temperature range. The pH optimum was 5.3—5.5 with the decrease being more precipitous on the acid side. Activity was half maximum at pH 4.6 and was negligible at pH 3.5. The activity profile was unaffected when the concentration of the buffer was varied between 0.05 and 0.5 M. Unlike the reaction with nitrocatechol sulfate which is characterized by an anomalous time course, the rate of reaction with L-tyrosine sulfate was constant with time up to 1 h and was proportional to enzyme concentration (Fig. 1). The hydrolysis was only moderately affected by NaCl: at 0.2 M there was 6% inhibition and at 2 M there was 37% inhibition. Na_2SO_4, however, showed strong inhibition: 7% at 0.25 mM, 20% at 2.5 mM, and 69% at 25 mM.

D-Tyrosine *O*-sulfate was also hydrolyzed by arylsulfatase A. The observed K_m of 40 mM and V of 333 μmol/h per mg (±0.65 relative standard error of the reciprocals) were not appreciably different from the kinetic data of the L-isomer.

Activity by arylsulfatase B

Arylsulfatase B catalyzed a slight hydrolysis of the tyrosine sulfate esters when assayed by the usual conditions employed with arylsulfatase A. When

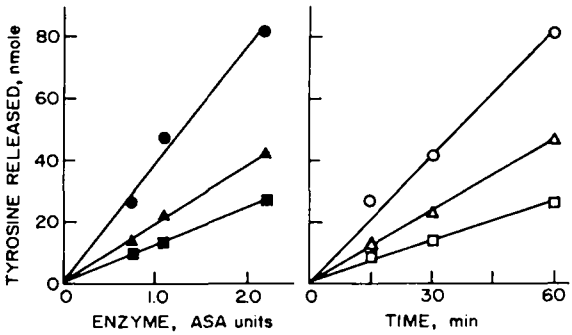

Fig. 1. The effect of enzyme concentration and time on the hydrolysis of L-tyrosine *O*-sulfate by arylsulfatase A. Enzyme activities were measured by the alternate assay procedure. Left Panel: Incubation times were 60 min, ●———●; 30 min, ▲- - - -▲; and 15 min, ■·———■. Right Panel: Arylsulfatase A levels/incubation mixture were 2.23 unit, ○———○; 1.12 unit, △- - - -△; and 0.75 units, □·——·□.

equivalent arylsulfatase activities were compared, the B enzyme cleaved the L- and D-tyrosine sulfates at 2 and 4% respectively, of the rates obtained with the A enzyme. These activities are near the limits of measurement and the difference between isomers is not significant. Tyrosine sulfate cleavage by arylsulfatase B increased with substrate concentration and the K_m was estimated to be between 300 and 1000 mM. The V was 13% of that for an equivalent activity of arylsulfatase A. These kinetics constants are imprecise because tyrosine sulfate concentrations above 100 mM could not be achieved. Nonetheless, the data do suggest that the difference in the action of arylsulfatase A and B is reflected in both the K_m and V parameters.

Urinary excretion

A patient with metachromatic leukodystrophy and 10 age-matched control children were examined for urinary excretion of tyrosine sulfate (presumably the L-isomer). The patient excreted 15.3 mg/24 h (3 specimens, range 11.9—20.4) and the average excretion by control subjects was 12.6 mg/24 h (range 5.4—22.7). Daily excretions of other amino acids were within normal limits. Expressed on the basis of creatinine exretion the patient urinary tyrosine sulfate was 112 mg/g creatinine (range 95—135) compared to 16 mg/g (range 8—33) for the controls.

Discussion

Pure human arylsulfatase A catalyzed the hydrolysis of L-tyrosine *O*-sulfate with a maximal turnover rate of 400 μmol/h per mg protein. This rate was considerably below the 3500—10 000 μmol/h per mg protein (depending on assay conditions) observed with nitrocatechol sulfate [7]. This basically confirms the observations of Dodgson et al. [6] who used a relatively crude preparation of human liver arylsulfatase A. Unexpectedly, the enzyme showed no stereospecificity, D-tyrosine *O*-sulfate being hydrolyzed with kinetic parameters indistinguishable from those for the L-isomer.

While the rate of tyrosine sulfate hydrolysis by arylsulfatase A was lower than that of nitrocatechol sulfate, it was comparable with a number of other substrates of this enzyme. The rate for cerebroside sulfate hydrolysis with optimal bile salt activation ranged from 300--500 μmol/h per mg protein [7]. A similar rate was observed with methylumbelliferone sulfate [7]. Ascorbic acid 2-sulfate was hydrolyzed at rates between 200 and 2000 μmol/h per mg protein [12]. Thus, one should not minimize the significance of tyrosine sulfate hydrolysis on the basis of catalytic efficiency.

The hydrolysis of tyrosine sulfate by arylsulfatase A followed normal Michaelis-Menten kinetics up to its limit of solubility. There was no evidence of substrate inhibition or unusual time course which are characteristic of this enzyme with nitrocatechol sulfate [7]. In this respect tyrosine sulfate resembled cerebroside sulfate [7] and methylumbelliferone sulfate [13]. The 12.5 cal/mol energy of activation was lower than the 21.5 cal/mol for cerebroside sulfate hydrolysis but was within the range observed for arylsulfates [7]. Tyrosine sulfate cleavage was relatively insensitive to NaCl. This was typical of arylsulfate substrates but contrasts with the salt sensitivity of cerebroside sulfate hydrolysis. Sulfate inhibited with a half effect around 10 mM.

The K_m for tyrosine sulfates was in the range of 30—40 mM; a higher value than typical for substrates of possible physiological significance. An arylsulfatase from *Aspergillus oryzae* with a similar K_m for tyrosine sulfate has been described by Burns and Wynn [14]. The tyrosine sulfate K_m was well above the tyrosine sulfate level expected within a cell, even if subcellular concentration by lysosomes were postulated. While the K_m cannot rule out tyrosine sulfate hydrolysis as a physiological significant reaction of arylsulfatase A, the value is atypical of metabolically important enzyme-substrate couples.

Tyrosine sulfate hydrolysis by arylsulfatase B was also observed, but significant rates were only achieved under extreme conditions. With standard assay conditions the activities were less than 5% of that exhibited by an equivalent number of arylsulfatase A units and were near the limits of detection. The reaction was enzyme and substrate concentration dependent and only by increasing tyrosine sulfate to its solubility limit was it possible to obtain rough estimates of the kinetic constants. The K_m values approached 1 M and the V was some 8-fold less than that for arylsulfatase A. While arylsulfatase B has some capacity for tyrosine sulfate hydrolysis it has almost no affinity for these compounds. The activity exhibited by arylsulfatase B is certainly beyond any consideration of biological relevance and under usual criteria the tyrosine sulfates would be considered relatively specific substrates of arylsulfatase A.

One way to evaluate any physiological significance of tyrosine sulfate breakdown by arylsulfatase A was to look at urinary excretion of tyrosine sulfate in the enzyme deficiency state. Arylsulfatase A is completely absent in late infantile metachromatic leukodystrophy [15]. The 24 h tyrosine sulfate excretion by a patient with this condition was in the same range as control subjects. If the comparison were made on the basis of creatinine excretion ratios, urinary tyrosine sulfate did appear to be increased by several fold in the patient. While creatinine levels are commonly used to normalize urinary excretion data, this was considered misleading in the present situation. Creatinine turnover is a

function of a muscle mass: the patient in this case was completely debilitated and had undergone extensive muscular degeneration. Other urinary amino acids were normal when compared on the basis of total 24-h excretions, supporting the validity of this method of comparison.

In conclusion, the tyrosine sulfates behave as fairly typical substrates of arylsulfatase A. Selectively for A and B enzymes was somewhat intermediate between those of common synthetic and physiological substrates. Even though the L-isomer occurs naturally, the physiological significance of the tyrosine sulfatase reaction is uncertain due to a high K_m, a lack of stereospecificity and the absence of an elevated tyrosine sulfate excretion by a metachromatic leukodystrophy patient. The tyrosine sulfates do interact differently with arylsulfatases A and B, a property which should make them useful for studying differential substrate binding and selection by the arylsulfatases.

Acknowledgements

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