Bile acid sulfotransferase I from rat liver sulfates bile acids and 3-hydroxy steroids: purification, N-terminal amino acid sequence, and kinetic properties[1]

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Abstract A bile acid:3'phosphoadenosine-5'phosphosulfate:sulfo-transferase (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-cholanoyl)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 $MgCl_2$, Mg^{2+} 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,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid) is an excellent substrate. The initial velocity curves for glycolithocholic and deoxycholic acid conjugates are sigmoidal rather than hyperbolic, suggestive of an allosteric effect. Maximum activity is observed at 80 μM for glycolithocholic acid. All substrates, bile acids and steroids, are inhibited by the 5β-bile acid, 3-keto-5β-cholanoic acid. The data suggest that BAST I is the same protein as hydrosteroid sulfotransferase 2 (Marcus, C. J., et al. 1980. *Anal. Biochem.* **107**: 296–304).—**Barnes, S., E. S. Buchina, R. J. King, T. McBurnett, and K. B. Taylor.** Bile acid sulfotransferase I from rat liver sulfates bile acids and 3-hydroxy steroids: purification, N-terminal amino acid sequence, and kinetic properties. *J. Lipid Res.* 1989. **30**: 529–540.

Supplementary key words hydroxysteroid sulfotransferases • affinity chromatography • amino acid composition • 3-keto-5β-cholanoate inhibition

The formation of sulfate esters of bile acids is considered to be an essential step in the prevention of toxicity

by monohydroxy bile acids in many species (1, 2). Bile acid: 3'phosphoadenosine-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 isoforms 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.

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, Stolz, 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-diphosphoadenosine-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-hydroxysteroid 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); ^{35}S-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 C_6 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 buffer, 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 dialyzate was centrifuged 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-ml 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 a conductance of 2 mmhos. 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 × 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 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 trichloracetic acid–sulfosalicylic acid. After drying the gel, protein bands were located with Coomassie blue. BAST activity was determined by removing a duplicate lane for each protein fraction analyzed before fixing. Each piece of gel was soaked in 100 mM sodium phosphate buffer, pH 7.0, for 1 hr at 4°C prior to assay of the resulting supernatant for BAST activity.

Amino acid analysis and partial sequencing

Purified BAST I (100 μg) was precipitated by methanol (80% by vol) overnight at −20°C. The precipitate was isolated by centrifugation and dissolved in 200 μl 6 M guanidine HCl, 200 mM Tris-HCl buffer, pH 8.3, containing 2 mM dithiothreitol, flushed with N_2, and was in-

cubated for 16 hr at 37°C. The cysteine residues were carboxymethylated by addition of iodoacetic acid (final concentration 4.4 mM) (20 min, room temperature) in the dark. The carboxymethylated BAST I was desalted by gel filtration through a 10 × 0.7 cm Sephadex G-25 column and equilibrated with 70% aqueous formic acid. After evaporation to remove the eluting solvent, the protein residue was used for N-terminal amino acid sequencing (17) using an Applied Biosystem model 470A automatic gas-phase sequencer (Applied Biosystems, Foster City, CA). Similarly prepared, carboxymethylated BAST I was subjected to hydrolysis in 6 M HCl under N_2 at 110°C for 24 hr. Corrections for incomplete hydrolysis or degradation were made using a known standard protein which was simultaneously hydrolyzed. The amino acids liberated by the Edman degradation or by hydrolysis were analyzed as their phenylthiohydantoin derivatives by reverse-phase HPLC on an ABI C_{18} column using a Perkin-Elmer Series 4 chromatograph (18).

The amino acid sequence of the N-terminal region of the BAST I was compared to all entries in the NBRF Protein Sequence Database (release 16.0, March 1988) with the sequence analysis software package (version 5.3, July 1988) of the Genetics Computer group of the University of Wisconsin Biotechnology Center (19).

Sulfotransferase assay

A radiometric assay for BAST activity was used as described in detail elsewhere (20). [35S]PAPS was the radioactive substrate. Total assay volume was 100 μl. The incubation medium contained (at final concentration) 100 mM sodium phosphate buffer, pH 6.5, 0.05 μCi [35S]PAPS, PAPS (5–500 μM), 2.5 mM $MgCl_2$, and bile acid (5–500 μM). In experiments with DHEA and 3β-hydroxy-5-cholenoic acid, much lower concentrations of substrates (0.05–5 μM) were used because of their higher affinities for BAST I and because of substrate inhibition. Incubations were carried out for 1 hr at 37°C for samples during purification and for shorter periods in kinetic studies; the reaction was terminated by placing the tubes in a boiling water bath for 3 min. Ammonium hydroxide (1 M; 0.4 ml) was added and the precipitate was removed by centrifugation (1000 g, 10 min at 4°C). The supernatant was extracted with 1 ml butan-1-ol; the butan-1-ol layer was backwashed with ammonium phosphate buffer, pH 10.8. An aliquot (0.8 ml) of the butanol phase was added to 4 ml Aquasol scintillation fluid (Amersham, Arlington Heights, IL) in a plastic mini-vial for determination of radioactivity by liquid scintillation counting.

In experiments where the sulfation of two substrates was carried out in the same incubation mixture to test for mutual inhibition, the incubation was stopped by boiling the sample. After allowing the sample to cool, ammonium hydroxide (10 μl) was added and the mixture was centrifuged (1000 g, 10 min at 4°C). Aliquots (30 μl) of the

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 Mg^{2+} 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 [^{35}S]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/V_{max} – v) vs. log GLC] (21). In kinetic experiments where DHEA or 3β-hydroxy-5-cholenoate 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
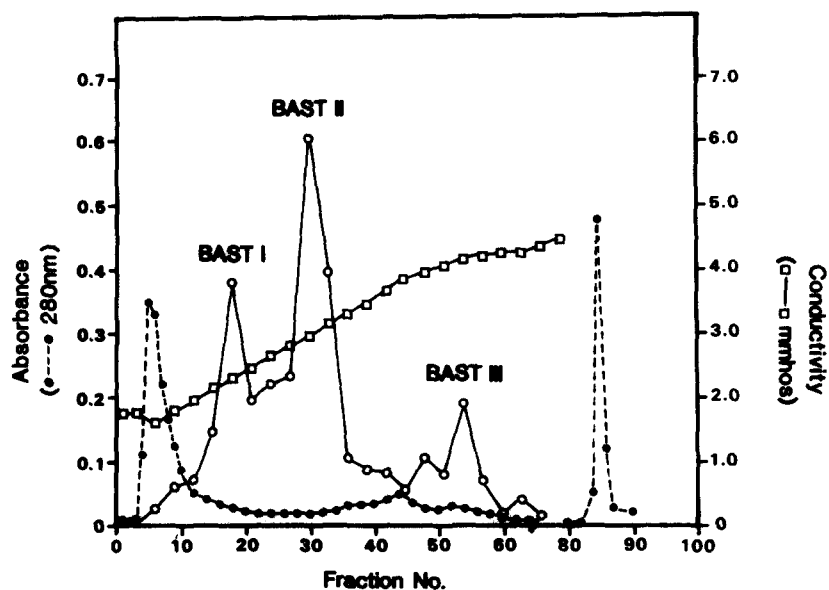

Fig. 1. DEAE-Trisacryl anion exchange chromatography of female rat liver cytosol BAST activities. Dialyzed cytosol (8 ml) was loaded onto a 20 × 1.5 cm DEAE-Trisacryl column equilibrated with 5 mM sodium phosphate buffer, pH 8.0 (buffer B). The column was washed with buffer B until the absorbance (at 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.
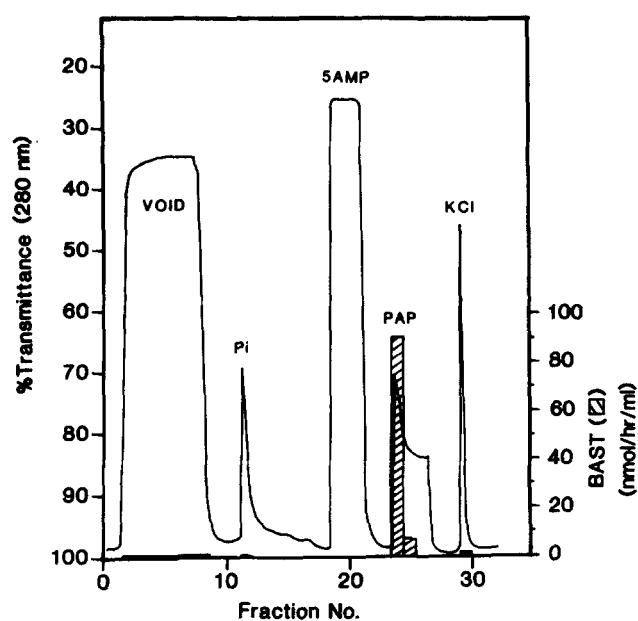
Fig. 2. PAP-hexane-agarose affinity column chromatography of DEAE-Trisacryl-purified BAST I. The column (6 × 1 cm) was equilibrated with buffer B (see Fig. 1). A pool of selected DEAE-Trisacryl column fractions, with the conductance adjusted to 1.8 mmhos, was loaded onto the affinity gel at 50 ml/hr. 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 μM PAP in buffer B; and finally, 20 ml of 1 M KCl in buffer B. BAST I activity (▨) was predominantly found in the PAP eluate, with a much smaller amount in the KCl fraction.

method was to elute the affinity column first with 50 mM sodium phosphate buffer, pH 8.0. This was followed by two washes with 5 mM 5'-AMP and 3'-AMP, respectively, in buffer B. The 50-mM wash step eluted many contaminating proteins from the affinity matrix. The 5'-AMP and 3'-AMP washes were quite selective with regard to the proteins that were eluted. Elution of BAST I from the affinity phase was carried out with 100 μM PAP in buffer B (Fig. 2).

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 immunoaffinity 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-

TABLE 1. Purification and recovery data for BAST I[a]

Fraction	Protein	BAST Activity[b]	Specific Activity	Purification
	mg	nmol/min	nmol/min per mg	-fold
Cytosol	1206	144	0.119	1
DEAE peak[c]	50	48	0.96	8.1
PAP-purified	0.385	7.2	18.7	157

[a]The DEAE fractions containing BAST I represent only a part of the total BAST activity. Since all the BAST activity in the cytosol was recovered from the DEAE column, BAST I recovery up to this point should be considered as being 100%. The final yield of affinity-purified BAST I is, therefore, 15%.
[b]Measured using 100 μM GLC and 100 μM PAPS as substrates.
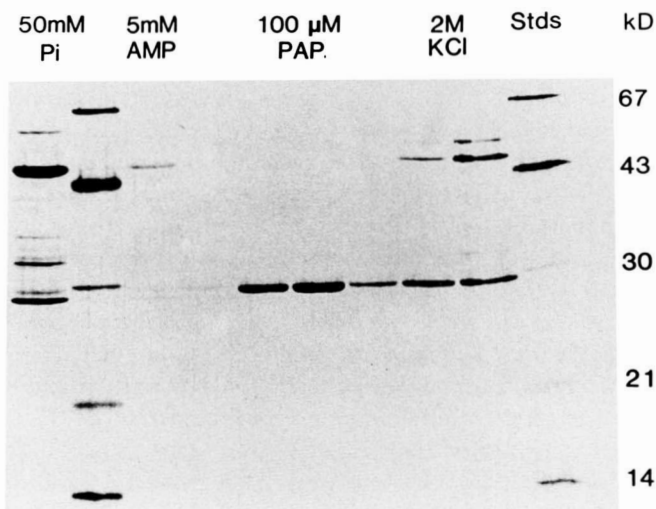
Fig. 3. SDS-PAGE analysis of reduced, denatured protein fractions from the purification of BAST I from rat liver cytosol as shown in Fig. 2. BAST activity in the eluted fractions was confined to the PAP eluate fractions. Proteins were visualized by the silver stain procedure. The protein molecular weight standards in ascending order are α-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).

HCl showed that it had a molecular weight of 30,000, consistent with SDS-PAGE analysis (**Fig. 4B**). When the elution medium was changed to 0.1 M sodium phosphate, pH 7.0, there was a broad peak of absorbance indicative of aggregation into several molecular weight forms, all of which were enzymatically active (Fig. 4A). When substrate (40 μM GLC) was added to the eluting buffer (5 mM sodium phosphate–100 mM NaCl, pH 7.0), the peaks were sharper and the molecular weight of the smaller first peak to be eluted increased to over 200,000 (data not shown). Each of the peaks was enzymatically active with the substrates GLC and DHEA. 2-Mercaptoethanol (20 mM) had no effect on molecular weights (data not shown). When 0.5 M NaCl was added to the eluting buffer, three molecular weight forms, 60,000, 130,000, and ~250,000, were observed in approximately equal amounts (data not shown). Furthermore, at this concentration of NaCl, BAST I slowly precipitated from solution, suggesting that NaCl-induced aggregation was occurring.

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-Gly-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
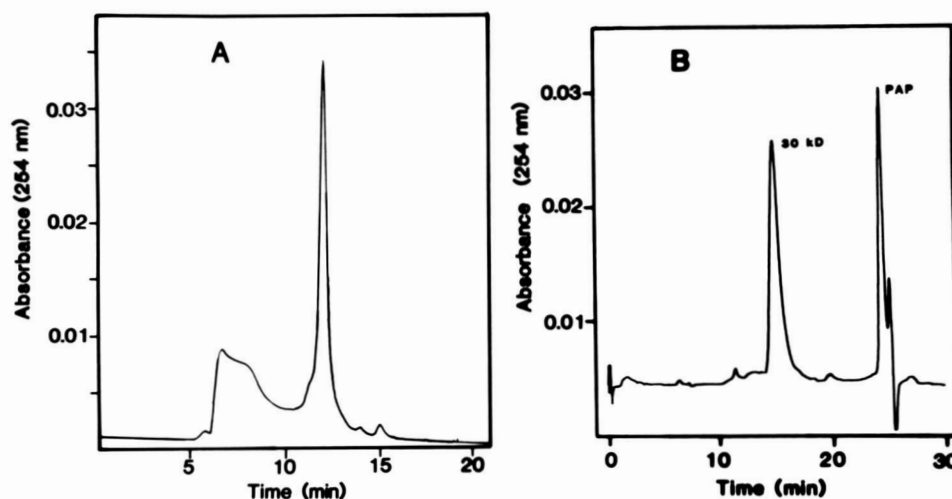
Fig. 4. HPLC permeation analysis of purified BAST I. In A, 100 μg of BAST I (in 50 μl buffer) was injected onto a 300 mm × 7.5 mm Altex Spherogel-TSK 2000SW column and eluted at a flow rate of 1 ml/min with 100 mM sodium phosphate buffer, pH 7.0, at 1 ml/min. In B, the column was equilibrated and eluted with 10 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.

TABLE 2. Amino acid composition of purified BAST I

Amino Acid	BAST I	Hydroxysteroid Sulfotransferase 2[a]
		residues/279 residues
Asx	27	31
Glx	23	25
Cys	2	0[b]
Ser	19	17
Gly	21	22
His	5	4
Arg	8	8
Thr	17	16
Ala	10	12
Pro	15	19
Tyr	11	12
Val	13	12
Met	11	8
Ile	9	14
Leu	30	29
Phe	20	19
Lys	37	26
Try	0[c]	5

[a]From Marcus et al. (24).
[b]Hydroxysteroid sulfotransferase was not carboxymethylated by Marcus et al. (24).
[c]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 Mg^{2+}, 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 Mg^{2+} (data not shown). However, at all pHs studied, BAST I activity was only slightly stimulated by Mg^{2+}. All subsequent experiments were carried out at pH 6.5 and a Mg^{2+} 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 glycodeoxycholic 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 K_m 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

Substrate	% Relative Activity[a]		
	5 μM	20 μM	100 μM
Bile acids			
3β-Hydroxy-5-cholenoate	37	33	32
Lithocholate			
Unconjugated	1	2	2
Glycine conjugate	5	21	47
Taurine conjugate	4	10	16
Hyodeoxycholate	<1	<1	<1
3α,6β-Dihydroxy-5β-cholanoate	<1	<1	<1
Chenodeoxycholate			
Unconjugated	<1	<1	2
Glycine conjugate	<1	2	13
Taurine conjugate	<1	2	17
7-Keto-lithocholate	1	2	2
Ursodeoxycholate	<1	<1	<1
Deoxycholate			
Unconjugated	2	5	27
Glycine conjugate	3	13	84
Taurine conjugate	2	8	75
12-Keto-lithocholate	2	3	7
Cholate			
Unconjugated	<1	<1	1
Glycine conjugate	<1	<1	1
Taurine conjugate	<1	<1	2
Hydroxysteroids			
Dehydroepiandrosterone	100	65	56
Estradiol	7	7	7
Testosterone	9	9	9
Cortisol	5	9	16

[a]Measured relative to that BAST activity with 5 μM DHEA and 40 μM PAPS (9.4 nmol/min per mg protein).
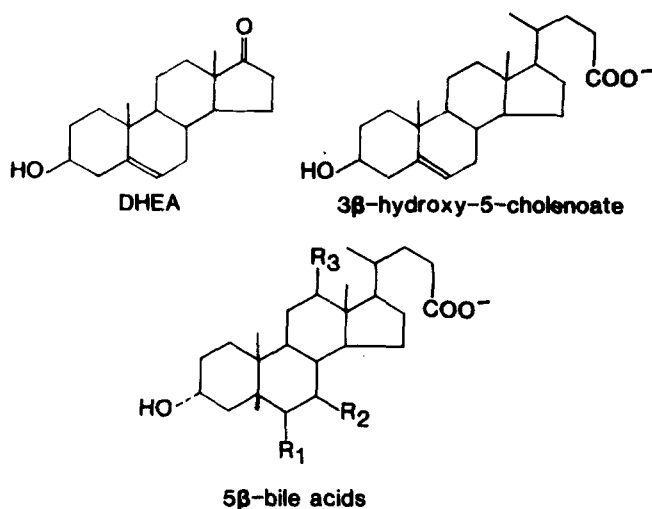
Fig. 5. Structures of dehydroepiandrosterone and 3β-hydroxy-5-cholenoate, and the 5β-bile acids tested with BAST I. The individual 5β-bile acids have the following substituents:

Bile Acid	R_1	R_2	R_3
Lithocholate	H	H	H
Hyodeoxycholate	αOH	H	H
3α,6β-Dihydroxy-5β-cholanoate	βOH	H	H
Chenodeoxycholate	H	αOH	H
7-Ketolithocholate	H	= O	H
Ursodeoxycholate	H	βOH	H
Deoxycholate	H	H	αOH
12-Ketolithocholate	H	H	= O
Cholate	H	αOH	αOH

moidal fashion from 3 to 60 μM (**Fig. 7A**), then peaked and subsequently fell (Fig. 7B). A Hill plot of the initial velocity data from 3 to 60 μM for both bile acids was linear with a slope of 1.45 (**Fig. 8**).

The initial velocity curve for DHEA did not have the sigmoidal component as seen for GLC or GDC. Instead, it was hyperbolic reaching a maximum at 2–3 μM and declining thereafter (**Fig. 9**). As for GLC, DHEA caused complete substrate inhibition of the enzyme (Fig. 9). The apparent K_m of DHEA in the presence of 2 μM PAPS (nonsaturating) was 0.72 ± 0.06 μM.

3β-Hydroxy-5-cholenoic acid, which has a steroid ring structure almost identical to that of DHEA (Fig. 5), had a V_{max} comparable to the steroid. However, the maximum enzyme activity with this bile acid was observed at 0.5 μM. Its apparent K_m in the presence of 2 μM PAPS was 0.24 ± 0.03 μM, far lower than that of 5β-bile acids (~ 50 μM). The K_m of the 5β-bile acids was inferred from the concentration of each bile acid which produced half the maximum enzyme velocity.

Two groups of experiments were carried out to test whether the enzyme activity which was sulfating DHEA was the same one as that which was sulfating 5β-bile acids. In the first group, 3KC, an inhibitor of other BASTs

(8, 23) was co-incubated with DHEA. As shown in **Fig. 10**, it is a potent competitive inhibitor of DHEA sulfation with a K_i of 0.26 ± 0.02 μM. In a similar experiment, 3KC was shown to be a competitive inhibitor of 3β-hydroxy-5-cholenoic acid sulfation; again the K_i of 3KC was 0.26 μM.

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 Δ^5 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

Bile acid	Inhibition[a]	
	30 μM	100 μM
3-Keto-5β-cholanoate	82.7	89.4
Hyodeoxycholate	45.8	52.2
3α,6β-Dihydroxy-5β-cholanoate	56.6	63.1
6-Ketolithocholate	49.0	71.4
3,6-Diketo-5α-cholanoate	49.0	57.9
3,6-Diketo-5β-cholanoate	62.4	80.0
Chenodeoxycholate	16.7	ND
Ursodeoxycholate	53.2	ND
7-Ketolithocholate	23.4	39.6
3,7-Diketo-5β-cholanoate	43.8	39.8
3,12-Diketo-5β-cholanoate	ND	2.7

[a]The 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.
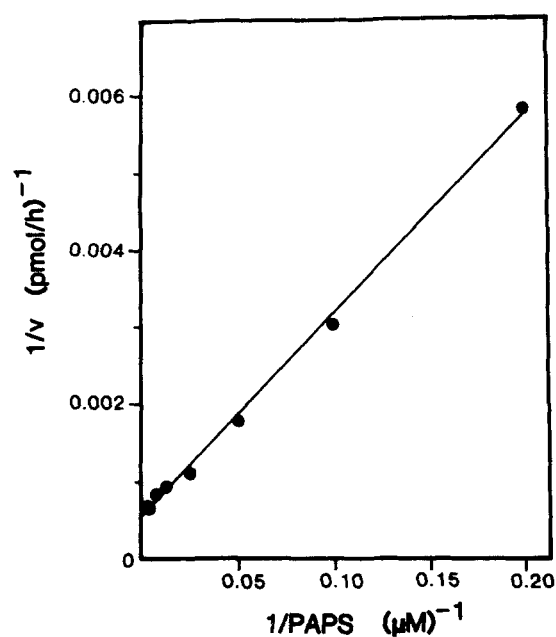
Fig. 6. Initial velocity curve versus the PAPS concentration at a fixed GLC concentration. BAST I (0.5 μg) was added to incubates containing 100 μM GLC and varying amounts of PAPS (2.5–500 μM) in 5 mM $MgCl_2$–100 mM sodium phosphate buffer, pH 6.5. Data are plotted in the form of a double reciprocal plot (1/v vs. 1/s).

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 chroma-

tography (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 BASTs 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
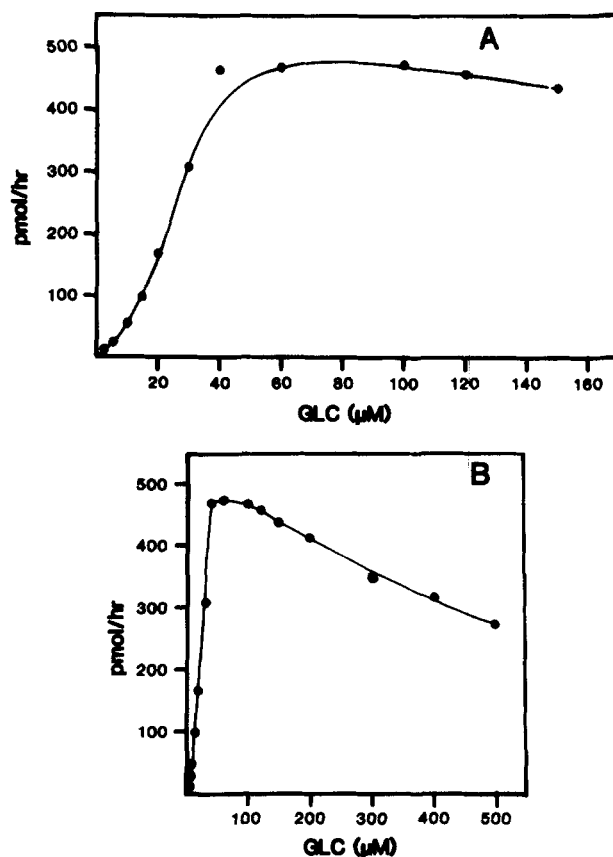
Fig. 7. Initial velocity curves versus GLC concentration at fixed PAPS concentration. BAST I (0.5 μg) was added to incubates containing 40 μM PAPS and varying amounts of GLC (3–500 μM) in 5 mM $MgCl_2$–100 mM sodium phosphate buffer, pH 6.5. In A, the sigmoidal part of the curve in the range from 3 to 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.
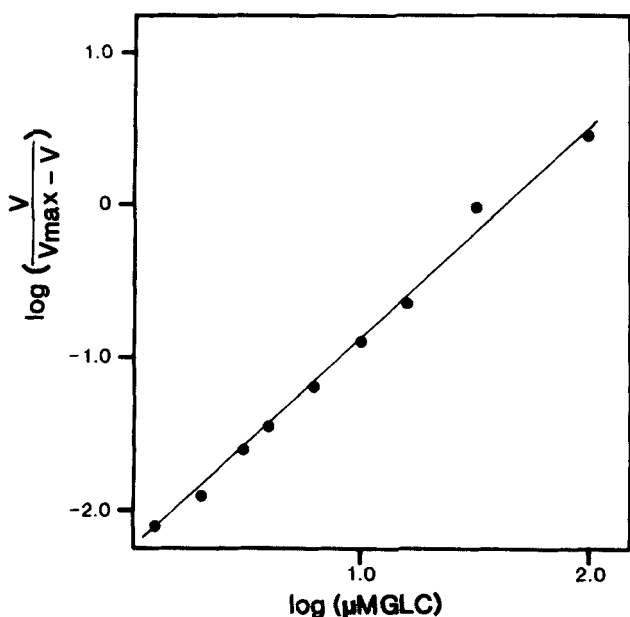
Fig. 8. A Hill plot of initial velocities over a GLC concentration range of 3–60 μM at fixed PAPS concentrations. Data from Fig. 7A were used in this figure. The slope of the line is 1.45.

Since the distance from the 3α-hydroxy group on the bile acid molecule to the 6($\alpha\beta$)-, 7($\alpha\beta$)-, and 12α-positions is approximately the same (4–5 Å), it is necessary to speculate that the bile acid molecule has to enter the active 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* configuration 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 lithocholic 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-oxygenated bile acids to be substrates for BAST I, nonetheless they must be able to approach the active site since each of them, with the possible exception of chenodeoxycholic acid, is able to inhibit sulfation of GLC when incubated with GLC on an equimolar basis.

The reaction mechanism for BAST I could not be determined 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-
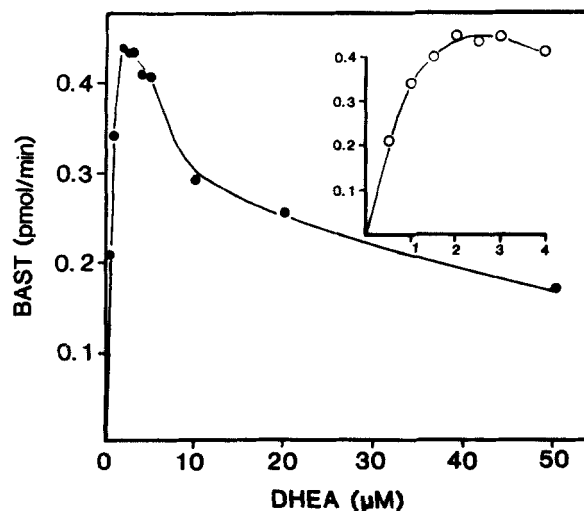
two cysteine residues in BAST I. Marcus et al. (24) did not carboxymethylate purified hydroxysteroid sulfotransferase 2 and therefore would not have measured the two cysteine residues.

Contrary to 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_{max} of this preparation 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). Unlike 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 preparation of male rat liver BAST. However, these investigators 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 hydroxtsteroid 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 concentrations (~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.

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.

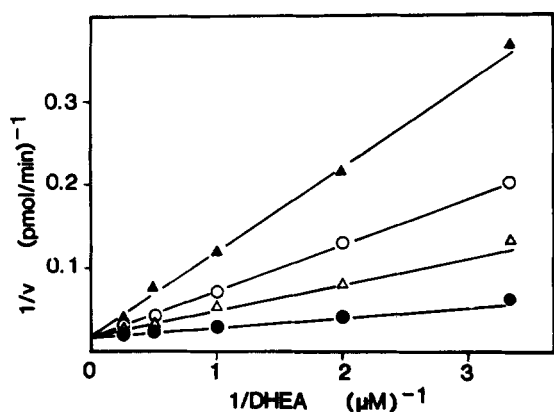**Fig. 10.** Inhibition of DHEA sulfation by 3-keto-5β-cholanoic acid. BAST I (0.1 μg) was incubated at 37°C for 20 min in 100 mM sodium phosphate buffer, pH 6.5, in the presence of 2 μM PAPS, varying concentrations of DHEA alone (●), and with 0.5 μM (△), 1.0 μM (○), and 2 μM (▲) 3-keto-5β-cholanoic acid.
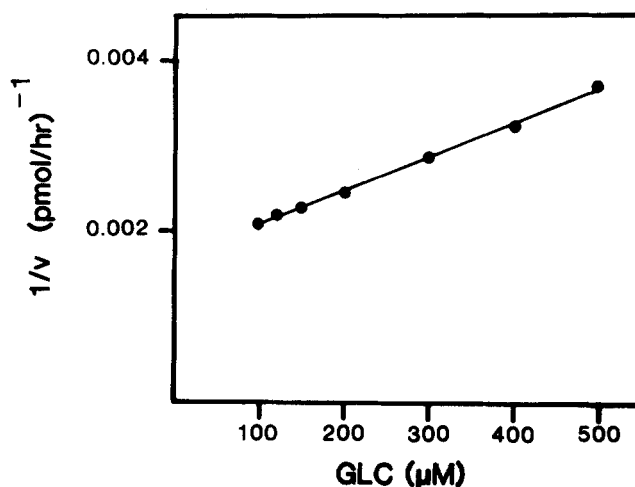

Fig. 12. A plot of the reciprocal initial enzyme velocities versus GLC concentration in the range from 100 to 500 μM. Data from Fig. 7B were used in this plot.
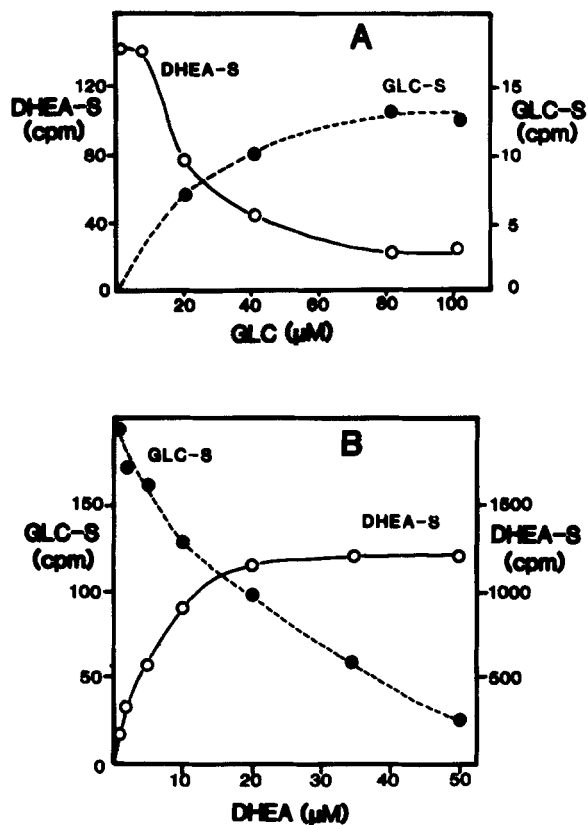

Fig. 11. Mutual inhibition of DHEA and GLC on the sulfation of each other by BAST I. In A, the concentration of DHEA was held constant (2.5 μM) and that of GLC was varied. In B, the concentration of GLC was held constant (60 μM) and that of DHEA was varied. More BAST I was added in B. The ^{35}S-labeled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

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). ■

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