Isolation and Characterization of an Alicyclic Amine N-Sulfotransferase from Female Rat Liver

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An alicyclic amine N-sulfotransferase sulfonating 4-phenyl-1,2,3,6-tetrahydropyridine (PTHP) was purified from female rat liver cytosol and showed a homogeneous band with a molecular weight of 30500 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The enzyme, designated NST-1, catalyzed sulfonation not only of the alicyclic amine but also dehydroepiandrosterone, a typical substrate of hydroxysteroid sulfotransferases (STs), but had little sulfonating activity towards 2-naphthol, a typical substrate of aryl STs. The N-terminal amino acid sequence for the first 24 residues had a high homology with those of rat liver hydroxysteroid STs. Therefore, it is suggested that NST-1 is classified as a member of the hydroxysteroid ST. Immunoblot analysis of male and female rat liver cytosol, carried out by using rabbit antisera raised against NST-1, indicated that the female cytosol contained a higher level of the enzyme than that of male. The marked sex difference in the expression level of NST-1 was in good accordance with the previous demonstration that female rat liver cytosol catalyzed sulfonation of PTHP to a greater extent than that of male.

Keywords: rat liver cytosol; sulfotransferase; alicyclic amine; dehydroepiandrosterone

In mammals, sulfotransferase (ST) plays an important role in the excretion of a wide variety of phenols, enols, alcohols and amines by transformation into their hydrophilic O- and N-sulfonates in the presence of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a co-factor. The enzyme has been classified into three groups; aryl, hydroxysteroid and amine STs, according to their distinct catalytic activities towards the corresponding xenobiotic and endogeneous substances.1–3) Much work has been done on the purification and characterization of phenol and hydroxysteroid STs. Recently, an amine N-sulfotransferase sulfonating arylamines was purified from guinea pig liver,4) however, there is little information as to the molecular origin and characterization of amine STs.

We have investigated the metabolism of tiaramide, a drug with anti-inflammatory, analgesic and antipyretic actions, and identified a novel sulfonjugate, namely, the N-sulfonate of the alicyclic amine in N-deethanolated tiaramide (DETR).5) We have also studied the incorporation of the sulfogroup from PAPS into various alicyclic amines6) and reported a marked sex difference (female >> male) in the sulfonation of alicyclic amines.7)

In this study, we purified an alicyclic amine N-sulfotransferase sulfonating 4-phenyl-1,2,3,6-tetrahydropyridine (PTHP) from female rat liver cytosol and characterized its enzymatic properties.

MATERIALS AND METHODS

Materials: Reagents and chemicals used in this work were obtained from the sources as follows: PTHP (Aldrich Chemical Co., Milwaukee, W1, U.S.A.). Tiaramide hydrochloride, DETR, desipramine hydrochloride (Fujisawa Pharmaceutical Co.). Aniline hydrochloride (Nakarai Tesque, Kyoto, Japan), 2-Naphthol, 2-naphthyl sulfate (Sigma Chemical Co., St. Louis, MO, U.S.A.).[14C]Dehydroepiandrosterone (New England Nuclear Corp., Boston, MA, U.S.A.). PAPS was synthesized according to the method of Sekura and Jakoby7) and Jakoby et al.8) Aniline N-sulfonate, desipramine N-sulfonate and PTHP N-sulfonate were synthesized as potassium salts according to the method of Boyland et al.9) Tiaramide O-sulfate and DETR N-sulfonate were synthesized as described previously.10,11) The following materials were used for chromatography: DEAE-cellulose DE-52 (Whatman, Maidstone, England), 3'-phosphoadenosine 5'-phosphate (PAP)-agarose (Sigma), hydroxylapatite–HPLC, KB-column (Koken, Tokyo, Japan).

Enzyme Assay: PTHP, tiaramide, DETR, 2-naphthol, desipramine, and aniline sulfonation were determined by HPLC as reported previously.7) Dehydroepiandrosterone sulfonation was determined by the method of Marcus et al.12)

Purification of Alicyclic Amine N-Sulfotransferase: All purification steps were carried out at 4°C except for step 4 in the following sequence.

Step 1. Cytosol Preparation: Sprague-Dawley 8-week-old female rats were killed by decapitation. Livers were removed and homogenized in buffer A (10 mM Tris–HCl (pH 7.4), 0.25 mM sucrose, 3 mM 2-mercaptoethanol). The homogenate was centrifuged at 9000 x g for 30 min and the supernatant was recentrifuged at 105000 x g for 1 h, and the resultant supernatant was used as the cytosol fraction.

Step 2. DEAE-Cliprone Exchange Chromatography: A DE-52 column (5 x 40 cm) was equilibrated with buffer A. The cytosol was applied and washed with 1.5 l of the same buffer, followed by linear gradient formed from 1.5 l of buffer A and 1.5 l of 0.3 M KCl in buffer A. The fractions containing enzyme activity were pooled, and concentrated by precipitating the enzyme with solid ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation at 10000 x g for 20 min. The
pellet was dissolved in a minimum volume of buffer B (50 mM Tris-HCl (pH 7.4), 0.25 mM sucrose, 3 mM mercaptoethanol), and dialyzed against a large excess of the same buffer overnight.

Step 3. PAP-Agarose Chromatography: The dialyzed fraction was loaded onto a column (1 x 25 cm) packed with PAP-agarose equilibrated with buffer B and eluted with 60 ml buffer B, followed by a linear KCl gradient established between 200 ml of buffer B and 200 ml of 20 mM adenosine diphosphate (ADP) in buffer B. The active fractions were pooled, concentrated by ultrafiltration through an Amicon YM10 membrane and dialyzed against buffer C (10 mM potassium phosphate (pH 6.8), 0.25 mM sucrose, 3 mM 2-mercaptoethanol).

Step 4. Hydroxylapatite-HPLC: The dialyzed fraction was loaded onto a hydroxylapatite-HPLC (KB-column, Type S, 0.6 x 10 cm). After washing with 30 ml of buffer C, the column was treated with a gradient of 60 ml buffer C and 60 ml 700 mM potassium phosphate with sucrose and 2-mercaptoethanol as above at a flow rate of 1 ml/min. The active fractions were pooled, concentrated by ultrafiltration through an Amicon YM10 membrane and dialyzed against buffer A.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)** The fraction from hydroxylapatite-HPLC was subjected to 15% SDS-PAGE as described by Laemmli.

**Antibody Preparation and Immunoochemical Quantitation** A New Zealand white rabbit (2.5 kg, male) was immunized intradermally with 60 µg of the purified protein in complete Freund’s adjuvant and boosted with 25 µg of the protein subdermally 4 weeks later. After an additional week, antisera were obtained and kept at -80°C until used. For Western blotting, 50 µg of the cytosol protein was electrophoretically transferred to a nitrocellulose sheet (Bio-Rad) and probed with antibody (1:200 dilution).

**NH2-Terminal Amino Acid Sequence Analysis** Edman degradation of the purified protein was carried out using an Applied Biosystems model 470A gas phase sequence analyzer.

**RESULTS**

**Isolation and Purification of Sulfotransferase Sulfonating PTHP** The PTHP sulfonating activity of female rat liver cytosol eluted as one major peak on a DE-52 column to which the cytosol had been directly applied (Fig. 1). The major peak of activity was subjected to PAP-agarose affinity column chromatography after salt precipitation. The PTHP-sulfonating activity was partly retained on the PAP-agarose column and eluted as a single peak with a linear gradient of ADP (Fig. 1). The concentrated active fraction from the above step was subjected to hydroxylapatite-HPLC. The application of a linear gradient of potassium phosphate from 10—700 mM on the hydroxylapatite-HPLC resulted in the effective purification of the enzyme (Fig. 1). The purified enzyme, designated NST-1, was obtained as a homogeneous protein (M.W. 30500 by SDS-PAGE) (Fig. 2) in 2% yield from female rat liver cytosol, with a purification factor of 52.5-fold (Table I).  

**TABLE I. Purification of Aliphatic Amino N-Sulfotransferase**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Protein (mg)</th>
<th>Total activity (nmol/min)</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Factor</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>8690</td>
<td>39182</td>
<td>4.5</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DE-52</td>
<td>2501</td>
<td>20727</td>
<td>8.3</td>
<td>1.8</td>
<td>52.9</td>
</tr>
<tr>
<td>Salt precipitation</td>
<td>1539</td>
<td>17404</td>
<td>11.5</td>
<td>2.5</td>
<td>44.4</td>
</tr>
<tr>
<td>PAP-agarose</td>
<td>46.5</td>
<td>4093</td>
<td>88.0</td>
<td>19.6</td>
<td>10.4</td>
</tr>
<tr>
<td>Hydroxylapatite-HPLC</td>
<td>3.3</td>
<td>779</td>
<td>236</td>
<td>52.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The activity was assayed with 100 mM glycine-NaOH buffer (pH 10) containing 0.2 mM PAPS and 2 mM PTHP.

**Substrate Specificity** A range of amines and non-amine compounds were tested as possible acceptor substrates for the purified enzyme (Table II). The data in Table II were obtained at two pH values because of the pattern observed for the optimum of the enzyme. The activity towards two aliphatic amines (PTHP and DETR), was higher at pH 10 than at pH 7.4. NST-1 catalyzed...
sulfonation not only of the alicyclic amines but also dehydroepiandrosterone. However, it had little sulfonating activity toward 2-naphthol, desipramine and aniline. The apparent kinetic parameters obtained for the enzymatic sulfonation of PTHP were as follows: $K_m$ 382 μM and $V_{max}$ 278 nmol/mg of protein/min.

**NH$_2$-Terminal Amino Acid Sequence** The results of the NH$_2$-terminal amino acid sequence analysis of NST-1 is shown in Fig. 3. The NH$_2$-terminal amino acid sequence of NST-1 exhibited a high homology with those of ST$_a^{14}$ and BAST I.$^{15}$ The sequence differed by two (2nd and 12th) and by four (2nd, 12th, 17th and 23rd) amino acids from those of ST$_a$ and BAST I, respectively.

**Sex Difference in Hepatic Level of NST-1** Immunoblot analysis of male and female rat liver cytosol was performed using rabbit polyclonal antibodies raised against purified NST-1, in order to obtain direct evidence as to whether the enzyme played an important role in the previously demonstrated sex difference (female ≪ male). The male and female rat liver cytosols contained a M.W. 30500 protein, corresponding to NST-1, and there was a marked difference in the cystosolic levels of NST-1 between female and male rat livers (female ≪ male) (Fig. 4). Other immunoreactive proteins were also observed in both cytosols.

**DISCUSSION**

We report here the purification of a form of female rat liver sulfotransferase conjugating PTHP. Purification of an alicyclic amine N-sulfotransferase was performed by a three-step procedure. NST-1 had a higher affinity towards PAP-agarose and hydroxylapatite-HPLC than the other alicyclic amine N-ST isozymes (Fig. 1).

NST-1 catalyzed sulfonation not only of the alicyclic amines but also the endogenous steroid dehydroepiandrosterone, which is a typical substrate for rat liver hydroxysteroid ST.$^{1,2}$ NST-1 had little sulfonating activity towards 2-naphthol, which is a typical substrate for rat liver aryl ST.$^{1,2}$ The aryl STs have been demonstrated to be unable to catalyze sulfonation of endogenous steroids.$^{1,2}$ Therefore, NST-1 could be an isozyme of hydroxysteroid ST. Thus, this study provides the first direct evidence that the hydroxysteroid ST participates in enzymatic alicyclic amine N-sulfonation. The purified enzyme exhibited no activity toward an alkylamine (desipramine) and an arylamine (aniline). These amines might be catalyzed by other isozymes. Recently, an amine N-sulfotransferase sulfonating 2-naphthylamine was purified from guinea pig liver. However, this purified enzyme catalyzed not only of arylamines but also alicyclic amines. These results suggest that this purified enzyme has different characteristics from NST-1.

Recently, Ogura et al. reported the N-terminal amino acid sequence of ST$_a$ and BAST I.

![Fig. 3. Comparison of NH$_2$-Terminal Amino Acid Sequences of NST-1, ST$_a$ and BAST I](image)

In the sequence of NST-1, "X" indicated an unidentified amino acid. The sequences of ST$_a$ and BAST I have been reported by Ogura et al. and Barnes et al., respectively.$^{14,15}$

**TABLE II. Substrate Specificity of Purified NST-1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (nm)</th>
<th>Activity of NST-1 (nmol/mg protein/min)</th>
<th>pH 7.4</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTHP</td>
<td>2.0</td>
<td>44</td>
<td>212</td>
<td></td>
</tr>
<tr>
<td>Tiaramide</td>
<td>5.0</td>
<td>120</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>DETR</td>
<td>5.0</td>
<td>18</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Desipramine</td>
<td>0.5</td>
<td>N.D.</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Aniline</td>
<td>5.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2-Naphthol</td>
<td>0.05</td>
<td>5.1</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0.05</td>
<td>391</td>
<td>368</td>
<td></td>
</tr>
</tbody>
</table>

The activity was assayed with 100 mM phosphate buffer (pH 7.4) or 100 mM glycine–NaOH buffer (pH 10) containing 0.2 mM PAPS and the indicated concentration of substrates. N.D.; not detected (<0.4 nmol/mg protein/min).
acid sequence of the hydroxysteroid sulfotransferase STa\(^{16}\) and the isolation of the cDNA screened with rabbit anti-serum raised against STa.\(^{14,17}\) Barnes et al. also reported the N-terminal amino acid sequence of a bile acid ST, named BAST1, which catalyzed not only the sulfonation of the 3-hydroxy groups of a variety of bile acids and their precursors but also the sulfonation of dehydroepiandrosterone.\(^{15}\) These N-terminal amino acid sequences have a high degree of homology with that of NST-1. This data is further evidence that NST-1 is an isozyme of hydroxysteroid ST, but NST-1 differs from the other isozymes in N-terminal to 24th amino acid residue. Our results indicate that we have isolated a previously unknown form of hydroxysteroid ST. We plan to isolate a cDNA of NST-1 to establish that it is a new isoform of hydroxysteroid ST.

PTHP was sulfonated approximately twenty-five times more effectively in female rat liver cytosol than in male liver cytosol.\(^{17}\) A similar difference was observed for dehydroepiandrosterone ST activity.\(^{1,2,18}\) Immunoblot analysis suggests that female rat liver cytosol contains a higher level of NST-1 than that of male rat liver cytosol. This is reflected in the sex differences of PTHP and dehydroepiandrosterone ST activity.

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REFERENCES