The magnesium-dependent, plasmamembrane-associated neutral sphingomyelinase (N-SMase) catalyzes hydrolysis of membrane sphingomyelin to form ceramide, a lipid signaling molecule implicated in intracellular signaling. We report here the biochemical purification to apparent homogeneity of N-SMase from bovine brain. Proteins from Nonidet P-40 extracts of brain membranes were subjected to four purification steps yielding a N-SMase preparation that exhibited a specific enzymatic activity 23,330-fold increased over the brain homogenate. When analyzed by two-dimensional gel electrophoresis, the purified enzyme presented as two major protein species of 46 and 97 kDa, respectively. Matrix-assisted laser desorption/ionization-mass spectrometry analysis of tryptic peptides revealed at least partial identity of these two proteins. Amino acid sequencing of tryptic peptides showed no apparent homologies of bovine N-SMase to any known protein. Peptide-specific antibodies recognized a single 97-kDa protein in Western blot analysis of cell lysates. The purified enzyme displayed a $K_m$ of 40 $\mu$M for sphingomyelin with an optimal activity at pH 7–8. Bovine brain N-SMase was strictly dependent on $\text{Mg}^{2+}$, whereas $\text{Zn}^{2+}$ and $\text{Ca}^{2+}$ proved inhibitory. The highly purified bovine N-SMase was effectively blocked by glutathione and scyphostatin. Scyphostatin proved to be a potent inhibitor of N-SMase with 95% inhibition observed at 20 $\mu$M. The results of this study define a N-SMase that fulfills the biochemical and functional criteria characteristic of the tumor necrosis factor-responsive membrane-bound N-SMase.

Ceramide belongs to the group of sphingosine-based lipid signaling molecules that are involved in regulation of diverse cellular responses to exogenous stimuli (for review Refs. 1–3). The mode of ceramide action and the regulation of its production have recently attracted great attention because of possible roles of ceramide in cellular differentiation, proliferation, and apoptosis (1–3). The catabolic pathway for ceramide formation involves the action of SMases, sphingomyelin-specific forms of phospholipase C, which hydrolyze the phosphodiester bond of sphingomyelin (N-acylsphingosine-1-phosphorylcholine), a phospholipid found in the plasma membrane of mammalian cells yielding ceramide and phosphorylcholine. There are several isoforms of SMase, distinguished by different pH optima, cellular topology, and cation dependence. A $\text{Mg}^{2+}$-dependent neutral (N-SMase) operates at the plasmamembrane (4), whereas an acid (A-SMase) is localized in the endosomal-lysosomal compartments (5). Further, a neutral, $\text{Mg}^{2+}$-independent neutral (N-SMase) was localized in the gastrointestinal tract (8). N-SMase and A-SMase are rapidly and transiently activated by diverse exogenous stimuli. N- and A-SMases appear to be responsible for stimulus-induced increases of ceramide within a time frame of seconds and minutes (9). Therefore, these forms of SMases are considered as principal pathways for production of ceramide in early signal transduction. However, direct links between SMases and specific signaling systems remain to be established. Clearly, the unambiguous assignment of specific signaling functions to SMases will require genetic models, specific SMase inhibitors, and the availability of monoclonal anti-SMase antibodies. This is exemplified by the progress made with regard to the functional characterization of A-SMase. Human and murine A-SMase (pH optimum 4.5–5.0) have been molecularly cloned and determined to be the products of a conserved gene (10–12). The A-SMase gene also directs, independent of alternate splicing, the synthesis of a $\text{Zn}^{2+}$-dependent secreted form of A-SMase (13). Cells from patients suffering genetically determined A-SMase deficiency (Niemann-Pick disease) (14) as well as cells from A-SMase knock-out mice (15, 16) will be instrumental for unraveling the role of A-SMase in signaling and apoptosis. With respect to N-SMase, it is important to note that Niemann-Pick patients as well as A-SMase knock-out mice retain N-SMase activity, indicating that the neutral forms are products of a distinct gene or genes (14–16).

In lieu of detailed information about the biochemical properties of N-SMase, the physiological function of N-SMase remains rather elusive. A vast array of biological functions has been ascribed to N-SMase. At the cellular level, N-SMase has been implicated in proliferation (17), differentiation (18), senescence (19), and apoptosis (for reviews see Refs. 1–3). As to

* This work was supported by grants from the Deutsche Forschungsgemeinschaft Kr10/12-114. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Roche Bioscience, Palo Alto, CA 94304.

‡‡ To whom correspondence should be addressed: Inst. of Medical Microbiology and Hygiene, Medical Center, University of Cologne, 50935 Köln, Germany, Institute of Physiological Chemistry, University of Bochum, 44780 Bochum, Germany, Department of Internal Medicine and ‡‡‡Department of Dermatology, Medical Center, University of Kiel, 24105 Kiel, Germany, †‡‡Kekule-Institute for Organic Chemistry and Biochemistry, University of Bonn, 53121 Bonn, Germany.

¶ The abbreviations used are: SMase, sphingomyelinase; TNF, tumor necrosis factor; Con A, concanavalin A; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; N-SMase, neutral SMase; A-SMase, acidic SMase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
more complex processes, N-SMase was suggested to regulate phagocytosis (20), lung development (21), or hepatic regeneration (22). In view of this plethora of putative functional activities, the activation requirements of N-SMase are the subject of extensive investigations. Transient and rapid activation of N-SMase is observed in response to many exogenous stimuli including cytokines like TNF and interleukin-1, cell surface molecules like CD40 ligand and CD95 ligand, growth factors like nerve growth factor, chemotherapeutic agents, etc. (1–3). In the paradigm of TNF signaling, we have identified a 9-amino acid residue motif at position 310–318 in the cytoplasmic tail of the p55 TNF receptor that is both necessary and sufficient for activation of N-SMase (23). A novel WD repeat protein, designated FAN, was described that specifically binds to this motif and functionally couples the p55 TNF receptor to N-SMase (24). TNF-induced activation of N-SMase was completely abolished in mice lacking a functional FAN protein because of targeted disruption of the FAN gene, confirming the essentiality of FAN for N-SMase activation (25). Whether FAN directly interacts with N-SMase or acts through yet to be described intermediates remains to be resolved. Further delineation of the activation requirements of N-SMase and its functional role in cellular signaling clearly requires its definitive biochemical characterization and the molecular cloning of N-SMase cDNA.

Stoffel and co-workers (26) described the cloning of a putative N-SMase cDNA that was based on homologies to phosphodiesterases. However, overexpression of this candidate N-SMase cDNA did not significantly increase intracellular ceramide levels. Furthermore, the gene product did not respond to TNF (27), suggesting that the TNF-responsive N-SMase remains to be identified. Hannun and co-workers (27) recently reported the partial purification of a Mg2+-dependent N-SMase from rat brain. Thorough functional characterization revealed that this type of N-SMase most likely represents the membrane-bound form of a stimulus-responsive N-SMase, which seemed to be clearly distinct from the gene product of the putative N-SMase cDNA (27).

In this study we describe the purification to homogeneity of a Mg2+-dependent N-SMase from bovine brain. Biochemical and functional characteristics of this highly purified isoform of N-SMase will be provided.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain tissue was obtained from a local slaughterhouse and was homogenized immediately. Methyl- Macroprep medium and low molecular weight protein standards were purchased from Bio-Rad. Nonidet P-40, hydroxypropyl methylcellulose, Con A-Sepharose, bovine brain t-a-phosphatidyl-t-serine, and other lipids were obtained from Sigma. N-methyl-3H(sphingomyelin from bovine brain, carbamylmethy fast flow-Sepharose, and rainbow colored protein standards (e.g., N-SMase) were eluted using a linear gradient of o-methylglucopyranoside (from 0 to 15%) in Con A buffer. This step was used to remove A-SMase activity, and the N-SMase activity was found in the flow-through.

Hydrophobic Interaction Chromatography on Methyl-Macroprep—The flow-through fraction of the Con A-Sepharose column was loaded onto a Con A-Sepharose column, which had been equilibrated with buffer A containing additionally 200 mM sodium chloride, 10 mM magnesium chloride, and 2 mM calcium chloride. EDTA and EGTA were omitted in this buffer (Con A buffer). Proteins (e.g., N-SMase) were eluted using a linear gradient of o-methylglucopyranoside (from 0 to 15%) in Con A buffer. Bound material was eluted from the column using a linear gradient of Nonidet P-40 (from 0 to 2%) in buffer C without ammonium sulfate and sodium chloride.

Preparative Isoelectric Focusing—Preparative isoelectric focusing was performed on a Tetrapak Octopus “free flow electrophoresis” (Weber GmbH, Muenchen FRG) apparatus according to the manufacturer’s instructions. The electrolyte solution including 0.1 M NaOH and 0.1 M phosphoric acid in 20% glycerol and 0.2% hydroxypropyl-methyl cellulose were used. The ampholyte solution contained Ampholine 3–10 (Serva, Heidelberg-FRG) and Ampholine 4–7 (Serva Heidelberg-FRG) (0.5% v/v), 0.2% hydroxypropyl methyl-cellulose, 20% glycerol (v/v), and 0.2% (v/v) Nonidet P-40. A suspension of 0.2% hydroxypropyl-methyl cellulose in 20% glycerol (v/v) was used as counterflow to avoid electroendosmosis. The ampholyte was prefocused by setting the current to 3000 V, 12 watt, and 15 mA prior to the application of the N-SMase preparation. Samples were collected into 96-well plates with a fraction size of 1.3 ml. After determining the pH of every second fraction, 30 μl of 0.1 M HEPES (pH 7.4) was added to each fraction. Fractions were stored at −20 °C until analysis for N-SMase activity and SDS-PAGE.

Purification of a Polyclonal Anti-p97-derived Peptide Antibody—A polyclonal antibody was raised against a p97-derived peptide (GLPYEQEQR) in rabbits. The antibody was purified using a 5-ml Hitrap N-hydroxy succinimide activated peptide affinity chromatography column (Amersham Pharmacia Biotech) according to the instructions of the manufacturer.

SMase Activity Assay—The activities of both N-SMase and A-SMase were measured using radiolabeled substrate in a mixed micelle assay system as described by Wiegmann et al. (9). For N-SMase, the reaction mixture contained 100 mM HEPES, pH 7.4, 3 nmol of [3H]sphingomyelin (80,000 cpm), 1 mM DTT, 2 mM EDTA, 2 mM EGTA, 0.1% Nonidet P-40 (1.54 μm), 10 mM sodium fluoride, 10 mM magnesium chloride and various dilutions of the enzyme preparation in a total volume of 50 μl. A-SMase activity was measured in a 50-μl reaction mixture consisting of enzyme preparations in 100 mM sodium acetate, pH 5.0, 2 nmol of [3H]sphingomyelin (80,000 cpm), 5 mM EDTA, and 0.1% Nonidet P-40. To examine the effects of other lipids, the substrate was mixed with these lipids before drying under nitrogen. Mixed micelle solutions were prepared by sonicating the tube for 5 min in a bath sonicator and vortexed for 5 min at room temperature. The mixture was incubated for 120 min at 37 °C, and the enzymatic reaction was stopped by the addition of 250 μl of water and 800 μl of chloroform/methanol (2:1 v/v). After vortexing and phase separation by centrifugation, 0.2 ml of the upper aqueous phase was removed and added to 2 ml of scintillation solution for radioactivity counting. The reaction was linear with incubation times up to 6 h. The amount of enzyme added to the reaction mixtures was chosen such that no more than 10% of the substrate would be degraded. Appropriate blanks containing denatured enzyme (30 min, 95 °C) were run with each reaction and subtracted from the experimental samples.

Possible inhibitors of the N-SMase were tested by adding them to the assay in aqueous solution. Alternatively, hydrophobic compounds were added to each assay, the samples were incubated for 60 min at 100,000 × g for 15 min to remove debris. The supernatant (postnuclear supernatant) was centrifuged at 100,000 × g for 1 h. The pellet was then resuspended by homogenization in 2 volumes of N-SMase buffer (20 mM HEPES (pH 7.2), 20% glycerol, 0.1% Nonidet P-40, 5 mM magnesium chloride, 2 mM EDTA, 2 mM EGTA) followed by solubilization with Nonidet P-40 (1% final concentration) and kept for 2 h at 4 °C with constant shaking. After 60 min at 100,000 × g the samples were subjected to column chromatography. All steps were carried out at 4 °C with buffers containing NaN3 (0.02% w/v) to prevent bacterial growth. Unless stated otherwise, the flow rate of the column chromatography steps was maintained at 30 ml/h.

Carboxymethyl Fast Flow-Sepharose (CM-Sepharose)—A CM-Sepharose column was equilibrated with buffer A consisting of 0.1 M sodium chloride, 10 mM sodium fluoride, 1 mM sodium molybdate, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin and pepstatin A). The Nonidet P-40 extract containing the membrane proteins was diluted with buffer A to a final concentration of 0.2% Nonidet P-40 and loaded onto the CM-Sepharose column, which was washed with a 100-ml linear gradient of 0–100% buffer A containing 1 M KCl.

Con A-Sepharose—The flow-through fraction of the CM-Sepharose column was loaded onto a Con A-Sepharose column, which had been equilibrated with buffer A containing additionally 200 mM sodium chloride, 10 mM magnesium chloride, and 2 mM calcium chloride. EDTA and EGTA were omitted in this buffer (Con A buffer). Bound material was eluted from the column using a linear gradient of o-methylglucopyranoside (from 0 to 15%) in Con A buffer. This step was used to remove A-SMase activity, and the N-SMase activity was found in the flow-through.
Characterization of Bovine Neutral Sphingomyelinase

Purification of neutral sphingomyelinase from bovine brain

| Purification step                  | Volume ml | Total protein mg | Total activity nmol/h | Specific activity nmol/h/mg | Yield % | Purification factor -fold |
|-----------------------------------|-----------|------------------|-----------------------|-----------------------------|---------|--------------------------|
| Whole homogenate                  | 1,000     | 88,625           | 51,700                | 0.6                         | 100     | 1                        |
| Postnuclear homogenate            | 270       | 25,770           | 48,481                | 1.9                         | 94      | 3.6                      |
| NP-40 extract                     | 200       | 5,685            | 32,067                | 5.6                         | 62      | 9.3                      |
| CM-Sepharose fast flow            | 250       | 925              | 29,500                | 31.9                        | 57      | 53.2                     |
| Concanavalin A-Sepharose          | 340       | 544              | 51,680                | 58.2                        | 61.3    | 97                       |
| Methyl-Macroprep                  | 50        | 5                | 1,855                 | 371                         | 3.6     | 618                      |
| Preparative isoelectric focusing  | 30        | 0.03*            | 420                   | 14,000                      | 0.8     | 23,330                   |

* The protein amount was determined by SDS-PAGE using bovine serum albumin as standard.

are representative for at least three experiments where each value represents the average of three independent samples.

SDS-PAGE—One-dimensional denaturing SDS-PAGE was performed on 10% polyacrylamide gels according to the method of Schagger and Jagow (28) in a Bio-Rad Protein-II electrophoresis system. Two-dimensional gel electrophoresis was performed according to O’Farrell (29) using the IPG-Phor (Amersham Pharmacia Biotech) system according to the instructions of the manufacturer. Isoelectric focusing was performed as described by Görg et al. (30) using 8 M urea, 2 M thiourea, 2% CHAPS, and 0.05% bromphenol blue. Focusing of the N-SMase was carried out in the buffer containing 8 M urea, 2 M thiourea, 2% CHAPS, and 0.05% bromphenol blue with increasing voltage (1 h, 100 V; 1 h, 200 V; 1 h, 500 V; 1 h, 1000 V; 1 h, 2000 V; and 3 h, 8000 V). Rod gels were soaked for 15 min at ambient temperature in equilibration buffer (50 mM Tris-HCl, pH 8.8, 8 M urea, 2 M thiourea, 30% glycerol, 2% SDS, 0.05% bromphenol blue, 10 mg/ml DTT). A second equilibration was performed for further 15 min in equilibration buffer containing 25 mg/ml iodoacetamide instead of DTT, and applied to a second dimension using a 10% Tris-Tricine SDS gel (16 cm × 16 cm × 1.0 mm).

The gels were stained for 1 h in 0.2% Coomassie Brilliant Blue R250 with 45% ethanol in water containing 15% acetic acid. Alternatively, silver staining was performed according to the method described by Mann and co-workers (31).

In Gel Preparation of Tryptic Peptides—In gel digestion with trypsin was performed according to standard protocols (31, 32). Coomassie Blue-stained protein spots were excised from the gel and washed three times for 10 min with water.

In situ reduction was achieved in a total volume of 50 µl containing 25 mM DTT, 4 mM guanidine hydrochloride, 100 mM Tris-HCl, pH 8.2, for 1 h at 37 °C. After cooling to room temperature, SH groups were alkylated by adding iodoacetamide to a final concentration of 50 mM followed by a 30-min incubation in the dark. Excess of iodoacetamide was neutralized by adding 5 µl of β-mercaptoethanol. The gel pieces were then washed (2 × 40 µl) for 10 min with water, equilibrated (2 × 40 µl) with 50 mM NH₄HCO₃ (pH 7.8), (1 × 40 µl) with 50 mM NH₄HCO₃/acetonitrile (1:1 v/v), and shrunk by dehydration with acetone. The gel pieces were reswollen in a digestion buffer containing 50 mM NH₄HCO₃, 5% acetonitrile and treated with 0.2 µg of trypsin (Promega) at 37 °C for 16 h.

Peptides were extracted by subsequent incubation for 15 min at room temperature with 50 mM NH₄HCO₃, two changes of trifluoroacetic acid and immediately analyzed by micro reverse phase-HPLC on a 180-µm capillary column. Prior to μHPLC analysis, aliquots of 0.5 µl of the combined extract were used for MALDI mass spectrometry to obtain MS fingerprints. All mass spectra were obtained with Bruker REFLEX III mass spectrometer (Bruker-Daltonik, Bremen, Germany). Control of all data acquisition parameters and the transfer and the subsequent averaging of the time-of-flight data, as well as all further data processing, were carried out using the XMASS 4.02 postanalysis software. MALDI-MS spectra were calibrated using several peaks as external standards. Obtained spectra were analyzed using the sequest algorithm against public data base.

RESULTS

Purification of a Membrane-bound N-SMase from Bovine Brain—The purification procedure of N-SMase from bovine brain is summarized in Table I. Protease inhibitors, phosphatase inhibitors, and reducing agents were used throughout the extraction and purification procedure to preserve and stabilize the N-SMase activity. Nonidet P-40 membrane protein extracts were applied to a carboxymethyl-Sepharose fast flow column. The flow-through from the CM-Sepharose column containing the major part of N-SMase activity was loaded to Con A-Sepharose, which served to effectively remove the acid SMase. The flow-through from the Con-A column was loaded to the hydrophobic interaction chromatography column using the methyl-Macroprep material (Bio-Rad) in the presence of 10% of saturated ammonium sulfate. The enzyme bound to the hydrophobic column and the majority (80%) of the N-SMase activity were eluted between 0.4 and 0.7% Nonidet P-40. Preparative isoelectric focusing by free flow electrophoresis eventually resulted in a ~23,300-fold purification of the N-SMase from bovine brain. Two-dimensional electrophoresis of the purified enzyme revealed two major protein species with molecular masses of ~97 and ~46 kDa, respectively (Fig. 1). In some preparations also minor protein spots corresponding to molecular masses of 14, 17, and 28 kDa, respectively, were also occasionally observed. Peptide MS fingerprint analysis and Edman microsequencing of these bands revealed their related-
ness to the 97-kDa as well to the 46-kDa protein (data not shown), indicating that these protein species are either degradation products or subunits of the mature N-SMase.

Polyclonal antibodies were raised against a synthetic 10-amino acid peptide based on the amino acid sequence of a p97-derived peptide. As shown in Fig. 2A, Western blot analysis of the purified enzyme revealed a 97-kDa protein species. To avoid proteolytic degradation processes in bovine brain cadaver we analyzed N-SMase in living cells. Within brain tissue the cell type expressing high N-SMase activity has not yet been determined. However, because N-SMase activity is ubiquitously expressed we have chosen bovine aortic endothelial cells (obtained from ATCC, Rockville). When extracts from bovine aortic cells were analyzed, a major 97-kDa protein was stained under nonreducing as well as under reducing conditions (Fig. 2B), indicating that the 97-kDa band is not composed of subunits. The reactivity of the antibody with a 97-kDa protein was efficiently competed by the peptide used for immunization (Fig. 2B), indicating nonspecific staining of proteins of lower apparent molecular mass. Because the polyclonal antibody does not specifically recognize a 46-kDa protein found in the two-dimensional gel electrophoresis (Fig. 1), these findings strongly suggest that the 46-kDa protein species represents a breakdown product rather than a subunit of the 97-kDa protein.

Functional Characterization of the Purified Bovine N-SMase—The methyl-Macroprep eluate as well as the isoelectric focusing purified N-SMase were used for further functional characterization. Michaelis-Menten kinetic analysis revealed an apparent $K_m$ of $40 \mu M$ (Fig. 3). The reaction was linear for up to 6 h. The purified enzyme displayed a pH optimum of 7.0–8.0 with half-maximal activity at pH 6.5. No activity was detected at acidic pH (4–6) or alkaline pH (Fig. 4). The pI of the purified enzyme has ranged from about a pI of 5.3 to 5.6 (Fig. 5). The purified enzyme did not cleave either phosphatidylcholine or ceramide (data not shown). When incubated at 56 °C for 5 min, the purified enzyme lost 95% of its activity indicating that N-SMase is a heat labile C-type phospholipase. In the presence of 10 mM EDTA, the purified N-SMase activity was reduced to 5% of its basal activity, indicating a metal ion dependence of N-SMase. In contrast 10 mM EGTA had no significant effect (data not shown). To examine metal ion dependences of N-SMase in greater detail, the purified enzyme was incubated with several cations like magnesium, calcium, manganese, iron, copper, and lithium. As shown Fig. 6, none of the tested cations was able to replace magnesium. In the presence of magnesium, zinc and calcium were inhibitory (Fig. 6).

Previous reports have demonstrated that glutathione (33) and scyphostatin (34) inhibit the magnesium-dependent, membrane-bound N-SMase. As shown in Fig. 7, a nearly complete inhibition of N-SMase was observed with 0.2 mM glutathione. Likewise, N-SMase was completely inhibited by scyphostatin.
Finally, several lipids were tested for possible modulation of the activity of the purified N-SMase. A 4-fold stimulation was observed by phosphatidylserine, whereas a 2-fold stimulation was observed with phosphatidylethanolamine (Table II).

**DISCUSSION**

In this report we describe the purification to apparent homogeneity of a 97 kDa, magnesium-dependent neutral sphingomyelinase from bovine brain. On two-dimensional gel electrophoresis the purified enzymatic activity was determined in the presence of the indicated cations. The basal activity of N-SMase was 2.5 μmol/mg/h. A 4-fold stimulation was observed by phosphatidylserine, whereas a 2-fold stimulation was observed with phosphatidylethanolamine (Table II).

**TABLE II**

| Lipid | Fold increase in N-SMase activity |
|-------|----------------------------------|
| Control | 1                      |
| PE     | 2.9                     |
| PS     | 3.8                     |
| PC     | 2.0                     |
| PI     | 1.9                     |
| AA     | 1.2                     |

**FIG. 6.** Effect of cations on bovine N-SMase activity. A, N-SMase enzymatic activity was determined in the presence of the indicated cations. The basal activity of N-SMase was 2.5 μmol/mg/h. B, N-SMase activity was determined in the presence of 1 mM magnesium. Indicated cations were added. The basal activity of N-SMase was 13 μmol/mg/h.

**FIG. 7.** Inhibition of bovine N-SMase by glutathione and scyphostatin. Purified N-SMase was preincubated for 15 min at 37 °C with scyphostatin (A) or glutathione (B), and N-SMase activity was determined.

**FIG. 5.** Determination of pI for the bovine N-SMase. The N-SMase activity eluted after the methyl-Macroprep column was applied to preparative isoelectric focusing as described under “Experimental Procedures.”
lysates from bovine aortic endothelial cells. At the amino acid sequence level no homologies were found in public data bases, indicating that we have purified a novel enzyme. The enzyme activity is optimal at pH 7–8; its pi is 5.3–5.6. The purified N-SMase proved magnesium-dependent and was inhibited by calcium and zinc, as well as by glutathione and scyphostatin. The properties of the purified bovine enzyme match those characteristic of the plasma membrane-associated N-SMase.

Tomiuk et al. (34) recently described a microbial compound, scyphostatin, as an inhibitor of N-SMase. Using rat brain microsome fractions as a source for N-SMase, these authors suggested that scyphostatin acts as a substrate or product analogue of the enzymatic reaction. Our study confirm this notion in that scyphostatin is shown to inhibit the activity of purified bovine N-SMase, thus any action on possible co-factors of the N-SMase activation pathway seems unlikely.

It is important to note that the 97-kDa bovine N-SMase is not related to a candidate N-SMase cDNA recently described by Tomiuk et al. (26). First, the amino acid sequences of bovine N-SMase-derived peptides do not reveal any homology to the sequences deduced from the N-SMase cDNA. Second, the bovine N-SMase-derived peptides do not reveal any homology to the signal-regulated kinases was not impaired in fibroblasts from kinase (43). However, TNF-induced activation of extracellular signal-regulated kinases was not impaired in fibroblasts from FAN knock-out mice (25). In addition, Müller et al. (44) reported that TNF does not activate Raf-1 kinase in a number of cell lines. In fact, TNF down-regulated Raf-1 kinase activation induced by EGF (44). Thus, the proposed link between N-SMase, CAP kinase, and Raf-1 needs to be revisited. At present the assessment of the biological significance of N-SMase is based on rather indirect and descriptive evidence. The purification to apparent homogeneity of N-SMase from bovine brain should be instrumental for the cloning of bovine N-SMase cDNA, which seems to be required for obtaining more pertinent evidence for possible roles of N-SMase in cellular signaling.

Acknowledgments—We thank Christiane Sandberg, Institute of Immunology, University of Kiel, and Gaby Becker, Institute of Physiological Chemistry, University of Bochum, for excellent technical assistance. Scyphostatin was a kind gift of Dr. T. Ogita and Dr. F. Nara, Sankyo Co LTD, Tokyo. We thank H. Korte, Institute of Physiological Chemistry, University of Bochum, for fruitful discussions and the critical interpretation of the amino acid sequence obtained by Edman degradation.

REFERENCES

1. Liu, B., Obeid, L. M., and Hannun, Y. A. (1997) Semin. Cell Dev. Biol. 8, 311–322
2. Kolesnick, R. N., and Krohne, M. (1998) Annu. Rev. Physiol. 60, 643–665
3. Spiegel, S., and Merril, A. H. Jr. (1996) FASEB J. 10, 1388–1397
4. Hostetler, K. Y., and Yazaki, P. J. (1979) J. Lipid Res. 20, 456–463
5. Ferlinz, K., Hurwitz, R., Vielhaber, G., Suzuki, K., and Sandhoff, K. (1994) Biochem. J. 301, 855–862
6. Yamaguchi, S., and Suzuki, K. (1978) J. Biochem. 83, 4909–4902
7. Okazaki, T., Bielsawksa, A., Domae, N., Bell, R. M., and Hannun, Y. A. (1994) J. Biol. Chem. 269, 4070–4077
8. Duan, R.-D., Nyberg, L., and Nilssen, A. (1995) Biochim. Biophys. Acta 1259, 49–55
9. Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krone, M. (1994) Cell 78, 1085–1015
10. Quinetters, L. P., Schuchman, E. H., Levan, O., Suchi, M., Ferlinz, K., Reineke, H., Sandhoff, K., and Desnick, R. J. (1989) EMBO J. 8, 2469–2473
11. Schuchman, E. H., Suchi, M., Takahashi, T., Sandhoff, K., and Desnick, R. J. (1991) J. Biol. Chem. 266, 4531–4539
12. Schissel, S. L., Keersler, G. A., Schuchman, E. H., Williams, K. J., and Tabas, I. (1998) J. Biol. Chem. 273, 18250–18259
13. Schissel, S. L., Schuchman, E. H., Williams, K. J., and Tabas I (1996) J. Biol. Chem. 271, 18431–18436
14. Brady, B. O., Kanfer, J. N., Mock, M., and Fredrickson, D. S. (1966) Proc. Natl. Acad. Sci. U. S. A. 53, 366–369
15. Tourno, K., Erlb, S., Peri, D. P., Ferlinz, K., Biegaar, C. L., Sandhoff, K., Richardson, R. J., Stewart, C. L., and Schuchman, E. H. (1995) Nat. Genet. 10, 288–293
16. Otterbach, B., and Stoffel, W. (1995) Cell 81, 1053–1061
17. Anagnost, N., Escargueil-Blanc, L., Szotek-Mazone, I., Sue, J., Andreu-Abadie, N., Pieraggi, M.-T., Chatelut, M., Thiers, J.-C., Jafrezou, J.-P., Laurent, G., Levaude, T., Negre-Salvayre, A., and Salvayre, R. (1998) J. Biol. Chem. 273, 12993–12990
18. Okazaki, T., Bell, R. M., and Hannun, Y. A. (1989) J. Biol. Chem. 264, 19767–19808
19. Venable, M. E., Lee, J. Y., Smyth, J. M., Bielawska, A., and Obeid, L. M. (1995) J. Biol. Chem. 270, 30701–30708
20. Suchard, S. J., Hinkovska-Galeva, V., Mansfeld, P. J., Boxer, L. A., and Shayman, J. A. (1997) Blood 89, 2139–2147
21. Longo, G. A., Tyler, D., and Mallampalli, R. K. (1997) Am. J. Respir. Cell Mol. Biol. 16, 605–612
22. Albi, E., and Magni, M. P. (1997) Biochem. Biophys. Res. Commun. 236, 29–33
23. Adam D., Wiegmann, K., Adam-Clages, S., Ruff, A., and Krohne, M. (1996) J. Biol. Chem. 271, 14617–14622
24. Adam-Clages, S., Adam, D., Wiegmann, K., Struve, S., Kolanus, W., Schneider-Mergener, J., and Kroene, M. (1996) Cell 86, 937–947
25. Kreder, D., Kruit, O., Adam-Clages, S., Wiegmann, K., Scherer, G., Pits, T., Jensen, J. M., Prakke, E., Steinmann, J., Pfeifer, K., and Krohne, M. (1999) EMBO J. 18, 2472–2479
26. Tomiuk, S., Hofmann, K., Nix, M., Zambmann, M., and Stoffel, W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3638–3643
27. Liu, B., Hassler, F. D., Smith, K. G., Weaver, K., and Hannun, Y. A. (1998) J. Biol. Chem. 273, 34472–34479
28. Schagger, H., and Jagow, G. (1987) Anal. Biochem. 166, 368–378
29. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4090–4092
30. Gorg, A., Postel, W., and Günter, S. (1988) Electrophoresis 9, 531–546
31. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Anal. Chem. 68, 650–659
32. Jeni, P., Mini, T., Moe, S., Hintermann, E., and Horst, M. (1995) Anal. Biochem. 224, 451–455
33. Liu, B., and Hannun, Y. A. (1997) J. Biol. Chem. 272, 16281–16287
34. Tomiuk, S., Nara, F., Suzuki, K., Hanaya, T., and Ogita, T. (1997) J. Am. Chem. Soc. 119, 7871–7872
35. Maruyama, E. N., and Arima, M. (1989) J. Neurochem. 52, 611–618
36. Carre, J. B., Morand, O., Homayoun, P., Roux, P., Boure, J. M., and
Characterization of Bovine Neutral Sphingomyelinase

Baumann, N. (1989) J. Neurochem. 52, 1294–1299
37. Tamiya-Koizumi, K., Umekawa, H., Yoshida, S., and Kojima, K. (1989) J. Biochem. (Tokyo) 106, 593–598
38. Yamaguchi, S., and Suzuki, K. (1977) J. Biol. Chem. 252, 3805–3813
39. Rao, B. G., and Spence, M. W. (1976) J. Lipid Res. 17, 506–515
40. Chatterjee, S., and Ghosh, N. (1989) J. Biol. Chem. 264, 12554–12561
41. Heinrich, M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Brunner, J., Kronke, M., and Schütze, S. (1999) EMBO J. 18, 5252–5263
42. Mathias, S., Dressler, K. A., and Kolesnick, R. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10009–10013
43. Zhang, Y., Yao, B., Delikat, S., Bayesuny, S., Lin, X. H., McGinley, M., Chan-Hui, P.-Y., Lichtenstein, H., and Kolesnick, R. (1997) Cell 89, 63–72
44. Müller G., Storv, P., Bourteel, S., Doppler, H., Pfizenmaier, K., Mischak, H., Philipp, A. Kaiser, C., and Kelch, W. (1998) EMBO J. 17, 732–742
45. Lineweaver, H., and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666
Purification and Characterization of a Magnesium-dependent Neutral Sphingomyelinase from Bovine Brain

Katussevani Bernardo, Oleg Krut, Katja Wiegmann, Dirk Kreder, Marta Micheli, Reiner Schäfer, Albert Sickman, Wolfgang E. Schmidt, Jens M. Schröder, Helmut E. Meyer, Konrad Sandhoff and Martin Krönke

J. Biol. Chem. 2000, 275:7641-7647.
doi: 10.1074/jbc.275.11.7641

Access the most updated version of this article at http://www.jbc.org/content/275/11/7641

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 24 of which can be accessed free at http://www.jbc.org/content/275/11/7641.full.html#ref-list-1