Biochimica et Biophysica Acta, 522 (1978) 443—451

BBA 68333

ENZYMATIC SULFATION OF BILE SALTS

II. STUDIES ON BILE SALT SULFOTRANFERASE FROM RAT KIDNEY

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(Received June 28th, 1977)

Summary

An enzyme system which catalyzes the transfer of the sulfate group from 3′-phosphoadenosine-5′-phosphosulfate to bile salts has been isolated and characterized from rat kidney. The enzyme is present in the cytosol fraction of kidney cells. It was purified by DEAE-Sephadex A-50, agarose-hexane-adenosine 3′,5′-diphosphate affinity chromatography and isoelectrofocusing electrophoresis. The apparent K_m values of the enzyme are $2 \cdot 10^{-6}$ M for 3′-phosphoadenosine-5′-phosphosulfate, and $4 \cdot 10^{-5}$ M for taurolithocholate. Sulfation occurred with conjugated as well as with unconjugated bile salts. The enzyme reacts with both primary bile salts (cholate, chenodeoxycholate and their conjugates), and secondary bile salts (lithocholate and its conjugates). The rates of reaction in decreasing order are monohydroxylated > dihydroxylated > trihydroxylated and glycoconjugates > tauroconjugates > unconjugates. The enzyme activity is inhibited by *p*-chloromercuri benzoate and iodoacetate indicating the possible requirement of a sulfydryl group for activity. A molecular weight of 80 000 was estimated by gel filtration techniques which is significantly smaller than the liver enzyme (130 000). The purified enzyme does not react with estrone or dihydroepiandrosterone.

Introduction

Lithocholate and its conjugates have been shown to cause hepatic damage in a variety of animal species [1]. Sulfation of bile salts has been implicated as a possible detoxification mechanism by increasing their renal clearances and fecal excretions [2,3]. Stiehl et al. [4] and Makino et al. [5] have shown that marked increase of urinary excretion of bile salt sulfate esters was found in patients with extra hepatic obstruction, hepatitis, cirrhosis and metastases. Therefore, urinary excretion of bile salt sulfates is an important mechanism for

the removal of these compounds under various patho-physiological situations. In our previous report we had identified an enzyme, bile salt sulfotransferase, which catalyzes the sulfation of bile salts in both the rat liver and kidney. Our earlier work also characterized this enzyme found in the rat liver tissue [6]. Additional work has shown that with increasing hepatic dysfunction in humans, the enzyme activity in the liver decreases [7]. Furthermore, by employing experimental cholestasis (complete bile duct ligation) in the rat, we observed that as the liver enzymatic activity decreased the activity in the kidney increased [8]. Therefore, the urinary bile salt sulfates may arise in part from de novo synthesis and excretion by kidney under pathological condition. The present paper describes the isolation and characterization of an enzyme from rat kidney responsible for the sulfation of bile salts.

Experimental Procedures

Materials

Non-radioactive bile salts were purchased from CalBiochem, La Jolla, Calif. and Supelco Inc., Bellefonte, Pa. [24-^{14}C]taurolithocholate and sodium [^{35}S]-sulfate were purchased from California Bionuclear Co., and New England Nuclear, respectively. 5-Cholenic acid-5β-ol and 5α-cholanic acid-3β-ol were obtained from Steraloids, Inc. (Wilton, N.H.). Adenosine 3′,5′-diphosphate (PAP), estrone sulfate and proteins used for the calibration of Sephadex G-100 chromatography column were obtained from Sigma Chemical Co. DEAE-Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia, Piscataway, N.J. Agarose-hexane-adenosine 3′,5′-diphosphate (PAP-agarose) was obtained from P-L Biochemicals, Inc., Milwaukee, Wisc.

Methods

Preparation of 3′-phosphoadenosine-5′-phosphosulfate (P-ado-P-S). *P*-ado-*P-S* was prepared according to the method of Gregory and Lipmann [9] with the exception that *P*-ado-*P-S* was separated from other nucleotides by paper electrophoresis. Paper electrophoresis was performed by streaking a 0.5 ml aliquot onto a 15 × 55 cm Whatman 3MM paper. Electrophoresis was carried out in 0.025 M potassium phosphate buffer (pH 5.8) for 1 h at 4°C with a voltage gradient of 45 V/cm. ^{35}S-labelled *P*-ado-*P-S* obtained from New England Nuclear was used as a standard. After electrophoresis, nucleotides were observed by ultraviolet light and the zone containing *P*-ado-*P-S* (the compound with the fastest mobility) was cut out and eluted with 0.5 ml cold water. Concentration of *P*-ado-*P-S* was measured by absorbance at 259 nm with a molar extinction coefficient of $14.7 \cdot 10^3$.

Radioactive *P*-ado-*P-S* was synthesized with ^{35}S-labelled sodium sulfate. The preparation usually had a specific activity of $1.2 \cdot 10^9$ cpm/μmol.

Preparation of bile salt sulfates. Sulfate esters of glycolithocholate and taurolithocholate were prepared by the method of Fieser [10]. Lithocholate sulfate was prepared according to the method of Mumma [11]. 3-α-Monosulfate of chenodeoxycholate was synthesized by the method of Haslewood and Haslewood [12] and 7-α-monosulfate was prepared according to the method of Summerfield et al. [13]. On thin-layer chromatography in a solvent system of

chloroform/methanol/acetic acid/water (65 : 24 : 10 : 5, v/v) the 3-α-monosulfate of chenodeoxycholate had an R_F value of 0.82 and 7-α-monosulfate had an R_F of 0.70. These compounds gave the same R_F values as the samples obtained from Professor Haslewood.

Assay of sulfotransferase. Bile salt sulfotransferase activity was measured as previously reported [6]. When the assay was performed with glycolithocholate, the substrate was made in methanol and the solution was evaporated to dryness prior to enzyme addition. The constituents and conditions were identical as described above. However, the thin-layer plate (silica G, Antech Inc.) was developed in a solvent system of chloroform/methanol/acetic acid/H_2O (65 : 24 : 10 : 5, v/v) for 1.5 h according to a modified system of Cass et al. [17].

Determination of protein. Protein concentrations were determined by the method of Lowry et al. [14] with bovine serume albumin as a standard. The method of Warburg and Christian [15] was used for the determination of protein concentrations of the purified enzyme.

Isoelectric focusing. The procedure was previously described in detail [6].

Gel electrophoresis. Gel electrophoresis was performed by a modification of the method of Ornstein and Davis [16]. The large-pore gel was substituted by layering 10 μl of the protein solution dissolved in 20% sucrose directly onto the running gel. The electrophoresis was carried out with an initial current of 0.5 mA/tube for 30 min and then increased to 5 mA/tube for 1 h.

Results

Isolation of the enzyme. All extractions and preparations were conducted at 4°C. Rat kidney (4 g) was removed and homogenized in 20 ml 0.25 M sucrose containing 5 mM Tris · HCl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol utilizing a Potter-Elvehjem homogenizer with a Teflon pestle. The supernatant was obtained by centrifuging the homogenate at 100 000 × *g* for 1 h in a Beckman Spinco L2B ultracentrifuge.

4 ml of the resulting solution was applied to a column (1.5 × 20 cm) of DEAE-Sephadex A-50 which had previously been equilibrated with 5 mM Tris · HCl (pH 7.5), containing 1 mM EDTA, and 10 mM β-mercaptoethanol. The column was washed with 20 ml of the same buffer followed by a 100 ml linear gradient of NaCl (0—0.5 M). Fractions of 2 ml were collected. A typical chromatogram is shown in Fig. 1. The fractions containing the major enzyme activity were combined and concentrated to one-fourth of the original volume by ultrafiltration with an Amicon apparatus. The solution was dialyzed against the starting buffer then placed on a PAP-agarose column (1.5 × 20 cm) which had been equilibrated with the same buffer. The enzyme was washed with 100 ml of the same buffer followed by a linear gradient of NaCl (0—1 M). As shown in Fig. 2, a small portion of the original activity was eluted out of the column during washing, and the majority of the activity was eluted off with 0.25 M NaCl. It does not appear that they were two separate entities, since reapplication of the first portion of the enzyme to the column was not washed out but eluted off with 0.25 M NaCl. The fractions containing the enzyme activity were combined, dialyzed, and subjected to isoelectric focusing. The peak of the enzyme

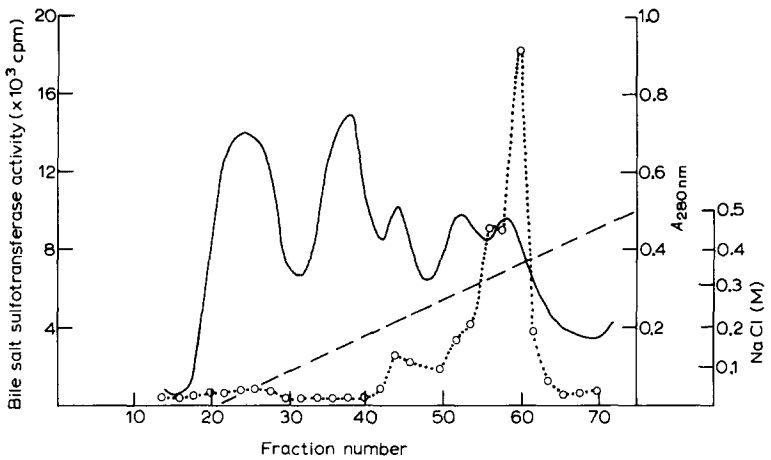

Fig. 1. Chromatography of bile salt sulfotransferase on DEAE-Sephadex A-50. Rat kidney cytosol (4 ml) was applied to a column (1.8 X 22 cm) of DEAE-Sephadex A-50. The column was equilibrated with 5 mM Tris · HCl buffer (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol. The column was washed with 20 ml of the same buffer followed by a 100 ml linear gradient of NaCl (0—0.5 M). Enzyme activity was assayed as described in the text except $1 \cdot 10^5$ cpm radioactive *P*-ado-*P*-*S* was used without cold carrier. Fractions of 2 ml were collected and monitored for protein and enzyme activity. o- - - - -o, enzyme activity; ———, absorbance at 280 nm; and - - - - - -, salt concentration.

activity corresponded to a pH value of 5.8 (Fig. 3). The fractions with the enzyme activity were pooled and dialyzed against the starting buffer and concentrated to 1/10 of the original volume by ultrafiltration. This enzyme preparation was used for the study of substrate specificity and subjected to polyacrylamide gel electrophoresis (one major and two minor protein bands were observed). A typical result of the enzyme purification is shown in Table I. An approximate 200-fold purification was achieved by this procedure.

General properties of the enzyme. In a typical enzyme assay the rate of tau-

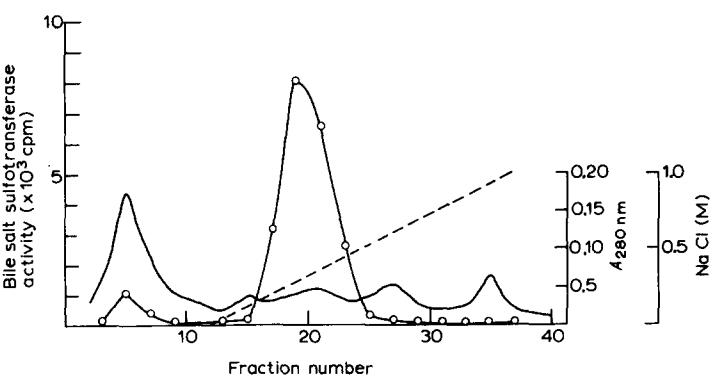

Fig. 2. Affinity chromatography of bile salt sulfotransferase on PAP-agarose. A 2.5 ml solution of the enzyme containing 7.5 mg of protein was applied to a column (2.0 X 10 cm) of PAP-agarose. The column was equilibrated with 5 mM Tris · HCl buffer (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol. The column was washed with 100 ml of the same buffer followed by a 200 ml linear gradient of NaCl (0—1.0 M). Enzyme activity was determined as described in Fig. 1. Fraction volume was 7.5 ml. o———o, enzyme activity; ———, absorbance at 280 nm; and - - - - - -, salt concentration.

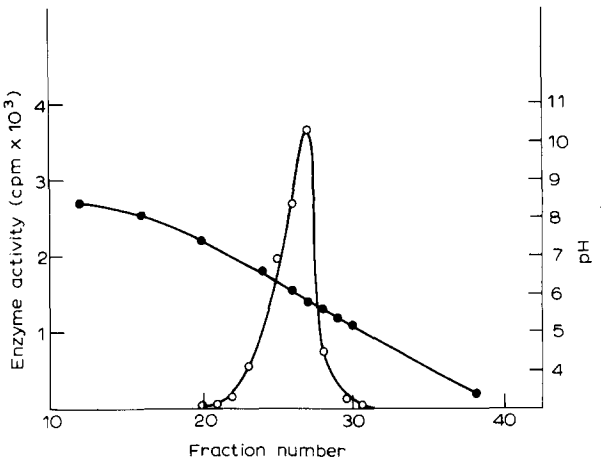

Fig. 3. Isoelectric focusing of bile salt sulfotransferase. Electrophoresis was performed utilizing ampholyte solution with a pH range of 3.5—10. Fractions (2 ml) were collected and assayed for pH (●———●) and enzyme activity (○———○).

rolithocholate sulfation was linear with respect to enzyme concentration (up to 0.4 mg of cytosol protein) and incubation time (up to 30 min). The Michaelis constants of the enzyme for taurolithocholate and 3′-phosphoadenosine-5′-phosphosulfate were $4 \cdot 10^{-5}$ and $2 \cdot 10^{-6}$ M, respectively. With a concentration of *P*-ado-*P*-*S* equal to $4 \cdot 10^{-4}$ M, the apparent K_m for glycolithocholate was $2 \cdot 10^{-5}$ M.

The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-100 column according to the method of Andrews [18]. The molecular weight estimated from known protein standards was 80 000.

Effects of various substances. Table II shows the effect of various compounds on the sulfation of bile salt. The enzyme was significantly inhibited by *p*-chloromercuribenzoate and iodoacetate indicating a possible requirement of a sulfhydryl group for enzyme activity. The activity was completely inhibited by adenosine 3′,5′-diphosphate whereas no inhibition was observed with a phenol concentration of $1 \cdot 10^{-4}$ M.

Substrate specificity of the enzyme. The rates of sulfation with various types of bile salts under standard assay conditions were investigated. The results are shown in Table III. Sulfation occurred with conjugated as well as with unconju-

TABLE I

PURIFICATION OF BILE SALT SULFOTRANSFERASE FROM RAT KIDNEY

One enzyme unit is defined as the amount of enzyme required to form 1 pmol of product per min under the assay condition described.

Stage	Volume (ml)	Activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Supernatant	4	1068	24.7	100	1
DEAE-Sephadex A-50	2.6	756	97	71	4
PAP-Agarose	1.5	549	572	51	23
Isoelectric focusing	1.7	250	4900	23	198

TABLE II

EFFECT OF VARIOUS SUBSTANCES ON THE INHIBITION OF BILE SALT SULFOTRANSFERASE ACTIVITY

The standard assay solution contained 10 nmol taurolithocholate, 5 μmol sodium phosphate buffer (pH 7.0), 50 nmol $MgCl_2$, 7.5 nmol 3′-phosphoadenosine-5′-phospho[^{35}S]sulfate ($2 \cdot 10^5$ cpm), and 1.4 μg of protein in a total volume of 0.1 ml.

Addition	Inhibition (%)	
	$1 \cdot 10^{-4}$ M	$1 \cdot 10^{-5}$ M
None	0	0
p-Chloromercuri benzoate	83	50
Iodoacetate	97	77
Adenosine 3′,5′-diphosphate	100	85
NaF	70	60
NaN_3	56	56
ATP	74	63
EDTA	30	—
Phenol	0	—

oxycholate) and secondary bile salts. The rates of sulfation in decreasing order are monohydroxylated > dihydroxylated > trihydroxylated. Furthermore, the enzyme did not react with bile acids with hydroxyl group in the β centrifugation. However, it was significantly inhibited by isolithocholate (Fig. 4).

When chenodeoxycholate was used as a substrate, a radioactive product was found in the same position as synthetic 7-α-monosulfates ester and no radioactivity was detected in the area corresponding to 3-α-monosulfate. No sulfation was observed when estrone or dehydroepiandrosterone was used as a substrate.

TABLE III

SULFATION OF VARIOUS TYPES OF BILE SALTS

Reaction mixtures contained 6 nmol 3′-phosphoadenosine-5′-phospho[^{35}S]sulfate ($5 \cdot 10^5$ cpm), 10 nmol bile salt, 5 μg of protein in a total volume of 0.1 ml. The solution was incubated for 10 min. The yield of ester sulfate was determined by autoradiogram after thin-layer chromatography as described in the text.

Substrate	pmol ester sulfate formed
Taurolithocholate	965
Taurochenodeoxycholate	435
Taurocholate	217
Glycolithocholate	955
Glycochenodeoxycholate	375
Glycocholate	177
Lithocholate	602
Chenodeoxycholate	252
5-Cholenic acid-3β-ol	0
5β-Cholanic acid-3β-ol (isolithocholate)	0

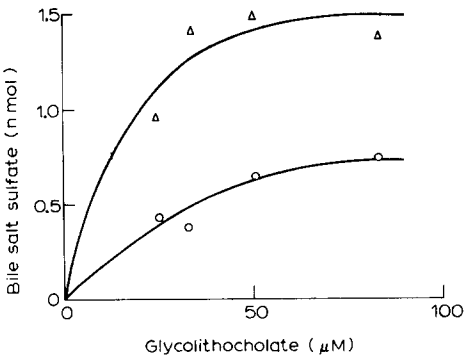

Fig. 4. Enzyme inhibition by isolithocholate. Enzyme (16 μg) activities were assayed in the absence (△———△) or presence of 0.12 mM isolithocholate (○———○) with varying concentrations of glycolithocholate and constant amount (0.06 mM) of ^{35}S-labelled *P*-ado-*P*-*S*.

gated bile salts, however, the rate was higher with conjugates than with unconjugates. The enzyme reacts with both primary bile salts (cholate and chenode-

Discussion

In our previous report [6], it was demonstrated that only liver and kidney had the ability to sulfate bile salts in the rat tissues examined. This paper described the method of isolation and properties of the enzyme from rat kidney. The existence of sulfate-activating systems (adenosine triphosphate sulfurylase, EC 2.7.7.4 and adenylylsulfate kinase, EC 2.7.1.25) have been demonstrated in rat kidney [19,20]. Farrell and McKahn [19] have synthesized 3′-phosphoadenosine-5′-phosphosulfate from cytosol of rat kidney. In addition, Rice et al. [20] showed that cholesterol could be sulfated by the kidney. Formation of sulfate ester of bile salts in isolated rat kidneys also has been demonstrated [13]. Therefore, sulfation of neutral and acid steroids may be a normal mechanism in the rat kidney.

Attempts to simplify the assay procedure such as employing $Ba(OH)_2$ precipitation to specifically remove unreacted *P*-ado-*P*-*S* as described by Wengle [21] were made, however, significant amounts (more than 80%) of sulfated bile salt was also precipitated out by this procedure. This is in accordance with the observation by Foldes and Meek [22] who showed that only 25—40% of sulfate esters of acidic phenolic compounds (homovanillic acid, dihydroxyphenylacetic acid and vanillylmandelic acid) remained in the supernatant fraction after $Ba(OH)_2$ precipitation. However, sulfate esters of neutral and basic phenolic compounds were not precipitated out. Methods employing charcoal to absorb unreacted *P*-ado-*P*-*S* or XAD-2 resin to specifically remove bile salt sulfates were also unsatisfactory.

There are several differences in bile salt sulfotransferase obtained from liver and kidney. In DEAE-Sephadex A-50 column chromatography, the liver enzyme was eluted out with approx. 0.2 M NaCl whereas the kidney enzyme required 0.3 M NaCl. Furthermore, the molecular weight of the kidney enzyme is smaller than the liver enzyme (80 000 vs. 130 000) as determined by Sepha-

dex gel filtration. A slight difference in isoelectric points between the two enzymes was also observed (5.3 for liver vs. 5.8 for kidney). Chemical and immunological comparison of bile salt sulfotranferase from rat kidney and liver are currently under investigation.

It has been suggested that renal clearance of bile salts depends on the polarity of the compounds [2]. The finding that bile salt sulfotransferase activity was more active toward less hydroxylated compounds may indicate the function of this enzyme is to introduce a polar group into a less polar substance for renal clearance. However, the rate of sulfation is more active toward conjugated bile salts than unconjugates. This may be due to decreased solubility of unconjugated compounds in the reaction mixture. Addition of albumin or ether to enhance the solubility of lithocholate did not increase the rate of sulfation.

The substrate specificity study of the enzyme showed that the enzyme did not react with compounds containing a hydroxyl group at the 3-β position. It is interesting to note that isolithocholate is not a substrate for the enzyme, but a potent inhibitor with a K_i of 0.026 mM. Similar observations were found with partially purified enzyme from rat liver (unpublished result). Norman and Palmer [23] demonstrated that about 50% of lithocholate was converted to isolithocholate in the body after oral administration of radioactive lithocholate. Although isolithocholate is less toxic and pyrogenic than lithocholate [23], increased isolithocholate concentrations in the body may indirectly augment the toxic effect of lithocholate by impairing its sulfation. Our results showed that chenodeoxycholate was mainly sulfated at the 7-α-position and lithocholate at the 3-α-position confirming the report by Summerfield et al. [13] using the isolated kidney perfusion technique. It is possible that the same enzyme possesses different specificity toward various types of bile salts, since we are unable to dissociate these two activities by electrophoresis or ion-exchange column chromatography techniques.

The existence of bile salt sulfotransferase activity in rat as well as human kidney (unpublished result), and a documented increase in sulfated bile salts appearing in the urine without concomitant increase in the serum bile acid sulfate concentration in pathological states [13], suggest that the renal sulfation mechanism plays a role in the detoxification and removal of these toxic compounds in altered states of hepatic function.

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