Purification and Activation of Brain Sulfotransferase*

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Galactosylceramide sulfotransferase (EC 2.8.2.11) catalyzes the biosynthesis of sulfatide from galactocerebroside and adenosine 3'-phosphate 5'-phosphosulfate (PAPS). This enzyme is developmentally controlled, reaching a maximum activity in the brains of mice corresponding to that of maximum myelination. The product, sulfatide, is an important component of myelin. This transferase from mouse brain has been purified 2600-fold using a combination of pyridoxal 5'-phosphate- and ATP-ligated columns. The purified enzyme yielded a single band following SDS-polyacrylamide gel electrophoresis with an apparent Mr of 31,000. The entire purification procedure can be completed in 1 day. The pH optimum for the enzyme is 7.0. The Kₚ for PAPS is 1.2 × 10⁻⁶ M, and the Kₚ for cerebroside is 2.6 × 10⁻⁶ M. Cerebroside concentrations >80 pmol/ml are inhibitory. Enzyme preparations were associated with several lipids. Vitamin K + P₄ activated purified preparations of the sulfotransferase and maintained enzyme activity during storage at −80 °C.

Galactosylceramide sulfotransferase (EC 2.8.2.11) catalyzes the formation of sulfatide from galactocerebroside and adenosine 3'-phosphate 5'-phosphosulfate. This enzyme is developmentally controlled, showing maximum activity 18–22 days after birth. Sulfatides are important components of myelin; they have also been implicated in the binding of thrombospondin (1), laminin (2), properdin (3), and CD62/P-selectin (4) and in the activation of protein kinase C by tumor promoters (5). Unregulated biosynthesis of sulfatide due to a defect in the catabolic enzyme arylsulfatase A results in metachromatic leukodystrophy, a fatal genetically determined disease (6).

Previous studies (7, 8) have shown that the activity of galactocerebroside sulfotransferase in the maturing mouse brain is regulated by vitamin K. We earlier reported a reduction in sulfatide levels in the brains of young mice following the administration of warfarin, the vitamin K antagonist. This effect of warfarin was reversed by the subsequent administration of vitamin K (9). Whereas most of these compounds stimulated activity, pyridoxal 5'-phosphate (PLP) was a strong inhibitor; 0.2 mM PLP completely inhibited the enzyme. This occurred with no preincubation and in the presence of 10 mM ATP. In contrast to the inhibitory action of PLP, pyridoxal and pyridoxamine were not inhibitory.

In previous studies by other workers, enzyme purification factors of up to 500-fold (10) have been reported, although a considerably lower purification factor was recently obtained (12). Here, we report a purification of ~2600-fold using PLP-ligated and ATP-ligated columns.

A preliminary report of these results has been presented (13).

EXPERIMENTAL PROCEDURES

Male Swiss mice (ICR) weighing 8–12 g were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were used at 16 days old post-weaned. Animals were killed by CO₂ asphyxiation, and the brains were removed. Brain microsomes were prepared as described previously (7, 8). The brains were homogenized in 0.32 M sucrose to give 40–60 mg/ml protein. After a 30-min centrifugation at 50,000 × g, the supernatant was centrifuged at 100,000 × g for 90 min. The microsomal pellet was stored suspended in 0.32 M sucrose at −80 °C at a concentration of 5 mg/ml protein. The microsomal preparation was extracted with an equal volume of 100 mM imidazole buffer (pH 7.2) containing 20 mM MgCl₂, 4 mM dithiothreitol, and 1% Triton X-100 (buffer A). The Triton-extracted material was centrifuged (100,000 × g, 30 min), and the supernatant was used for further purification.

Cerebroside sulfotransferase assay was performed as described by Sakac and Lingwood (14) and as modified (7, 8). Galactocerebroside (20 nmol) was dissolved in 25 μl of chloroform/methanol (2:1, v/v) containing 4% Triton X-100; evaporated under N₂; and then resuspended in 0.2 ml of buffer A containing 10 mM ATP, 2 μM [³⁵S]PAPS (200,000 cpm), and 50 μl of enzyme preparation in a total volume 0.25 ml. After incubation for 1 h at 37 °C, the reaction was terminated by addition of 5 ml of chloroform/methanol, and the mixture was partitioned into 0.2 ml of aqueous 0.88% KCl. The lower phase was washed twice with 1 volume of methanol/H₂O (1:1, v/v) and concentrated under N₂. The concentrated extract was passed through a Sephadex G-25 column (0.5-cm diameter, 1 g of Sephadex) equilibrated with chloroform/methanol/H₂O (120:80:9, v/v) and further eluted with 2 column volumes of the solvent. This effectively removed the unused [³⁵S]PAPS and any inorganic ³⁵S-labeled compounds from the lipid products. The radiolabeled products were separated by TLC on silica gel, detected by reference to standards after exposure to iodine vapors, scraped from the plates, and counted.

Enzyme Purification—Gel filtration was performed at 6–8 °C using Sephacryl S-200 in a column (2.5 × 80 cm) calibrated with proteins.

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† The abbreviations used are: PLP, pyridoxal 5'-phosphate; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; PAGE, polyacrylamide gel electrophoresis.
of known Stokes radii. Fractions of the supernatant, eluted with buffer A, containing sulfotransferase activity were pooled, concentrated by ultrafiltration (Amicon PM-10 membrane), and analyzed further by SDS-PAGE or by PLP- and ATP-ligated column chromatography.

**PLP-ligated Column Chromatography**—PLP was ligated to Sepharose by the method of Churchich (15). EAH-Sepharose 4B (50 ml) was suspended in 50 ml of 10 mM Tris (pH 7.0), mixed with 1 g of pyridoxal 5'-phosphate, and left at 25 °C overnight in the dark. Later it was reduced by addition of 20 mg of sodium borohydride at 4 °C, washed several times with 300 mM KCl and water, and kept at 4 °C in the dark. The active fraction from the Sepharose S-200 column was passed through a 3-ml column of PLP-Sepharose that was washed three times with buffer A. Less than 5% galactocerebroside sulfotransferase activity was contained in the eluate. The column was then washed with 5 column volumes of buffer A containing 100 mM KCl. The galactocerebroside sulfotransferase retained by the column was eluted with 5 column volumes of buffer containing 100 mM KCl and 5 mM PLP. The eluate from this procedure was desalted by passage through a Pharmacia LKB Biotechnology PD-10 column.

**ATP-ligated Column Chromatography**—A 3-ml column of ATP-ligated agarose (Sigma, C₆ linked to 4% beaded Agarose) was used. The active fraction from Sepharose S-200 and PLP-ligated columns was applied to the ATP column and washed with 5 column volumes of buffer A. The enzyme was eluted with 5 column volumes of buffer containing 50 mM KCl and 5 mM ATP. The eluate was desalted by passage through a Pharmacia PD-10 column.

**PAGE Analysis**—Purified sulfotransferase was examined by SDS-PAGE as follows. Lyophilized enzyme (1.5-13 pg of protein) was added to a slot in a minigel system (3.5% acrylamide), and run at a constant 200 V for 30 min. The gels were stained with silver stain (Bio-Rad).

**Photoaffinity Labeling**—The procedure of Otterness et al. (16) was used in attempts to photoaffinity label the enzyme.

**Materials**—[3H]SAP (1.6 Ci/mmol) was obtained from Du Pont-New England Nuclear; AquaMEPHYTON (colloidal suspension of vitamin K₁ in detergent/glucose/soybean alcohol) was from Merck; and EAH-Sepharose 4B and PD-10 columns were from Pharmacia. Galactocerebroside was obtained from Matreya, Inc. (Pleasant Gap, PA) and was a mixture of α-hydroxy and non-hydroxy fatty acids. Other chemicals were from Sigma. All the chemicals were reagent grade or the best quality available.

**RESULTS**

**Sepharose S-200 Chromatography**—The enzyme eluted from a Sepharose S-200 column as a single peak with an apparent Mₐ of 28,000. A 5-fold purification resulted from this procedure.

**Pyridoxal 5'-Phosphate-ligated Column Chromatography**—We have shown previously that PLP is a strong inhibitor of galactocerebroside sulfotransferase; 0.2 mM completely inhibited enzyme activity. This occurred with no preincubation and in the presence of 10 mM ATP (9). This property of PLP was used to purify the enzyme further. Elution from a PLP-ligated Sepharose column (15) resulted in a 250-fold purification of brain sulfotransferase (Table I).

**ATP-ligated Agarose Column Chromatography**—The activity of the sulfotransferase by ATP suggested the application of an ATP-ligated column in the purification of the enzyme. When a solubilized microsomal extract was passed through the ATP-ligated column, the enzyme was bound, whereas the majority of other proteins were eluted. The enzyme was then eluted using 50 mM KCl + 5 mM ATP. When a microsomal extract was applied, a 400-fold purification of the enzyme was obtained by this procedure: an increase in specific activity from 4.8 to 1868 pmol of sulfatide/mg/h.

The sequential use of the PLP- and ATP-ligated columns resulted in a 2.5-fold enhancement of purification over PLP column chromatography alone, yielding a final purification factor of 2600. The entire purification procedure was completed in 1 day.

**Gel Electrophoresis**—Since undelipidated enzyme yielded a streak following SDS-PAGE, enzyme prepared by PLP and ATP column chromatography was delipidated and subjected to SDS-PAGE under reducing conditions. This procedure yielded a single band following silver staining (Fig. 1). The band has an apparent Mₐ of 31,000.

**pH Dependence of Sulfotransferase Activity**—The activity of the purified enzyme prepared by PLP chromatography was determined at various pH values; enzyme activity was diminished at acidic pH values. The optimum appears to be at pH 7.0; however, alkaline values showed a gradual reduction in activity over the range tested (to pH 7.8).

**Kᵩ for PAPS**—The Kᵩ for PAPS was determined as shown in Fig. 2a and yielded a value of 1.2 × 10⁻⁶ M. The data points fit the theoretical curve at the concentrations tested.

**Response of Sulfotransferase to Added Cerebroside**—The effect of cerebroside concentration on enzyme activity was determined as described under “Experimental Procedures.” The response of the enzyme to added cerebroside varied; in some experiments using microsomal preparations, added cerebroside did not significantly affect enzyme activity over a range of concentrations (to 75 μmol/reaction); approximately maximum sulfatide yield occurred with no added galactocerebroside. With the purified enzyme preparation, the optimum level for enzyme activity occurred at 20 nmol of cerebroside;

**TABLE I**

| Purification step | Protein | Specific activity | Recovery | Purification |
|------------------|---------|------------------|----------|--------------|
| Brain homogenate | 36.5 mg | 1.2 pmol/SO₄/mg | 100%     | 1-fold       |
| Microsomes       | 13.1    | 4.8 pmol/SO₄/mg | 100%     | 4-fold       |
| PLP-Sepharose    | 0.028   | 1380 pmol/SO₄/mol| 61.5%    | 1150-fold    |
| ATP-agarose      | 0.009   | 3130 pmol/SO₄/mol| 44.8%    | 2610-fold    |

* Recovery from the column purification is based on the microsomal preparation.

**FIG. 1.** SDS-PAGE of sulfotransferase on 7.5% gel at stages in purification. Details are described under “Experimental Procedures.” Lane A, standards (phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa; and lysozyme, 14 kDa); lane B, microsomal extract (13 μg of protein); lane C, Sepharyl S-200 fraction (5 μg of protein); lane D, PLP-column-eluted purified sulfotransferase (31 kDa; arrow) (~1.5 μg of protein).
higher concentrations were less active. The $K_m$ for cerebroside was determined to be $2.6 \times 10^{-6}$ M. Fig. 2 shows the theoretical curve for the enzyme activity calculated from the data obtained from the plot in the inset; the points represent the observed values that diverge from the theoretical curve at concentrations $>80$ μM. The purified enzyme preparation contained ~20 pmol/reaction, determined as described below.

Lipid Analysis of Crude and Purified Enzyme Preparations—The inability of added cerebroside to enhance enzyme activity suggested that a significant quantity of cerebroside could be associated with the nonpurified preparations. Purified enzyme was lyophilized, and lipids were extracted and analyzed as described previously (7, 8). Cholesterol, phosphatidylycerol, and cerebrosides were detected in trace amounts by TLC. Approximately 25 ng of cerebroside were associated with 0.5 μg of purified enzyme protein/reaction mixture, whereas the crude microsomal preparation contained 55 μg of cerebroside/reaction mixture (250 μg of protein). Attempts were made to delipidate the enzyme by extraction with cold (~20 °C) acetone. This procedure resulted in loss of enzyme activity, which could not be restored by adding back the extracted lipids (data not shown).

Stability of Purified Galactocerebroside Sulfotransferase—Previous reports have indicated that this enzyme is unstable on storage at −40 °C (10). We found a 35% reduction in activity after 7 days at −80 °C. Addition of vitamin K$_1$ + P$_i$ (5 mM) maintained the original activity of the sulfotransferase for the 14-day period tested. Ethylene glycol, Me$_2$SO, ATP (10 mM), and a mixture of phosphatidylethanolamine and phosphatidylcholine (1:1, 100 μg/reaction mixture) did not improve enzyme stability at −80 °C.

Activation of Purified Enzyme by ATP, Orthophosphate, Vitamin K$_1$, and Menadione—This experiment was performed to determine if the purified enzyme was activated in a manner similar to that reported using brain microsomal extracts (9). The purified enzyme showed the same degree of activation with orthophosphate + vitamin K$_1$ or with ATP as did the crude preparations.

**DISCUSSION**

Brain galactocerebroside sulfotransferase is a membrane enzyme that hitherto has not been purified. Tennekoon and McKhann (10) reported a 500-fold purification of galactocerebroside sulfotransferase from rat brain, which included a 136-fold purification step in the preparation of microsomes from brain homogenate. This degree of purification for this procedure was not obtained by Fressinaud et al. (12) or by us (Table I). Fressinaud et al. (12) have described 7-9-fold lower purification factors using a number of column purification procedures. They attributed their inability to purify galactocerebroside sulfotransferase further to the instability of the enzyme, the necessity for nonionic detergents, and the denaturation following delipidization.

Galactocerebroside sulfotransferase was retained by an ATP-ligated column, and elution from this column resulted in a purification of ~400-fold. Our observation that galactocerebroside sulfotransferase is sensitive to pyridoxal 5'-phosphate has enabled us to use a PLP-ligated column to purify the enzyme 250-fold. The combination of both columns gave a purification of 2600. The PLP column was used prior to the ATP column to avoid degradation of the latter by microsomal ATPases. The advantages of the very rapid purification procedure, which can be completed in 1 day, are obvious.

SDS-PAGE of column-purified galactocerebroside sulfotransferase resulted in a diffuse band. Following lipid extraction of the column-purified material, a single well-defined
band was found. However, attempts to confirm the identity of the single band by photoaffinity labeling \[^{35}S\]PAPS to the enzyme were not successful since after photoaffinity labeling and PAGE, the compound formed did not enter the gel. Similar difficulties with photoaffinity labeling this enzyme have been reported (12). Our results indicate an \( M_c \) of 28,000 based on column chromatography and 31,000 based on SDS-PAGE. Since these results are comparable, subunits of the enzyme apparently are not produced by SDS-PAGE. The molecular weight of brain sulfotransferase is reported for the first time; molecular weights for the kidney galactocerebrosidase sulfotransferase have been reported as 42,000 (17) and 64,000 (18). The kidney enzyme thus differs from that of the brain in size as well as in its response to vitamin K (7).

A number of differing \( K_m \) values for PAPS (0.9 \( \times 10^{-4} \) M and 0.15 \( \mu \text{Ci/ml} \)) (10) and for galactocerebroside (2.0-80 \( \times 10^{-6} \) M) (11) have been reported. Of these, the \( K_m \) values for PAPS (10) and cerebroside (11) are similar to our values of 1.2 \( \times 10^{-4} \) and 2.6 \( \times 10^{-5} \) M, respectively. The response to added cerebroside is of interest; with crude microsomal extracts, addition of cerebroside increased sulfatide formation in size as well as in its response to vitamin K (7).

The ability of ATP and vitamin K \( _1 \) \( _2 \) to activate the enzyme was judged too small to influence the enzyme activity. There is some specificity regarding this enzyme-activating property of vitamin K since other compounds tested, addition of vitamin K showed a response to added cerebroside up to a 20-nmol level, and Pi activate the enzyme by phosphorylating the enzyme and that vitamin K has a role involving Pi; vitamin K alone had no effect on enzyme activation (9). In this regard, it is of interest to note that protein kinases or protein kinase inhibitors did not affect sulfotransferase activity.\(^2\) Whereas these experiments do not completely rule out the involvement of a kinase, the activation of brain sulfotransferase thus differs considerably from that of the sulfogalactosylglycerolipid sulfotransferase in the testis (21, 22). Lingwood and co-workers (23) have investigated the galactolipid sulfotransferase from rat testis and have shown that phosphorylation is required for activation. This enzyme has recently been purified to homogeneity by use of 3'-phosphoadenosine 5'-phosphosulfate-agarose chromatography and shown to be a 56-kDa protein. Although there are similarities to the brain sulfotransferase in the requirement for phosphorylation, the testis enzyme is not activated by vitamin K (24).

The availability of purified galactocerebrosidase sulfotransferase will facilitate study of the vitamin K-mediated regulatory mechanism and its significance in vivo in brain maturation.

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\(^2\) K. Soma Sundaram and Meir Lev, unpublished data.