Purification and Characterization of Rat Kidney Sphingosine Kinase*

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Sphingolipid metabolites, such as ceramide, sphingosine, and sphingosine 1-phosphate (SPP), 1 are members of a novel class of lipid second messengers (1–4). Ceramide is an important regulatory component of stress responses and programmed cell death, known as apoptosis (2, 5, 6). In contrast, we have implicated a further metabolite of ceramide, SPP, as a second messenger in cellular proliferation and survival induced by platelet-derived growth factor, nerve growth factor, and serum (7–9). Previously, we showed that SPP protects cells from apoptosis resulting from elevations of ceramide (7, 9) and proposed that the dynamic balance between levels of the sphingolipid metabolites (ceramide and SPP) and consequent regulation of opposing signaling pathways is an important factor that determines whether a cell survives or dies (7). Recently, we demonstrated that this ceramide/SPP rheostat is an evolutionarily conserved stress regulatory mechanism influencing growth and survival of yeast (10). A variety of stress stimuli, including Fas ligand, tumor necrosis factor-α, interleukin-1, growth factor withdrawal, anticancer drugs, oxidative stress, heat shock, and ionizing radiation, stimulate sphingomyelinase, leading to increased ceramide levels (2, 5, 6), whereas platelet-derived growth factor and other growth factors stimulate ceramidase and sphingosine kinase and elevate SPP levels (3, 4, 8, 11). Progress in determining the importance of these sphingolipid metabolites has been hampered because most of the relevant metabolic enzymes have not yet been purified or cloned.

The level of SPP in cells is low and determined by the relative contributions of its formation, mediated by sphingosine kinase (12), and its degradation, catalyzed by an endoplasmic reticulum pyridoxal phosphate-dependent lyase and specific phosphatases (10, 13, 14). SPP was initially described as an intermediate in the degradation of long-chain sphingoid bases (15). However, the roles of SPP in cellular proliferation, survival, and other cellular responses (reviewed in Ref. 16), as well as the observations that SPP triggers novel signal transduction pathways of calcium mobilization (17, 18), activation of phospholipase D (19), and the Raf/MKK/ERK signaling cascade (20, 21), suggest that the importance of sphingosine kinase is not restricted to the catabolism of sphingolipids but as was originally proposed nearly 20 years ago (22).

Sphingosine kinase is a ubiquitous enzyme found in yeast (22); Tetrahymena pyriformis (23); rat liver, kidney and brain (24, 25); bovine brain (26); and human and porcine platelets (25, 27). Although it is known that sphingosine kinase activity increases in response to certain growth-promoting agents, such as platelet-derived growth factor (8, 28, 29), phorbol esters (3, 7, 30), the B subunit of cholera toxin (31), and nerve growth factor (9), little is yet known about the properties or mechanism of regulation of sphingosine kinase. Thus, purification and characterization of sphingosine kinase are important goals to gain understanding of its physiological roles. We have now succeeded in purifying sphingosine kinase from rat kidneys by >6 × 105-fold to apparent homogeneity.
**Experimental Procedures**

Materials—Frozen rat kidneys were purchased from Pel-Freez Biologicals (Rogers, AK). DEAE-cellulose (DE52) was purchased from Whatman. Dye-ligand gel chromatography medium (blue 3GA and green A) and Centriprep, Centricron, and Microcon sample concentrators were from Amicon, Inc. (Beverly, MA). The prepacked hydroxylapatite column was purchased from Tonen Chemical Co. (Tokyo, Japan). EAH-Sepharose, calmodulin-Sepharose 4B, prepacked Mono Q, and Superdex 75 columns and silver staining reagents were purchased from Pharmacia Biotech (Uppsala, Sweden). Alkyl-agaroses were from Sigma. [32P]ATP was from ICN (Costa Mesa, CA). Sphingosine was obtained from Matreya, Inc. (Pleasant Gap, PA). Coomassie Plus protein reagent was from Pierce. Molecular mass markers for polycrylamide gels and the gel electrophoresis apparatus were from Bio-Rad.

Assay of Sphingosine Kinase Activity—Sphingosine kinase activity was determined as described previously with minor modifications (12). Samples (up to 40 μl) were mixed with buffer A (20 mM Tris (pH 7.4), 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor, 1 μM p-nitrophenylsulfonyl fluoride, and 0.5 mM 4-deoxyxypyradoxidine) in a total volume of 190 μl, and reactions were started by addition of 10 μl of [32P]ATP (10 μCi, 20 μm) containing MgCl₂ (200 mM) and incubated for 5 or 15 min at 37 °C. Reactions were terminated by addition of 20 μl of 1 N HCl followed by 0.8 ml of chloroform/methanol/HCl (100:200:1, v/v). After vigorous vortexing, 240 μl of chloroform and 240 μl of 2 M KCl were added, and phases were separated by centrifugation. The labeled lipids in the organic phase were resolved by TLC on Silica Gel G60 with 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v) and visualized by autoradiography. The radioactive spots corresponding to authentic SPP were identified as described (32), scraped from the plates, and counted in a scintillation counter or, alternatively, quantified with a Molecular Dynamics Storm PhosphorImager. Sphingosine kinase specific activity in a scintillation counter or, alternatively, quantified with a Molecular Dynamics Storm PhosphorImager. Sphingosine kinase specific activity is expressed as pmol of SPP formed per min (unit)/mg of protein. At each purification step, linearity of the enzymatic reaction with time of incubation and protein concentration was observed.

**Extraction and Ammonium Sulfate Fractionation**—Frozen rat kidneys (0.5 kg) were thawed in 0.5 liter of cold 20 mM Tris (pH 7.4) containing 20% glycerol, 1 mM diithiothreitol, 1 mM EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotonin, 0.5 μg/ml soybean trypsin inhibitor, and 0.2 mM phenylmethylsulfonyl fluoride (buffer B), decapsulated, transferred to fresh buffer (2 ml/g), minced, and then homogenized in a blender. After centrifugation at 5000 × g for 15 min, the supernatant fraction was filtered through glass wool, centrifuged at 20,000 × g for 1 h, filtered through glass wool again, and centrifuged at 100,000 × g for 90 min. This supernatant was then centrifuged at 100,000 × g for 1 h to obtain the cytosolic fraction, which was fractionated by precipitation with ammonium sulfate. The 25–45% ammonium sulfate precipitate was resuspended in 150 ml of buffer B and dialyzed overnight against the same buffer.

DEAE-cellulose Chromatography—The dialyzed was clarified by centrifugation for 15 min at 100,000 × g and applied overnight at a flow rate of 70 ml/h to a DEAE-cellulose column (5-cm diameter, 500-ml bed volume) equilibrated with buffer B. After washing with buffer B, proteins were eluted with a linear gradient to 0.5 M NaCl in the same buffer at a flow rate of 250 ml/h. Sphingosine kinase activity was determined on aliquots of each fraction, and peak activity fractions were pooled.

**Dye-Ligand Affinity Chromatography on Green A- and Blue A-Sepharose Columns**—The pooled DEAE fractions were applied to a green A-Sepharose column (5-cm diameter, 200–250-ml bed volume) equilibrated with buffer B containing 0.2–0.22 M NaCl in the same buffer at a flow rate of 120 ml/h. After the sample was applied, the flow rate was increased to 300 ml/h, and stepwise elution was performed with 3 bed volumes of buffer B containing 0.2 and 0.4 M NaCl, respectively, and then 6 bed volumes of buffer B containing 1 M NaCl. The 1 M NaCl fraction contained most of the sphingosine kinase activity and was diluted 1:1 with buffer B and applied at 120 ml/h to a blue A-Sepharose column (5-cm diameter, 125-ml bed volume) equilibrated with buffer B containing 0.5 M NaCl. The blue A-Sepharose was washed at 250 ml/h with 3 bed volumes of buffer B containing 0.5 M NaCl and with 3 bed volumes of buffer B containing 0.7 M NaCl, and the sphingosine kinase activity was eluted with 6 bed volumes of buffer B containing 2 M NaCl.

**EAH-Sepharose Chromatography**—Half of the 2 M NaCl fraction was concentrated 100-fold in Centricon Plus-20 concentrators (M, 10,000 cutoff) and then dialyzed against buffer B. The dialyzed was concentrated to remove precipitated proteins and, after addition of Triton X-100 to a final concentration of 0.05%, was loaded onto a 20-ml EAH column pre-equilibrated with buffer B containing 0.05% Triton X-100. The EAH column was washed stepwise with 60-ml fractions of buffer B and applied at 120 ml/h to a green A-Sepharose column (5-cm diameter, 200–250-ml bed volume) equilibrated with buffer B containing 0.5 M NaCl. Most of the sphingosine kinase activity was eluted with 150 ml NaCl.

**Affinity Chromatography on Calmodulin-Sepharose 4B—CaCl₂ was added to the EAH fraction to a final concentration of 4 mM and immediately applied to a calmodulin-Sepharose 4B column (12 ml) pre-equilibrated with buffer B without EDTA and containing 100 mM NaCl, 0.05% Triton X-100, 2 mM CaCl₂, and 10% sucrose. The column was washed successively with 30 ml of equilibration buffer and 60 ml of the same buffer containing 2 mM EGTA. Sphingosine kinase activity was purified.

**TABLE I**

Comparison of sphingosine kinase activity from various sources

| Cytosolic fractions of various tissues and *S. cerevisiae* | Specific activity | Cytosolic activity (%) |
|----------------------------------------------------------|------------------|-----------------------|
| Rat kidney                                               | 100              | 57                    |
| Rat spleen                                               | 84               | 23                    |
| Rat liver                                                | 18               | 60                    |
| Bovine kidney                                            | 40               | 49                    |
| *S. cerevisiae*                                          | 20               | 16                    |

**TABLE II**

Purification of sphingosine kinase from rat kidney

Sphingosine kinase was purified from 2.5 kg of rat kidneys (≈2500 kidneys) as described under “Experimental Procedures.” One unit of sphingosine kinase activity is expressed as pmol of SPP formed from sphingosine per min. Up to the ammonium sulfate fractionation step, sphingosine kinase activity was determined by incubation for 5 min at 37 °C to ensure linearity of reactions. With more purified fractions, assays were carried out for 15 min. After the blue A-Sepharose step, the activity was frozen and stored at −70 °C. This fraction was usually thawed within several weeks and used for the next purification steps. It should be noted that concentration and dialysis of the blue A-Sepharose eluate resulted in loss of 30–50% of the sphingosine kinase activity.

| Steps                      | Activity (units × 10⁶) | Protein (mg) | Specific activity | Recovery (%) | Purification fold |
|----------------------------|------------------------|--------------|-------------------|-------------|------------------|
| Total homogenate           | 12,654                 | 163,000      | 78                | 100         | 1                |
| Supernatant                | 8658                   | 114,000      | 76                | 68          | 1                |
| Ammonium sulfate (25–45%)  | 8554                   | 15,100       | 568               | 68          | 7                |
| DEAE-cellulose             | 7400                   | 3550         | 2085              | 58          | 5                |
| Green A-Sepharose          | 7384                   | 4935         | 14,917            | 58          | 191              |
| Blue A-Sepharose           | 4569                   | 215          | 21,253            | 36          | 273              |
| EAH-Sepharose              | 1040                   | 34.7         | 30,000            | 8.2         | 385              |
| Calmodulin-Sepharose       | 670                    | 0.94         | 71.3 × 10⁴        | 5.3         | 9141             |
| Hydroxylapatite            | 380                    | 0.084        | 45.2 × 10⁴        | 3.0         | 57,949           |
| Mono Q                     | 138                    | 0.0075       | 184 × 10⁴         | 1.1         | 235,897          |
| Calmodulin-Sepharose       | 133                    | 0.007        | 190 × 10⁴         | 1.1         | 243,589          |
| Superdex 75                | 77                     | 0.001        | 50 × 10⁴          | 0.6         | 641,026          |
The eluate from the calmodulin affinity column was concentrated to 200 mM ammonium sulfate precipitate was applied to a DEAE-cellulose column pre-equilibrated with buffer B, and the proteins were eluted with a linear gradient (0–0.5 M) of NaCl, collecting 20-ml fractions. Sphingosine (Sph) kinase activity (units/fraction; ●) and protein (mg/fraction; □) were measured as described under “Experimental Procedures.” Similar results were obtained in at least six experiments.

Hydroxyapatite Chromatography—The concentrated calmodulin-Sepharose fraction was injected onto a hydroxyapatite column equilibrated with buffer B containing 0.05% Triton X-100, 10% sucrose, and 25 mM potassium phosphate and was eluted at a flow rate of 0.4 ml/min with a linear gradient of 0.025–0.5 M potassium phosphate in the same buffer (Waters HPLC system). Fractions (0.4 ml) were collected in tubes containing 100 μl of 5 M NaCl, and sphingosine kinase activity was determined.

Mono Q Anion Exchange Chromatography—The pooled hydroxyapatite fraction was concentrated and desalted using Microcon-10 concentrators and injected onto a Mono Q 5/5 column (Waters HPLC system) equilibrated with buffer B containing 0.05% Triton X-100, 10% sucrose, and 15 mM potassium phosphate. The column was washed for 10 min with equilibration buffer at 1 ml/min, and then a linear gradient of 0.015–0.5 M potassium phosphate was applied. Fractions of 1 ml were collected in tubes containing 250 μl of 5 M NaCl. Sphingosine kinase activity was eluted as two broad peaks. Each peak was pooled and reapplied to a small calmodulin-Sepharose column (1 ml) to concentrate the sample since concentration by ultrafiltration usually resulted in a marked loss of activity. Furthermore, this second calmodulin-Sepharose column decreased the Triton X-100 concentration, which would interfere with further purification.

Gel Filtration Chromatography on Superdex 75—The eluate from the calmodulin affinity column was concentrated to 200 μl using Microcon-10 concentrators and then injected onto a Superdex 75 gel filtration column (Waters HPLC system) pre-equilibrated with buffer B containing 0.05% Triton X-100, 10% sucrose, and 1 M NaCl. Proteins were eluted at a flow rate of 0.4 ml/min, and 0.4-ml fractions were collected. The column was calibrated using bovine serum albumin, ovalbumin, chymotrypsinogen, and lysozyme as standard proteins to determine the apparent molecular mass of native sphingosine kinase.

Protein Determination—Proteins were determined with the Lowry procedure after precipitation with 7% trichloroacetic acid in the presence of 0.015% deoxycholate (Peterson variation (34)). After Superdex 75 gel filtration, the protein concentration was too low for determination by these methods and was estimated from optical densities obtained by scanning silver-stained gels using bovine serum albumin as a standard.

Characterization—Purified sphingosine kinase obtained after the gel filtration step was used for characterization studies. To determine pH dependence, the following buffers were used: pH 4–5, 200 mM sodium acetate; pH 6.0–6.6, 200 mM MES; pH 7.0–7.5, 200 mM HEPES; and pH 7.4–9.0, 200 mM Tris-HCl. For inhibition studies, sphingosine kinase was incubated with sphingosine and the indicated concentrations of N,N-dimethylsphingosine or dl-threo-dihydrosphingosine dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in assays was <1%.
RESULTS AND DISCUSSION

Sphingosine Kinase Activity in Various Tissues—It has been reported that sphingosine kinase is a ubiquitous enzyme (12, 22–27, 35, 36). Using our recently developed quantitative sphingosine kinase assay (12, 35), we measured sphingosine kinase activity in various rat tissues to determine which source would be most appropriate for purification. In agreement with a previous qualitative study (24), we found that spleen and kidney have higher specific activities than liver, which has about twice the activity of brain (Table I). More than 50% of the

FIG. 3. Separation of sphingosine kinase by chromatography on EAH-Sepharose and calmodulin-Sepharose columns. A, a portion of the blue A-Sepharose 2 M NaCl eluate was concentrated and dialyzed. Triton X-100 was added to a final concentration of 0.05% before application to EAH-Sepharose. The column was eluted stepwise with increasing NaCl concentrations. B, the sphingosine (Sph) kinase-containing fractions from the EAH column were applied to a calmodulin-Sepharose column after addition of CaCl₂, and the proteins were then eluted stepwise with buffer B containing 10% sucrose, 0.05% Triton X-100, and 1 mM EGTA without and then with 1 M NaCl added. Results are expressed as percentage of the total protein or sphingosine kinase activity applied to each of the columns. Similar results were obtained in at least six experiments. RT, run-through.

FIG. 4. Purification of sphingosine kinase by hydroxylapatite chromatography. A, sphingosine (Sph) kinase activity eluted from the calmodulin-Sepharose column was loaded onto a hydroxylapatite column and then eluted with a gradient of potassium phosphate buffer (pH 7.4). Sphingosine kinase activity in the fractions was immediately measured as described under “Experimental Procedures.” B, aliquots of fractions were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by silver staining. The molecular masses of standard proteins are indicated. The second lane contains the calmodulin-Sepharose eluate.

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sphingosine kinase activity in kidney, liver, and brain was in the cytosolic fraction, independently of ionic strength of the extraction buffer. SPP levels have also been measured in these tissues (37). However, there does not appear to be a good correlation between sphingosine kinase activity and SPP levels, as the highest levels of SPP were found in brain and spleen. Furthermore, kidney has more SPP than liver, which contains only very low levels (37, 38). Although we found that spleen has high sphingosine kinase activity and SPP levels, we selected kidneys as the source for the purification of sphingosine kinase since initial experiments demonstrated that the majority of the sphingosine kinase activity in spleen was associated with membranes (Table I) and unstable. Bovine kidneys were also examined as a potential source for purification of sphingosine kinase since they are much larger. However, the specific activity in the cytosolic fraction from bovine kidneys was lower than that in rat kidney cytosol (Table I), and very poor recoveries were found after preliminary ammonium sulfate fractionations (data not shown). Interestingly, we have found that most of the sphingosine kinase in Saccharomyces cerevisiae is in the microsomal fraction and not in the cytosol (Table I). These results suggest that various forms of sphingosine kinase may exist depending on the tissue and species.

**Purification of Rat Kidney Sphingosine Kinase**—Table II summarizes the purification of sphingosine kinase from 2500 rat kidneys. Sphingosine kinase was purified 6 × 10^5-fold to near homogeneity, with a total activity recovery of 0.6%. After an initial 20,000 × g centrifugation of the homogenate, it was important to carry out two subsequent centrifugations at 100,000 × g since after ammonium sulfate precipitation of the 20,000 × g supernatant and consequent dialysis, 60% of the sphingosine kinase activity became insoluble and could not be solubilized by detergents or in different buffers with pH values ranging from 6.0 to 8.0. However, when the second 100,000 × g supernatant was fractionated with ammonium sulfate, most of the sphingosine kinase activity could be resolubilized, and very little was irreversibly associated with insoluble material.

In contrast to bovine brain sphingosine kinase (26), rat kidney sphingosine kinase binds to DEAE-cellulose at pH 7.5 in Tris buffer, but not in phosphate buffer. The activity was eluted with 0.2 M NaCl from DEAE-cellulose as a single broad peak (Fig. 1). As expected for a nucleotide-binding protein, sphingosine kinase binds tightly to both green A and blue A dye-matrix columns (Fig. 2, A and B), even in the presence of relatively high salt concentrations (0.2 M for the green A column and 0.5 M for the blue A column). This makes it possible to directly apply the pooled DEAE fractions containing sphingosine kinase activity to these dye-matrix columns in a sequential manner. This was advantageous since either concentration or dialysis of sphingosine kinase activity resulted in considerable loss of activity. Sphingosine kinase was purified 270–400-fold after the dye-ligand chromatography steps, with a yield of 30–50%. Sphingosine kinase activity eluted from the blue A column in 2 M NaCl could be stored for several weeks at −70 °C after quick freezing in liquid nitrogen. It should also be noted that concentration and dialysis of the blue A column eluate resulted in a loss of 30–50% of the activity. Subsequent purification steps were then repeated two times with half of this fraction since poor recoveries were found when larger columns were used for the chromatography separations described below. Addition of 0.05% Triton X-100, but not Nonidet P-40 or β-octyl glucopyranoside, markedly improved both the recovery and the resolution of proteins and was thus included in subsequent steps. Triton X-100 has previously been successfully used to stabilize a number of other lipid enzymes, such as phospholipase A_2_, ceramidase, acid sphingomyelinase, and phosphoinositide 4-kinase, since it prevents aggregation and nonspecific adsorption to surfaces (39–42).

Several attempts were made to purify sphingosine kinase by affinity chromatography on immobilized sphingosine (43) or ATP and by hydrophobic chromatography, but they were un-
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FIG. 7. Characterization of sphingosine kinase. A, pH dependence of sphingosine (Sph) kinase activity. The activity of purified sphingosine kinase was measured as described under “Experimental Procedures.” The pH was adjusted by addition of the following buffers: 200 mM sodium acetate (pH 4–5), 200 mM MES (pH 6.0–6.6), 200 mM HEPES (pH 7.0–7.5), and 200 mM Tris-HCl (pH 7.4–9.0). B, concentration-dependent activation of sphingosine kinase by MgCl₂. Sphingosine kinase activity was measured in the presence of increasing concentrations of MgCl₂. C, Michaelis-Menten and Lineweaver-Burk (inset) plots for sphingosine kinase with d-erythro-sphingosine. The activity of purified sphingosine kinase was determined by gel filtration was identical to that of sphingosine synthetase (47), to buffers containing 0.05% Triton X-100 and concentrations of ATP. The Km for ATP was 93 μM.

successful probably due to the necessity of maintaining the enzyme in solutions containing high salt concentrations and detergent. We found that sphingosine kinase binds tightly to 1,6-diaminohexane covalently linked to Sepharose 4B (EAH-Sepharose), but could not be eluted from this matrix by substrates containing primary amino groups, such as sphingosine or choline, in a similar manner, as was previously found for purification of choline kinase (44). In contrast, at least 70% of the applied sphingosine kinase activity was eluted from EAH-Sepharose with 0.15 M NaCl, whereas only 14–18% of the applied proteins were eluted in this fraction, resulting in 5–fold purification (Fig. 3A).

This EAH fraction was immediately applied to a calmodulin-Sepharose column after addition of CaCl₂. Most of the protein applied (96–98%) did not bind and was eluted with the wash buffer. In contrast, most of the sphingosine kinase activity was tightly retained, and only a small fraction of the activity could be eluted with EGTA in the absence of CaCl₂ (Fig. 3B). However, >95% of the activity was eluted when the calmodulin column was eluted with 2 mM EGTA solution containing 1 M NaCl (Fig. 3B), resulting in at least 20-fold purification. Similarly, another calmodulin-binding lipid kinase, inositol-1,4,5-trisphosphate 3-kinase, from either human platelets (45) or rat brain (46) could not be eluted from a calmodulin-Sepharose column unless the elution buffer contained EGTA as well as 0.5% Triton X-100 or 0.2% SDS, respectively. Despite purification of sphingosine kinase by >9000-fold at this stage, several protein bands were still evident on silver-stained SDS-polyacrylamide gels. Detectable amounts of calmodulin also coeluted with the sphingosine kinase activity (Fig. 4B). One of the difficulties we encountered in later steps of the purification of sphingosine kinase was that the kinase rapidly lost activity. However, we found that addition of 10% sucrose, which has been shown to stabilize other lipid enzymes, including squalene synthetase (47), to buffers containing 0.05% Triton X-100 and high salt concentrations further increased stability at these stages of purification.

Sphingosine kinase was then further purified by hydroxylapatite chromatography followed by HPLC on a Mono Q column. As shown in Fig. 4, sphingosine kinase activity was eluted from a hydroxylapatite column as a single peak with a gradient of increasing concentrations of potassium phosphate at ~0.06 M, resulting in 6-fold additional purification. Although sphingosine kinase is tightly bound to the strong anion exchanger Mono Q and can be eluted at an ionic strength of 0.15 M with a salt gradient (data not shown), it is not as tightly bound when applied in the presence of 15 mM potassium phosphate. We found that most of the activity could be eluted with the wash buffer alone in this case, greatly improving separation from other more tightly bound proteins (Fig. 5). Sphingosine kinase was resolved into two activity peaks by chromatography on Mono Q. For further purification, only the first activity peak was utilized since the major protein band in this fraction was a 49-kDa polypeptide (Fig. 5B), and preliminary experiments utilizing several different sequences of purification suggested that this 49-kDa polypeptide correlated with the sphingosine kinase activity.

Because SDS-polyacrylamide gel electrophoresis analysis suggested that sphingosine kinase was highly purified at this stage, we decided to do a final purification by gel filtration chromatography. However, it was necessary to concentrate the Mono Q eluate to a small volume before injection onto a Superdex 75 gel filtration HPLC column. Ultrafiltration at this stage resulted in large losses of activity and high concentrations of Triton X-100, which interfere with the gel filtration. Thus, we used a 1-ml calmodulin-Sepharose column to concentrate the sample 9-fold, eluted exactly as above described for the large-scale calmodulin-Sepharose column. Further concentration of the calmodulin eluate by ultrafiltration did not result in major losses of activity, and the resulting concentration of Triton X-100 did not interfere with the resolution of the gel filtration column.

As shown in Fig. 6, the sphingosine activity was eluted from a Superdex 75 gel filtration column at a volume corresponding to an apparent native molecular mass of ~59 kDa when compared with standard proteins (Fig. 6B). Silver-stained SDS-polyacrylamide gels revealed that the fractions with the highest sphingosine kinase activity (fractions 23–25) contained a single 49-kDa polypeptide under both reducing and nonreducing conditions. Thus, sphingosine kinase isolated from rat kidney is likely active as a monomer since the apparent native molecular mass of sphingosine kinase was similar to its molecular mass on SDS-polyacrylamide gel. However, the possibility that sphingosine kinase purified to homogeneity from rat kidney cytosol may be an active fragment of membrane-bound sphingosine kinase cannot be excluded. It should be noted that the native molecular mass of Swiss 3T3 fibroblast sphingosine kinase determined by gel filtration was identical to that of...
highly purified rat kidney sphingosine kinase (data not shown). The specific activity of highly purified rat kidney sphingosine kinase (100 μmol/min/mg) is ~10–100-fold higher than that of several other highly purified lipid kinases, including phosphoinositide 3-kinase (48, 49), phosphoinositide 4-kinase (40), phospholipase D (50), and phospholipase A2 (41), but it is the same order of magnitude as that reported for acid sphingomyelinase from human urine (51).

Characterization of Sphingosine Kinase—To characterize purified sphingosine kinase, a number of experiments were carried out to examine the pH dependence, substrate specificity, and enzyme kinetics. Purified sphingosine kinase was active from pH 6 to >8, with a broad pH optimum between pH 6.6 and 7.5 (Fig. 7A). At pH 7.4, activity increased linearly with the incubation time for the first 60 min of the reaction and then gradually decreased. Sphingosine kinase activity was maximal at a concentration of 5–10 mM MgCl₂ (Fig. 7B), whereas physiological concentrations of calcium (1–100 μM) had no effect on its activity. Activity with ν-erythro-sphingosine showed typical Michaelis-Menten kinetics, with $K_m = 5.1 ± 1.7$ μM and $V_{max} = 101 ± 22$ μmol/mg/min (Fig. 7C). The $K_m$ for ATP was 93 μM (Fig. 7D), similar to $K_m$ values for other lipid kinases (40, 48). Next, we examined the substrate specificity for sphingosine kinase. The naturally occurring $\Delta^\text{1,2}$-trans-sphingosine was the best substrate for purified rat kidney sphingosine kinase. $\Delta^\text{1,2}$-trans-Dihydrosphingosine was also phosphorylated, but to a lesser extent (30% compared with $\Delta^\text{1,2}$-erythro-sphingosine), whereas $\Delta^\text{1,2}$-trans-dihydrosphingosine, $\Delta^\text{1,2}$-trans-dihydrosphingosine, ceramide, diacylglycerol, and phosphatidylinositol were not phosphorylated (Fig. 8A). $N,N$-Dimethylsphingosine and $\Delta^\text{1,2}$-trans-dihydrosphingosine have previously been used to decrease SPP levels stimulated by various physiological stimuli (7, 8, 18). We have now found that both $N,N$-dimethylsphingosine and $\Delta^\text{1,2}$-trans-dihydrosphingosine are potent competitive inhibitors of purified sphingosine kinase, with $K_i$ values of 9.9 ± 1.0 and 5.2 ± 0.5 μM, respectively (Fig. 8B and C). These results further substantiate the usefulness of these compounds as tools to inhibit sphingosine kinase activity in vivo and to examine the role of SPP in diverse cellular responses.

In summary, rat kidney sphingosine kinase has been purified to homogeneity by >6 × 10⁵-fold, indicating that sphingosine kinase is a low abundance protein in rat kidney and probably in other tissues as well. Similarly, another kinase, choline kinase, has been partially purified from rat kidney by >200,000-fold (44). This study provides the basis for molecular characterization of sphingosine kinase and will aid in elucidation of its role in various physiological processes.

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