Bile acid sulfotransferase I from rat liver sulfates bile acids and 3-hydroxy steroids: purification, N-terminal amino acid sequence, and kinetic properties

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Abstract  A bile acid:3-phosphoadenine-5'-phosphosulfate:sulfotransferase (BAST I) from adult female rat liver cytosol has been purified 157-fold by a two-step isolation procedure. The N-terminal amino acid sequence of the 30,000 subunit has been determined for the first 35 residues. The $V_{max}$ of purified BAST I is 18.7 nmol/min per mg protein with N-(3-hydroxy-5β-cholan-24-oyl)-glycine (glycolithocholic acid) as substrate, comparable to that of the corresponding purified human BAST (Chen, L.-J., and I. H. Segel, 1985. Arch. Biochem. Biophys. 241: 371-379). BAST I activity has a broad pH optimum from 5.5-7.5. Although maximum activity occurs with 5 mM MgCl₂, Mg²⁺ is not essential for BAST I activity. The greatest sulfotransferase activity and the highest substrate affinity is observed with bile acids or steroids that have a steroid nucleus containing a 3β-hydroxy group and a 5-6 double bond or a trans A-B ring junction. These substrates have normal hyperbolic initial velocity curves with substrate inhibition occurring above 5 μM. Of the saturated 5β-bile acids, those with a single 3-hydroxy group are the most active. The addition of a second hydroxy group at the 6- or 7-position eliminates more than 99% of the activity. In contrast, 3α,24-dihydroxy-5β-cholestan-24-0ic acid (deoxycholic acid) is an excellent substrate. The initial velocity curves for glycolithocholic and deoxycholic acid conjugates are sigmoidal curves with substrate inhibition occurring above 5 μM. Although maximum activity occurs with 5 μM MgCl₂, Mg²⁺ is not essential for BAST I activity. The greatest sulfotransferase activity and the highest substrate affinity is observed with bile acids or steroids that have a steroid nucleus containing a 3β-hydroxy group and a 5-6 double bond or a trans A-B ring junction. These substrates have normal hyperbolic initial velocity curves with substrate inhibition occurring above 5 μM. Of the saturated 5β-bile acids, those with a single 3-hydroxy group are the most active. The addition of a second hydroxy group at the 6- or 7-position eliminates more than 99% of the activity. In contrast, 3α,24-dihydroxy-5β-cholestan-24-0ic acid (deoxycholic acid) is an excellent substrate. The initial velocity curves for glycolithocholic and deoxycholic acid conjugates are sigmoidal curves with substrate inhibition occurring above 5 μM.

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Supplementary keywords hydroxy steroid sulfotransferases • affinity chromatography • amino acid composition • 3-keto-5β-cholanolic inhibition

The formation of sulfate esters of bile acids in many species (1, 2). Bile acid: 3-phosphoadenine-5'-phosphosulfate:sulfotransferase enzymes (BASTs) have been demonstrated in the livers of rats (3, 4), hamsters (5), guinea pigs (6), humans (7), and rhesus monkeys (8), but not in rabbits or in pigs (Barnes, S., R. Waldrop, and R. J. King, unpublished observations).

BAST activity in hamsters (5) and rats (9) is sexually differentiated. Ion exchange chromatography of BAST activity from male and female rat liver cytosol has revealed that at least two charge isomers exist (4, 10), varying amounts of which may account for the sex difference in BAST activity. Kane, Chen, and Thaler (10) have shown that there are substantial differences in substrate specificity between the two peaks of BAST activity, although they did not carry out any further purification.

Abbreviations: BAST, bile acid:3-phosphoadenine-5'-phosphosulfate:sulfotransferase; DEAE, diethylaminoethyl; PAP-hex-agarose, 3',5'-diphosphoadenosine-5'-phosphosulfate:sulfotransferase; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; HPLC, high pressure liquid chromatography; buffer A, 250 mM sucrose-5 mM sodium phosphate buffer, pH 8.0; buffer B, 5 mM sodium phosphate, pH 8.0; 3'-AMP, adenosine-3'-monophosphate; PAP, 3',5'-diphosphoadenosine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris-HCl, tri(hydroxymethyl)aminomethane hydrochloride; cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-0ic acid); chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-0ic acid); deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-0ic acid); hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-0ic acid); ursodeoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-0ic acid); cholylcholic acid (3α,7β-dihydroxy-5β-cholan-24-0ic acid): GLC, glcyolithocholic acid (N-[3α-hydroxy-5β-cholan-24-0yl]-glycine); LC, lithocholic acid 3α-hydroxy-5β-cholan-24-0ic acid; DHEA, dehydroepiandrosterone (androst-5-ene-3β-01-17-one); TLC, thin-layer chromatography; 3KC, 3-keto-5β-cholanolic acid; GDC, glycocylocholic acid.

1 Some of the data in this manuscript was presented during Digestive Disease Week in Chicago, IL, in May 1987 and has appeared in abstract form (Gastroenterology. 1987; 92: 1648).

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Only a few attempts to purify BASTs from rat liver have been reported. Chen, Bolt, and Admirand (3) obtained 33-fold purification of BAST from male rat liver using a combination of ion exchange chromatography, gel filtration and isoelectric focusing. Takikawa, Stoiz, and Kaplowitz (11) used a complex purification scheme which, in addition to the methods used by Chen et al. (3), also involved Affigel Blue affinity chromatography, chromatofocusing, and hydroxyapatite chromatography; however, the procedure suffered from a very low recovery (1%) of BAST activity, which in any case was only purified 10-fold. The specific activity of the BAST preparation was low, suggesting that enzyme inactivation was a major problem. Collins et al. (12) used an immunoaffinity procedure to purify BAST up to 75-fold from male and female rat liver. These authors prepared a monoclonal antibody to a 29,500 subunit of BAST. SDS-PAGE analysis indicated that BAST isolated by this method is composed of at least three subunits (29,500, 32,000, and 34,000), of which the 29,500 subunit predominated. However, these investigators made no attempt to separate any isoforms that are separable by ion exchange chromatography and, therefore, may have isolated more than one form of the 29,500 subunit.

In the present study we have carried out the isolation from female rat liver of BAST I, so-called because it is the first peak of enzyme activity to be eluted from a DEAE-Trisacryl anion exchange column. The enzyme has been further purified by 3,5-diposphoadenosine-hexane-agarose affinity chromatography using a sequential elution procedure. This two-step purification procedure is efficient and yields a highly purified preparation of BAST I which is fully stable for at least 4 weeks when kept at 4°C. Purified BAST I has been used for determination of its amino acid composition and N-terminal amino acid sequence.

Purified BAST I has also been used to study the specificity and kinetics of this sulfotransferase. These studies have revealed that the BAST I is both a bile salt and a 3-hydroxy steroid sulfotransferase. The bile acid, 3β-hydroxy-5-cholenoic acid, has kinetic properties similar to that of DHEA, but unlike those of the 5β-bile acids. Nonetheless, DHEA and a 5β-bile acid, glycolithocholic acid, mutually inhibit each other's sulfation. The kinetics of this enzyme with 5β-bile acid substrates is complex. The data are consistent with an allosteric effect, although other more complex mechanisms are possible.

**MATERIALS AND METHODS**

**Materials**

PAPS was purchased from Pharmacia Fine Chemicals (Piscataway, NJ) and from Dr. S. Singer, University of Dayton (Dayton, OH); 35S-Labeled PAPS (1–3 Ci/mmol) was bought from New England Nuclear Corp. (Boston, MA). The radioactive material was freeze-dried to remove the ethanol added by the manufacturer. It was reconstituted in water, divided into small aliquots, and frozen at −70°C. Its purity was regularly checked by paper electrophoresis in a pyridine-acetic acid buffer (4).

Unlabeled PAPS was greater than 95% pure as assessed by ion-pair, reverse phase HPLC on a 250 mm × 4.6 mm 5-μm Spherisorb C4 column using a Laboratory Data Control series 7800 HPLC chromatograph. The eluting solvent was methanol–3 mM tetrabutylammonium hydroxide/40 mM potassium phosphate buffer (pH 7.0) 1:4 (v/v). Eluted peaks were detected at 254 nm.

The bile acids, steroids, and the nucleotides other than PAPS were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-Trisacryl was obtained from LKB (Gaithersburg, MD). PAP-hex-agarose was purchased from Pharmacia Fine Chemicals.

**Purification of the enzyme**

Adult female Sprague-Dawley rats (200–225 g, Charles River, Wilmington, DE) were fed a laboratory chow diet ad libitum and were killed by decapitation. The livers from four to six animals were quickly excised and placed on ice. They were homogenized (2 vol/g tissue) in ice-cold 5 mM sodium phosphate, pH 8.0, containing 250 mM sucrose (buffer A). The homogenate was filtered through cheesecloth and centrifuged at 1000 g for 10 min. The supernatant was decanted and the pellet was rehomogenized in 1 vol of buffer A and centrifuged again. The combined supernatants were centrifuged at 105,000 g for 60 min at 4°C.

The supernatant fraction was aspirated and dialyzed overnight against 4 liters of 5 mM sodium phosphate, pH 8.0 (buffer B). The dialyze was centrifugated at 105,000 g for 30 min and the supernatant was filtered through a 0.22-μm membrane filter (Nalgene). The filtrate was loaded onto a 30 cm × 2 cm DEAE-Trisacryl column, equilibrated with buffer B. The column was washed with buffer B (120 ml) to remove unbound and weakly bound proteins. BAST activity was eluted with a 500-m1 gradient of 5–100 mM sodium phosphate buffer, pH 8.0. BAST I was eluted in fractions between 50 and 150 ml.

Fractions containing BAST I activity were pooled and diluted with distilled water to conductance of 2 mmos. The diluted pool was passed over a 6 × 1 cm PAP-hex-agarose column, which was then washed exhaustively with buffer B to remove unbound proteins. It was eluted successively with 20 ml of 50 mM sodium phosphate buffer, pH 8.0, 5 mM 5’-AMP and 5 mM 3’-AMP in buffer B. The BAST activity was finally eluted with 100 μM PAP in buffer B. In one experiment individual fractions from DEAE-Trisacryl chromatography were separately purified on 1 × 1 cm PAP-hex-agarose columns.
PAP was removed from the purified BAST I preparation by adding the chaotrope NaSCN to a final concentration of 0.5 M, leaving the mixture to stand for 6 hr at 4°C, and dialyzing (3 x 1 liter) overnight against 5 mM sodium phosphate buffer, pH 7.0. Alternatively, the enzyme preparation was passed over a Sephadex G-10 column equilibrated with buffer B.

The specific activity of BAST in each fraction was estimated by using glycolithocholate (GLC) as substrate under saturating conditions (100 μM GLC and 100 μM PAPS). Protein concentration was determined using the method of Lowry et al. (13) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis

Purity of individual fractions was assessed by SDS-PAGE as described by Laemmli (14), using 0.75-mm-thick 12.5% (w/v) polyacrylamide gels made in 0.4 M Tris-HCl buffer, pH 8.8, containing 0.1% (w/v) SDS. Samples were denatured at 100°C for 3 min in 0.0625 M Tris-HCl buffer, pH 6.8, containing 1.25% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 7.5% (w/v) sucrose, and 0.002% (w/v) bromophenol blue. Aliquots (20 μl) were placed in the wells of a 3% (w/v) polyacrylamide stacking gel formed on top of the analytical gel. SDS-running buffer, used to fill the upper and lower reservoirs of the vertical electrophoresis apparatus (Model SE 600, Hoefer, San Francisco, CA), consisted of 0.384 M glycine, 0.05 M Tris, and 0.1% (w/v) SDS, pH 8.3. Samples were electrophoresed through the stacking gel at a constant current of 15 mA and subsequently through the analytical gel at 35 mA. After fixing the gel in 50% methanol-10% acetic acid, the protein bands were located using the silver stain procedure of Merril et al. (15).

Isoelectric focusing

Isoelectric focusing of purified BAST I was carried out on a flat-bed multiphor apparatus (LKB) in a 1% (w/v) agarose gel containing ampholytes (16). The pH gradient in the gel was determined by cutting out a blank lane adjacent to the purified protein fraction prior to fixing the gel in trichloroacetic acid-sulfosalicylic acid. After drying the gel, protein bands were located with Coomassie blue. BAST activity was determined by removing a duplicate piece of gel and was in-
resulting supernatant were applied to 2-cm-wide lanes on a silica gel G TLC plate (Rediplates, Fisher, Norcross, GA). After careful drying, the plates were developed in chloroform-methanol-glacial acetic acid-water 65:24:10:5 (by vol) (5). Zones (0.5 cm) were carefully scraped off into plastic scintillation vials and their position was compared chromatographically to synthetic bile acid and steroid sulfate esters. Before adding the scintillant (4 ml), the scrapings were soaked in methanol (0.5 ml) to solubilize the bound components.

Optimization of enzyme activity

To test the effect of pH on purified BAST I activity, the pH of the phosphate buffer was varied from pH 5 to 8. The role of Mg²⁺ was examined in the range from 0 to 10 mM. One group of samples was isolated in buffers containing EDTA and then dialyzed against 1 mM EDTA prior to assay. Substrate specificity studies were carried out with various bile acids and steroids at 5, 20, and 100 μM.

Kinetic experiments

The initial enzyme velocities were measured at several concentrations of bile acids and steroids (0.1–500 μM, where appropriate) and PAPS (1–500 μM). The amount of BAST I used, or the incubation time, was adjusted so that less than 5% of the [³⁵S]PAPS or of the bile acid/steroid was converted to the sulfate ester. This ensured that the reaction rate was linear with time. In experiments where the concentration of GLC was varied, the initial velocity data (v) were examined by a Hill plot [log v/Vₘₐₓ − v] vs. log GLC (21). In kinetic experiments where DHEA or 3β-hydroxy-5-cholestanate were the substrates, data were analyzed with the program COMPET as we have previously described (8) using the methods of Cleland (22).

RESULTS

Purification of BAST I

BAST activity was completely absorbed from dialyzed cytosol onto the DEAE-Trisacryl column in low ionic strength buffer at pH 8. Elution with a 5–100 mM gradient of sodium phosphate buffer, pH 8.0, resulted in two large peaks of BAST activity (BAST I and BAST II) (Fig. 1). A third peak (BAST III) was eluted at higher ionic strength (Fig. 1).

The fractions containing the BAST I peak were combined and passed over the PAP-hex-agarose affinity column. Less than 4% of the loaded BAST activity appeared in the eluate and in the washings (Fig. 2). SDS-PAGE analysis of the affinity column eluate showed that the major band absorbed from the proteins in the DEAE pooled fractions had a molecular weight of 30,000.

Elution of bound BAST I from the affinity phase could be directly accomplished with 0.5 M NaCl or 100 μM PAPS. However, this led to coelution of many other proteins that had bound to the column. A far superior

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Fig. 1. DEAE-Trisacryl anion exchange chromatography of female rat liver cytosol BAST activities. Dialyzed cytosol (8 ml) was loaded onto a 20 × 1.5 cm DEAE-Trisacryl column equilibrated with 5 mM sodium phosphate buffer, pH 8.0 (buffer B). The column was washed with buffer B until the absorbance at 280 nm returned to within 0.01 absorbance units of the initial baseline. The retained BAST activity (O), measured with GLC as substrate, was eluted with a 500-ml linear gradient of 5–100 mM sodium phosphate buffer, pH 8.0; absorbance at 280 nm (●) and conductivity (□) are shown. The fraction sizes were 6 ml.
Fig. 2. PAP-hexane-agarose affinity column chromatography of DEAE-Trisacryl-purified BAST I. The column (6 x 1 cm) was equilibrated with buffer B (see Fig. 1). A pool of selected DEAE-Trisacryl column fractions, with the conductance adjusted to 1.8 mhos, was loaded onto the affinity gel at 50 ml/hr. Nonadsorbed proteins (void fraction) were washed off with buffer B (50 ml). The bound proteins were eluted sequentially using first 50 ml of 50 mM sodium phosphate buffer, pH 8.0; second, 20 ml of 5 mM 5'-AMP in buffer B (note: the 280 nm absorbance is mostly due to 5'-AMP); third, 20 ml of 100 pM PAP in buffer B; and finally, 20 ml of 1 M KCl in buffer B. BAST I activity (m) was predominantly found in the PAP eluate, with a much smaller amount in the KCl fraction.

The apparent recovery of the BAST I activity through the PAP-hex-agarose affinity column was 15–20% (Table 1, 40 different preparations). However, in the radioassay for purified BAST I, the enzyme activity increased in a sigmoidal fashion with increased total protein. This made it difficult to compare the enzyme activity at each stage of the purification. Since the eluted protein (0.1 mg/ml solution) retained 100% of its enzyme activity over a 3-week period stored at 4°C, it seemed unlikely that there was a loss of enzyme activity during the affinity purification. Furthermore, elution of the affinity column with 2 M NaCl, or with the chaotrope 1 M NaSCN, produced only small amounts of additional enzyme activity, or BAST I protein, as assessed by SDS-PAGE analysis.

One freeze–thaw cycle destroyed greater than 90% of the purified enzyme activity. However, this was mostly prevented in the presence of PAP (100 μM). The observed specific enzyme activity of the homogeneous preparation with GLC as substrate was 18.7 nmol/min per mg protein, a 157-fold purification relative to cytosol, which must be regarded as a minimum (Table 1).

Physical properties of BAST I

SDS-PAGE analysis of the various fractions from the purification steps showed that reduced, denatured BAST I had an apparent molecular weight of 30,000 (Fig. 3), similar to the major BAST subunit (29,500) isolated by immunofinity chromatography by Collins et al. (12). Omission of 2-mercaptoethanol during the denaturation of BAST I by boiling in SDS did not alter the apparent molecular weight. However, the native protein would not enter a polyacrylamide gel that did not contain SDS. Variable but small amounts of proteins with molecular weights of 32,500 and 34,000 were also found in some preparations. However, when BAST I was isolated from individual DEAE-Trisacryl chromatography fractions instead of from pooled fractions, the 32,500 and 34,000 protein bands did not coincide with BAST I activity (data not shown).

HPLC gel permeation analysis of the native protein in 10 mM sodium phosphate buffer (pH 6.0)–4 M guanidine-
The amino acid composition of carboxymethylated BAST I is given in Table 2. The N-terminal amino acid sequence of BAST I was carried out on 0.5 nmol of BAST I and yielded the first 35 residues: Pro-Asp-Tyr-Thr-Trp(His)-Phe-Glu-Ile-Pro-Phe-Pro-Ala-Phe-Gly-Ile-Ser-Lys-Glu-Thr-Leu-Gln-Asp-Val-Asn-Lys-Ser-Val-Lys-Asp-?-Asp-Leu-Ile. The same sequence through the first 26 residues from the N-terminus was found using a different preparation of BAST I which was not carboxymethylated prior to sequencing. A search of the NBRF Amino acid composition and partial sequence data

Agarose isoelectric focusing of affinity-purified BAST I yielded a single band containing BAST activity, corresponding to a pI of 5.3 (data not shown), as was reported by Chen et al. (3) for BAST activity isolated from male rat liver. Attempts to focus BAST I in polyacrylamide gels was quite unsuccessful either with soluble ampholytes or chemically bonded Immobiline gels.

Amino acid composition and partial sequence data

The amino acid composition of carboxymethylated BAST I is given in Table 2. The N-terminal amino acid sequence of BAST I was carried out on 0.5 nmol of BAST I and yielded the first 35 residues: Pro-Asp-Tyr-Thr-Trp(His)-Phe-Glu-Ile-Pro-Phe-Pro-Ala-Phe-Gly-Ile-Ser-Lys-Glu-Thr-Leu-Gln-Asp-Val-Asn-Lys-Ser-Val-Lys-Asp-?-Asp-Leu-Ile. The same sequence through the first 26 residues from the N-terminus was found using a different preparation of BAST I which was not carboxymethylated prior to sequencing. A search of the NBRF Amino acid composition and partial sequence data

Fig. 4. HPLC permeation analysis of purified BAST I. In A, 100 µg of BAST I (in 50 µl buffer) was injected onto a 300 mm × 7.5 mm Altex Spherogel-TSK 2000SW column and eluted at a flow rate of 1 ml/min with 100 mM sodium phosphate buffer, pH 7.0, at 1 ml/min. In B, the column was equilibrated and eluted with 100 mM sodium phosphate (pH 6.0)-4 M guanidine HCl. The column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A. The broad peak eluted between 6 and 10 min contained BAST activity. The tall narrow peak was PAP.

Fig. 3. SDS-PAGE analysis of reduced, denatured protein fractions from the purification of BAST I from rat liver cytosol as shown in Fig. 2. BAST activity in the eluted fractions was confined to the PAP eluate fractions. Proteins were visualized by the silver stain procedure. The protein molecular weight standards in ascending order are a-lactalbumin (14,000), soybean trypsin inhibitor (20,000), carbonic anhydrase (30,000), ovalbumin (44,000), bovine serum albumin (67,000) and phosphorylase B (94,000).
TABLE 2. Amino acid composition of purified BAST I

<table>
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<th>Amino Acid</th>
<th>BAST I residues</th>
<th>Hydroxysteroid Sulfotransferase residues</th>
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</table>

*From Marcus et al. (24).

†Hydroxysteroid sulfotransferase was not carboxymethylated by Marcus et al. (24).

†Tryptophan is destroyed by the procedure used in this study.

protein sequence data bank revealed no significant sequence homology with known proteins.

Optimization of enzyme activity

In the absence of added Mg²⁺, maximum BAST I activity occurred at pH 6.0, but the pH profile was broad. The highest BAST I activity was observed at pH 6.5 in the presence of 5 mM Mg²⁺ (data not shown). However, at all pHs studied, BAST I activity was only slightly stimulated by Mg²⁺. All subsequent experiments were carried out at pH 6.5 and a Mg²⁺ concentration of 5 mM.

Substrate specificity

Substrate specificity studies showed that the concentration of the substrate dramatically affected the relative enzymatic activity (Table 3). At 5 μM, BAST I was most active with DHEA and 3β-hydroxy-5-cholenoic acid, 115 times and 43 times greater than with lithocholic acid (LC), and 22 times and 8 times greater than with GLC, respectively. At 20 μM, DHEA and 3β-hydroxy-5-cholenoic acid were still the best substrates, but with lower initial enzyme velocities than at 5 μM. At 100 μM there was a sharp increase in the activity of BAST I with GLC, but the largest increase occurred for deoxycholate and its conjugates, which were the best substrates at this concentration. Further, at 100 μM GLC was better substrate than 3β-hydroxy-5-cholenoic acid. Of the unconjugated bile acids tested, only 3β-hydroxy-5-cholenoic acid, deoxycholic acid, and 3α-hydroxy-12-keto-5β-cholanoic acid (Fig. 5) were substrates. Except for cholic acid, glycine or taurine conjugation substantially increased the rate of sulfation.

Some bile acids, although not being sulfated by BAST I, nonetheless were effective inhibitors (Table 4). The most potent was 3-keto-5β-cholanoic acid (3KC), a simple oxidation product of LC, which inhibited 80-90% of BAST I activity when it was incubated in equimolar amounts with GLC. Most of the dihydroxy bile acids were excellent inhibitors (40-80% inhibition) of BAST I activity, with the exception of chenodeoxycholic acid which caused only 17% inhibition. None of the tri-oxygenated bile acids had inhibitory activity (data not shown).

Kinetic experiments

At constant GLC or glycodychoxylic acid (GDC) concentrations, the initial enzyme velocity increased monotonically as the PAPS concentration was raised from 5 to 500 μM (Fig. 6). The data was consistent with an enzyme behaving with Michaelis-Menten kinetics. The apparent Kₘ of PAPS was 43 ± 2 μM under a saturating concentration of GLC (60 μM). However, when the GLC or GDC concentration was varied at constant PAPS concentrations, the initial enzyme velocity increased in a sig-

**TABLE 3. Substrate specificity of BAST I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Relative Activity</th>
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*Measured relative to that BAST activity with 5 μM DHEA and 40 μM PAPS (9.4 nmol/min per mg protein).
FIG. 5. Structures of dehydroepiandrosterone and 3β-hydroxy-5-cholenoate, and the 5β-bile acids tested with BAST I. The individual 5β-bile acids have the following substituents:

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<thead>
<tr>
<th>Bile Acid R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithocholate</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Hyodeoxycholate</td>
<td>αOH</td>
<td>H</td>
</tr>
<tr>
<td>3α,6β-Dihydroxy-5β-cholanoate</td>
<td>βOH</td>
<td>H</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>H</td>
<td>αOH</td>
</tr>
<tr>
<td>7-Ketolithocholate</td>
<td>H</td>
<td>βOH</td>
</tr>
<tr>
<td>Ursodeoxycholate</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>H</td>
<td>αOH</td>
</tr>
<tr>
<td>12-Ketolithocholate</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Cholate</td>
<td>H</td>
<td>αOH</td>
</tr>
</tbody>
</table>

Fig. 5. Structures of dehydroepiandrosterone and 3β-hydroxy-5-cholenoate, and the 5β-bile acids tested with BAST I. The individual 5β-bile acids have the following substituents:

- Lithocholate: R1 = R2 = H
- Hyodeoxycholate: R1 = αOH, R2 = H
- 3α,6β-Dihydroxy-5β-cholanoate: R1 = βOH, R2 = H
- Chenodeoxycholate: R1 = H, R2 = αOH
- 7-Ketolithocholate: R1 = H, R2 = βOH
- Ursodeoxycholate: R1 = H, R2 = H
- Deoxycholate: R1 = H, R2 = αOH
- 12-Ketolithocholate: R1 = H, R2 = H
- Cholate: R1 = H, R2 = αOH

In a second experimental approach, DHEA and GLC were incubated together in varying concentrations with BAST I. It was clearly demonstrated that DHEA inhibited GLC sulfation (Fig. 11A) and GLC inhibited DHEA sulfation (Fig. 11B). In each of these experiments, the conversion of substrates was limited to less than 5% of the starting concentrations.

DISCUSSION

In this study, BAST I from adult female rat liver was purified 157-fold by a simple two-step procedure involving anion exchange chromatography and affinity chromatography. The advantages of this procedure over published methods for BASTs (3, 11) and hydroxysteroid sulfotransferase 2 (24) are that the number of steps is reduced to a minimum and concentration or undue dilution of the enzyme is avoided. As we had previously found, steps involving chromatofocusing or hydroxyapatite chromatography led to large scale losses of BAST activity (4).

The most striking feature of this enzyme is that it sulfates the 3-hydroxy group of both 5β-bile acids and bile acids and steroids containing a Δ5 or 5α-configuration at the A-B ring junction (Fig. 5). Inhibition experiments with both nonsulfatable (3KC) and sulfatable substrates (DHEA and GLC) have confirmed the dual role of this enzyme in sulfating both bile acids and steroids. In fact,

TABLE 4. Inhibition of BAST I by nonsulfatable bile acids

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>Inhibitiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 μM</td>
</tr>
<tr>
<td>3-Keto-5β-cholanoate</td>
<td>82.7</td>
</tr>
<tr>
<td>Hyodeoxycholate</td>
<td>45.8</td>
</tr>
<tr>
<td>3α,6β-Dihydroxy-5β-cholanoate</td>
<td>56.6</td>
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<tr>
<td>6-Ketolithocholate</td>
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<td>3,6-Diketo-5α-cholanoate</td>
<td>50.6</td>
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<td>3,6-Diketo-5β-cholanoate</td>
<td>62.4</td>
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<td>Chenodeoxycholate</td>
<td>16.7</td>
</tr>
<tr>
<td>Ursodeoxycholate</td>
<td>53.2</td>
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<tr>
<td>7-Ketolithocholate</td>
<td>23.4</td>
</tr>
<tr>
<td>3,7-Diketo-5β-cholanoate</td>
<td>43.8</td>
</tr>
<tr>
<td>3,12-Diketo-5β-cholanoate</td>
<td>ND</td>
</tr>
</tbody>
</table>

aThe inhibition by a bile acid represents the percentage reduction in the conversion of GLC to its sulfate in the presence of an equimolar amount of the inhibiting bile acid compared to the conversion observed without the second bile acid; ND, not determined.
Fig. 6. Initial velocity curve versus the PAPS concentration at a fixed GLC concentration. BAST I (0.5 µg) was added to incubates containing 100 µM GLC and varying amounts of PAPS (2.5-500 µM) in 5 mM MgCl₂-100 mM sodium phosphate buffer, pH 6.5. Data are plotted in the form of a double reciprocal plot (1/v vs. 1/p).

Fig. 7. Initial velocity curves versus GLC concentration at fixed PAPS concentration. BAST I (0.5 µg) was added to incubates containing 40 µM PAPS and varying amounts of GLC (3-500 µM) in 5 mM MgCl₂-100 mM sodium phosphate buffer, pH 6.5. In A, the sigmoidal part of the curve in the range from 3 to 100 µM is shown; whereas in B the entire range from 3 to 500 µM is presented, showing the inhibition by GLC at higher concentrations.

we suggest that BAST I is the same protein as hydroxysteroid sulfotransferase 2, previously purified 47-fold from female rat liver cytosol (24). The failure of Marcus, Sekura, and Jakoby (24) to recognize that their preparation could sulfate bile acids was a result of choosing chenodeoxycholic acid as the bile acid test substrate, since this bile acid is a poor substrate of BAST I (see Table 3). BAST I and hydroxysteroid sulfotransferase 2 also share the characteristic of being unable to enter a 7% polyacrylamide gel in the absence of a detergent such as SDS. This may be due to concentration-dependent aggregation that occurs in the polyacrylamide gel (24). This phenomenon also occurred when attempts were made to isolate BAST I by isoelectric focusing in polyacrylamide gels.

When reduced denatured BAST I is analyzed by SDS-PAGE, it migrates as a 30,000 subunit. The preparation of BAST I produced in the present study has many of the characteristics of the BAST activity purified by immunoaffinity chromatography by Collins et al. (12), including the copurification of minor 32,500 and 34,000 protein bands. However, in the present experiments these minor bands do not appear to be associated with BAST I activity and may instead be considered as minor variable contaminants. The apparent molecular weight of the 30,000 polypeptide band is not affected by whether or not BAST I is reduced by 2-mercaptoethanol during denaturation; this was previously observed for the subunits of the major form of BAST purified by immunoaffinity chromatography (12). This suggests that the subunits in the native form of BAST I are held together by hydrophobic forces rather than by disulfide bridges. This was further supported by the observation that in 4 M guanidine-HCl native BAST I is eluted from a gel permeation HPLC column as a 30,000 monomer.

We have previously shown that reduction of the native forms of BAST I by 2-mercaptoethanol causes an increase in apparent molecular weight, which was stabilized by carboxymethylation (4). It appears that carboxymethylation breaks the single intramolecular disulfide bridge in BAST I and exposes hydrophobic residues. Changes in ionic strength of the buffer, in substrate concentration, and in the concentration of the enzyme may therefore affect the molecular weight of the native form. As a result, none of the molecular weights reported by various investigators (3, 4, 11, 12) are necessarily correct.

The amino acid composition of BAST I is, within experimental error, the same as for hydroxysteroid sulfotransferase 2 (24). The major difference is the presence of
twocysteineresiduesinBASTI. Marcusetal. (24)did
not carboxymethylate purified hydroxysteroid sulfotrans
erase 2 and therefore would not have measured the two
cysteineresidues.

Contraryto expectation, BAST I is fully stable in the
purified state, sustaining constant enzyme activity over a
3-4-week period when stored at 4°C in 5 mM sodium
phosphate buffer, pH 8.0. Estimated $V_{\text{max}}$ of this prepa-
ration of BAST I with GLC as substrate was 18.7 nmol/min
per mg, similar to that of 760-fold purified human liver
BAST (23) and immunoaffinity-purified BAST (12). Un-
like hamster liver BAST (5) and rhesus monkey liver
BAST (8), rat liver BAST I is only slightly stimulated by
Mg$^{2+}$ ions. In the absence of Mg$^{2+}$ ions the pH optimum
is 6.0, whereas in the presence of Mg$^{2+}$ ions it is pH 6.5,
as noted by Chen et al. (3) for their 33-fold purified prepa-
ration of male rat liver BAST. However, these investiga-
tors reported an optimum Mg$^{2+}$ concentration of 0.5 mM
at pH 6.5, whereas in the present study it is 5 mM. The
pH optimum for hydroxysteroid sulfotransferases is pH
6.0 (25, 26).

The two best naturally occurring substrates of BAST I
at low substrate concentrations (<1 μM) are DHEA and
3β-hydroxy-5-cholenoic acid; the initial velocities of these
two substrates at these concentrations exceed those of the
5β-bile acids by at least 500-fold. At higher substrate con-
centrations (~100 μM), lithocholic acid conjugates and
deoxycholic acid conjugates become the best substrates.
Other dioxygenated bile acids, hyodeoxycholic acid,
chenodeoxycholic acid, and ursodeoxycholic acid, are not
significantly sulfated.

Since the distance from the 3α-hydroxy group on the
bile acid molecule to the 6(αβ)-, 7(αβ)-, and 12α-
positions is approximately the same (4–5 Å), it is neces-
sary to speculate that the bile acid molecule has to enter
theactive site of the enzyme in a particular orientation.
This orientation may be made more stringent because of
the kink in the bile acid molecule caused by the cis
configuration at the A–B ring junction. Support for this
concept comes from the observation that the 3α-hydroxy-
6-keto-5α-cholan-24-oic acid, which has a trans con-
figuration at the A–B ring junction and therefore is a planar
molecule, is a substrate for BAST I, whereas its 5β-
counterpart is not sulfated at all (Barnes, S., and R. J.
King, unpublished observations). The 5α-isomer of litho-
cholic acid and 3β-hydroxy-5-cholenoic acid, both of
which have an A ring which is coplanar with the B, C and
D rings, are 5–20 times better substrates than LC. Although
the bile acid side chain is unlikely to be near the
enzyme active site, it too has some influence on BAST I
activity. GLC is a 10–25 times better substrate than LC,
and GDC is three times better than DC. The side chain
may bind to hydrophobic regions close to the active site
on BAST I. Despite the failure of the 3,6- and 3,7-oxy-
genated bile acids to be substrates for BAST I, nonethe-
less they must be able to approach the active site since
each of them, with the possible exception of chenodeoxy-
cholic acid, is able to inhibit sulfation of GLC when in-
cubated with GLC on an equimolar basis.

The reaction mechanism for BAST I could not be de-
termined from experiments conducted in this study.
However, it is different from those reported for BASTs
from rhesus monkey liver (8) and human liver (23). Un-
Fig. 8. A Hill plot of initial velocities over a GLC concentration range
of 3–60 μM at fixed PAPS concentrations. Data from Fig. 7A were used
in this figure. The slope of the line is 1.45.

Fig. 9. Initial velocity curve versus DHEA concentration at 2 μM
PAPS. BAST I (0.1 μg) was incubated for 20 min in 100 mM sodium
phosphate buffer, pH 6.5. The inset shows an expanded range from 0 to
4 μM DHEA.
Fig. 10. Inhibition of DHEA sulfation by 3-keto-5β-cholanoic acid. 
BAST I (0.1 µg) was incubated at 37°C for 20 min in 100 mM sodium phosphate buffer, pH 6.5, in the presence of 2 µM PAPS, varying concentrations of DHEA alone (○), and with 0.5 µM (△), 1.0 µM (○), and 2 µM (△) 3-keto-5β-cholanoic acid.

Fig. 11. Mutual inhibition of DHEA and GLC on the sulfation of each other by BAST I. In A, the concentration of DHEA was held constant (2.5 µM) and that of GLC was varied. In B, the concentration of GLC was held constant (60 µM) and that of DHEA was varied. More BAST I was added in B. The 35S-labeled products were separated by TLC and the appropriate zones, identified with standards, were scraped into scintillation vials for counting.

Fig. 12. A plot of the reciprocal initial enzyme velocities versus GLC concentration in the range from 100 to 500 µM. Data from Fig. 7B were used in this plot.

like rhesus monkey liver BAST(s), BAST I binds to the PAP-hex-agarose affinity phase.

The sigmoidal initial velocity curves, similar to those reported for rat liver deoxycortisone sulfotransferase (27), may be due to positive cooperativity. When initial velocity data are analyzed by the Hill plot, n is 1.45. This could arise when the substrate (i.e., the bile acid) binds at an allosteric site and increases the affinity of the substrate for binding to the active site of BAST I. The positive cooperativity may also be due to the interaction of two BAST I molecules. However, HPLC permeation of BAST I under different conditions suggests that this is an oversimplification since several molecular weight forms of BAST I are observed. The large losses of enzyme activity during purification of sulfotransferases have been attributed to aggregation (24).

Since there is marked substrate inhibition by bile acids or DHEA, but not by PAPS, this would further imply that a significant proportion of BAST I:product complexes consist of the BAST I:PAP complex. Addition of bile acids or DHEA to this complex would yield an unproductive, dead-end complex. If the dissociation of the products from the BAST I:products complex is ordered, i.e., the BAST I:bile acid sulfate complex does not exist, then complete inhibition of BAST I activity would be predicted at infinite concentration of GLC. When the reciprocals of the initial velocities at GLC concentrations from 100 to 500 µM are plotted against the GLC concentration, a straight line with a positive slope is obtained (Fig. 12). This result is consistent with ordered dissociation of the BAST I:products complex. A similar result was obtained for other bile acids and DHEA (data not shown).
REFERENCES


