A novel mass spectrometric assay for the cerebroside sulfate activator protein (saposin B) and arylsulfatase A

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Abstract A mass spectrometric method is described for monitoring cerebrosides in the presence of excess concentrations of alkali metal salts. This method has been adapted for use in the assay of arylsulfatase A (ASA) and the cerebroside sulfate activator protein (CSAct or saposin B). Detection of the neutral glycosphingolipid cerebroside product was achieved via enhancement of ionization efficiency in the presence of lithium ions. Assay samples were extracted into the chloroform phase as for the existing assays, dried, and diluted in methanol-chloroform-containing lithium chloride. Samples were analyzed by electrospray ionization mass spectrometry with a triple quadrupole mass spectrometer in the multiple reaction monitoring tandem mass spectrometric mode. The assay has been used to demonstrate several previously unknown or ambiguous aspects of the coupled ASA/CSAct reaction, including an absolute in vitro preference for CSAct over the other saposins (A, C, and D) and a preference for the nonhydroxylated species of the sulfatide substrate over the corresponding hydroxylated species. The modified assay for the coupled ASA/CSAct reaction could find applicability in settings in which the assay could not be performed previously because of the need for radiolabeled substrate, which is now not required.— Norris, A. J., J. P. Whitelegge, A. Yaghoubian, J-R. Alattia, G. G. Privé, T. Toyokuni, H. Sun, M. N. Brooks, L. Panza, P. Matto, F. Compostella, N. Remmel, R. Klingenstein, K. Sandhoff, C. Fluharty, A. Fluharty, and K. F. Faull. **A novel mass spectrometric assay for the cerebroside sulfate activator protein (saposin B) and arylsulfatase A. *J. Lipid Res.* 2005.** 46: **2254–2264.**

Supplementary key words saposins A, B, C, and D • electrospray ionization • tandem mass spectrometry

Metachromatic leukodystrophy (MLD) is a neurodegenerative autosomal recessive disease in which affected individuals most commonly have a defect in arylsulfatase A (ASA) enzyme activity originating from ~60 different known mutations (1). MLD can also arise as a result of a defective cerebroside sulfate activator protein (CSAct), also known as saposin B (2), as the sphingolipid activator protein 1 (3), or as the nonspecific activator protein (4). In this and other publications from the University of California Los Angeles (UCLA) group, this protein is referred to as CSAct to reflect the activity that is measured. The function of ASA is to metabolize intracellular sulfatide (sulfogalactosyl cerebroside) via enzymatic hydrolysis of the 3-*O*-galactosyl-sulfate group. However, ASA is apparently unable to perform this function as long as the lipid substrate is confined within the membrane. CSAct is thought to sequester sulfatide from membrane fragments incorporated into lysosomes by a poorly understood lipid-exchange mechanism (5), and in the resulting CSAct-sulfatide com-

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Abbreviations: ASA, arylsulfatase A; CSAct, cerebroside sulfate activator protein; ESI, electrospray ionization mass spectrometry; IS, internal standard; MALDI, matrix-assisted laser desorption ionization; MLD, metachromatic leukodystrophy; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; TOF, time-of-flight; U, unit of enzyme activity (the amount of enzyme activity that will catalyze the transformation of 1 μmol of the substrate per minute under standard conditions); UCLA, University of California Los Angeles.

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plex, the polar sugar sulfate moiety is sufficiently exposed for hydrolysis catalyzed by lysosomal ASA (**Fig. 1**). During this process, the hydrophobic tail of the lipid is confined to a pocket within the dimeric protein shell and is shielded from the hydrophilic environment (6). Thus, the true in vivo substrate for ASA is the CSAct-sulfatide complex, and in this process CSAct serves as the lipid carrier.

Humans deficient in ASA and/or CSAct activity are unable to catabolize sulfatide, leading to sulfatide accumulation and, subsequently, neurological damage (7). In MLD-affected individuals, progressive demyelination of central and peripheral axonal myelin sheaths occurs via an unclear mechanism. This results in various debilitating clinical symptoms, including loss of previously achieved milestones in infants such as walking, crawling, and chewing, along with symptoms of hypotonia, hyporeflexia, optic atrophy, and eventually death.

In principle, both CSAct and ASA activity could be measured in vitro by measuring the temporal change in concentration of one or more of the participating reactants/products in the coupled reaction (Fig. 1). This would involve measuring the rate of either substrate (sulfatide) depletion or product (cerebroside and/or inorganic sulfate) formation. In practice, ASA activity can be measured using artificial substrates (*p*-nitrocatechol sulfate or 4-methylumbelliferyl sulfate) by following the formation of colored [*p*-nitrocatechol, λ_{max} = 550 nm (8)] or fluorescent [4-methylumbelliferol, λ_{ex} = 365 nm, λ_{em} = 450 nm (9)] products, respectively. These assays are performed without difficulty in a homogeneous milieu because all participants in the reaction are water-soluble. However, assays using natural sulfatide substrate are more complicated, because of the poor water solubility of monomeric sulfatide and its propensity to form aggregates. In this case, ASA activity can be measured using detergent-solubilized sulfatide (10), whereas CSAct activity can be measured using CSAct-solubilized sulfatide (11). As done previously in the UCLA laboratory, both of these procedures involve measuring the rate of inorganic sulfate formation. Because existing assays for inorganic sulfate were insufficiently sensitive, ^{35}S-radiolabeled sulfatide was used and the production of radioactive inorganic sulfate was measured. ^{35}S-radiolabeled sulfatide is prepared by in vivo administration of [^{35}S]sulfate to rat pups, followed by extraction and purification of the radiolabeled lipid (12). This radiolabeled lipid is not available commercially, and the tedious and time-consuming preparation requires relatively large amounts of radioactivity to yield a product with adequate specific activity. Furthermore, the short ^{35}S half-life of 87.32 days means that the effective shelf life of the [^{35}S]sulfatide is ~6 months (unpublished observation), necessitating the periodic preparation of new material. More recently, ^{14}C-radiolabeled sulfatide has been used as substrate and the production of [^{14}C]cerebroside measured (6), but this substrate is expensive and until recently was not generally available.

There is a need for a more convenient assay with which the natural substrate system can be monitored. Mass spectrometry provides a valuable tool for the analysis of lipids and lipid-metabolizing enzymes (13). Previous work using mass spectrometry for the analysis of lipid-metabolizing enzymes has involved the use of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization mass spectrometry (ESI) (14, 15). In the present case, the possibility of following sulfatide depletion by ESI has been tested by taking advantage of the strong signals that these lipids yield in the negative ion mode (16, 17); more importantly, the possibility of following cerebroside formation has been tested by taking advantage of the strong signals that these lipids yield as their lithiated adducts in the positive ion mode (18). These two approaches have been tried, and here we present the results of this comparison, which showed that monitoring cerebroside formation by ESI coupled to tandem mass spectrometry (MS/MS) provides a reliable, fast, specific, nonradioactive method for assays involving both CSAct- and detergent-solubilized sulfatide. The assays have been used to reveal hitherto obscure but potentially important details of the sulfatide specificity for binding by CSAct and the saposin specificity for the ASA-catalyzed reaction.

MATERIALS AND METHODS

Chemicals and reagents

Bovine brain sulfatide and cerebroside and semisynthetic [$^{2}H_{35}$] stearoyl cerebroside ([$^{2}H_{35}$]C_{18}:0 cerebroside) were obtained from Matreya, Inc. (State College, PA). Taurodeoxycholate, lithium chloride, and BSA were from Sigma Aldrich (St. Louis, MO). Quartz-distilled water (<16 mΩ/cm) was produced in house, and all other reagents and solvents were of analytical grade or better.

Preparation of synthetic sulfatides

Nonhydroxylated sulfatides with fatty acyl chain (R moiety; Fig. 1) compositions of C_{16}:0 (palmitic), C_{18}:0 (stearic), C_{22}:0

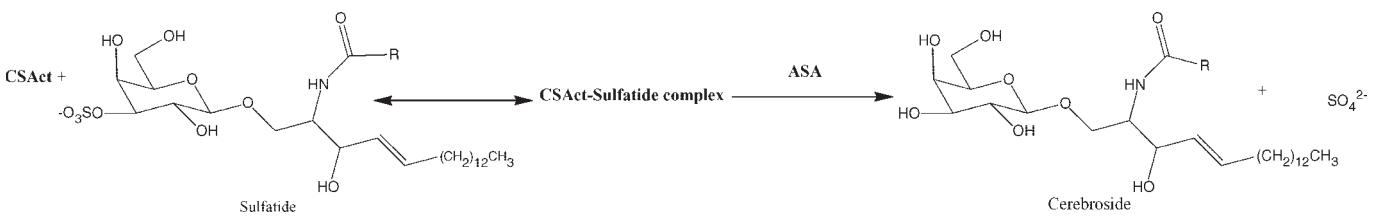

Fig. 1. Scheme of the arylsulfatase A/cerebroside sulfate activator protein (ASA/CSAct) coupled reaction in which sulfatide (cerebroside sulfate) is converted to cerebroside with the release of inorganic sulfate. In this reaction, CSAct presents the sulfatide substrate to ASA, with subsequent sulfate hydrolysis.

(behenic), and C_{24}:1^{15} (nervonic) were synthesized as described (19). Hydroxylated sulfatides with fatty acyl chain (R moiety; Fig. 1) compositions of α-hydroxylated C_{18}:0 (hC_{18}:0, α-hydroxy-stearic) and hC_{22}:0 (α-hydroxy-behenic) were synthesized from racemic mixtures of the corresponding hydroxylated fatty acids according to the following procedure. First, β-D-galactosylsphingosine was prepared through stereoselective glycosylation of 3-*O*-benzoyl azidosphingosine (20) with 2,3,4,6-tetra-*O*-pivaloyl-α-D-galactopyranosyl trichloroacetimidate, followed by removal of the protecting groups and azide reduction (19). Then, the corresponding fatty acid (1.5 eq.), with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.0 eq.) and *N*-hydroxybenzotriazole (1.5. eq.), was added to a solution of the β-D-galactosylsphingosine (0.03 g) in dichloromethane (2 ml). The mixture was refluxed (1 h) and diluted with methanol, and then the solvent was removed under vacuum. After purification by flash chromatography with dichloromethane-methanol (85:15, v/v), the galactosylceramide was selectively sulfated at position 3 as described previously (19) and the 3-*O*-sulfated products were recovered in 50% yields.

Negative ion ESI of the final products (17; see below) from solutions in chloroform-methanol-triethylamine-water (4:5:1:0.05, v/v) showed intense signals for each negatively charged molecule (mass spectra not shown) corresponding to the $(M\text{-}H)^-$ anions: C_{16}:0, observed *m/z* 778.2, calculated 778.51 Da; C_{18}:0, observed *m/z* 806.4, calculated 806.54 Da; C_{22}:0, observed *m/z* 862.8, calculated 862.61 Da; C_{24}:1^{15}, observed *m/z* 888.9, calculated 888.62 Da; hC_{18}:0, observed *m/z* 822.6, calculated 822.54 Da; hC_{22}:0, observed *m/z* 878.7, calculated 878.60 Da. Based on the negative ion ESI spectra, the purity of each sample was estimated to be in excess of 90%.

Aqueous sulfatide suspensions were prepared as described previously (21). Briefly, aliquots of stock solutions in chloroform-methanol-water (63:32:5, v/v) were dried under nitrogen. The dried residue was redissolved in 40 μl of the same solvent. Sufficient boiling water was then added to bring the mixture to 1 mg/ml, and it was then vigorously mixed, sonicated briefly, and vigorously stirred for 2 h, during which time the temperature of the mixture slowly returned to room temperature. The result was a faintly turbid suspension that was stored frozen at −80°C. Before use, it was brought to room temperature and vigorously stirred for 30 min.

Detergent-solubilized sulfatide was prepared from bovine sulfatide [125 μl, 12 mg/ml in chloroform-methanol (2:1, v/v)] and dried to near dryness under a stream of nitrogen gas in a 2 ml conical glass vial. The white precipitate was then redissolved in 250 μl of taurodeoxycholate (5 mg/ml in 4 mM sodium sulfate containing 0.73% sodium chloride) by vigorous stirring for 1 h at room temperature.

ASA

ASA was purified from human liver as described previously (22) and stored at −80°C in 25 mM Tris-Cl at pH 7.5 with a spe-

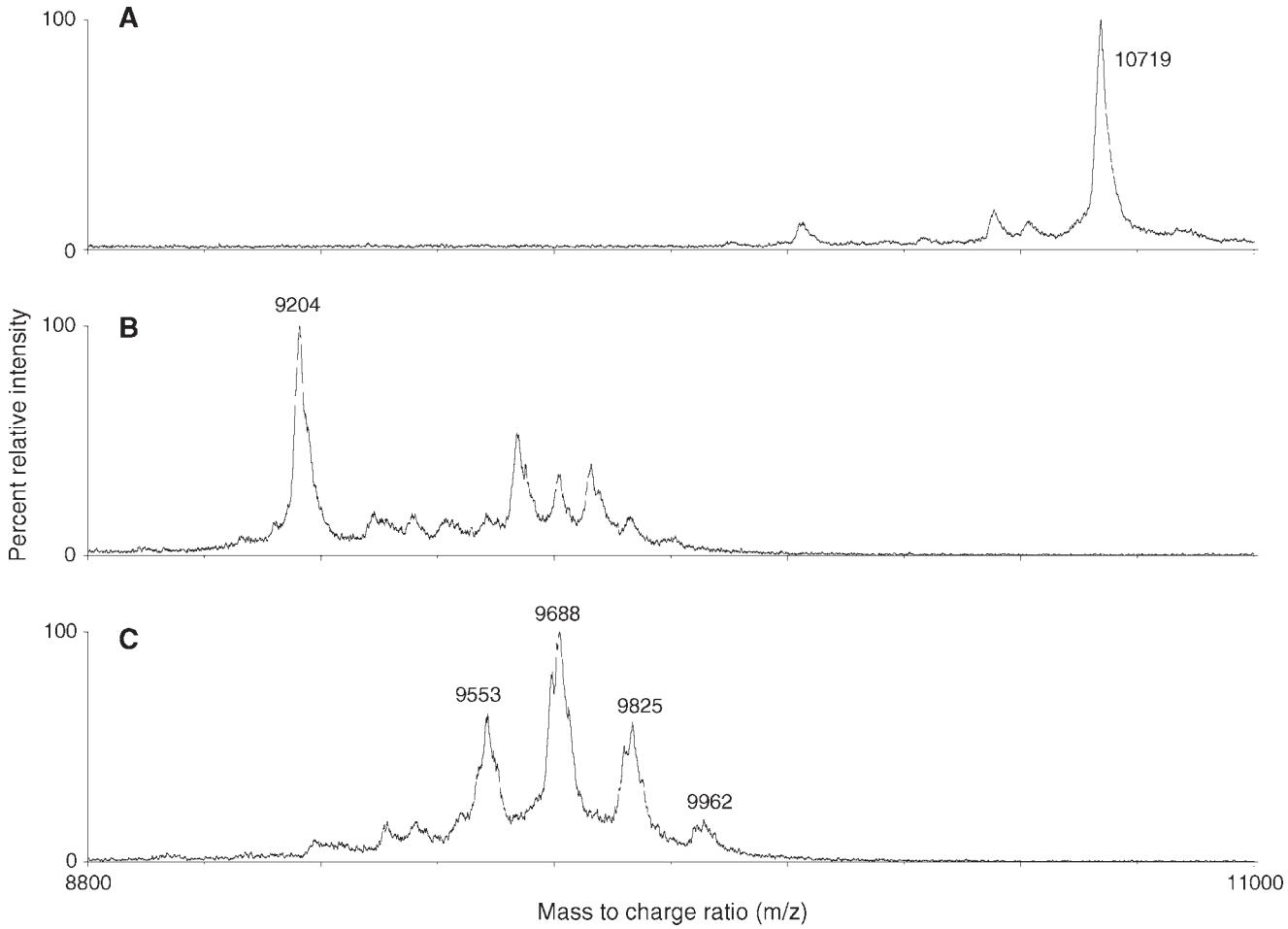

Fig. 2. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectra of recombinant human saposins A (A), C (B), and D (C) produced by the *P. pastoris* expression system. The measurement errors are within the accuracy typically obtained with MALDI-TOF of proteins (±10 Da at 10 kDa). See Materials and Methods for details.

cific activity of ~3,000 units (U) of enzyme activity (the amount of enzyme activity that will catalyze the transformation of 1 μmol of the substrate per minute under standard conditions)/mg protein as determined by a modified Baum, Dodgson, and Spencer (8) assay using *p*-nitrocatechol sulfate as substrate. When needed for the CSAct and ASA assays, the stock was diluted in 25 mM Tris-HCl, pH 7.5, containing 1 mg/ml BSA to an activity of 250 U/ml.

CSAct and saposins A, C, and D

Native CSAct was purified from pig kidney as described previously (11). The preparation contained predominantly a glycosylated 79 amino acid residue protein and has been characterized extensively by chromatographic and mass spectrometric analysis (23), with biological activity demonstrated by in vitro and in vivo assays (21, 24, 25). The material was stored as a 27 mg protein/ml solution in 25 mM Tris-HCl, pH 7.4, at −80°C and diluted as indicated for the various assays.

Two different sources of recombinant human saposins were used. The human prosaposin sequence numbering will be used throughout (Swiss-Prot primary accession number P07602 at http://us.expasy.org). One set of proteins was prepared as nonglycosylated hexahistidine-tagged molecules in a yeast (*Pichia pastoris*) expression system according to standard Invitrogen™ protocols and purified by ion exchange and affinity chromatography (26). Saposins A and C were stored as 0.66 and 0.60 mg protein/ml solutions, respectively, in 50 mM sodium phosphate buffer containing 300 mM sodium chloride, pH 4.0, at −80°C. Saposin D was stored as a 1.0 mg protein/ml solution in 10 mM citrate buffer, pH 4.0, at −80°C. MALDI-time-of-flight (TOF) mass spectrometry (Applied Biosystems DE STR; linear mode with time-lagged focusing using internal calibration with bovine myoglobin) showed agreement within experimental error between the measured and calculated masses of the principal components in each preparation after consideration for some N- and C-terminal proteolytic trimming, which presumably occurred during sample purification: saposin A (**Fig. 2A**), measured *m/z* 10,719; calculated 10,716.3 Da for MH^+ [EAEAYV-human saposin A (S_{60}–K_{143})-HHHHHH; three disulfide bonds]; saposin C (Fig. 2B), measured *m/z* 9,204, calculated 9,200.7 Da for MH^+ [V-human saposin C (S_{311}–G_{390})-R; three disulfide bonds]; and saposin D (Fig. 2C), measured *m/z* 9,962 (10%), 9,825 (50%), 9,688 (100%), and 9,553 (60%), calculated 9,963.5, 9,826.4, 9,689.3, and 9,552.2 Da for MH^+ [EAEA-human saposin D (G_{407}–S_{484})-nH; three disulfide bonds] for six, five, four, and three C-terminal histidine residues, respectively]. The differences between measured and calculated molecular masses (0.8–3.3 Da) are within the accuracy typically obtained with MALDI-TOF of proteins (±10 Da at 10 kDa) (27). The purified materials were biologically active, as demonstrated by their support of the efficient hydrolysis of lactosylceramide by activator protein-deficient cultured human fibroblasts (26). The stock solutions were diluted in 25 mM Tris-HCl buffer (pH 7.5) for use in the CSAct assay.

The other set of nonglycosylated human recombinant proteins was produced by the Toronto group without any purification tags in a thioredoxin reductase-deficient strain of *Escherichia coli* and purified by heat treatment followed by sequential ion exchange, gel filtration, and hydrophobic interaction chromato-

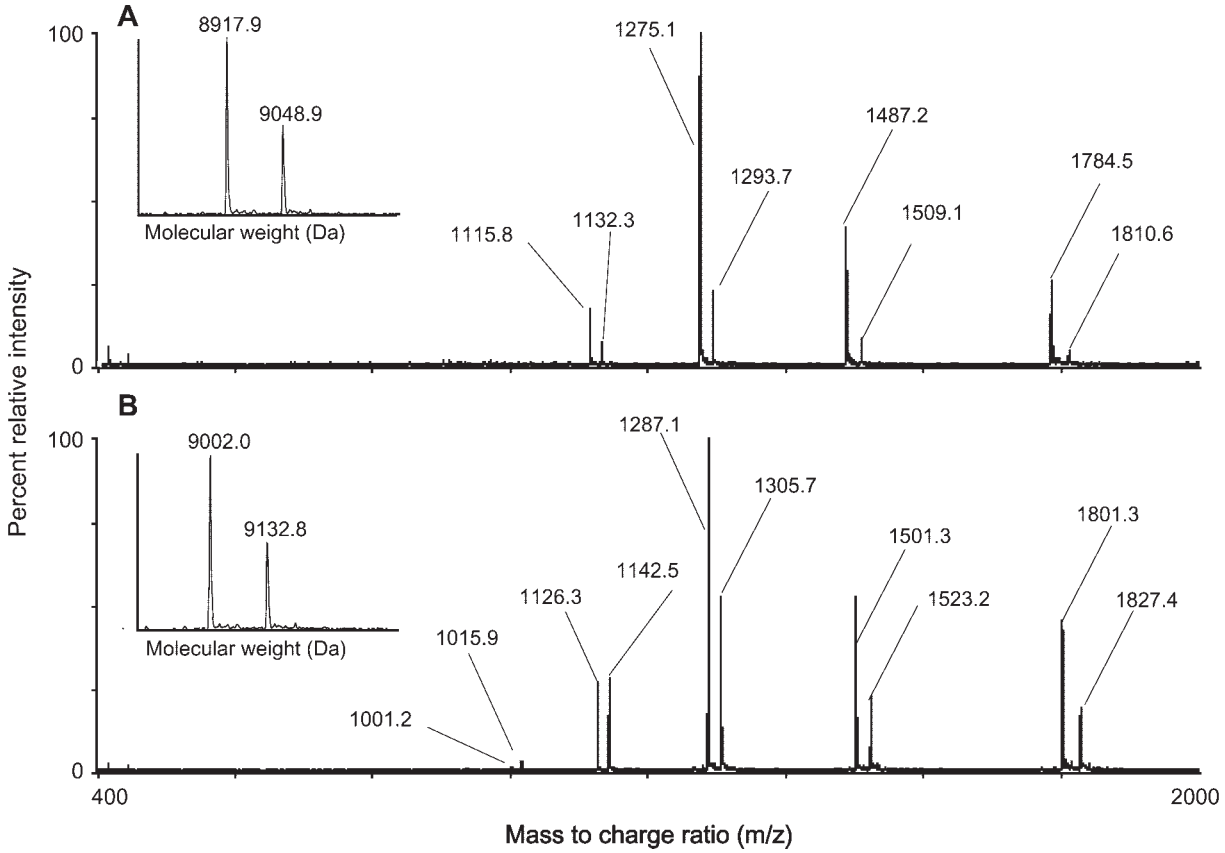

Fig. 3. Electrospray ionization mass spectrometry (ESI) spectra of recombinant human saposins A (A) and C (B) produced by the *E. coli* expression system. The insets represent the computer-generated molecular mass profiles of the two samples. The molecular mass measurement errors are within the accuracy typically obtained with ESI of proteins on a standard quadrupole mass spectrometer (±1 Da at 10 kDa). See Materials and Methods for details.

graphy using a previously described system (28). The final preparations were stored as 7.5 (saposin A), 39 (CSAct), and 28 (saposin C) mg/ml aqueous solutions at −80°C. Stock solutions were diluted in 25 mM Tris-HCl buffer (pH 7.5) for use in the ASA/CSAct assays. ESI analysis by flow injection (see below) of stock solutions diluted to 20 pmol/μl in water-acetonitrile-formic acid (50:50:0.1, v/v) with molecular mass calculations made from the spectra (MacSpec HyperMass; Perkin-Elmer Sciex Instruments, Thornhill, Canada) and percentage relative intensity estimates made from the reconstructed molecular mass profiles (BioMultiView, version 1.3.1; Perkin-Elmer Sciex Instruments) showed agreement between the calculated and measured molecular masses: saposin A (**Fig. 3A**), measured 9,048.9 (20%) and 8,917.9 (100%) Da, calculated 9,049.6 Da [MG-human saposin A (S_{60}–S_{140}); three disulfide bonds] and 8,917.4 Da [G-human saposin A (S_{60}–S_{140}); three disulfide bonds], respectively; CSAct (28), measured 9,115.2 Da, calculated 9,115.48 Da [MG-human CSAct (G_{195}–E_{273}); three disulfide bonds]; and saposin C (Fig. 3B), measured 9,132.8 (50%) and 9,002.0 (100%), calculated 9,132.6 [MG-human saposin C (S_{311}–G_{390}); three disulfide bonds] and 9,001.4 Da [G-human saposin C (S_{311}–G_{390}); three disulfide bonds], respectively. The differences between measured and calculated molecular masses (0.2–0.7 Da) are within the accuracy typically obtained with ESI of proteins on quadrupole mass spectrometers (±1 Da at 10 kDa) (29).

CSAct-potentiated ASA assay

A microtized version of an established procedure (11, 21) used 0.2 ml polypropylene tubes and a total volume adjusted to 20 μl with 25 mM Tris-HCl buffer, pH 7: sodium acetate buffer (3.3 μl, 1 M, pH 4.5); CSAct or saposin A, C, or D (10 μl containing up to 6 μg of protein in 25 mM Tris-HCl buffer, pH 7.5); and aqueous sulfatide suspension (3.3 μl, 1 μg/μl). The reaction was commenced by the addition of ASA enzyme solution (3 μl, 250 U/ml).

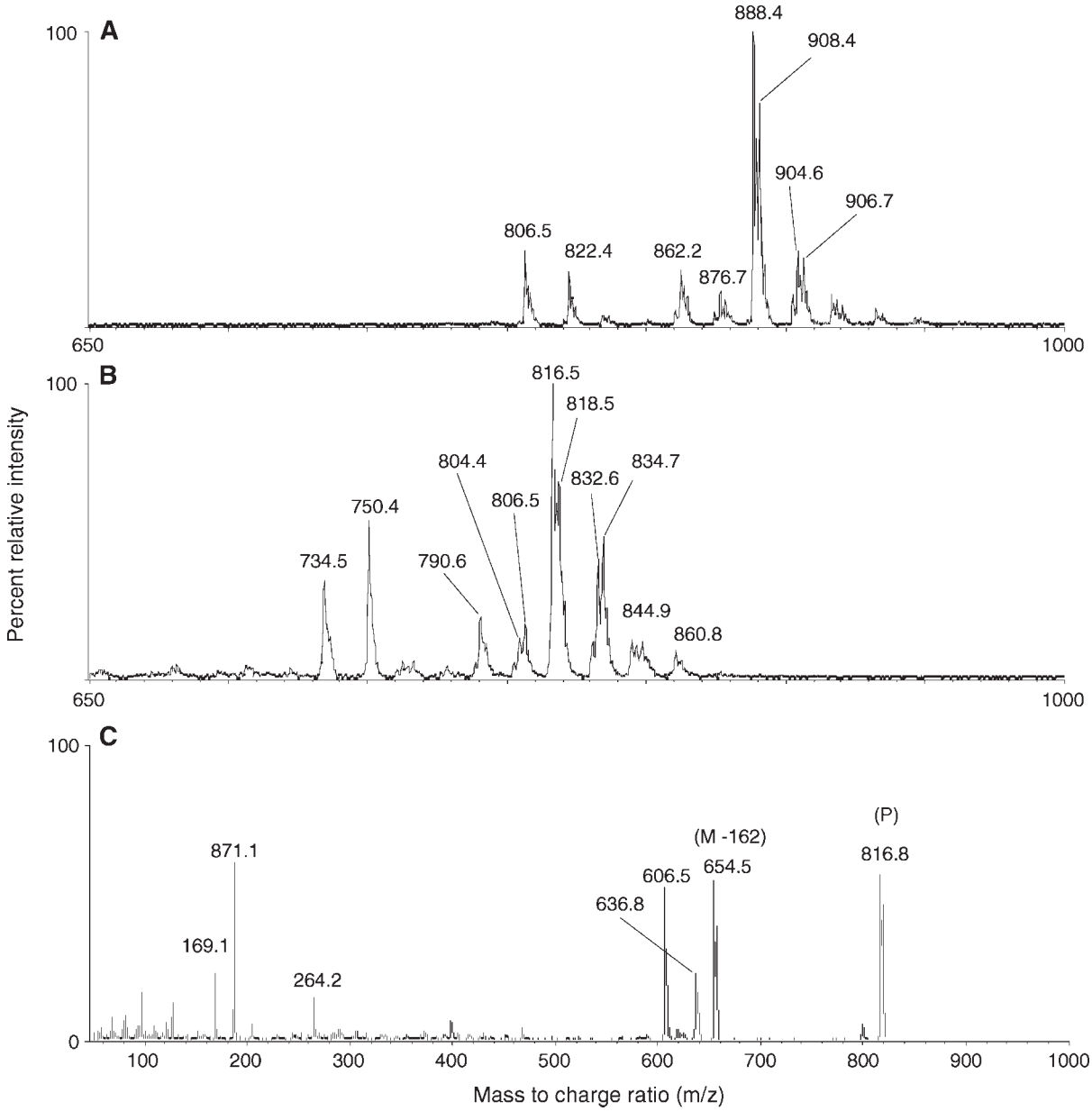

Fig. 4. ESI spectra of bovine brain sulfatide (A; negative ion mode) and bovine brain cerebroside (B; positive ion mode, lithium adducted) and the tandem mass spectrometry spectrum (C) of the *m/z* 816.5 parent ion from the spectrum in B.

Blank reactions contained no saposin. After incubation (2 h, 37°C), chloroform-methanol (300 μl; 2:1, v/v) was added, the samples were mixed vigorously (30 s), and aqueous sodium sulfate (200 μl, 4 mM containing 0.12 M sodium chloride) was added. The samples were mixed vigorously again (30 s) and centrifuged (3,000 *g*, 5 min), and an aliquot of the lower phase (200 μl) was removed with a 1 ml glass gas-tight syringe fitted with a Chaney adaptor, placed into a new tube, and dried with a stream of dry nitrogen gas. For injection into the mass spectrometer, the dried samples were redissolved in 100 μl of chloroform-methanol (1:4, v/v) containing 250 pmol of [$^2H_{35}$]stearoyl cerebroside internal standard (IS) and 100 mM lithium chloride.

Taurodeoxycholate-potentiated ASA assay

A microtized variation of the established procedure for this detergent-based assay (10) used 0.2 ml polypropylene tubes and a total volume adjusted to 50 μl with 25 mM Tris-HCl buffer, pH 7: sodium acetate buffer (5 μl, 1 M, pH 4.5); 10 μl of taurodeoxycholate-solubilized bovine sulfatide substrate (60 μg of bovine sulfatide; see above); and 25 μl of 17 mM Tris-HCl (pH 7.5; to substitute for the saposin protein used in the CSAct assay). The reactions were started by the addition of 5 μl of ASA enzyme (250 U/ml); blank reactions with no added ASA used 5 μl of 1 mg/ml BSA in 25 mM Tris-HCl (pH 7.5). During incubation (37°C), 5 μl aliquots were removed at 0.5, 1, 1.5, and 2 h and placed into new 1 ml tubes containing 330 μl of chloroform-methanol (2:1, v/v) and 70 μl of 4 mM sodium sulfate containing 0.12 M sodium chloride. These mixtures were vortexed (30 s) and then centrifuged (3,000 *g*, 5 min). An aliquot of the lower phase (200 μl) was then removed with a 1 ml glass gas-tight syringe fitted with a Chaney adaptor, placed into a new tube, and dried with a stream of dry nitrogen gas. For injection into the mass spectrometer, the dried samples were redissolved in 100 μl of chloroform-methanol (1:4, v/v) containing 250 pmol of [$^2H_{35}$]stearoyl cerebroside IS and 100 mM lithium chloride.

ESI

A Perkin-Elmer Sciex API III$^+$ triple quadrupole mass spectrometer equipped with an Ion Spray™ source was tuned and calibrated in the positive ion mode as described previously (30). Under these standard resolution conditions, the polypropylene glycol/NH_4^+ singly charged ion clusters were resolved with a 40% valley. Flow injection was used for sample introduction (20 μl per injection), with water-acetonitrile-formic acid (50:50:0.1, v/v) as solvent for proteins, and methanol-chloroform (4:1, v/v) as solvent for lipids, both constantly infused into the source (15 μl/min). For proteins, an *m/z* 400–2,000 range was scanned (positive mode, orifice 90 V, 0.3 Da step size, 5.61 s/scan). For lipids, an *m/z* 100–1,000 mass range was scanned (0.3 Da step size, 6.66 s/scan). For cerebrosides, the positive ion mode was used with the orifice set to +120 V. For sulfatides, the instrument polarity was reversed and the orifice was set to −90 V. Fragment ion spectra of Q1 preselected cerebroside parent ions [$(M+Li)^+$, 99.999% argon collision gas with a collision gas thickness instrument setting of 200 and a rod offset (R_0-R_2) of 40 V] were recorded by scanning Q3 from *m/z* 50 to 1,000 (0.3 Da step size, 7.43 s/scan). Up to eight of the following cerebroside transitions were recorded for multiple reaction monitoring (MRM) measurements: C_{16}:0, 706.4→544.4; C_{18}:1, 732.6→570.6; C_{18}:0, 734.6→572.6; hC_{18}:0, 750.6→588.6; [$^2H_{35}$]C_{18}:0, 769.7→607.7; C_{22}:0, 790.7→628.6; hC_{22}:0, 806.7→644.6; C_{24}:1[15], 816.7→654.6; C_{24}:0, 818.7→656.6; hC_{24}:0, 834.7→672.6; C_{26}:1, 844.7→682.6; C_{26}:0, 846.7→684.7.

Data processing

Representative spectra were computed as the average of all spectra accrued from each injection using instrument-supplied software (MacSpec™, version 3.3; Perkin-Elmer Sciex). MRM pro-

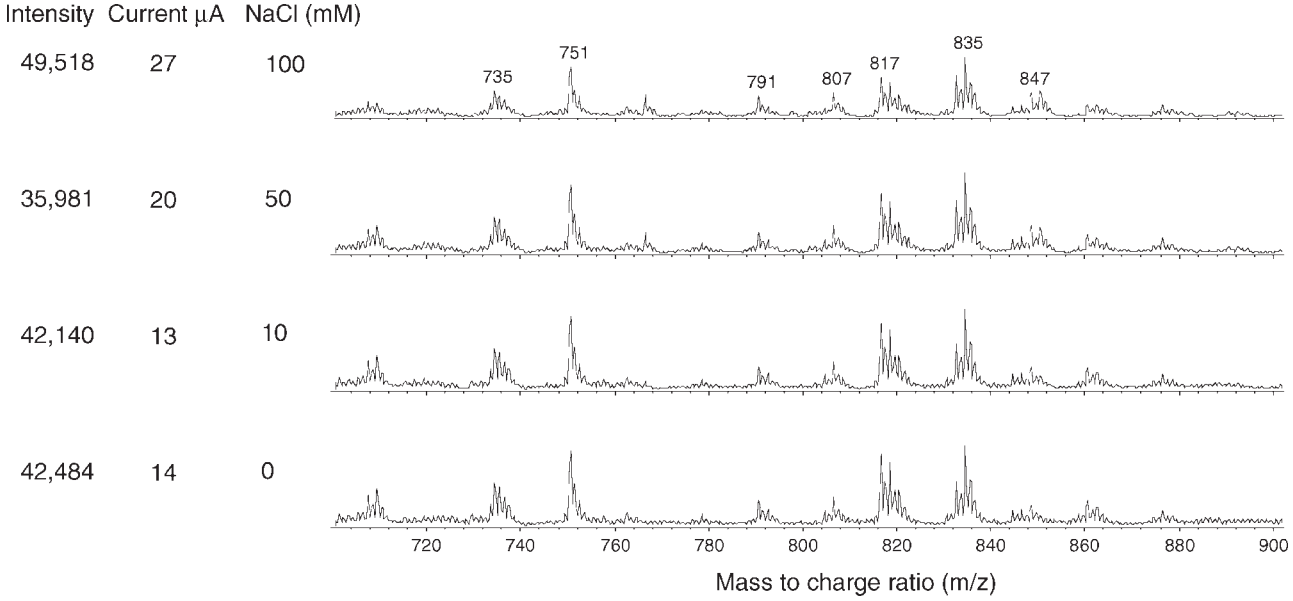

Fig. 5. ESI spectra of bovine brain cerebrosides (positive ion mode, lithium adducted) in the presence of increasing concentrations of sodium chloride, demonstrating the absence of ion suppression by salt. Bovine cerebrosides, 100 pmol/μl in methanol-chloroform (4:1, v/v) containing 100 mM lithium chloride, were analyzed in the presence or absence of the indicated concentrations of sodium chloride or the lithiated solvent alone (bottom spectrum). Intensity (in arbitrary units) refers to the maximal signal recorded during the collection of each spectrum; current (μA) refers to the maximal observed read-back of the electrospray needle current during the collection of each spectrum. The cerebroside masses are identified by their aminoacyl-linked fatty acid (R) chains. The calculated *m/z* values for the various lithiated species are as follows: C_{18}:0, 734.6; hC_{18}:0, 750.6; C_{22}:0, 790.7; hC_{22}:0, 806.7; C_{24}:1, 816.7; hC_{24}:0, 834.7; and C_{26}:0, 846.7.

files were smoothed and peak areas determined using the IGOR Pro™ software package (version 3; WaveMetrics, Inc., Lake Oswego, OR) and converted to moles of cerebroside using the response from the IS. Reaction rates were expressed as moles of product per hour per milligram of saposin.

RESULTS AND DISCUSSION

As reported previously, the negative ion ESI spectra of brain sulfatide reveal a series of ions from *m/z* 806 to 950, reflecting the relative abundance of the various species of this lipid in the brain (**Fig. 4A**) (16, 17). The positive ion spectra of brain cerebrosides in the presence of lithium (18) reveal a corresponding series of ions from *m/z* 734 to 860, reflecting a similar pattern of relative abundance to that of the sulfatides (Fig. 4B). The 72 Da difference between the signals from corresponding sulfatide and cerebrosides is accounted for by the mass difference attributable to sulfation (80 Da), the loss of a proton in the negative ion sulfatide spectra, and lithium adduction (7 Da) in the positive ion cerebroside spectra. Surprisingly, the intensity of the lithiated adducted cerebroside signals did not diminish in the presence of up to 100 mM sodium chloride (**Fig. 5**).

The lack of ion suppression by the alkali metal salt prompted investigation into the utility of these signals for monitoring cerebroside formation in the ASA/CSAct enzymatic assays. The MS/MS spectra of the $(M+Li)^+$ cerebroside ions show several intense fragment ions suitable for MRM, including a base peak corresponding to the loss of the galactosyl moiety (−162 Da), which was common to all cerebroside spectra (Fig. 4C). For MRM experiments, the loss of the galactosyl moiety (−162 Da) was chosen because it was the consistent dominant fragment. The raw MRM data $[(M+Li)^+ \rightarrow (M+Li-162)^+$ transitions] for cerebroside accumulation in the in vitro ASA/CSAct assay with increasing concentrations of CSAct reveal increasing formation of seven different cerebrosides in the presence of relatively uniform signals for the added IS ($[^2H_{35}]C_{18}$:0 cerebroside; **Fig. 6**). There was no detectable formation of cerebroside in the absence of added CSAct. The limitation of data accumulation to eight separate MRM transitions is a consequence of the data system used for this work, and with contemporary systems this could be expanded to include the less abundant cerebrosides, should that be necessary. A refinement to the procedure would be IS addition at the end of the incubation rather than immediately before mass spectrometry. A further refinement would be the use of a specific IS for each cerebroside monitored. However, at present, only $[^2H_{35}]C_{18}$:0 cerebroside is commercially available. Nevertheless, the specific activity for the data represented in Fig. 6 for the combined seven most abundant cerebroside species in the assay is 579.2 nmol cerebroside formed/h/mg CSAct, which is in good agreement with data from the radioactive-based assay, which varies at ~600 nmol sulfate formed/h/mg CSAct, when run under similar conditions (11, 24, 31).

In the MRM assay, it is possible to individually monitor the saturated (C_{18}:0, C_{22}:0, and C_{24}:0) as well as the hydroxylated-saturated (hC_{18}:0 and hC_{24}:0) and the monounsaturated (C_{24}:1 and C_{26}:1) cerebrosides in the presence of the IS. This allowed a comparison of the specific activity for each cerebroside, which is impossible with the radioactivity-based methodology. Notable differences in specific activity were found among the seven cerebrosides analyzed (**Fig. 7**). However, the bovine brain sulfatide substrate is a mixture of >10 different species (17) in varying relative abundance. Therefore, the differences observed in the rate of formation of the various cerebrosides could arise as a consequence of either differential substrate preference and/or abundance. To address this question, the data were reexpressed relative to the abundance of the corresponding substrate species. The activity profile then showed no significant differences between the individual species, with the exception that the hC_{18}:0 and hC_{24}:0 species had slightly lower specific activities relative to the corresponding nonhydroxylated species (data not shown). To resolve this issue, six representative sulfatide species were synthesized (C_{16}:0, C_{18}:0, C_{22}:0, C_{24}:1[15], hC_{18}:0, and hC_{22}:0) and used in an equimolar mixture of substrate in

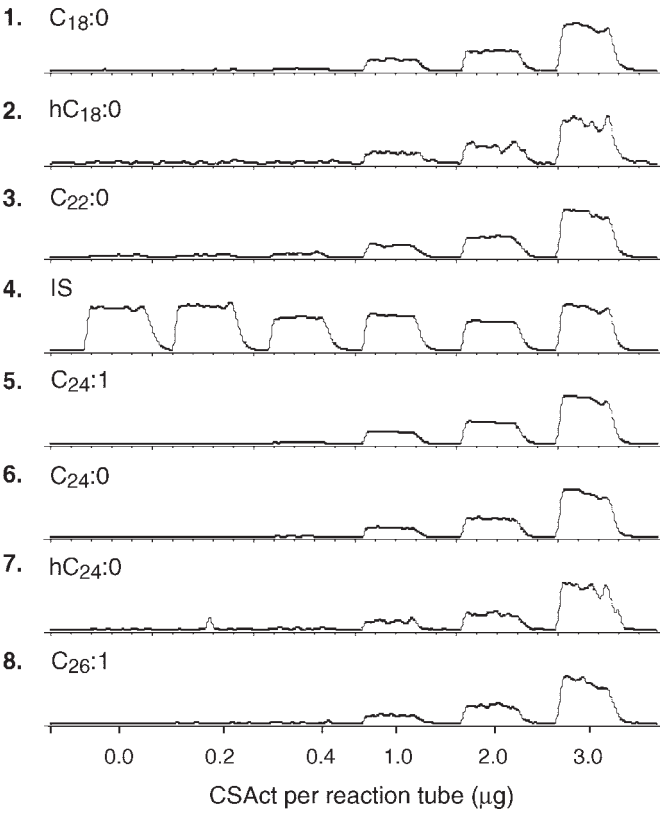

Fig. 6. Multiple reaction monitoring analysis of cerebroside product formation in the coupled ASA/CSAct reaction. At the indicated amounts of CSAct (abscissa) in the reaction mixture, the following transitions were measured: C_{18}:0, 734.6→572.6 (trace 1); hC_{18}:0, 750.6→588.6 (trace 2); C_{22}:0, 790.7→628.6 (trace 3); $[^2H_{35}]C_{18}$:0, 769.7→607.7 (trace 4); C_{24}:1, 816.7→654.6 (trace 5); C_{24}:0, 818.7→656.6 (trace 6); hC_{24}:0, 834.7→672.6 (trace 7); and C_{26}:1, 844.7→682.6 (trace 8). The data show increasing signal intensity for each of the seven product transitions recorded (traces 1–3 and 5–8) in the presence of a relatively constant response from the internal standard (IS; trace 4).

the assay system. The results showed no significant difference in the rate of cerebroside formation between the C_{16}:0, C_{18}:0, C_{22}:0, and C_{24}:1[15] substrates (data not shown) but significantly lower rates of cerebroside formation from the hC_{18}:0 and hC_{22}:0 substrates compared with their nonhydroxylated counterparts (**Fig. 8**).

The interaction between CSAct and lipids has been evaluated previously by several different paradigms. Exchange between bound and unbound lipids can be observed under acidic conditions, but at neutral pH the CSAct-lipid complex is stable and can be isolated free of unbound ligand by several techniques, including size-exclusion chromatography, gel electrophoresis, and thin-layer chromatography (32–34). These in vitro studies have shown that CSAct binds a varied but limited repertoire of lipids in addition to sulfatide, including gangliosides (G_{D3} and G_{m1}), phosphoinositols (tri-, di- and monophosphates), globosides (Gb_3 and Gb_4), sphingomyelin, and cerebroside, in addition to some other lipids (35). It appears from these studies that ligands with longer and/or more complex lipoidal and polar components are favored. However, any preference of CSAct for any one of the many naturally occurring sulfatide molecular species has not been investigated previously. This is because homogeneous preparations of the individual sulfatide species have not been available and because it is impossible to study such preferences with a heterogeneous substrate and the radioactivity-based assay. In this new mass spectra-based assay, several activity profiles are individually measured, and the results reveal a subtle but measurable preference for the nonhydroxylated over the hydroxylated substrates (Fig. 8). Any possible relationship between this substrate preference and the large increase in hydroxylated over nonhydroxylated sulfatides observed in myelin from MLD patients (36), and the increase in hydroxylated over nonhydroxylated sulfatides reported previously by the UCLA group in plaque tissue from multiple sclerosis patients (16), is obscure. However, in this regard, it would be of interest to investigate the substrate preference of the various mutant forms of MLD-specific CSAct.

To address the question of the saposin specificity in the ASA-catalyzed reaction, two different sets of recombinant saposins were compared. The results show that cerebroside formation occurred only with recombinant CSAct (saposin B), and there was no detectable cerebroside formation when recombinant saposins A, C, or D from the *P. pastoris* expression system (**Fig. 9**), or when recombinant saposins A and C from the *E. coli* expression system (data not shown), were used. The earlier observations that saposins C and D have low but measurable activity in this assay were made with proteins purified from natural sources (37). We conclude that this result is a consequence of incomplete separation of the various proteins using the conventional chromatography that was available at the time, resulting in a low level of CSAct contamination in the saposin C and D samples. The in vitro data presented here

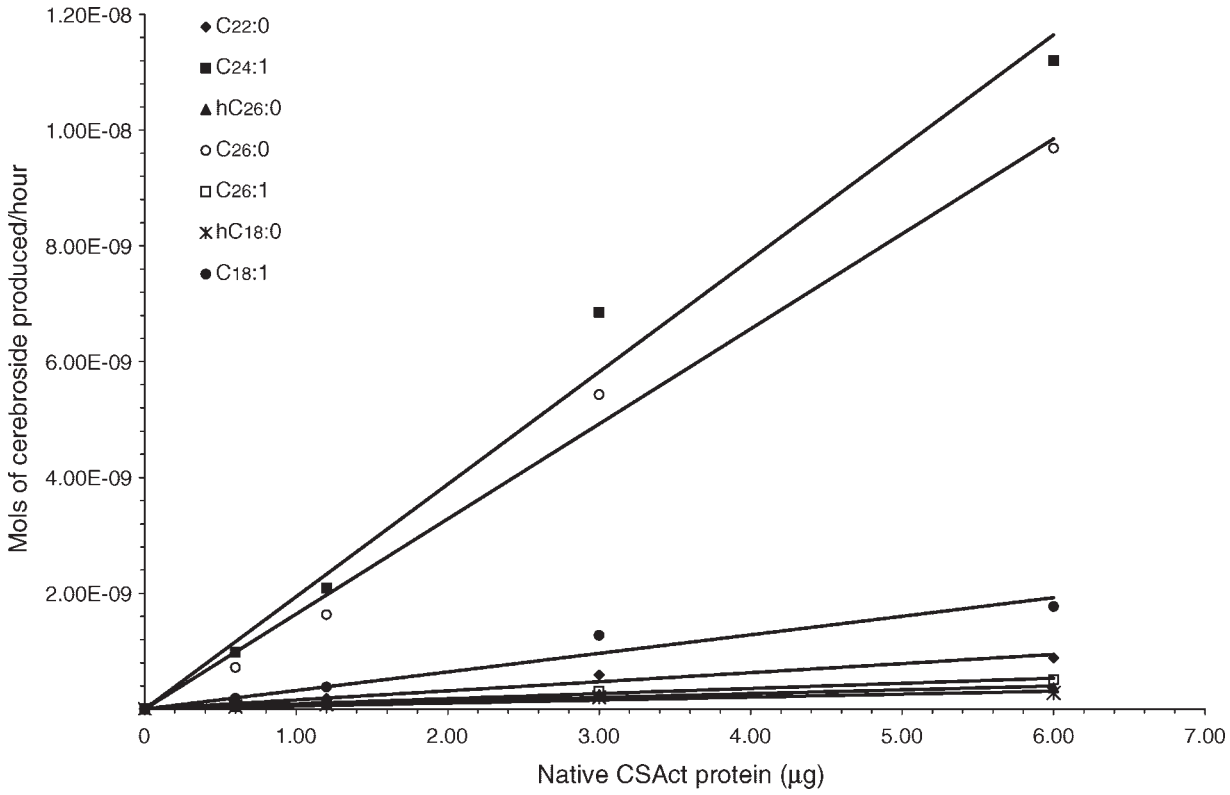

Fig. 7. Effect of the concentration of native (pig kidney) CSAct on the rate of formation of seven different cerebrosides in the coupled ASA/CSAct reaction in which bovine sulfatides were used as substrate. The fatty acyl compositions of the seven cerebroside products are indicated.

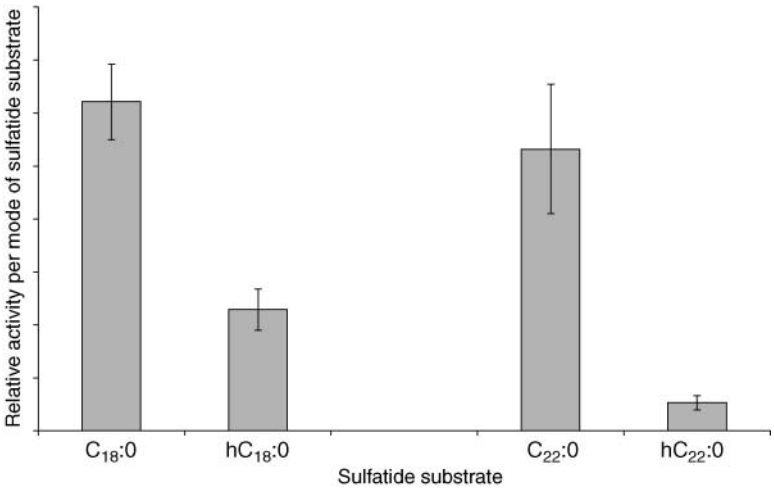

Fig. 8. Comparison of the rate of cerebroside formation from a near-equimolar mixture of the indicated synthetic sulfatide substrates. Ordinate values were obtained from activity measurements as shown in Fig. 7 (slopes) after normalization by the corresponding sulfatide substrate abundance. Each data point represents the mean of three experiments with the error bars indicating standard deviation within each respective data set.

clearly indicate that saposins A, C, and D cannot substitute for CSAct. However, caution should be exercised when extrapolating to the in vivo situation, because the in vitro assay does not exactly reflect the catabolic situation of sulfatides within the lysosomal compartment. The assay presents the sulfatide substrates in a micellar form in the absence of any other membrane lipids. In the lysosomal compartment, sulfatide substrates are presumably presented as components of inner lysosomal membranes rich in bis(monoacylglycero)phosphate. This lipid is specific for the inner membranes and stimulates sphingolipid hydrolysis. The topology of lysosomal digestion (38) may affect the specificity of CSAct and ASA, and especially their variants present in patients, in a different way than in the micellar situation used in the in vitro assay.

To adapt the system for the study of ASA rather than CSAct, the detergent-based assay was run using the MRM method, and the limits for the detection of enzymatic activity were assessed. It was found that the presence of the detergent taurodeoxycholate at up to 5 mg/ml and 0.12 M NaCl in the reaction mixture did not interfere with the ability to assay the enzyme. Assaying ASA under these conditions using between 0.025 and 0.0025 U/tube gave linear increases in cerebroside formation during incubation times from 0 to 90 min. We concluded that the assay had a limit of detection for ASA of ~0.0025 U/tube. The tauro-

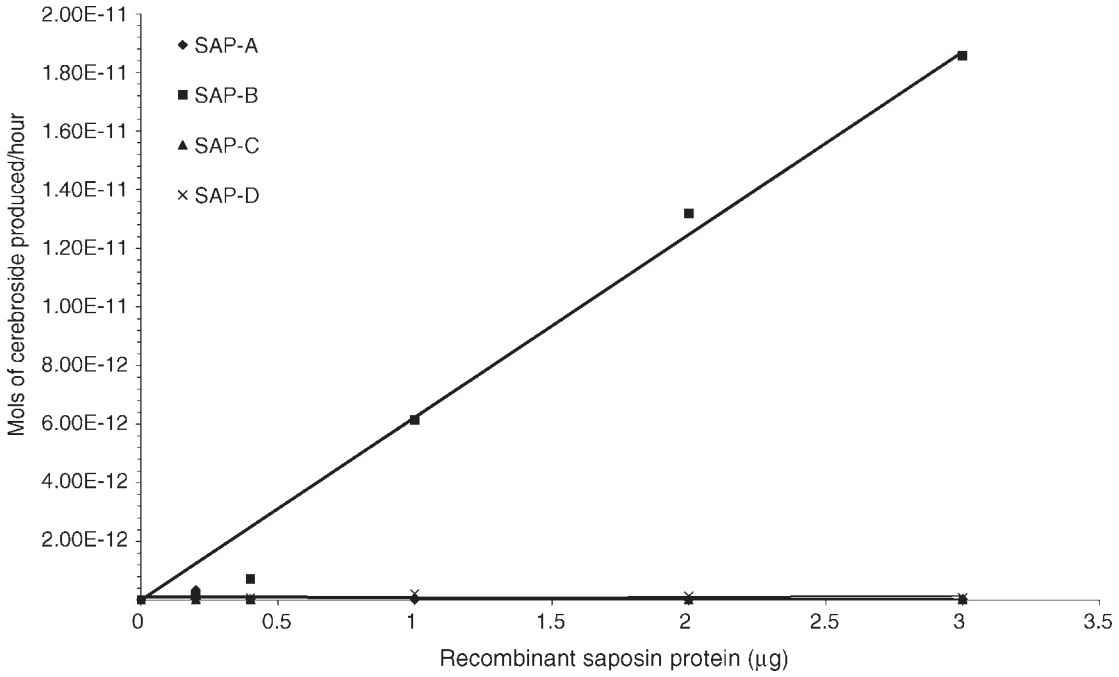

Fig. 9. Effect of the concentration of recombinant CSAct and saposins (SAP) A, C, and D on the rate of cerebroside formation from bovine sulfatide substrate in the in vitro assay. The ordinate represents the summed formation of the C_{18}:1 + hC_{18}:0 + C_{22}:0 + C_{24}:1 + C_{24}:0 + hC_{24}:0 + C_{26}:1 cerebrosides.

deoxycholate-based assay is used clinically to assess potential defects in ASA activity and as such has significant diagnostic importance for the evaluation of MLD. The development of an MRM-based procedure that avoids the need for radiolabeled substrate could find clinical utility.

Finally, it should be noted that earlier efforts focused on using the strong negative ESI signals obtained with sulfatide for the development of an MRM assay for the ASA/CSAct coupled reaction (39). The procedure was based on measuring the temporal decline in sulfatide (substrate) concentration. With the conventional Ion Spray™ source, concentrations of nonvolatile inorganic salt in excess of 1 mM typically result in significant reductions in ion current from organic analytes. Although this is true for the detection of negative sulfatide ions, it is paradoxically not true for the detection of positively charged lithiated cerebroside ions, as already mentioned. To avoid changing the reaction conditions, it was necessary to develop a method for the effective removal of inorganic salt while maintaining reasonable sulfatide recovery. Several methods were tried, including dialysis against water (Spectra/Por™ membrane), hexane/2-proponal extraction (40), and size-exclusion chromatography (Toso Haas Toyopearl™ 40 HW HW 40S resin and elution with hexane/2-proponal). None of these methods adequately removed the inorganic salt without a significant loss of sulfatide. The final method of repeated aqueous 0.1 M ammonium acetate (pH 6.5) extractions gave a recovery of 65–70% of radiolabeled sulfatide through the entire sample preparation procedure, and the resulting extracts gave sulfatide MRM signals that were not suppressed. This procedure yielded comparable data to those reported above for the positive ion MRM assay in which the lithiated cerebroside ions were monitored. However, in addition to being a simpler and more comprehensive procedure, the measurement of product (cerebroside) formation is preferred over the measurement of substrate (sulfatide) depletion, particularly in the ASA/CSAct coupled assay that is used in the range of 0–10% substrate hydrolysis (12).

We have developed a mass spectrometric method for assaying the coupled ASA/CSAct reaction that provides information on the rate of formation of specific cerebroside products, thus allowing a more complete understanding of the kinetic properties of the reaction than is possible with the conventional radioactive method. The procedure can be used in CSAct-potentiated and detergent-potentiated reactions. No modifications to the standard assay protocols are needed. Future studies will include application of this method for the characterization of CSAct mutants as a way of better understanding the molecular basis of MLD.

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