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CHEMICAL CHARACTERIZATION AND SUBSTRATE SPECIFICITY OF RABBIT LIVER ARYL SULFATASE A

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Summary

Rabbit liver aryl sulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1) is a glycoprotein containing 4.6% carbohydrate in the form of 25 residues of mannose, seven residues of *N*-acetylglucosamine, and three residues of sialic acid per enzyme monomer of molecular weight 140 000. Each monomer consists of two equivalent polypeptide chains. The protein has a relatively high content of proline, glycine and leucine, and the amino acid composition of rabbit liver aryl sulfatase A is similar to that of other known liver sulfatases. Rabbit liver aryl sulfatase A catalyzes the hydrolysis of a wide variety of sulfate esters, although is appears possible that cerebroside sulfate is a physiological substrate for the enzyme because the K_m is very low (0.06 mM). The turnover rate for hydrolysis of nitrocatechol sulfate or related synthetic substrates is much higher than the rate with most naturally occurring sulfate esters such as cerebroside sulfate, steroid sulfates, L-tyrosine sulfate or glucose 6-sulfate. However, the turnover rate with ascorbate 2-sulfate is comparable to the rates measured using most synthetic substrates. These results are discussed in relationship to several previously described sulfatase enzymes which were claimed to have unique specificities.

Introduction

Aryl sulfatase A enzymes (aryl sulfate sulfohydrylase, EC 3.1.6.1) from vertebrates have not been extensively characterized. They generally show a pH-dependent polymerization [1—7] and some of them are found to show two pH optima in pH vs. activity profiles [3,8]. Apparently irrespective of the animal source, aryl sulfatase A enzymes are found to be inactivated during the hydrolysis of certain substrates [3,9—11], and under some conditions the

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inactive turnover-modified enzyme can be partially reactivated by SO_4^{2-} in presence of substrates [3,12,13]. The turnover-modification process has been shown to involve a sulfation of the enzyme [14]. The physiological substrate of aryl sulfatase A is considered to be cerebroside sulfate [15] and a deficiency of the enzyme is associated with the genetic disease known as metachromatic leucodystrophy [16]. In recent years the recognition of new potential substrates such as ascorbate 2-sulfate and L-tyrosine *O*-sulfate in animal tissues [17] and in excretion products [18], respectively, has raised questions about the physiological significance of these substrates.

In addition, only limited data are available on the chemical composition of aryl sulfatase A enzymes from mammalian tissues [5,19—21]. Comparison of the amino acid composition of the human liver enzyme with that of the human urine enzyme reveals significant differences [5]. There is also little information available about the carbohydrate residues of these glycoproteins. Comparisons of the limited data suggest the possibility of significant variations in the carbohydrate content of aryl sulfatase A enzymes [4,21,22]. In view of these facts we have studied the chemical composition and substrate specificity of aryl sulfatase A from rabbit liver. The present paper presents data for the amino acid and the carbohydrate composition of homogeneous rabbit liver aryl sulfatase A. In addition, substrate specificity studies are presented which suggest that the rabbit liver enzyme can cleave virtually all sulfate esters, although the turnover rates do vary widely. To permit accurate comparison, both K_m and V were measured at several pH values, in many cases both by measurement of sulfate release and by measurement of phenol or alcohol release.

Experimental

Materials. Aryl sulfatase A was isolated from frozen rabbit liver (Pel-Freez Biological, Inc., Rogers, AR) by the procedure of Lee and van Etten [3]. The enzyme sample used in these studies was homogeneous on the basis of polyacrylamide gel electrophoresis at pH 8.0. The specific activity of the enzyme preparation was 350 μmol/min per mg using nitrocatechol sulfate as a substrate [3]. Acetic acid/pyridine buffers (pH 4.5 and pH 5.5) were prepared by titrating 0.1 M acetic acid with 0.1 M pyridine.

L-Tyrosine *O*-sulfate was synthesized by the method of Tallan et al. [23] as modified by Dodgson et al. [24]. Despite repeated recrystallizations the elemental analysis indicated either some disulfation or that inorganic sulfate was present.

Calculated for $C_9H_{10}O_6NSK \cdot 2\ H_2O$: C, 32.2; H, 4.2; N, 4.2; S, 9.6
Found: C, 30.4; H, 3.55; N, 4.0; S, 10.2

The spectrum of the resulting L-tyrosine *O*-sulfate was identical with that previously reported [24].

Nitrocatechol sulfate, *p*-nitrophenyl sulfate, α-naphthyl sulfate, 4-methylumbelliferone sulfate, dehydroisoandrosterone sulfate, estrone 3-sulfate, estriol 3-sulfate and glucose 6-sulfate were purchased from Sigma Chemical Co., St. Louis, MO. Ascorbate 2-sulfate was a gift from Hoffmann-La Roche, Inc. Cerebroside sulfate was obtained from Supelco, Bellefonte, PA. Rhodizonate dipotassium salt was purchased from Eastman Chemical Co. 2,6-Dichloroindo-

phenol dye was from Sigma Chemical Co. Other reagents were of analytical reagent grade. The purity of all commercial substrates was established by direct sulfate assay [25] before they were used for the determination of the enzyme activity.

Determination of protein concentration. The protein concentrations of stock solutions of the pure enzyme were determined from the absorbance at 280 nm using $A^{1\%}_{1cm} = 10.0$ at pH 7.5 [26]. The method of Lowry et al. [27] was used to determine the protein concentration with bovine serum albumin as standard.

Substrate specificity of rabbit liver aryl sulfatase A. Enzymatic activities with the various substrates including cerebroside sulfate preparations were determined in 0.05 M acetic acid/pyridine pH 4.5 and 5.5 buffers at 37°C as previously described [25]. Enzyme concentrations and incubation times in the hydrolysis of different substrates were selected such that the amount of sulfate released during the reaction could be precisely measured. Under identical experimental conditions the rates of hydrolysis of nitrocatechol sulfate, *p*-nitrophenyl sulfate and α-naphthyl sulfate were also determined by measuring phenol release using a discontinuous method involving quenching the reaction in 0.2 M NaOH [3]. The amounts of nitrocatechol, *p*-nitrophenol and α-naphthol were determined using their molar extinctions in alkali: 12 600 at 515 nm for nitrocatechol [3], 18 400 at 402 nm for *p*-nitrophenol [28] and 7800 at 335 nm for α-naphthol [29].

L-Ascorbate sulfatase activity was determined by method B of Stevens et al. [30]. The molar extinction coefficient of the 2,6-dichloroindophenol-ascorbic acid complex was determined to be 8600 $M^{-1} \cdot cm^{-1}$ at 520 nm.

4-Methylumbelliferone sulfatase activity was determined by both continuous and discontinuous methods. In the substrate concentration range 0.1—1.2 nM a continuous procedure was used. Substrate solution (2.0 ml) was incubated at 37°C in required buffer in a thermostated cell of the fluorimeter and the enzyme reaction was initiated by mixing 2.1 μg (in 25 μl) of aryl sulfatase A, with stirring. Progress of the reaction was recorded at 445 nm on a Perkin-Elmer MPF-44A fluorescence spectrophotometer following excitation at 337 nm. The amount of 4-methylumbelliferone was calculated from the relative molar fluorescence which was determined to be $2621 \cdot 10^8$. The initial slope of the reaction curve was used to calculated the kinetic parameters.

In the discontinuous procedure of activity measurement, 4-methylumbelliferone sulfate (2.0 ml) in the required buffer was incubated at 37°C and 1.87 μg (20 μl) of aryl sulfatase A was added to the initiate the reaction. After 3 min the reaction was quenched by addition of 2 ml of 0.4 M glycine-NaOH buffer, pH 10.5. The fluorescence of the sample was measured at 450 nm while the sample was excited at 365 nm. The concentration of the product formed was determined by using the molar relative fluorescence of $5.55 \cdot 10^8$ at 405 nm for 4-methylumbelliferone in 0.4 M glycine-NaOH buffer, pH 10.5.

Spectrophotometric measurements. All absorption measurements at fixed wavelength, such as that of enzyme activity, were made on a Gilford 2000 Spectrophotometer. For spectral characterization of substrates, a Beckman Acta V recording spectrophotometer was used.

Amino acid analysis. The enzyme samples (100 μg in deionized water) were hydrolyzed under vacuum in 6 N HCl at 110 ± 5°C for 24, 48 and 72 h, respec-

tively. HCl was removed either under vacuum or at 50 °C under a stream of dry nitrogen gas. Hydrolyzates were dissolved in 0.2 M citrate buffer, pH 2.2, and amino acids analyzed on a Durrum amino acid analyzer. The values of amino acid residues for unstable amino acids were corrected for decomposition by extrapolation to zero time of the values obtained at 24, 48 and 72 h. The number of amino acid residues was rounded to the nearest integer.

Carbohydrate composition. An extensively dialyzed sample of aryl sulfatase A in water was lyophilized and 1 mg protein was used for carbohydrate analysis. Following methanolysis, re-N-acetylation, and conversion to the trimethylsilyl derivatives [31] the sample was analyzed by gas liquid chromatography on a 6 ft OV-17 column using a temperature program as follows: started at 120 °C, raised 4 °C/min for 15 min; raised 2 °C/min for 15 min and raised 5 °C/min for 6 min.

Results and Discussion

The results of amino acid composition determinations for rabbit liver aryl sulfatase A are given in Table I. A total of 1034 amino acid residues (not including tryptophan) were observed per monomer of enzyme *. The molecular weight of the monomer due to these amino acids should be 111 428. However, the molecular weight of aryl sulfatase A at pH 7.5 (where it exists in the monomeric form) has been determined by zonal and frontal gel chromatography to be 140 000 [3] and 132 000 ± 3000 [7], respectively. The difference in the molecular weight determined from the amino acid analysis and that determined by gel filtration techniques is due to the presence of carbohydrate residues on rabbit liver aryl sulfatase A.

The amino acid composition reveals some interesting features of aryl sulfatase A and permits an understanding of some previous observations on the behavior of the enzyme molecule in solution. The presence of a high content of hydrophobic amino acid residues (leucine, isoleucine, tyrosine, phenylalanine) and of proline in the enzyme molecule may explain the relative instability at 0 °C or below and the greater stability at elevated temperatures [6,22]. It should be noted that a number of hydrophobic proteins and enzymes are found to be less stable at reduced temperatures.

The proline content is quite high in rabbit liver aryl sulfatase A and in fact contributes approx. 10% of the total amino acid residues present in the protein molecule. The relatively low content of α-helix and the higher amount of β-sheet and unordered structure in the enzyme molecule [26] can be attributed to this high content amount of proline. A high content of glycine may also contribute to the fact that the sturcture of rabbit liver aryl sulfatase A is low in helix content, since there is a 10% glycine content in the enzyme molecule (Table I).

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* Numerous preparations of apparently pure rabbit liver aryl sulfatase A showed an unusual peak for an amino acid near the ascending edge of the histidine peak. We were not able to fully characterize the new peak. However, it did not appear to correspond to either 3-methyl- or 1-methylhistidine. A qualitative comparison of amino acid analysis data, obtained for casein heated in a pH 10.6 buffer [32], with our results for amino acid analysis suggests the possibility of lysinoalanine, since this was found to emerge very close to the new peak in the amino acid analysis profile of arylsulfatase A.

TABLE I

AMINO ACID COMPOSITION OF RABBIT LIVER ARYL SULFATASE A

n.d., not determined.

Amino acid	Residues/monomer	Amino acid	Residues/monomer
Aspartic acid	78	Methionine	24
Threonine	64	Isoleucine	18
Serine	[illegible]	Leucine	128
Glutamic acid	86	Tyrosine	26
Proline	100	Phenylalanine	48
Glycine	118	Histidine	36
Alanine	94	Lysine	20
1/2-Cystine	20	Arginine	48
Valine	54	Tryptophan	n.d.
Total number of residues:			1 034
Molecular weight contribution of amino acids:			111 428

The present data may be compared with data available for four other aryl sulfatase A enzymes the amino acid compositions of which have been reported. They are ox liver [19], human liver [5], human urine [20] and rabbit testis [21]. To permit such a comparison the results of amino acid analyses for these aryl sulfatase A enzymes have been normalized to residues per 100 000 g of protein (Table II). The aryl sulfatase A enzymes from liver are quite comparable with respect to several amino acids: all aryl sulfatase A enzymes have relatively high contents of proline, glycine and leucine. However, the human urine

TABLE II

COMPARISON OF AMINO ACID COMPOSITIONS OF ARYL SULFATASE A ENZYMES

n.d., not determined. Sources: ox liver [19], human liver [5], human urine [20] and rabbit testis [21].

Amino acid	Residues per 100 000 g				
	Rabbit liver	Ox liver	Human liver	Human urine	Rabbit testis
Aspartic acid	70	67	61	67	45
Threonine	58	52	43	34	91
Serine	65	55	49	67	130
Glutamic acid	77	82	65	102	121
Proline	90	84	86	135	89
Glycine	106	101	85	113	105
Alanine	85	87	77	56	85
1/2-Cystine	18	20	27	n.d.	n.d.
Valine	49	46	43	45	91
Methionine	22	15	15	11	20
Isoleucine	16	15	15	23	35
Leucine	115	124	100	45	82
Tyrosine	23	26	26	11	35
Phenylalanine	43	48	37	23	38
Histidine	32	36	26	35	25
Lysine	18	13	20	45	22
Arginine	43	31	37	34	34
Tryptophan	n.d.	10	19	n.d.	n.d.

and rabbit testis enzymes have distinctly different compositions. The amounts of proline and glycine are high (as for the liver enzymes) but leucine is significantly lower.

Ox liver lysosomal aryl sulfatase A is a glycoprotein [22]. Rabbit liver aryl sulfatase A is also a glycoprotein and contains 4.6% (w/w) carbohydrate. The results of carbohydrate analyses showed that rabbit liver aryl sulfatase A contains sialic acid/*N*-acetylglucosamine/mannose in the ratio of 1 : 2.3 : 8.3. This suggests that rabbit liver aryl sulfatase A is a glycoprotein of the 'high mannose' class which typically contains 5—8 residues of mannose, plus two *N*-acetylglucosamine residues. However, the carbohydrate analysis of ox liver aryl sulfatase A [22] indicated a 10% carbohydrate content consisting of 2.2 sialic acid, 3.0 glucosamine, 2.5 mannose, 1.35% galactose and traces of fucose and glucose. Carbohydrate analysis data for sheep brain aryl sulfatase showed 25% neutral sugars and 0.5% sialic acid [4], values which are not much different from the carbohydrate content of the rabbit testis enzyme [21]. Therefore, these findings suggest the possibility of considerable variation in the carbohydrate composition of aryl sulfatase A enzymes from mammalian sources.

The substrate specificity of rabbit liver aryl sulfatase A was tested using various sulfate esters at pH 4.5 and pH 5.5. Measurements of kinetic parameters and specific activity of the enzyme are shown in Table III and Table IV, respectively. The enzyme assay was performed by two procedures as discussed in the Experimental section: one in which alcohol or phenol was monitored and the other one where SO_4^{2-} was determined. In cases where K_m and V determinations were done by both methods the results with the same substrate are in agreement (Table III). This further supports the validity of our relatively convenient spectrophotometric assay for sulfate [25] as used in kinetic studies of aryl sulfatase A.

TABLE III

KINETIC PARAMETERS FOR HYDROLYSIS OF SUBSTRATES BY RABBIT LIVER ARYL SULFATASE

Units of K_m are mM, while V has units of μmol product formed per min per mg of protein. All measurements were made at 37°C.

	Assay procedure							
	Determination of alcohol or phenol				Determination of SO_4^{2-}			
	pH 4.5		pH 5.5		pH 4.5		pH 5.5	
	K_m	V	K_m	V	K_m	V	K_m	V
p-Nitrocatechol sulfate	0.46	205	0.73	420	0.35	254	0.60	454
p-Nitrophenol sulfate	3.11	5.26	80	100	3.00	5.56	81	100
α-Naphthyl sulfate	15.9	0.76	28	3.85	15.9	0.76	29.0	4.00
4-Methylumbelliferone sulfate	3.52	3.45	4.86	7.14	4.00	3.85	5.00	7.14
Ascorbate 2-sulfate	2.00	100	0.8	20	—	—	—	—
Tyrosine *O*-sulfate	—	—	—	—	31.8	4.00	32.8	7.69
Cerebroside sulfate	—	—	—	—	0.06	0.20	0.19	0.315

TABLE IV

SUBSTRATE SPECIFICITY OF RABBIT LIVER ARYL SULFATASE A AT 37°C

Substrate	Spec. act. nmol/min per mg of enzyme	
	pH 4.5	pH 5.5
Dehydroisoandrosterone sulfate	20	40
Estrone 3-sulfate	40	48
Estriol 3-sulfate	42	52
Glucose 6-sulfate	220	280

It can be seen from Table III that the lowest K_m value at pH 4.5 is 0.06 mM for cerebroside sulfate. This observation appears consistent with the general contention that cerebroside sulfate is a natural substrate for aryl sulfatase A [15]. Except for ascorbate 2-sulfate, the K_m values for all substrates were larger at pH 5.5 relative to the values at pH 4.5. The turnover rates of commonly used synthetic substrates are often much higher than those of naturally occurring sulfate esters (Table III and Table IV). Rabbit liver aryl sulfatase A was able to catalyze the hydrolysis of all of the sulfate esters used in the present study.

Ascorbate 2-sulfatase activity has been observed with ox liver [33], human urine [30] and rabbit kidney [34] aryl sulfatase A. Carlson et al. [35] isolated a putative ascorbate 2-sulfate sulfohydrolase enzyme from bovine liver and found that it catalyzed the hydrolysis of ascorbate 2-sulfate. They determined K_m for ascorbate 2-sulfate at pH 4.8 to be 1.2 mM, a value which is similar to the K_m determined for rabbit liver aryl sulfatase A (2 mM). Recently, Fluharty et al. [36] also reported that human urine aryl sulfatase A hydrolyzed L-tyrosine *O*-sulfate. However, the physiological significance of the hydrolysis of L-tyrosine *O*-sulfate by aryl sulfatase A is uncertain.

Rabbit liver aryl sulfatase A is able to hydrolyze steroid sulfate esters as well as glucose 6-sulfate (Table IV). The turnover rates for steroid sulfates are very low. Iwamori et al. [37] isolated a putative estrone sulfatase from rat liver and the specific activity using estrone sulfate as a substrate was 87 nmol/min per mg of protein, a value which is only 2—4-fold higher than the specific activity for rabbit liver aryl sulfatase A (Table IV). It appears possible that their enzyme was merely an aryl sulfatase. Aryl sulfatase A enzymes from other mammalian sources were usually not established to catalyze the hydrolysis of steroid sulfates although Jerfy and Roy [38] have demonstrated the hydrolysis of estrone sulfate with a specific activity of 1 nmol/min per mg.

Rabbit liver aryl sulfatase catalyzes the hydrolysis of glucose 6-sulfate at a rate which is 5 times faster than the steroid sulfate hydrolysis rate (Table IV). We have reported earlier the hydrolysis of chondroitin sulfate [25]. However, aryl sulfatase A enzymes from other mammalian source were not found to hydrolyze carbohydrate sulfate [21,34]. Aryl sulfatase B has been reported to hydrolyze a few carbohydrate sulfate esters [34]. Basner et al. [39] isolated an enzyme termed *N*-acetylglucosamine-6-sulfate sulfatase from human urine. The relationship of that enzyme to aryl sulfatases is not yet clear. Also unclear is the relationship between aryl sulfatases and enzymes active against *N*-acetyl-

galactosamine 6-sulfate and its derivatives (such as are found in chondroitin sulfate and keratan sulfate). These enzymes have been considered unique and their absence is implicated in the genetic disease characterized by Morquio's syndrome. A careful comparison of activities using homogeneous enzyme preparations and galactose, glucose and phenolic sulfate substrates is needed.

Sulfate esters of steroids and carbohydrates are sometimes unstable or difficult to prepare and always difficult to purify. Sulfate analysis [25] demonstrates that many commercial sulfate esters are highly impure. Particularly in the case of substrates having high K_m values and/or low turnover rates it is important to have substrates free from sulfate. SO_4^{2-} acts as a competitive [3] or a noncompetitive [38] inhibitor of aryl sulfatase A activity. From our experience, the presence of traces of free sulfate (which can be established by direct assay [25] prior to activity measurements) in steroid sulfates and carbohydrate sulfates, may inhibit the enzyme to such a degree that one may not be able to demonstrate the hydrolysis of such substrates (see also the Appendix in Ref. 40).

Since it appeared that the anomalous kinetic behavior of aryl sulfatase A was related to the initial velocity of the enzymatic reaction (Waheed, A. and van Etten, R.L., unpublished results), we attempted to test this hypothesis by examining the time course for the hydrolysis of 4-methylumbelliferone sulfate since this substrate has a V which is 60 times lower than nitrocatechol sulfate at pH 5.5 (Table III). The results of the kinetic experiment at pH 5.5 are shown in Fig. 1. It is evident that the initial velocity for the hydrolysis of 4-methylumbelliferone sulfate is much lower than that for the nitrocatechol sulfate.

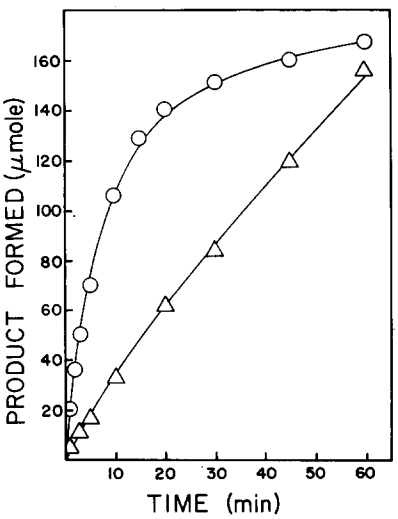

Fig. 1. Progress curves for the hydrolysis of nitrocatechol sulfate and 4-methylumbelliferone sulfate by aryl sulfatase A. The reactions were carried out at 37°C, I = 0.5 M at pH 5.5 using an enzyme concentration of 0.5 μg/ml. Upper curve (○———○), the anomalous kinetic behavior with 4 mM nitrocatechol sulfate plus 4 mM $BaCl_2$ (the decrease in turnover velocity with time is only slightly less pronounced in the absence of $BaCl_2$). Lower curve (△———△) the turnover reaction with 15 mM 4-methylumbelliferone sulfate.

Interestingly, this reduced velocity is also associated with an almost complete absence of the anomalous kinetic behavior during the hydrolysis reaction. Although aryl sulfatase A is significantly inactivated during the hydrolysis of nitrocatechol sulfate there is almost no detectable inactivation of the enzyme during the hydrolysis of comparable amounts of 4-methylumbelliferone sulfate. Fluharty and Edmond [41] have also observed such normal kinetic behavior in the case of the human liver enzyme during the hydrolysis of methylumbelliferyl sulfate. We suggest that this behavior can be easily rationalized on the basis that those substrates exhibiting the anomalous kinetic behavior are precisely those substrates (typically activated aryl derivatives) which would undergo decomposition by a transition state with a high degree of unimolecular character, equivalent to postulating a transition state involving sulfur trioxide as a potential leaving.group [14].

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