ARYL SULFOTRANSFERASE IN RAT LIVER: MULTIPLICITY AND SUBSTRATE SPECIFICITY

JUNKO NAKAMURA, TAKASHI MIZUMA, TOSHIHARU HORIE, MASAKIROS HAYASHI* AND SHOJI AWAZU

Department of Biopharmaceutics, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo, 192-03, Japan

(Received June 25, 1987)

Rat liver aryl sulfotransferase was purified by chromatography on diethylaminoethyl-cellulose or chromatofocusing and three fractions, referred to by Sekura and Jäckoby as I, II and IV, were obtained in the order of their elution, each containing sulfation activity. p-Nitrophenol (PNP) at mM order and β-naphthol were substrates common to all three fractions, but PNP at μM order and tyramine were substrates only for IV. IV corresponded to the enzyme designated M by Rein et al. and was active with monoamine, as predicted from our previous results with rat liver cytosol. However, the effectiveness of IV in bringing about the sulfation of PNP at mM order was not evident from our previous results. The characteristics of aryl sulfotransferase multiplicity on the basis of thermostability of sulfation activity could not be determined since essentially the characteristics were the same for all three purified fractions. The multiplicity of aryl sulfotransferase purified from rat liver was different from that of human platelets, indicating possible species and/or tissue differences in this enzyme.

Keywords — aryl sulfotransferase; rat liver; human platelet; p-nitrophenol; sulfate conjugation; multiplicity; substrate inhibition

INTRODUCTION

Sulfate conjugation is an important detoxication pathway for a variety of phenolic and catechol drugs and monoamines. For aryl sulfotransferase (EC 2.8.2.1) which catalyzes sulfation, multiple forms have been reported in mammalian tissues.1, 2 Mizuma et al. reported the following results for p-nitrophenol (PNP) sulfate conjugation in isolated rat liver cells and rat liver cytosol:3–5 (1) There is substrate inhibition in PNP sulfation when PNP was used at μM order followed by a second increase in sulfation activity when PNP was used at mM order. Thus, at least two types of aryl sulfotransferase may catalyze PNP sulfation. (2) One fraction which is effective for bringing about PNP sulfation at μM order is thermolabile but the other at mM order is not. Sekura and Jäckoby separated this enzyme into four fractions (I–IV) and determined their amino acid composition, optimum pH and substrate specificity.6, 7 Using β-naphthol as a representative substrate, I and II were found to have an optimal pH of 6–7 and IV, an optimal pH of 5.5. Furthermore, the values of the Michaelis-Menten constant (K_m) for PNP and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) at mM order of PNP in rat liver cytosol, obtained by Mizuma et al.,5 were very similar to those of I and II.6

Rein et al. found aryl sulfotransferase activity in human platelets, the jejunum, adrenal and brain and functionally separated this enzyme into two fractions, M which is active with monoamines and P which is active with phenol.2 The phenol derivatives, however, were confirmed to be substrates not confined to the P enzyme but serving as substrates for both enzymes.8 Reiter et al. partially purified aryl sulfotransferase from human platelets and separated it into the two fractions by ion exchange chromatography; one of which was thermolabile and active with PNP at mM order and monoamines, and the other, thermostable and active with PNP at μM order.8 Accordingly, the substrate specificity and thermostability for multiple forms of aryl sulfotransferase in rat liver cytosol and human platelets, differed considerably, thus indicating possible tissue and species differences for this enzyme.

In the present study, using the three fractions
separated by partial purification of rat liver aryl sulfotransferase, the characteristic relation between multiplicity and PNP sulfate conjugation, dependent on PNP concentration, was investigated in detail.

MATERIALS AND METHODS

Chemicals — The chemicals used in this study and the sources from which they were obtained are as follows: $^{35}$S-PAPS (1.0—1.5 Ci/mmol) from New England Nuclear, Boston, MA; bovine serum albumin (BSA, fraction V), dithiothreitol and tyramine from Sigma Chemical Co., St Louis, MO and Polybuffer 74 from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of reagent grade.

Preparation of Liver Cytosol — The livers of male Wistar rats (body weight, 300 g) were freshly excised after perfusing cold saline solution through the portal vein and homogenized in cold 0.154 M isotonic KCl solution using a Teflon-glass homogenizer to give a 25% (w/v) homogenate. The homogenate was centrifuged at 79000 × g for 20 min at 4 °C and the resulting supernatant fluid was further centrifuged at 100000 × g for 60 min at 4 °C. The final supernatant fluid was diluted with 0.062% BSA in 5 mM potassium phosphate buffer (pH 7.5) to give a cytosol fraction for conducting an assay of aryl sulfotransferase activity.

Purification of Aryl Sulfotransferase — Aryl sulfotransferase was partially purified from rat liver (male Wistar rat, 300 g) by the method of Sekura and Jakoby. The purification procedure was carried out at 4 °C as follows: (1) Collection of the supernatant fluid following centrifugation of the liver homogenates at 140000 × g and for 90 min (extraction), (2) chromatography on Affi-Gel Blue (Bio-Rad, 100 to 200 mesh), (3) salt precipitation with ammonium sulfate and (4) chromatography on diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman). Purification by chromatofocusing instead of DEAE-cellulose was also carried out as follows: a column (1 × 30 cm) of PBE 94 gel (Pharmacia) was equilibrated with 0.025 M imidazole-HCl buffer (pH 7.4) and Sephacore G-25 coarse (Pharmacia) was overlaid on the gel to a thickness of 1 cm. The protein solution obtained from step (3) with an ultrafilter (CF-25, Amicon) and 0.025 M imidazole-HCl buffer was added again and the concentration step repeated.

Finally, the protein solution was applied onto the gel column and the column eluted with Polybuffer (pH 4.0) at a flow rate of 30 ml/h.

For assay of the aryl sulfotransferase activity at each purification step, the protein concentration in the solution obtained from extraction and Affi-Gel Blue was diluted to obtain 0.25 protein μg/100 μl with 0.0625% BSA in potassium phosphate buffer. The fractions from DEAE-cellulose and chromatofocusing were similarly diluted 100-fold for the sulfation reactions of β-naphthol and PNP, and diluted 10-fold for sulfation reaction of tyramine.

Thermal Stability of Aryl Sulfotransferase — The thermal stability of aryl sulfotransferase activity in the purified fraction from DEAE-cellulose was examined by preincubation at different temperatures such as 37, 41 and 45 °C for 15 min as described before. The fraction which was maintained at 4 °C for thermal treatment was used as the control. After thermal treatment, each reaction system was immediately brought to 4 °C and then to 37 °C to initiate the reaction which then proceeded for 30 min.

Aryl Sulfotransferase Assay — Aryl sulfotransferase activity was measured by the method of Anderson and Weinshilbaum. Two hundred μl of the above enzyme solution and 20 μl of PNP (final concentration, 1 μM and 7.5 mM), β-naphthol (final concentration, 2 mM) or tyramine (final concentration, 2 mM) were introduced into glass tubes. The reaction was initiated by the addition of 100 μl of PAPS solution; potassium phosphate buffer (final concentration, 8.3 mM) and $^{35}$S-PAPS (final concentration, 0.4 μM; 0.4—0.6 μCi/ml) and dithiothreitol (final concentration, 8 mM). The final pH of the reaction medium was 6.8. After 30 min of incubation, the reaction was stopped by the addition of 100 μl of 0.1 M barium acetate and 100 μl of saturated barium hydroxide followed immediately by the addition of 100 μl of 0.1 M zinc sulfate to precipitate any excess barium. The mixture was centrifuged at 800 × g for 10 min, whereupon 100 μl of 0.1 M zinc sulfate were added to the supernatant fluid followed by centrifugation.

A 500 μl aliquot of the supernatant fluid was placed in 10 ml scintillation fluid (naphthalene 100 g, diphenyloxazol 4 g, dimethyl POPOP 0.04 g, dioxane 750 ml and toluene 150 ml) and radioactivity was measured in an Aloka 903
liquid scintillation counter. The reaction rate was previously found to be linear for 30 min of incubation under this experimental condition. All results were corrected for sample quenching and counting efficiency. The blank radioactivity originating from the remaining $^{35}$S-PAPS, slightly less than 0.1% of the initial amount of $^{35}$S-PAPS, was subtracted from the measured radioactivity. Enzyme activity was expressed as nmol sulfate formed per min of incubation at 37 °C and per mg of protein. The activity in the elution profile obtained by chromatography was shown as nmol sulfate formed per min and per unit volume (ml) of each fraction.

Identification of $^{35}$S-Sulfate — The radioactive product in PNP sulfation was identified by radio-high performance liquid chromatography (Shimadzu LC2A-Aloka RLCR 17-748) with a reversed phase C-18 column (JASCO octadecylsilane bonded silica, 4.6 × 250 mm). The mobile phase was a water and methyl alcohol (7:3 v/v) mixture containing 26 mg/ml tetraethylammonium bromide and 0.1 mg/ml potassium nitrate. The flow rate was 1 ml/min. For the mobile phase of the radioactivity detector, the scintillation fluid described above was used at a flow rate of 8 ml/min. The peak of PNP $^{35}$S-sulfate was identified using authentic PNP sulfate detected with a ultraviolet (UV)-monitor (Shimadzu, UVD-II, at 310 nm). Two peaks were obtained and identified as PNP $^{35}$S-sulfate conjugate (retention time, 12 min) and $^{35}$S-PAPS (retention time, 5 min). The peak area of the latter was constant for the most part, regardless of substrate concentration.

Protein Determination — Protein concentration was determined by the method of Lowry et al. using BSA as the standard.11)

Kinetic Analysis — $K_m$ values were obtained from a nonlinear least squares fit (MULTI program12) of the data to the Michaelis-Menten equation. The sulfation rates were linear at less than 1 μg protein in the reaction medium for 30 min of reaction time, confirming the rates obtained were initial rates.

RESULTS

The PNP sulfation activity in the liver cytosol before purification, as determined by the present assay method, is shown in Fig. 1. Substrate inhibition was found to occur at a lower concentration (μM order) of PNP followed by a second increase in sulfation activity at a higher concentration (mM order), as was also noted in the previous paper.8)

The enzyme elution pattern from a DEAE-cellulose column is shown in Fig. 2. Use of β-naphthol as the substrate resulted in the isolation of three fractions, each containing sulfation activity under a linear gradient of sodium salt (Fig. 2a). Sulfation activity for tyramine was found in the third fraction only (Fig. 2a). It is thus evident that the peaks in the elution order corresponded to fractions I, II and IV, as designated by Sekura and Jakoby.7) Although III may possibly be included in IV, it was not considered in the present study because it was present in small amounts and has not yet been characterized, as pointed out by Sekura and Jakoby.7) Elution profiles for these three fractions with sulfation activity at 7.5 mM PNP were exactly the same as those of β-naphthol. At 1 μM PNP, however, the main activity was found in the third fraction with β-naphthol and a very small activity in the second fraction (Fig. 2b).

Also, when subjected to chromatofocusing between pH 4 and 7.4, the three peaks for β-naphthol sulfation were obtained in the order of decreasing isoelectric points (Fig. 3). The separation of the three peaks appeared to be better than that for the three peaks obtained by DEAE-cellulose. The activity peaks for tyramine and PNP coincided with those for DEAE-cellulose.
FIG. 2. *Elution Profiles of the Multiple Forms of Aryl Sulfotransferase from a DEAE-Cellulose Chromatography*

Sulfotransferase activity was measured with 2 mM β-naphthol (○), 2 mM tyramine (■), 1 μM PNP (△) and 7.5 mM PNP (▲) as sulfate acceptors. Protein concentration is shown as absorbance at 280 nm (A_280, ●) and NaCl concentration gradient, as a broken line.

FIG. 3. *Elution Profiles of the Multiple Forms of Aryl Sulfotransferase from Chromatofocusing between pH 4 and 7.4*

All symbols are the same as those in Fig. 2.

Figures 4 and 5 show sulfation rates at 0.1 μM–10 mM PNP using the peak fraction of three active fractions obtained from DEAE-cellulose, fractions 211, 218 and 226, and from chromatofocusing, fractions 25, 34 and 49. Specific sulfation activity (nmol/mg protein/min) in the extract (step 1) was 0.2 at 1 μM PNP and 0.1 at 7.5 mM PNP (not shown in the Fig-
FIG. 4. PNP Sulfation for Aryl Sulfotransferase Fractions I (a), II (b) and IV (c) Purified and Separated by DEAE-Cellulose Chromatography

For I, II and IV, the peak fractions in Fig. 2 was used; fraction 211 for I, 218 for II and 226 for IV. Final protein amount in each fraction was 0.0067 μg in I, 0.027 μg in II and 0.034 μg in IV.

FIG. 5. PNP Sulfation for Aryl Sulfotransferase Fraction I (a), II (b) and IV (c) Purified and Separated by Chromatofocusing

For I, II and IV, the peak fractions in Fig. 3 was used; fraction 25 for I, 34 for II and 49 for IV. Final protein amount in each fraction was 0.011 μg in I, 0.014 μg in II and 0.26 μg in IV.

ures). Thus, each enzyme type was purified 30 to 60-fold at the lower PNP concentration and 40 to 130-fold at the higher PNP concentration. The lower activity in chromatofocusing was due to the use of samples preserved for two weeks before the final chromatography. Accordingly, aryl sulfotransferase was very unstable as shown by Sekura and Jakoby, but both methods gave separation of the active forms to a degree sufficient to examine substrate specificity. I and II showed sulfation activity for PNP when used at mM order but IV showed activity when PNP was used at both μM and mM orders. This biphasic activity for IV was quite similar to that in liver cytosol (Fig. 1). The minor activity of II at μM order was observed more clearly than that in Fig. 2b when it was expressed as activity per mg protein in each fraction (Fig. 4b).

Apparent K_m values for PNP obtained from sulfation rates at the PNP concentration at which substrate inhibition does not occur, as shown in Figs. 4 and 5, are listed in Table I. The K_m values (0.6–1.7 mM) for PNP sulfation at mM order appeared essentially the same for all three fractions and became greater than 1000 times that of IV (about 0.5 μM) at μM order.

Pretreatment at 37, 41 and 45 °C lowered the activity of I, II and IV at 7.5 mM PNP and that of IV at 1 μM PNP to basically the same extent (Fig. 6). That is, different thermostabilities in PNP sulfation at μM and mM orders were not observed following partial purification.
TABLE I. Apparent $K_m$ Values for PNP of Aryl Sulfotransferase Fractions I, II and IV, Measured at Low and High PNP Concentrations

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Apparent $K_m$ (mM) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low PNP Conc. (µM order)</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>—</td>
</tr>
<tr>
<td>IV</td>
<td>0.00058 ± 0.00011</td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>—</td>
</tr>
<tr>
<td>IV</td>
<td>0.00049 ± 0.00029</td>
</tr>
</tbody>
</table>

Values are means ± S.D.  $^a$ Obtained from the sulfation rates at a PNP concentration at which no substrate inhibition was observed (see Figs. 4 and 5).

DISCUSSION

The partial purification of aryl sulfotransferase from rat liver by DEAE-cellulose and chromatofocusing gave in each case three fractions, I, II and IV, all possessing β-naphthol sulfation activity (Figs. 2 and 3), as previously reported by Sekura and Jakoby. Although each of these fractions may still have slight contamination of other fractions, the present results indicate the following differences among these fractions: The activity for tyramine and PNP of µM order could be found only in IV (Figs. 2 and 3). Reiter et al. found that the fraction with monoamine sulfation activity in human platelets was capable of catalyzing PNP sulfation at mM order but not at µM order. This clearly demonstrates species and/or tissue differences in aryl sulfotransferase multiplicity in human platelets and rat liver. I and II showed PNP sulfation activity at mM order but not at µM order, and IV had biphasic activity at both µM and mM orders (Figs. 4 and 5). The minor activity of II at µM order was considered to be due to the slight contamination of IV (Figs. 2b and 4b). Thus, the capacity for sulfation at higher PNP concentration appears to be a property common to all three fractions of aryl sulfotransferase in rat livers (Figs. 4 and 5).

No fractional differences could be found in apparent $K_m$ values (0.6–1.7 mM) for PNP at mM order (Table I). The $K_m$ value (approximately 0.5 µM) at µM order for IV was about 1000 to 2000-fold smaller than that at mM order (Table I). The $K_m$ for I and II were essentially the same as those reported by Sekura and Jakoby (1.6 mM for I and 2.5 mM for II) but that for IV was nearly 300 times less than their reported value (170 µM). This difference in IV may be due in part to differences in the pH of the enzyme reactions. Their pH was 5.5 while that in the present study, 6.8. This possibility is also supported by data from our previous report in which substrate inhibition for PNP sulfation failed to occur in liver cytosol at pH 5.6 up to 200 µM PNP and the $K_m$ value at pH 5.6 (about 40 µM) exceeded that at pH 6.8, as shown in Table I (0.5–0.6 µM). The $K_m$ value for IV at µM order of PNP and those for the other fractions at mM order of PNP were quite similar to the corresponding values of the pool I and II in human platelets, being 0.38 µM and 1.5 mM, respectively. The results of kinetic analysis thus indicate that multiple forms of rat liver and human platelets have essentially the same affinity for PNP, in spite of differences in the monoamine sulfation activity described above.

We have already reported the sulfation activity for PNP used at µM order to be more thermolabile than that at mM order in liver cytosol. From this, IV, which effectively brings about PNP sulfation at µM order, appears to be thermolabile, but it has not actually been proven to be more so than the other fractions (Fig. 6). That is, the characteristics in aryl sulfotransferase multiplicity could not be determined on the basis of thermostability following purification of this
enzyme. The reason for the difference between liver cytosol and the purified system is not clear, but some endogeneous factor which is lost by the purification may determine thermostability.

Acetaminophen, an effective analgesic agent, was considered a possible substrate for IV at the therapeutic plasma level (μM order) since sulfation was inhibited in the presence of PNP of μM order in rat liver cytosol. Thus IV may possibly perform a most important function for sulfoconjugation in vivo. However, multiplicity of human liver aryl sulfotransferase remains to be clarified. Reiter and Weinshilboum have demonstrated that the relative levels of human platelet aryl sulfotransferase activity for PNP reflect individual variations in the urinary excretion of acetaminophen sulfation. They noted the correlation of sulfation activities in human platelets from PNP sulfation and the urinary excretion of acetaminophen sulfate to be statistically significant but there was also a certain degree of variability. Their results may be due to lack of correspondence of aryl sulfotransferase multiplicity in platelets and the liver as well as individual variation in acetaminophen absorption. Thus, for a more exact estimation of hepatic aryl sulfotransferase activity from platelets, species and tissue differences in multiplicity should be studied in greater detail.

Acknowledgements The authors wish to thank Miss Michi Nishiyama, Mr. Tokutaro Ito and Mr. Toshiyuki Sumida for their technical assistance.

REFERENCES