MULTIPLE FORMS OF ARYL SULFOTRANSFERASE FOR ACETAMINOPHEN SULFATE CONJUGATION IN RAT LIVER CYTOSOL

TAKASHI MIZUMA, HIROSHI ARAYA, MASAHIRO HAYASHI AND SHOJI AWAZU*

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

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The multiplicity of aryl sulfotransferase (phenol sulfotransferase, PST) in acetaminophen (APAP) sulfate conjugation was studied in rat liver cytosol. The sulfation rate showed the optimal pH of about 9 similar to that in the phenolic monoamine sulfation. And another optimal pH of about 6.4 was found at the higher APAP concentration such as 16 mM, suggesting the existence of the PST multiplicity in the APAP sulfation. The effect of thermal treatment at various temperatures, 37 to 41°C, showed that PST catalyzing the sulfation at the lower APAP concentration (about less than 1 mM) is more thermostable and has the lower $K_m$ for APAP than at the higher APAP concentration. The APAP sulfation at $\mu$M order APAP in the presence of $p$-nitrophenol (PNP) was shown to be decreased by the substrate inhibition of PNP to PST. Consequently it is considered the sulfation at the lower APAP concentration ($\mu$M order) is mainly catalyzed by the thermostable PST with the lower $K_m$ for APAP, and at the higher APAP concentration the thermostable PST with the higher $K_m$ partially contributes to the sulfation.

**Keywords** — acetaminophen; sulfate conjugation; rat liver cytosol; aryl sulfotransferase; multiplicity; thermostability; Michaelis constant

INTRODUCTION

Aryl sulfotransferase (phenol sulfotransferase, EC 2.8.2.1., abbreviated as PST in this paper) catalyzing the sulfation conjugation for many phenolic compounds has been shown to have multiple forms. Recently Rein et al.,1,2) and Reiter and Weinshilboum3) reported that there are two functionally different forms of PST, the thermostable M enzyme with the sulfation activity for monoamines such as dopamine and tyramine and the thermostable P enzyme for phenol in human platelet. Anderson et al. also reported that these two forms exist in other organs.4)

On the other hand, Sekura and Jakoby,5,6) and Duffel and Jakoby7) purified four types named as type I to IV from rat liver. In the previous paper,8) we studied the form of PST catalyzing $p$-nitrophenol (PNP) sulfate conjugation in rat liver cytosol fraction. Our conclusion is as follows: Type IV or M enzyme works at $\mu$M order PNP since the sulfation has the substrate inhibition and is thermostable at such low PNP concentration. Further $K_m$ for $3'$-phosphoadenosine-$5'$-phosphosulfate (PAPS) was in agreement with that in tyrosine methyl ester sulfation catalyzed by type IV.9) And PST catalyzing at mM order PNP was suggested to be thermostable P enzyme or the mixture of type I and II from $K_m$ for PAPS and PNP.

In the present study, the function of PST multiple forms was examined for the acetaminophen (APAP) sulfation in rat liver cytosol fraction. Namely, the effects of pH, thermal treatment and the coexistence of PNP on the APAP sulfation rates were studied. And further the selection of an endogeneous substrate for predicting individually the drug sulfation activity was

* To whom all correspondence should be addressed.
briefly discussed on such complexed multiplicity of PST.

MATERIALS AND METHODS

Chemicals and Reagents — APAP was purchased from Wako Pure Industries Co., Ltd. (Osaka, Japan). Acetaminophen sulfate (APAP-Sul) was synthesized according to the modified Burkardt and Wood’s method. All other chemicals and reagents were the same in the previous paper.

Preparation of Liver Cytosol Fraction — All procedures followed the method in the previous paper. The liver freshly excised from the Wistar male rat (250–300 g) was homogenized in 0.154 M cold KCl solution using Teflon-glass homogenizer to give 10% (w/v) homogenate. And 40% (w/v) homogenate was prepared with the high-speed homogenizer (Physcotron Homogenizer, Nichion Irikasesiakusho, Tokyo, Japan). The homogenate was centrifuged at 10000 × g for 10 min and the resultant supernatant was further centrifuged at 105000 × g for 60 min. The final supernatant was used under the reaction conditions in the following section.

PST Reaction Conditions — (i) pH Profile in PST Reaction of APAP: To 0.25 ml of the cytosol fraction prepared from 40% (w/v) homogenate incubated at 37°C for 5 min, 2 ml of APAP buffer solution (pH 5.1–9.2), and 0.25 ml of 200 μM PAPS buffer solution (0.4 M Tris-HCl buffer of pH 7.4) generated according to Kempen and Jansen, were added simultaneously. Thereafter the mixture was incubated at 37°C for 5 min. The buffer solutions containing APAP were 0.1 M sodium acetate-acetic acid for the reaction at pH 5.1–7.3 and 0.4 M Tris-HCl buffer for that at pH 7.1–9.2. The final pH values were measured after preparing the reaction medium.

(ii) Thermostability of PST: After 0.25 ml of cytosol fraction from 40% (w/v) homogenate was first preincubated at 37, 39, 41 or 43°C for 15 min, the PST reaction was carried out at 37°C and pH 7.4 in the presence of 20 μM PAPS as described in (i). For the control, the cytosol fraction stored in ice-water (0°C) was used in the PST reaction as described in (i) without pretreatment at the above various temperatures.

(iii) Effects of Thermal Treatment on K_m Values for the PST Reactions: The cytosol fractions from 10% (w/v) homogenates were diluted two-fold with 0.4 M Tris-HCl buffer (pH 7.4), incubated at 37, 39 or 41°C as described in (ii) and then put in ice-water (0°C). Thereafter to 1 ml of the cytosol fraction, 1 ml of APAP buffer solution (0.4 M Tris-HCl, pH 7.4) containing 1.2 mM KH_2PO_4 and 2.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 ml of 150 μM PAPS buffer solution were added and the mixture was incubated at 37°C for 5 min. K_m values were calculated by the nonlinear least square method (Gauss-Newton method).

(iv) APAP Sulfate Conjugation in the Presence of PNP: After 1 ml of the cytosol fraction from 10% (w/v) homogenates diluted eight-fold with 0.4 M Tris-HCl buffer (pH 7.4) was incubated at 37°C for 5 min, 1 ml of the mixed solution of APAP and PNP containing 1.3 mM KH_2PO_4 and 2.5 mM EDTA and 0.5 ml of 200 μM PAPS were added to the fraction and the mixture was incubated at 37°C for 5 min.

Assay — After 0.5 ml of the incubation mixture for APAP sulfation was added into 0.1 ml of 25% (v/v) HClO_4 including 1.79 mM p-fluorophenol as an internal standard (I.S.) to stop the reaction, 0.2 ml of 0.8 M BaCl_2 was mixed and the mixture was centrifuged at 3000 rpm for 10 min. Forty to 90 μl of the supernatant was applied for reversed phase high performance liquid chromatography (HPLC) assay. The mobile phase was composed of water-methanol-acetic acid (79.5: 19.0:1.5 v/v/v) containing 100 mg/l KNO_3 and 26 mg/l tetrabutylammonium bromide as an ion pair agent. Ultraviolet absorption was measured at 250 nm. The chromatographic system was the same described in the assay of PNP conjugated metabolites. The capacity factors (k') of APAP sulfate and I.S. were 7.3 and 10.8, respectively. For the APAP sulfation in the presence of PNP, only the mobile phase was slightly changed to
water-methanol-acetonitrile-acetic acid (80.0:16.0:2.5:1.5 v/v/v/v) containing 50 mg/l KNO₃ and 45 mg/l tetrabutylammonium bromide to avoid the interference from PNP and its conjugates. The capacity factors \((k')\) of APAP sulfate and I.S. were 6.7 and 10.4, respectively. And the assay of PNP sulfate followed the HPLC method reported by Mizuma \textit{et al.}^{12b)}

RESULTS

Effect of pH on APAP Sulfate Conjugation

The pH profiles of APAP sulfate conjugation rates at 0.1, 1 and 16 mM APAP were obtained at pH 5.1 to 9.2 (Fig. 1). Although the effects of buffer components were observed between Tris-HCl buffer (open symbol) and acetate buffer (closed symbol), the APAP sulfation rates increased with pH and attained the maxima at about pH 9 for all APAP concentrations tested except a slight intermediate maximum at pH 6.4 for 16 mM APAP.

Thermostability of PST

As shown in Fig. 2, all the remaining activities of PST after preincubation at 37, 39, 41 and 43°C were the highest in 16 mM APAP and the lowest in 0.1 mM APAP. The result shows that the PST reaction is more thermolabile by lowering the APAP concentration.

Effect of Thermal Treatment on \(K_m\) Values for APAP

Double reciprocal plots of APAP sulfation rate (pH 7.4) versus APAP concentration of 0.05 to 16 mM in the cytosol fraction preincubated at 37, 39 and 41°C were shown in Fig. 3 together with the results in control experiment. The plots in control, 37 and 39°C-treatment were convex. Then, approximating the linearity at less than 0.2 mM APAP and more than 1 mM APAP, each \(K_m\) value for APAP was obtained as shown in Table I. In 41°C-treatment, although the linearity was observed at more than 0.2 mM APAP where the sulfation rates were detectable, \(K_m\) was calculated from the data at more than 1 mM APAP in the same manner as above (Table I). The \(K_m\) value obtained in the higher APAP concentration region in control, 37 and 39°C-treatment, were approximately twice as large as that in the lower region. And the 41°C-treatment gave a little higher \(K_m\).

FIG. 1. Acetaminophen (APAP) Sulfation Rate in Cytosol Fraction of Rat Liver versus pH at Various APAP Concentrations

\(\bigcirc\) \(\bullet\), 0.1 mM APAP; \(\square\) \(\blacksquare\), 1.0 mM APAP; \(\triangle\) \(\Delta\), 16 mM APAP. Open symbols and solid symbols indicate the sulfation in the Tris-HCl buffer and acetate buffer, respectively. The PAPS concentration is 20 \(\mu\)M. Data represent typical one of five experiments.

FIG. 2. Thermostability of Acetaminophen (APAP) Sulfation Rate (pH 7.4) in Cytosol Fraction of Rat Liver at Various APAP Concentrations

\(\bigcirc\), 0.1 mM APAP; \(\square\), 1.0 mM APAP; \(\triangle\), 16 mM APAP. The PAPS concentration is 20 \(\mu\)M. Data represent mean \(\pm\) S.E. of three experiments.
**APAP Sulfation in the Presence of PNP**

The APAP sulfation rates at 0.5 mM APAP in the presence of PNP of various concentrations were shown in Fig. 4. The PNP sulfation rates in the presence and absence of 0.5 mM APAP were depicted together. The APAP sulfation rates were remarkably decreased with increase in the PNP concentration and were less than detectable level in the presence of more than 50 μM PNP. On the other hand, neither the PNP sulfation rate nor substrate inhibition at more than 10 μM PNP was significantly affected by 0.5 mM APAP.

**DISCUSSION**

The pH optimum of about 9 in the APAP sulfation (Fig. 1) is in agreement with the reported optimal pH of 9.2 to 9.4 for tyramine sulfation and pH 9.0 for adrenaline sulfation in liver cytosol by Wong,13,14 and pH 8.0 for L-tyrosine methyl ester sulfation using purified PST (type IV) by Sekura and Jakoby.6 Accordingly since the APAP sulfation has the pH optimum similar to phenolic monoamine sulfation, the PST for the APAP sulfation can be suggested to be M enzyme1−3 or type IV5,6 which is active for monoamine sulfation.

On the other hand, in the PST reaction when increasing the APAP concentration to 16 mM, another pH optimum appeared at 6.4. Similar phenomenon is found in 4-hydroxybiphenyl

**TABLE I. K_m (mM) for Acetaminophen (APAP) in APAP Sulfation after Preincubation at Various Temperatures**

<table>
<thead>
<tr>
<th>APAP concentration region</th>
<th>Preincubation temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lower</td>
<td>0.167 ±0.014 b)</td>
</tr>
<tr>
<td>Higher</td>
<td>0.367 ±0.020 c)</td>
</tr>
</tbody>
</table>

a) APAP concentration region indicating linearity of double reciprocal plots in Fig. 3 was divided into lower and higher region.

b) Calculated from three data points in the lower concentration region (less than 0.2 mM APAP).

c) Calculated from three data points in the higher concentration region (more than 1 mM APAP).

d) Not calculated.

Data represent mean ± S.E. of three experiments.
sulfation using guinea-pig intestinal cytosol fraction by Dawson and Bridges regardless of the differences in species and organs.\textsuperscript{15} who presented the existence of PST multiplicity based on the result that optimal pH is 8.0 at 5 μM substrate and 7.0 and 8.5 at 500 μM. Consequently it was also suggested that the different form of PST works at the higher APAP concentration such as 16 mM in addition to the active form in the lower concentration.

The remaining activity of PST after preincubation at 37 to 43°C shows that PST working at the lower APAP concentration (less than 1 mM) is more thermolabile (Fig. 2). And $K_m$ for APAP obtained by the preincubation at 0°C (control), 37 and 39°C show that PST with the lower $K_m$ catalyzes the APAP sulfation below about 0.2 mM APAP but PST with the higher $K_m$ above about 1 mM APAP (Fig. 3 and Table I).

The results that the APAP sulfation was remarkably inhibited by PNP while the PNP sulfation was not inhibited even by APAP of 400-fold concentration at the lowest PNP concentration (1.25 μM) (Fig. 4) suggest that the inhibition in the APAP sulfation was not due to the competitive consumption of PAPS but due to the substrate inhibition by PNP.\textsuperscript{8} Then the APAP sulfation at 0.5 mM APAP is considered to be mainly catalyzed by M enzyme or type IV, which has been shown to have substrate inhibition of PNP.\textsuperscript{8} Similar inhibition by PNP was reported in the harmol sulfation\textsuperscript{17} with the optimal pH between 9.0 and 9.6\textsuperscript{18} without the detailed discussion. Our consideration are supported by Mattock et al.’s results where the sulfation rates of L-tyrosine methyl ester and tyramine catalyzed by partially purified PST (M enzyme) were inhibited by PNP.\textsuperscript{16}

Sekura and Jakoby reported that $K_m$ for the APAP sulfation at pH 6.5 was 2.1 mM for type I and 1.6 mM for type II.\textsuperscript{9} These values are larger than our $K_m$ (0.332 mM) at the higher APAP concentration (more than 1 mM) and at pH 7.4 (Table I). These differences may be partly due to the different pH of the reaction medium as shown in Fig. 1 and also suggest that type IV (or M enzyme) with the lower $K_m$ actually works together with type I and/or type II (or P enzyme) at the higher APAP concentration. This suggestion coincides with the result from the thermal treatment in our study. Namely $K_m$ (0.565) in 41°C-treatment was a little higher than that in the treatments at lower temperatures (Table I), suggesting that the contribution of the thermolabile PST with the lower $K_m$ (M enzyme or type IV) became smaller.

Consequently our result on the multiplicity of PST catalyzing the APAP sulfation in rat liver cytosol agrees with the report that in human platelet, APAP is a substrate predominantly for M enzyme and also for P enzyme.\textsuperscript{19} On the other hand, we reported that PNP is mainly sulfated by type IV (M enzyme) at μM order but not sulfated by this type at such high PNP concentrations as mM order.\textsuperscript{8} These differences between PNP and APAP sulfation suggest that PST has a substrate-dependent activity.

In clinical pharmacokinetics, it is valuable to estimate previously the drug metabolism rates in

![Graph](https://example.com/graph.jpg)

**FIG. 4.** Acetaminophen (APAP) and p-Nitrophenol (PNP) Sulfation Rates (pH 7.4) in Cytosol Fraction of Rat Liver under Coexistence of APAP and PNP

- □, APAP sulfation rates at 0.5 mM APAP in the presence of various PNP concentrations; ●, PNP sulfation rates in the presence of 0.5 mM APAP; ○, PNP sulfation rates in the absence of APAP.

The PAPS concentration is 40 μM. Data represent mean ± S.E. of three experiments.
Multiplicity of Aryl Sulfotransferase

each individual man using endogeneous index compounds such as 6β-hydroxycortisol in oxidation\(^\text{20}\) and 17-hydroxycorticosteroids glucuronide in glucuronidation.\(^\text{21}\) However, such index compound for sulfation has not been found. Considering that the sulfation of an endogeneous monoamine such as tyramine is catalyzed only by M enzyme or type IV,\(^b\) and the APAP sulfation at the therapeutic APAP blood level (μM order) is also catalyzed by the same form of PST, it is suggested that an endogeneous monoamine sulfate may be a candidate as an index compound for drug sulfation.

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REFERENCES


