FEBS Letters 531 (2002) 38–46 FEBS 26648

Minireview

Sphingomyelinases: enzymology and membrane activity

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Received 17 June 2002; revised 1 August 2002; accepted 9 September 2002

First published online 27 September 2002

Edited by Felix Wieland

Abstract This paper reviews our present knowledge of sphingomyelinases as enzymes, and as enzymes acting on a membrane constituent lipid, sphingomyelin. Six types of sphingomyelinases are considered, namely acidic, secretory, Mg^{2+}-dependent neutral, Mg^{2+}-independent neutral, alkaline, and bacterial enzymes with both phospholipase C and sphingomyelinase activity. Sphingomyelinase assay methods and specific inhibitors are reviewed. Kinetic and mechanistic studies are summarized, a kinetic model and a general-base catalytic mechanism are proposed. Sphingomyelinase–membrane interactions are considered from the point of view of the influence of lipids on the enzyme activity. Moreover, effects of sphingomyelinase activity on membrane architecture (increased membrane permeability, membrane aggregation and fusion) are described. Finally, a number of open questions on the above topics are enunciated.

Key words: Phospholipase; Sphingomyelinase; Sphingomyelinase–membrane interaction; Ceramide; Membrane signalling; Membrane fusion

1. Introduction and scope

Sphingomyelinases are enzymes that catalyze the hydrolysis of sphingomyelin (ceramide phosphorylcholine) into ceramide and phosphorylcholine. The reaction is formally similar to that of a phospholipase C. Sphingomyelinases have been known for many years [1,2], but in the last decade they have become the object of renewed interest after the discovery of the sphingomyelin signal transduction pathway [3–6]. Ceramide appears to be a lipid second messenger in programmed cell death (apoptosis), cell differentiation and cell proliferation (see reviews in [7–12]; but see also, for the opposite point of view, [13]), and sphingomyelinase is probably a major source of ceramide in the cells, although this is also a debated point.

In the midst of the advances, doubts, mistakes and corrections that mark a living scientific field, the last 12 years have witnessed a turmoil in the area of sphingomyelinases; known enzymes have been re-examined in the light of new physiological evidence, novel sphingomyelinases have been purified, some have been cloned and overexpressed … and the storm is far from dying down. Thus, the present review cannot be but a provisional assessment of the state of the art in 2002.

We have chosen two main aspects of sphingomyelinases to be covered in this review, one corresponds to the point of view of 'classical enzymology', with a description of the known sphingomyelinases, and of their properties. The second is more directly related to our experimental work, and describes the interaction of sphingomyelinases with phospholipid bilayers, and the mutual influence of enzyme and substrate properties. One important function of sphingomyelinases may be related to changing membrane properties (charge, fluidity, permeability) by transforming sphingomyelin into ceramide. The present work does not cover the cell physiological effects of sphingomyelinases, these are described in detail in the above mentioned reviews.

2. Sphingomyelinases

2.1. Types of sphingomyelinases

Samet and Barenholz [14] proposed a useful classification of sphingomyelinases into five categories. We shall follow rather closely their description, adding a sixth group for a specific kind of bacterial sphingomyelinases.

2.1.1. Acid sphingomyelinase (aSMase). This soluble glycoprotein was the first described sphingomyelinase [1]. Its name refers to the fact that its optimum activity is at pH ~5, as befits its localization in lysosomes. It is number EC 3.1.4.12 in the Enzyme Commission classification. The absence of this enzyme is responsible for the neurological disorder known as Niemann–Pick syndrome. It is the best characterized of the sphingomyelinases [14]. It has been cloned from human DNA and expressed in *Escherichia coli* [15–17] and in insect Sf21 cells [18,19]. In the latter case the recombinant protein is secreted as a 72 kDa protein, as opposed to the 75 kDa molecule purified from human placenta, both proteins having similar sphingomyelinase activity. Human aSMase has also been overexpressed in Chinese hamster ovary cells [20], and the authors have described the preparation and use of a fluorescent FITC-labelled aSMase. Acid sphingomyelinases have been recently characterized from leukemia HL-60 cells [14], and thrombin-activated human platelets [21,22]. The latter enzyme (70–73 kDa) may not originate in the lysosomes [22]. A 58 kDa aSMase has been located in the lesional skin of atopic dermatitis patients [23].

Grassme et al. [24] described the overexpression of human aSMase in JY B cells and in aSMase-deficient lymphocytes from patients with Niemann–Pick disease type A. These authors made the important observation that, upon activation of the surface receptor Fas (CD95), a SMase was translocated from its intracellular location to the plasma membrane outer

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0014-5793/02/$22.00
PII: S0014-5793(02)03482-8

leaflet, which is rich in sphingomyelin. aSMase activity induced clustering of CD95 in sphingolipid-rich membrane rafts, and subsequent apoptosis. Clustering was probably the result of lateral segregation of ceramide (see Section 3.2). In a related observation Cremesti et al. [25] showed that the anti-Fas antibody-induced lateral segregation ('capping') of Fas into one pole of the cell membrane was ceramide-dependent, and that $SMase^{-/-}$ hepatocytes were defective in antibody-induced ceramide generation, capping and apoptosis.

2.1.2. Secretory sphingomyelinase (sSMase). This enzyme arises from the acid sphingomyelinase gene via differential protein trafficking of a common protein precursor; this precursor can be targeted either to lysosomes (aSMase) or to the Golgi secretory pathway (sSMase) [26,27]. The secretory enzyme is typically activated by physiological concentrations of Zn^{2+}, but Zn^{2+}-independent forms may exist [27]. sSMases may be involved in signal transduction via the sphingomyelin pathway. A review of sSMases can be found in [28].

Secretory sphingomyelinase has been proposed to have a role in atherogenesis. It was known that serum low density lipoprotein (LDL), when treated with bacterial sphingomyelinase, gave rise to LDL aggregates that became retained on the extracellular matrix and stimulated macrophage foam cell formation [29]. Schissel et al. [26] were able to show that sSMase from arterial wall cells could hydrolyze and aggregate LDL modified by oxidation, or extracted from human atherosclerotic lesions, at pH 7.4, while native LDL could only be cleaved by sSMase at pH 5.5. Thus sSMase is likely to be the arterial wall enzyme responsible for the hydrolysis of sphingomyelin in LDL and subendothelial LDL aggregation, a key event in atherogenesis. In addition, macrophages from aSMase knockout mice, thus deprived of both aSMase and sSMase, displayed defective cholesterol trafficking and efflux [30]. This may implicate sSMase deficiency as a new atherosclerosis risk factor.

2.1.3. Neutral, Mg^{2+}-dependent sphingomyelinases (nSMase) Neutral Mg^{2+}-dependent sphingomyelinases are integral membrane proteins in mammals, and soluble proteins in bacteria. The mammalian enzymes have pH optima near 7, and require millimolar concentrations of Mg^{2+} or Mn^{2+} for activity. nSMase appears to be ubiquitous in mammalian tissues, but the highest activities occur in brain. The molecular mass is about 92 kDa, although smaller isoenzymes are found, which may result either from proteolysis or from alternative processing of common transcripts. nSMase is activated, either directly or indirectly, by a number of molecules including oxidized LDL and tumor necrosis factor-α (TNF-α), so that the enzyme may be related, among others, to atherosclerosis and apoptosis. A review of neutral sphingomyelinases, stressing the physiological aspects, can be found in Chatterjee [31].

The first nSMases of mammalian origin were characterized by Gatt [32] and by Rao and Spence [33]. Liu et al. [34] purified by 3030-fold a Mg^{2+}-dependent, nSMase activity (molecular mass ca. 60 kDa) from rat brain membranes. Enzyme activity was highly dependent on phosphatidylserine, and it was 95% inhibited by 3 mM glutathione [35]. Glutathione inhibition suggested that nSMase was activated by stress [36]. More recently, Bernardo et al. [37] purified by 23 330-fold a Mg^{2+}-dependent nSMase from bovine brain that was inhibited by glutathione. The enzyme consisted of two polypeptides, of 97 kDa and 46 kDa, that appeared to be structurally related, the latter being probably a degradation product. The bovine enzyme shares many properties with the one purified by Liu et al. [34] from rat brain (Mg^{2+} dependence, substrate specificity, activation by phosphatidylserine, inhibition by glutathione). However, the respective molecular masses were different, 60 vs. 97 kDa, thus the relationship between both enzymes remains to be established.

Ghosh et al. [38] isolated two isoforms of nSMase from rabbit skeletal muscle, of molecular masses 92 and 53 kDa respectively. Peptide mapping revealed important structural similarities, and the catalytic activities were also similar, except that the 53 kDa protein was Mg^{2+}-independent. The location of these nSMases is in the transverse tubules of the muscle cells, which may be related to the observation that sphingosine modulates calcium release from sarcoplasmic reticulum membranes [39]. Two Mn^{2+}- and Mg^{2+}-dependent nSMases located in the microsomal membranes of seminiferous tubes of immature Wistar rats have been characterized [40] whose properties do not appear to differ significantly from other mammalian nSMases. To end this short reference to nSMases purified from eukaryotic natural sources, we shall mention the Mg^{2+}-dependent nSMase isolated from *Saccharomyces cerevisiae* [41], and the one obtained from membrane fractions of intraerythrocytic *Plasmodium falciparum*, the malaria parasite [42]. The latter enzyme was activated by phosphatidylserine and other anionic phospholipids, and was sensitive to scyphostatin, an inhibitor of mammalian nSMase (see below).

The cloning of mammalian nSMase has not been free from debate. Tomiuk et al. [43] announced the cloning, overexpression and functional characterization of murine and human Mg^{2+}-dependent nSMase. Cloning was achieved by searching for sequences similar to those of bacterial nSMase. Overexpressing cells were treated with TNF-α but no clearly elevated ceramide levels or apoptosis were observed, which argued against the proposed role of nSMase in apoptosis. However, several concerns were soon raised, particularly with respect to the specificity of the cloned protein, i.e. it was not clear whether sphingomyelin was the natural substrate or not. Shortly afterwards Sawai et al. [44] demonstrated that although the cloned nSMase had sphingomyelinase activity in vitro, cells overexpressing the protein did not show marked changes in sphingomyelin metabolism. Furthermore 1-*O*-alkyl-lyso-phosphatidylcholine (lyso-platelet activating factor, or lyso-PAF) was metabolized into 1-*O*-alkylglycerol in overexpressing cells, but not in vector cells. Sawai et al. [44] concluded that the protein acted as lyso-PAF phospholipase C rather than as nSMase in cells. More recently Neuberger et al. [45] demonstrated the localization of the enzyme cloned by Tomiuk et al. [43] in the endoplasmic reticulum of cells. This location would argue against a role of this enzyme in sphingomyelin signalling.

In a further paper Tomiuk et al. [46] also reached the conclusion that the enzyme they had previously cloned (nSMase 1 in their nomenclature) was predominantly localized in the microsomal fraction (endoplasmic reticulum and Golgi). The observation that anti-nSMase 1 antibodies did not affect the nSMase activity in membrane fractions from murine brain [46] was interpreted as an indication that nSMase 1 was one of at least two mammalian nSMases. In fact, shortly afterwards the same group published the cloning and characterization of a 71 kDa mammalian brain-specific, Mg^{2+}-dependent nSMase [47], which they called nSMase 2. This enzyme

colocalized with a Golgi marker in several cell lines, according to immunofluorescence methods.

The enzyme with lyso-PAF phospholipase C and nSMase activities (nSMase 1) has been the object of attention by several groups. Katan and co-workers confirmed its localization in the endoplasmic reticulum, and showed that the enzyme had a strong requirement for reducing agents, and was reversibly inhibited by reactive oxygen species and by oxidized glutathione [48]. The same laboratory reported an essential role for two His residues (136 and 272 in nSMase 1) that were conserved in sequences from bacteria to human, and demonstrated that the transmembrane region contained determinants for the endoplasmic reticulum localization [49]. Mizutani et al. [50] cloned and expressed an essentially similar enzyme from rat, which they located afterwards in the nuclear matrix of ascites hepatoma AH7974 cells [51].

Meanwhile, Chatterjee et al. [52] had cloned a putative human nSMase that was activated by Mg^{2+}, inhibited by glutathione, and used sphingomyelin as its preferred substrate. The enzyme contained a 'death domain' analogous to those of TNF-α receptor-1 and Fas/Apo-I, and had no homology with the nSMase 1 from Tomiuk et al. [43]. The 'Chatterjee enzyme' overexpressed in human aorta smooth muscle cells stimulated apoptosis [52] while, as mentioned above, the 'Tomiuk enzyme' overexpressed in HEK cells or U937 cells did not induce apoptosis in the presence of TNF-α [43]. But Rodrigues-Lima et al. [49] indicated that expression of the 'Chatterjee enzyme' resulted only in a small increase in hydrolysis of exogenous sphingomyelin, and they even pointed out the sequence similarity of that enzyme with isochorismate synthetase. In addition, the 'Chatterjee enzyme' has not yet been found in the human genome data bank. In summary, it is not clear whether any of the putative nSMases cloned up to now have a physiological role as a sphingomyelinase and it is doubtful that any of them participate in the sphingomyelin signalling pathway.

Sphingomyelin signalling in response to external agents (TNF-α, oxidized LDL, ...) evokes the existence of a SMase at the plasma membrane level. It is thus disappointing that none of the cloned nSMases is localized there. In contrast, two recent reports characterize plasma membrane-associated nSMases. Veldman et al. [53] described a nSMase in caveolae of human skin fibroblasts. This enzyme was inhibited by a peptide that corresponded to the scaffolding domain of caveolin, which suggested a direct molecular interaction of both proteins. Such interaction would in turn imply a cytosolic orientation of the caveolar nSMase. Stimulation of overexpressing fibroblasts with TNF-α resulted in the appearance of caveolin-sensitive nSMase activity in the non-caveolar fractions. These results suggest that caveolar nSMase may be involved in TNF-α signalling. Working on a different system, namely pig liver plasma membranes, Martin et al. [54] isolated a Mg^{2+}-dependent nSMase that was not inhibited by glutathione but was inhibited by ubiquinol and other lipophilic antioxidants. Also relevant in this context is the somewhat forgotten report of a latent nSMase in chicken erythrocyte membranes [55].

A recent significant discovery has been the identification of a nSMase activity *in* LDL [56,57]. These authors have demonstrated that apolipoprotein B-100, the protein moiety of LDL, has a nSMase activity, which is also reflected by partial sequence homology between apolipoprotein B-100 and bacterial SMases. The physiological role of this activity could be related to non-receptor-mediated endocytotic entry of LDL into cells. Apart from that, this observation may open a new mechanistic link between elevated plasma levels of LDL, apoptosis and atherosclerosis.

A whole group of sphingomyelinases found in bacteria are structurally related to nSMase 1 [43,49]. In this sense they can be called neutral sphingomyelinases, although in practice they show a rather wide range of pH optima, and hydrolyze phospholipids other than sphingomyelin. These enzymes have been found in *Bacillus cereus*, *Staphylococcus aureus* (β-toxin), *Listeria ivanovi, Leptospira interrogans, Chromobacterium violaceum, Helicobacter pylori* and *Pseudomonas* TK4 [58–62]. Of these, the nSMase from *B. cereus* is the best known one. A distant evolutionary relationship has been noted between bacterial sphingomyelinase and mammalian DNase I [63]. These authors found that residues of DNase I that are involved in the active center (including His 134 and His 252) are conserved in bacterial sphingomyelinase, and are essential for activity. They correspond to the essential His 136 and His 272 in nSMase 1 [49]. Bacterial sphingomyelinases may be descendants of the eukaryotic enzymes, as proposed by Heinz et al. [64] for certain phospholipases C. In favor of this hypothesis is the fact that these enzymes are not usually required for the bacterial life cycle, and are often found in pathogens functioning as bacterial toxins [49]. The similarity between bacterial and mammalian nSMases has made it possible to subclone chimeras of bacterial SMase and green fluorescent protein into mammalian vectors containing sequences that target the enzyme to several subcellular organelles, with the result that only when bacterial SMase was targeted to mitochondria did cells undergo apoptosis [65].

2.1.4. Mg^{2+}-independent neutral sphingomyelinases. This small and barely known group of sphingomyelinases includes an enzyme found in the cytosol of HL-60 cells by Okazaki et al. [66], and the 53 kDa isoform of nSMase found by Ghosh et al. [38] in rabbit skeletal muscle.

2.1.5. Alkaline sphingomyelinase from the intestinal tract
The best characterized enzyme in this group has been an alkaline sphingomyelinase (bSMase), purified ca. 1600-fold from rat intestine [67]. The enzyme is not expressed in other organs, requires bile salts for activity, is Mg^{2+}-independent, and not inhibited by glutathione. The lack of this enzyme may be related to colon carcinogenesis. For a review, see Nilsson and Duan [68].

2.1.6. Bacterial sphingomyelinase-phospholipase C. This category was not included in the classification of Samet and Barenholz [14]. At least two representatives are known of this group, namely the so-called α-toxin from *Clostridium perfringens* [69], and the product of the *plcB* gene from *Listeria monocytogenes* [70]. Both can use phosphatidylcholine and sphingomyelin as substrates, and perhaps other glycerophospholipids as well [71]. Both enzymes are structurally related to the phosphatidylcholine-preferring phospholipase C from *B. cereus* but the latter has no SMase activity.

2.2. Sphingomyelinase mechanism

2.2.1. Binding of magnesium ions. Activation by Mg^{2+} is observed in most, but not all, nSMases of mammalian and bacterial origin. The Mg^{2+} effect on these enzymes becomes saturated at ca. 10^{-3} M. The role of Mg^{2+} in the activation of *B. cereus* SMase was analyzed in detail by Fujii et al. [58]. The

enzyme had two classes of binding sites for Mg^{2+}, with binding constants 2.1×10^{2} and 5.0×10^{6} M^{-1} at pH 6.0. The high-affinity site was saturated with a single Mg^{2+} ion. The functional meaning of the high-affinity binding is unclear. Low-affinity Mg^{2+} binding was essential for catalysis, but unrelated to substrate binding. Mg^{2+} binding also protected the enzyme against alkaline denaturation.

2.2.2. Binding of substrate. As with all other lipases, substrate binding is only one part of a more complex phenomenon, involving surface adsorption and perhaps other precatalytic stages. The interaction of SMase with its substrate has been carefully studied by Fanani and Maggio [72] using sphingomyelin monolayers at an air–water interface. The enzyme (*B. cereus*) was injected into the aqueous phase, from which it could interact with the substrate. Three stages could be distinguished in the enzyme activity, (i) a latency period, during which interfacial adsorption and other precatalytic steps took place, (ii) a period of steady-state enzyme activity, i.e. a constant rate of substrate degradation (pseudo zero-order kinetics), and (iii) a period of gradual halting of product formation.

During the latency period the amount of SMase adsorbed onto the lipid monolayer increased up to a plateau, the latter marking the start of the constant velocity region. The amount of adsorbed enzyme did not vary with the presence of product. An interface/subphase partition coefficient $K_P = 7\times10^{-4}$ was calculated for the enzyme, indicating a high tendency of SMase to associate with the lipid interface. Enzyme partition to the interface and interfacial activation were essentially irreversible.

Fanani and Maggio [72] analyzed quantitatively the activation phase of the SMase reaction, and compared the experimental results with six different kinetic models. The best fit was found for the so-called model V, whose kinetic scheme is as follows:

$$2E \overset{K_p}{\rightleftarrows} 2E_i + 2S \overset{K}{\rightleftarrows} 2E_iS \overset{K_d}{\rightleftarrows} (E_iS)_2 \xrightarrow{k_{act}} (E_i^*S)_2 + S \overset{K_S}{\rightleftarrows} (E_i^*S)_2S$$

$$\xrightarrow{k_{act}} (E_i^*S)_2 + P$$

After the partition step the model involves association of the enzyme with the substrate and subsequent dimerization before activation. The proportion of activated enzyme (E_i*) was assumed to be very small in relation to the total amount of enzyme at the interface (E_i+E_i*). Enzyme activation was the rate-limiting step for the reaction. The kinetics of activation was slow ($k_{act} = 3\times10^{-3}$ s^{-1}) as compared to the rate of catalysis in the steady-state regime ($k_{cat} = 4\times10^{2}$ s^{-1}), the latter estimated on the basis of classic Michaelis–Menten analysis. The authors conclude that the lag time observed in SMase catalysis is the result of an equilibrium between monomolecular and bimolecular activated states of the enzyme in the interface, and of a slow activation process.

2.2.3. Mechanism of catalysis. A model for the interaction of SMase with sphingomyelin (Fig. 1) and a catalytic mechanism (Fig. 2) have been proposed for *B. cereus* SMase respectively by Matsuo et al. [63] and by Fujii et al. [58], on the basis of four lines of evidence: (i) mutational analysis suggesting that Asp 126 and Asp 156 are involved in substrate recognition, and that Asp 295, His 151 and His 296 are essential for the hydrolytic activity [73]. Note that His 136 and His 272

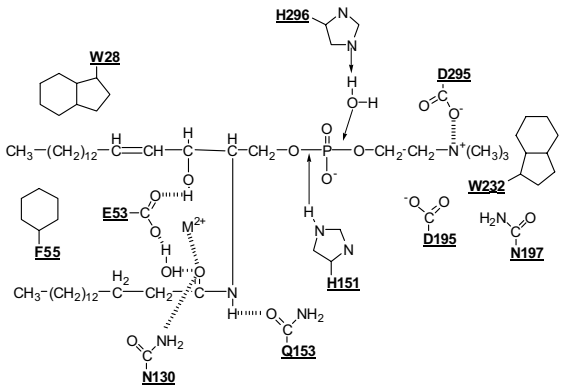

Fig. 1. A model for the interaction between SMase and sphingomyelin. These residues are all conserved in homologous bacterial SMases. Residues W28, E53, F55, N130, Q153, W232 and D295 may be involved in substrate recognition. E53 and D295 would be involved in hydrolysis of the phosphodiester bond. M^{2+} denotes the metal ion that is essential for catalysis. Redrawn from [63].

are essential for the activity of nSMase 1 [49,50]. His 134 and His 252 of DNase correspond to histidines 136 and 272 of nSMase 1, and to histidines 151 and 296 of *B. cereus* SMase. (ii) The prediction by Matsuo et al. [63] of the three-dimensional structure of the enzyme using a protein fold recognition method, which suggested that SMase adopts a structure similar to that of bovine pancreatic DNase I. This prediction included the proposal of several amino acid residues involved in catalysis and enzyme recognition, in agreement with the results of mutational analysis. (iii) The suggestion by Weston et al. [74], on the basis of their X-ray studies of a DNase I–substrate complex, that His 252 of DNase I acts as a general base to accept a proton from a water molecule, which then, as a nucleophile, attacks the phosphoryl group, and His 134 functions as a general acid to protonate the leaving oxygen of the phosphoester. His 252 and His 134 from DNase I correspond to His 296 and His 151, respectively, in *B. cereus* SMase. (iv) The studies by Fujii et al. [58] on the pH dependence of kinetic parameters of the enzyme. Fujii et al. [58] conclude that the mechanism of SMase appears to be one of general-base catalysis, with His 296 acting as a general base (Fig. 2).

2.3. Sphingomyelinase assay

Two different situations must be distinguished, (i) assays of pure enzyme and (ii) assays of cell extracts. When purified enzyme preparations are available, no labeled substrate is required. Natural or synthetic sphingomyelin is prepared, pure or mixed with other lipids, in the form of extruded large unilamellar vesicles (LUV) ca. 100 nm in diameter [75]. When pure sphingomyelin vesicles are used extrusion must take place at a temperature close to or above the gel–fluid transition temperature of the lipid, i.e. often in the 45–50°C range. LUV and enzyme are mixed in the appropriate assay

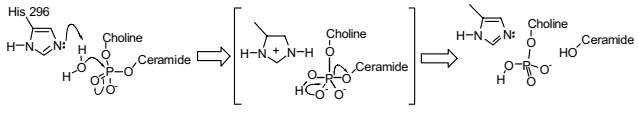

Fig. 2. A general-base catalytic mechanism for bacterial sphingomyelinase. Redrawn from [58].

buffer and aliquots are removed at fixed time intervals. The aliquots are mixed with chloroform–methanol and, after phase separation, phosphorus (from phosphorylcholine) is assayed in the aqueous phase. A detailed description of the procedure can be found in Ruiz-Argüello et al. [76].

When LUV are assayed with sphingomyelinase, ceramide production in the bilayers leads to vesicle aggregation, which in turn produces an increase in turbidity or light scattering in the suspension. Thus the reaction can be followed in real time just by measuring the increase in turbidity (absorbance at 500 nm) or in light scattering (e.g. with a fluorometer with both the excitation and emission monochromators adjusted at 500 nm). The chemical assay of the water-soluble product phosphorylcholine and the turbidity assay give superimposable results [76,77].

In some commercial preparations of purified sphingomyelinase from *B. cereus* a contamination by phospholipase C may occur. This is irrelevant when the substrate consists of pure sphingomyelin. But when the LUV contain glycerophospholipids, e.g. phosphatidylcholine, the contamination poses a serious problem, because both enzymes will be giving off the same water-soluble end-product phosphorylcholine. When this is the case, the assays must be performed in the presence of *o*-phenanthroline, a specific inhibitor of phospholipase C that is innocuous for SMase [77–79].

Assaying SMase activity in cell extracts requires that sphingomyelin be labeled, radioactively, fluorescently or otherwise. Also, and because of the difficulty in bringing together the enzyme and substrate molecules in the presence of cell homogenates, the assays are often carried out in Triton X-100 or other suitable detergents. It should be noted that, apart from emulsifying the substrate, the detergents bind and modify the enzyme activity, thus they are better avoided whenever possible. If their use is required, detergent concentration and initial detergent:substrate ratio must be kept constant for reproducibility of assays. After catalysis has taken place, the labeled enzyme products are separated, most often by thin-layer chromatography, and quantitated. A general description of SMase assays in cell extracts has been provided by Santana et al. [80], and by Gatt [81]. Samet and Barenholz [14] describe in detail assay conditions for aSMase and nSMase using a fluorescent sphingomyelin derivative. An alternative fluorescence assay is given by Loidl et al. [82]. Liu and Hannun [83] provide a detailed description of a SMase assay using a radioactively labelled substrate, namely *N*-[methyl-^{14}C]sphingomyelin. A novel non-radioactive approach is presented by Zhou et al. [84] who use biotinylated substrate conjugates. Then they purify the enzyme products by bioaffinity capture on streptavidin-agarose beads and, after release, they analyze them by electrospray ionization mass spectroscopy. High-throughput methods for sphingomyelinase screening, using *N*-[methyl-^{14}C]sphingomyelin, have also been published [85,86].

A SMase assay kit is commercially available, based on the use of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), a sensitive fluorogenic probe for H_2O_2. First, SMase hydrolyzes the sphingomyelin to yield ceramide and phosphorylcholine. After the action of alkaline phosphatase, which hydrolyzes phosphorylcholine, choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of a peroxidase, reacts with Amplex red to generate a highly fluorescent product. However, it should be noted that any enzyme generating phosphorylcholine will give off the fluorescent product, thus the assay does not discriminate SMase from phospholipase C.

2.4. Sphingomyelinase inhibitors

Some physiological SMase inhibitors, e.g. glutathione, have been mentioned in Section 2.1. Among the pharmacological inhibitors scyphostatin, obtained from mycelial extracts from the discomycete *Trichopeziza mollissima* is becoming widely accepted as a neutral sphingomyelinase inhibitor [37,42,87–89]. Other natural inhibitors of mammalian nSMase include macquarimicin A [90], alutenusin [91], chorogentisylquinone [92], and manumycin A [93]. Some synthetic substrate analogues can also be used as SMase inhibitors, e.g. a sphingomyelin methylene analogue [94], or a difluoromethylene analogue [95]. Arenz and Giannis [96] have produced the first synthetic irreversible inhibitor of nSMase.

Inhibitors of aSMase are also known, such as the xanthone α-mangostin, isolated from the bark of *Garcinia speciosa* [97]. α-Mangostin has recently been obtained by organic synthesis [98].

3. Sphingomyelinase–membrane interactions

At least two obvious aspects must be considered in this section, namely the influence of the membrane lipids on sphingomyelinase activity, and the effects of the enzyme on the membrane properties. Among the latter, we shall deal separately with enzyme effects on membrane permeability, and effects on membrane architecture.

3.1. Lipid effects on sphingomyelinase activity

The studies on this subject are scarce, particularly for substrates in bilayer form. Linke et al. [99] have recently published data that show stimulation of recombinant human acid sphingomyelinase by lipids that occur frequently in lysosomes. The substrate was present in the form of liposomes, and no detergents were used. Liposomes contained basically phosphatidylcholine, cholesterol and sphingomyelin. To this mixture other lipids were added as required. Lysosomally occurring lipids, e.g. phosphatidylinositol and bis-(monoacylglycero)-phosphate, were effective stimulators (by up to three-fold) of aSMase activity. Dolichol, and in particular dolicholphosphate, which occur in both Golgi and lysosomal membranes, were also effective aSMase activators. Interestingly, Linke et al. [99] also studied the effect of membrane curvature on aSMase activity, by preparing unilamellar liposomes of varying diameters, in the sub-50 to 200 nm range. Enzyme activity increased remarkably with increasing curvature (decreasing size) of the vesicles. However, it should be noted that, in the smaller liposomes tested, the vesicles are subjected to a considerable lateral tension, which could by itself have an influence on enzyme activity. Finally, Linke et al. [99] were able to demonstrate, using surface plasmon resonance, that binding of a SMase to lipid bilayers was actually increased by anionic lipids such as bis-(monoacylglycero)phosphate.

In another recent paper, Ruiz-Argüello et al. [100] have noted the unusual fact that most molecular species of sphingomyelin are in the gel state in the physiological range of temperatures, i.e. most naturally occurring sphingomyelins have gel-to-fluid transition temperatures in the 35–45°C range. These authors used liposomes (LUV) composed of pure egg sphingomyelin (gel-to-fluid transition temperature ca. 39°C)

or of binary mixtures of sphingomyelin and glycerophospholipids (phosphatidylcholine or phosphatidylethanolamine). The enzyme was of bacterial (*B. cereus*) origin. SMase was active on pure sphingomyelin bilayers but activity was exceedingly low when the lipid was in the gel state. It increased by over one order of magnitude when the substrate went from the gel to the fluid phase. SMase activity was found to exhibit lag times, followed by bursts of activity. Lag times decreased markedly when the substrate went across the phase transition to the fluid state. When egg phosphatidylcholine, or egg phosphatidylethanolamine was included in the bilayer composition together with sphingomyelin, sphingomyelinase activity at 37°C, which was negligible for the pure sphingolipid bilayers, was seen to increase with the proportion of glycerophospholipid, while the latency times became progressively shorter. A differential scanning calorimetry study of the mixed-lipid vesicles revealed that both phosphatidylcholine and phosphatidylethanolamine decreased in a dose-dependent way the transition temperature of sphingomyelin. Thus, as those glycerophospholipids were added to the membrane composition, the proportion of sphingomyelin in the fluid state at 37°C increased accordingly, in this way becoming amenable to rapid hydrolysis by the enzyme. Thus sphingomyelinase requires the substrate in bilayer form to be in the fluid state, irrespective of whether this is achieved through a thermotropic transition or by modulating bilayer composition.

Also relevant in this context is the observation by Jaffrezou et al. [101] that ceramide enhances neutral sphingomyelinase activity in myeloid leukemia cells and in normal skin fibroblasts. However, these results were obtained with short-chain, cell-permeant ceramides, whose physical effects on membranes may be different from the more frequent long-chain ones [9]. The subject is interesting enough to deserve a more detailed investigation.

3.2. *Effects of sphingomyelinase activity on membrane properties*

3.2.1. Effects on membrane permeability. Ruiz-Argüello et al. [76] were the first to point out that generation of ceramides in the bilayer induced efflux of vesicle or cell contents. They treated either LUV consisting of sphingomyelin, phosphatidylethanolamine and cholesterol (at a 2:1:1 molar ratio), or resealed erythrocyte ghosts, both loaded with low molecular mass fluorescent dyes, with bacterial SMase. In both cases, rapid efflux ensued in parallel with enzyme activity. This was in contrast to the situation of phosphatidylcholine:phosphatidylethanolamine:cholesterol (2:1:1) vesicles treated with phospholipase C, in which case no release of aqueous contents was observed.

The subject has been explored in more detail by Montes et al. [102]. They entrapped in liposomes fluorescent solutes of varying sizes, up to 20 kDa, and complemented the experiments in which ceramide was generated by SMase, with others in which ceramide was added in organic solvent to the preformed liposome suspension. Small proportions of ceramide (10 mol% of total lipid) that may exist under physiological conditions of ceramide-dependent signalling were used. When long-chain (egg-derived) ceramides were used, both externally added and enzymatically produced ceramides induced release of vesicle contents. However, the same proportion of ceramides generated by SMase induced faster and more extensive efflux than when added in organic solution to the vesicles. Remarkably, SMase treatment of bilayers containing 50 mol% sphingomyelin gave rise to release of fluorescein-derivatized dextrans of molecular mass ~20 kDa, i.e. larger than cytochrome *c*. Furthermore, some experiments in the same paper were designed to explore the mechanism of ceramide-induced efflux. Two properties of ceramide appear to be important in the process of membrane restructuring that leads to solute efflux. One is the capacity of ceramide to induce a 'negative' monolayer curvature, i.e. a curvature of the opposite sign to that found in the outer monolayer of cell membranes [9]. The other is its tendency to segregate into ceramide-rich domains [103]. The interface between ceramide-rich and -poor domains is probably the high-efflux region (Fig. 3). Very recently Siskind et al. [104] have shown that ceramide increases the permeability of the mitochondrial outer membrane to proteins of molecular mass up to 60 kDa.

3.2.2. Effects on membrane aggregation and fusion. Previous studies from this laboratory ([105]; review in [106]) had shown that phospholipase C induced aggregation and fusion of vesicles composed of phosphatidylcholine, phosphatidylethanolamine and cholesterol, in a 2:1:1 molar ratio. Interestingly, bacterial SMase acting on vesicles containing sphingomyelin, phosphatidylethanolamine and cholesterol (2:1:1 molar ratio) produced (apart from solute efflux, as discussed above) vesicle aggregation, but not fusion, the latter being defined as intervesicular mixing of lipids and of aqueous contents. However, when the sphingomyelin-containing vesicles were treated with low amounts of SMase, so that the rate of sphingomyelin hydrolysis was reduced considerably [107], as compared to that of the previously discussed experiments, a situation was found in which about one third of the vesicles were clearly larger in size (by ca. six-fold) than the original ones. Fusion had taken place together with extensive leakage, and because of this and other reasons, it is possible that SMase-induced fusion occurs via a different mechanism than phospholipase C-induced fusion [106,107].

Other lines of evidence concur in pointing towards a fusogenic capacity of SMase. Holopainen et al. [108] showed microscopic images of 'endocytic' budding of vesicles composed of phosphatidylcholine and sphingomyelin, upon addition of sphingomyelinase. Budding and fission are probably mirror image processes of fusion, and they may share the same kind of intermediates. Holopainen et al. [108] attributed the observed budding to the tendency of ceramide to separate into domains, and to its negative spontaneous curvature that would lead to membrane invagination (Fig. 3).

Also relevant is the observation by Zha et al. [109] that endocytotic vesicles are formed in the absence of ATP when fibroblasts and macrophages are treated with exogenous SMase (or ceramides). According to those authors, addition of *B. cereus* SMase to ATP-depleted cells induces the rapid formation of vesicles, approximately 400 nm in diameter, not enriched in clathrin or caveolin, that pinch off from the plasma membrane and go into the cytosol. Zha et al. [109] speculate that hydrolysis of sphingomyelin in the plasma membrane causes inward curvature and subsequent formation of sealed vesicles, in agreement with the biophysical data [106]. Moreover, entry of *Neisseria gonorrhoeae* into non-phagocytic cells is mediated by the activation of an acidic sphingomyelinase [110], while SMases in the genus *Listeria* mediate bacterial escape from phagocytic vacuoles and cell-to-cell propagation [60,70]. These examples of bacterial movements across

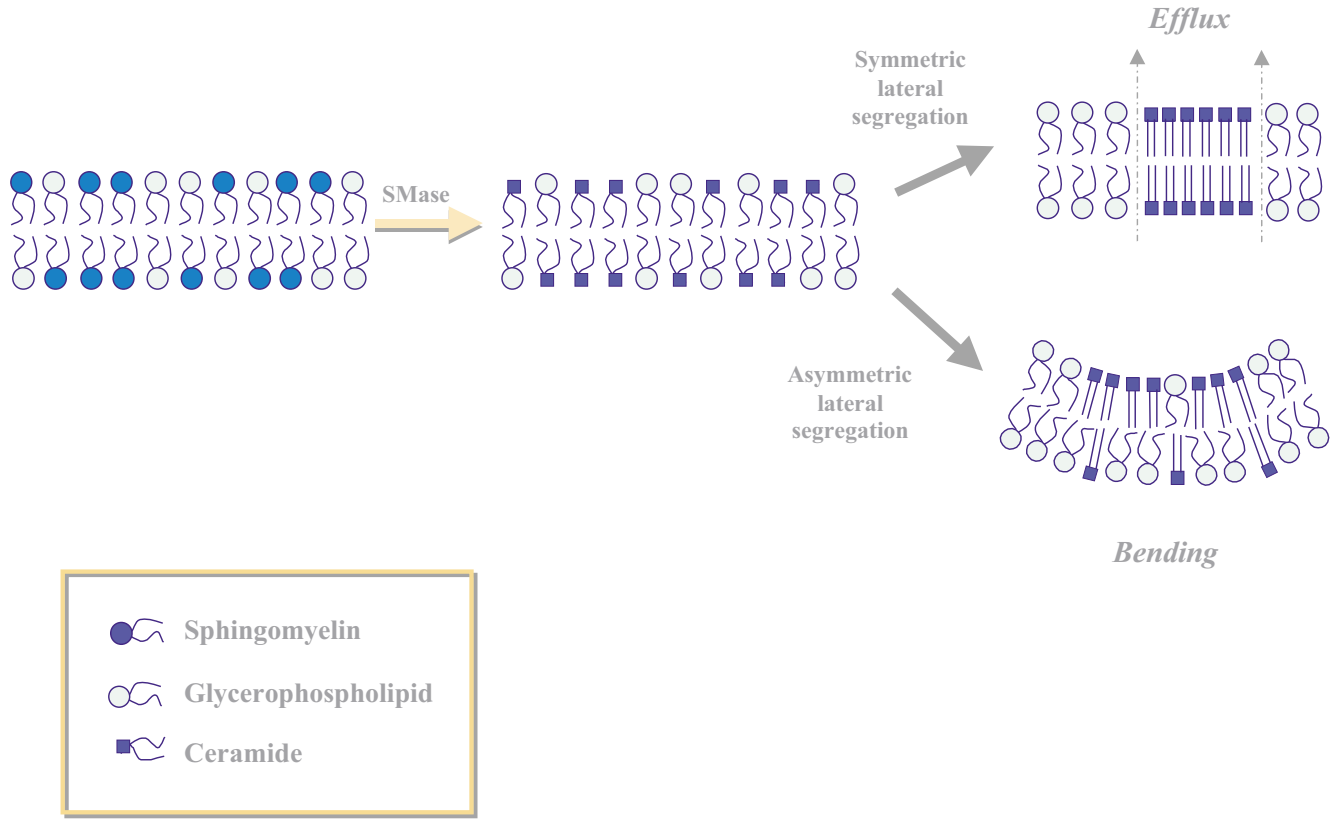

Fig. 3. A schematic drawing of the effects of SMase on lipid bilayers. In a bilayer containing both sphingomyelin and glycerophospholipids, sphingomyelin will be in the fluid state [100]. After SMase action, ceramides are still mixed with the glycerophospholipids, but the bilayer is in a metastable state. Even if SMase is acting only on one side of the bilayer, ceramide flip-flop tends to distribute ceramide molecules more or less evenly across the bilayer [79,106]. Simultaneously, and perhaps competing with flip-flop, lateral segregation forms patches of solid ordered ceramide-rich phases. The outcome of these events is the increased membrane *efflux* through the solid–fluid interface [76,102] and/or the *bending* of the bilayer, perhaps contributing to the formation of fusion/fission intermediates of the 'stalk' type [79,106].

membranes require either true membrane fusion or events mechanistically related to fusion, all of them induced by a SMase. In a non-membranous system, SMase was seen to induce aggregation and fusion of LDL particles [111]. In turn, the modified LDL particles bound the human aortic proteoglycans with increased strength.

Coming back to studies in model membranes, Ruiz-Argüello et al. [79] were able to observe the cooperative action of ceramide and diacylglycerol, generated in situ respectively by SMase and phospholipase C, to induce aggregation and fusion of vesicles containing equimolar mixtures of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and cholesterol. Neither enzyme was able to produce aggregation or fusion on its own under those conditions, but when both enzymes were added together their activities appeared to be mutually potentiated. In a conceptually related study, carried out on lipid monolayers at the air–water interface, Fanani and Maggio [112] demonstrated that the activities of SMase and phospholipase A_2 could be mutually modulated. The activity of one enzyme was affected by its own reaction products and by substrates and products of the other enzyme. These authors concluded that the mutual lipid-mediated interfacial modulation between both phosphohydrolytic pathways indicated that phospholipid degradation might be self-amplified or dampened depending on subtle changes of surface pressure and composition.

4. Concluding remarks. The 'To Do' list

In the nearly 40 years elapsed since the first descriptions of sphingomyelinases, and particularly in the last decade, enormous progress has been made. Several animal and bacterial SMases have been cloned and overexpressed in a variety of cells, a mechanism of action has been proposed and tested with good results, and the process of interfacial activation is understood to a reasonable extent. Most importantly, SMases have been related to cell processes (apoptosis, cell proliferation, cell differentiation, membrane fusion/fission) that go well beyond their originally proposed role in the intermediary metabolism of lipids. Moreover, biophysical studies on model membranes have provided plausible explanations for the physiological effects of these enzymes. However, a number of important questions remain still (and pressingly) unanswered, we shall close this review by listing a few of them. (i) The topology of eukaryotic SMases, and, in particular, their possible changes in location between the resting and activated states. (ii) Cloning of mammalian neutral SMases: are the cloned proteins the physiological neutral SMases in the cell? (iii) The relationship between SMases, cholesterol homeostasis, and atherogenesis. (iv) The phylogeny of the known mammalian SMases. What are the SMases in non-mammalian cells?

Acknowledgements: The authors are grateful to the Ministerio de

Ciencia y Tecnología (Grant BMC2001-0791) and Universidad del País Vasco (Grant 13552/2001) for continuing support. The assistance of Dr. M.I. Collado with the reaction mechanisms is greatly appreciated.

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