Structural Requirements for Catalysis and Membrane Targeting of Mammalian Enzymes with Neutral Sphingomyelinase and Lysophospholipid Phospholipase C Activities

Analysis by Chemical Modification and Site-directed Mutagenesis

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Abstract
The sequence similarity with bacterial neutral sphingomyelinase (NSM) resulted in the isolation of putative mammalian counterparts and, subsequently, identification of similar molecules in a number of other eukaryotic organisms. Based on sequence similarities and previous characterization of the mammalian enzymes, we have chemically modified specific residues and performed site directed mutagenesis in order to identify critical catalytic residues and determinants for membrane localization. Modification of histidine residues and the substrate protection experiments demonstrated the presence of reactive histidine residues within the active site. Site directed mutagenesis suggested an essential role in catalysis for two histidine residues (His136 and His272) which are conserved in all sequences. Mutations of two additional histidines (His138 and His151), conserved only in eukaryotes, resulted in reduced NSM activity. In addition to sphingomyelin, the enzyme also hydrolyzed lysophosphatidylcholine. Exposure to an oxidizing environment or modification of cysteine residues using several specific compounds also inactivated the enzyme. Site-directed mutagenesis of eight cysteine residues and gel-shift analysis demonstrated that these residues did not participate in the catalytic reaction and suggested the involvement of cysteines in the formation/breakage of disulfide bonds which could underlie the reversible inactivation by the oxidizing compounds. Cellular localization studies of a series of deletion mutants expressed as GFP-fusion proteins, demonstrated that the transmembrane region contains determinants for the ER localization.
Introduction

Hydrolysis of sphingomyelin (SM) by sphingomyelinas (SMases), with the subsequent generation of ceramide, is a signalling pathway implicated in a number of cellular responses (1-3). Ceramide has been suggested to play important roles in cell cycle arrest, apoptosis, inflammation and the regulation of the eukaryotic stress response. Although ceramide can be generated by *de novo* synthesis through ceramide-synthase, for the majority of cellular responses it is generated from sphingomyelin by the action of neutral or acidic sphingomyelinas (1-3). These enzymes are sphingomyelin-specific phosphodiesterases that hydrolyze the phosphodiester bond of sphingomyelin yielding ceramide and phosphocholine. Different SMase activities have been described in eukaryotes and prokaryotes and are distinguished by their localization, pH optima and requirement for metal ions. However, only a few enzymes have been characterized at the molecular level. The best characterized of these enzymes is the acidic sphingomyelinase, which is deficient in patients with Niemann-Pick disease (2), and the bacterial Mg$^{2+}$-dependent neutral sphingomyelinas (NSM) (4).

Purification of mammalian NSM involved in signalling and subsequent determination of its primary structure, has proved very difficult. Recently, one putative clone has been isolated, however, the expression resulted in a very modest increase in hydrolysis of exogenous SM (5). The clone has no obvious sequence similarity with other SMases, but is surprisingly similar to isochorismate synthase*; the significance of this similarity needs to be further explored. Other mammalian clones (mouse and human) have been isolated based on their sequence similarity to bacterial NSM (6). This is potentially a powerful approach since, at least for some C-type phospholipases (7), there is an evolutionary link suggesting that the bacterial enzymes could be descendants from eukaryotic proteins as they are usually not required for the bacterial life-cycle and are often found in pathogens functioning as bacterial toxins. Properties of mammalian clones with similarity to bacterial NSM have been studied further, demonstrating NSM activity *in vitro*, Mg-dependence and the localization to the ER (6,8-10). It has also been suggested that in brain, the cloned enzyme represents one of several activities that can hydrolyze SM *in vitro*, while it seems to be the main activity in many other tissues (8,10). We have also shown that the murine enzyme has a requirement for reducing agents and is reversibly inhibited by oxidized
glutathione and reactive oxygen species (8). However, the function of this enzyme in signalling and its activity towards cellular SM has not been clearly demonstrated and would require further studies (9,10). Nonetheless, using an antisense strategy, Tonnetti et al. (11) showed that the cloned enzyme could be involved in ceramide-mediated apoptosis triggered by TCR activation. Recently, it has also been shown that the enzyme has phospholipase C activity towards specific lysophospholipids (9). Based on activities detected in vitro, we refer to the mouse and human clones as mammalian enzymes with neutral sphingomyelinase and lysosphospholipid phospholipase C activities (NSM/LysoPLC).

Although some insights into the properties of the cloned enzyme have been described, the functional significance of sequence similarities and differences between bacterial and mammalian proteins has not been investigated. In this study we aimed to identify residues that are essential for catalysis and those involved in reversible inhibition by reactive oxygen species. Determinants for localization to the ER, a specific property of the eukaryotic enzymes, have also been analyzed. Our data suggest that bacterial and mammalian enzymes have a common catalytic mechanism involving conserved histidine residues. A property that is not shared with the bacterial enzymes is the redox state dependent reversible regulation of activity which could involve the formation and breakage of S-S bonds between cysteine residues, while the transmembrane region contains the main determinants for the ER localization.
Experimental Procedures

Materials- $[^{14}\text{C}}$-methyl]-sphingomyelin, $[^{14}\text{C}}$-palmitoyl]-lyso-3-phosphatidylcholine and pGEX-2T vector were supplied by Amersham Pharmacia. Lipofectamine was obtained from Gibco-BRL. Oligonucleotides were purchased from Oswel. The vector pRSET was from Invitrogen, pEGFP-C1 from Clontech and myc-PLINK vector was a gift from Dr. R. Marais. Anti-GST monoclonal antibody was purchased from Santa Cruz Biotechnology, monoclonal anti-polyHis antibody from Qiagen, anti-GFP antibody was from Clontech and anti-myc was prepared in house. Probond Nickel resin was from Invitrogen. Complete protease inhibitor tablets were from Roche. All other chemicals were obtained from Sigma.

Sequence alignments- The protein sequences of human, mouse, C. elegans, S. cerevisiae, S. pombe and B. cereus enzymes were compared and aligned using BLAST 2 (12), SIM (13), CLUSTAL W1.8 (14), Multalin (15) and Dialign 2 (16) computer algorithms. Secondary structure elements were predicted with the Predator (17) and PHDsec (18) algorithms. Fold recognition analyses were carried out with 3D-PSSM (19) and FRSDP (20) algorithms.

Plasmid Construction and Site-directed Mutagenesis- The mouse NSM/LysoPLC cDNA (8) was subcloned into pGEX-2T, pRSET and PLINK vectors, which encodes the enzyme as a glutathione S-transferase fusion protein (GST-NSM/LysoPLC), as a polyhistidine-tagged fusion protein (polyHis-NSM/LysoPLC) or as a myc-tagged protein (Myc-NSM/LysoPLC) respectively. Single point mutations were introduced into the constructs (specified in the figure legends) using a cassette mutagenesis strategy employing an overlap extension PCR protocol. All oligonucleotides used in this study are listed in Table IA. A similar strategy was used to generate a construct in which the eight conserved cysteine residues were mutated and this construct was termed "super mutant" (Smut). First, several single mutations were combined using unique restriction sites and the resulting construct subsequently used as a template to generate further mutations. Constructs were then transformed into E. coli XL1-Blue. All constructs were subsequently sequenced to check that only the desired mutation had been introduced into the sequence.
A series of deletion mutants were also made by a PCR approach using pEGFP-C1 as a vector, generating N-terminally GFP-tagged proteins. The oligonucleotides are listed in Table IB.

**Heterologous Expression and Purification of Proteins**—Transformed bacteria were induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside and grown at 18 °C for 18 hours. GST-fusion proteins were prepared as described previously (8). Eluted GST-fusion protein was buffer exchanged into 25 mM Tris-HCl, pH 7.4, 1 mM DTT, 0.1% Triton X-100, 10% glycerol and stored at -20°C. Purity of the preparation was more than 90% and only minor protein contaminants could be detected.

PolyHis-NSM/LysoPLC protein was prepared essentially as described for GST-NSM/LysoPLC but no DTT was included in the buffers. The supernatant was added to 2 ml of nickel resin incubated with rotation at 4 °C for 90 minutes. Subsequently, the resin was washed with 20 mM sodium phosphate, 500 mM NaCl, 0.2% Triton X-100, pH 6 and proteins eluted with 250 mM imidazole in the same buffer. The eluate was buffer exchanged into 25 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 10% glycerol and stored at -20°C.

Myc-tagged proteins were expressed in Cos cells as previously described (8). A post-nuclear supernatant was prepared and used in the disulfide bond-sensitive electrophoretic shift assay described below.

**Analysis of subcellular localization**—Constructs of various deletion mutants with the GFP tag at the N-terminus, were transfected into Cos cells as previously described (8). Twenty four hours after transfection, the cells were fixed with 4% formaldehyde for 10 minutes, washed in PBS and mounted prior to visualisation using confocal microscopy.

For expression studies (Western blotting) and activity measurements of the deletion mutants, the post-nuclear supernatant (PNS) was prepared (8) and analysed as described below.

**Measurement of Sphingomyelinase and Lysosphospholipid Phospholipase C Activities**—For the analysis of the wild type and mutant enzymes in most experiments, measurements of neutral magnesium-dependent sphingomyelinase activity (NSM activity) were performed. The activities
were measured using radiolabelled substrate as previously described (8). Briefly, enzyme preparations were incubated with \(^{14}\)C-sphingomyelin for 1 hour at 37 °C. Concentration of Triton X-100 in the assay was 0.1% and the substrate concentration relative to the detergent was 22 mol%. Following quenching and phase separation, a sample of the aqueous phase was analyzed by scintillation counting.

For measurement of lyso-phosphatidylcholine (lysoPC) hydrolyzing activity the assay was performed as described by Sawai et al. (9) with \(^{14}\)C-palmitoyl]-lyso-3-phosphatidylcholine as substrate. The activity was measured in the absence or in the presence Triton X-100 at the concentrations used in the NSM assay.

**Chemical Modifications using DEPC and Cysteine-specific Reagents**- Purified recombinant protein (15 µl) was preincubated with various concentrations of DEPC or sulfhydryl-reagents (freshly diluted in 25 mM Tris-HCl, pH 7, 0.05% Triton X-100) in a final volume of 20 µl for 15 min at room temperature and the remaining activity assayed using the standard assay in the presence of DTT. For controls, the enzyme was preincubated in the presence of buffer only. In the case of DEPC, reactions were quenched by 500 µM imidazole. The specific reaction of DEPC with His residues was monitored using a Beckman spectrophotometer by scanning UV absorbance from 220 to 300 nm at the times specified in the figure legend. Protection against inhibition by DEPC was performed by preincubating purified GST-NSM/LysoPLC with increasing concentrations of SM, PC (0.5, 1 and 2 mM final concentrations) or MgCl\(_2\) (2, 5 and 10 mM final concentrations) in a total volume of 7 µl for 10 min at room temperature, followed by treatment by DEPC (200 µM final concentration, total volume 8 µl) as described above.

**Kinetic Analysis of Wild Type and Mutant Enzymes**- The WT and mutant proteins were subjected to analysis by steady-state kinetics. Reactions were performed under the standard assay conditions with initial velocities measured at SM concentrations ranging from 22 to 350 µM (1.4-22 mol%). The kinetic data were fitted to the Michaelis-Menten equation and kinetic parameters (\(K_m\), \(V_m\), \(k_{cat}\), \(k_{cat}/K_m\)) were determined from secondary Lineweaver-Burk plots.
Intrinsic Fluorescence of Wild Type and Mutant Enzymes- The WT and mutant enzymes were examined by intrinsic fluorescence spectroscopy using a Proton Technology Instruments fluorimeter. Equal concentrations of enzymes (3 µM) in 25 mM Tris-HCl, pH 7.4, 2 mM DTT, 1 mM CHAPS were used. Fluorescence emission spectra were recorded between 300 and 400 nm with an excitation wavelength of 295 nm. Buffer alone was used for a blank and subtracted from the other spectra.

Disulfide Bond-sensitive Electrophoretic Shift Assay- Breakage or formation of disulfide bonds in purified polyHis-tagged NSM/LysoPLC or myc-tagged enzymes were monitored by SDS-PAGE under non reducing conditions as described by Mahoney et al. (21) followed by Western-blotting with anti-polyHis antibody or anti-myc antibody. Aliquots (5-10 µl) of purified polyHis-NSM/LysoPLC were reduced by increasing concentrations of DTT for 15 min at room temperature, mixed with SDS-sample buffer without reducing agent and subjected to SDS-PAGE and Western-blotting. For reversible breakage/formation of disulfide bonds, aliquots of polyHis-NSM/LysoPLC were first reduced or oxidized by 1 mM DTT or 1 mM H$_2$O$_2$ (final concentrations) for 15 min at room temperature. To assess the reversibility of the reaction, the excess of the reagent from the first reaction (reduction or oxidation) was removed by ultrafiltration with Microcons (Millipore) before addition of the reducing or oxidizing reagent in a second incubation. The protein mobility was analysed by SDS-PAGE and Western-blotting.

Protein Determination, SDS-PAGE and Western-Blotting- Protein concentrations were determined from a standard curve generated from BSA standards using a Bradford assay (Bio-Rad). Samples for gel electrophoresis were combined with reducing 4x SDS sample buffer, unless otherwise noted, and separated by SDS-PAGE. Gels were stained by Coomassie Brilliant Blue R-250. For Western-blotting, following separation by SDS-PAGE, proteins were electrotransferred to polyvinylidene difluoride membrane. The membrane was blocked with Tris-buffered saline/Tween containing 5% dried milk powder for 1 hour. Primary antibody, anti-GST (1:15,000), anti-polyHis (1:1000), anti-GFP (1:1000) or anti-myc (1:2000), was added for 1 hour in Tris-buffered saline/Tween followed by washing. Secondary antibody (anti-mouse,
1:2000) was added for 30 min. Following washing, ECL (Amersham Pharmacia Biotech) was used for detection.
Results

Sequence Alignments of Eukaryotic Enzymes with Bacterial NSM. In addition to the mammalian enzymes cloned according to sequence similarity to bacterial NSM, other eukaryotes including yeast, nematodes, fruit fly and silk worm, have homologous sequences. Several of these sequences were aligned in Fig. 1 showing that the mouse enzyme, used in our studies, shared 20%, 28%, 42% and 81% identity with Bacillus cereus, yeast (S.pombe and S.cerevisiae), C.elegans, and human enzymes, respectively. The regions of strongest conservation (shaded in Fig. 1) are also found in other phosphodiesterases although overall similarity was very low. Furthermore, the 3-D fold of the B. cereus enzyme has been predicted and modeled using the structure determined for DNAse I (4). Based on this prediction and supporting experimental evidence (4,22,23) the function of several residues in the B. cereus enzyme have been suggested. For example, His 151 is likely to function as a general acid and His 296 as a general base in the catalytic reaction while Asp 195 and Asn 197 have been implicated in the interaction with the phosphate group of SM. A comparison of predicted secondary structure and the fold recognition for mammalian enzymes (Fig. 1) with bacterial NSM and DNAse I strongly suggests that the large N-terminal part (residues 1-290 in the mouse enzyme) adopts the same general fold. Furthermore, several residues (His136, His272, Asp178, Asn180) are in a similar position as the critical residues implicated in catalysis in the bacterial NSM. However, there are examples where the function of similarly positioned residues in prokaryotic and eukaryotic enzymes is not conserved; in PI-PLCs, the function of general base His in the bacterial enzyme is not carried out by a His in a similar position in the mammalian sequence but by a residue unique to eukaryotic enzymes (7). The comparison of NSM sequences reveals the presence of two His residues (His138 and 151) in the vicinity of His 136, which are only present in eukaryotic sequences (Fig. 1).

In addition to the regions of similarity with bacterial NSM, all eukaryotic sequences have a C-terminal extension (Fig. 1). This region is predicted to incorporate two transmembrane domains (residues 325-346 and 353-375 in mouse sequence); this is consistent with mammalian enzymes being integral membrane proteins (8, 10). Despite some similarities, there are other
specific features of different sequences. For example, murine and human enzymes are characterized by a high content of Cys residues, eight of which are conserved between the two sequences (Fig. 1). Our previous study has demonstrated that some Cys residues are highly reactive and that the reduced state is essential for activity (8). These properties are not shared with the bacterial enzymes and in the case of *B. cereus* (23) formation of one disulfide bridge is required for the enzyme activity while the addition of reducing agents has an inhibitory effect.

To examine the functional significance of structural similarities and differences described above, we analysed the murine enzyme using chemical modification of specific residues and mutagenesis. In particular, we focused on the function of His and Cys residues and the C-terminal extension present in eukaryotic enzymes.

*Chemical Modification of Histidine and Cysteine Residues.* For the chemical modification studies, mouse enzyme expressed as a GST fusion protein was used. This recombinant enzyme was produced and purified from bacteria and has been shown to have properties identical to protein expressed in either insect or mammalian cells (8). For the specific modification of His residues, DEPC is the most widely used reagent (24). To determine whether His residues are important for the enzyme activity towards SM, recombinant enzyme was preincubated with increasing concentrations of DEPC and residual NSM activity measured. As shown in Fig. 2A, the enzyme was fully inhibited by DEPC at concentrations ranging from 100 to 500 µM. Even at a low concentration of DEPC (20 µM), 40% inhibition of enzyme activity was observed. The specific reaction of DEPC with His residues can be determined spectrophotometrically by monitoring the increase in absorbance at 240 nm. The spectra (Fig. 2A, inset) recorded at 5 and 15 min intervals during the modification of the enzyme confirmed that His residues were being specifically modified, since only an increase in absorbance at 240 nm was observed. For Cys residues, chemical modification was carried out with different sulfhydryl reagents: Iodoacetamide (IAD), HgCl₂, p-chloromercuribenzoate (PCMB), iodoacetic acid (IAA) and N-ethylmaleimide (NEM). All these compounds were able to significantly inhibit the NSM activity with total inhibition obtained using 100 and 200 µM of HgCl₂ and NEM (Fig. 2B). Strong inhibition (>80% of control) was also obtained under the same conditions with IAA. With IAD
and PCMB approximately 60% inhibition of enzyme activity was observed at a concentration of 250 µM. Taken together these chemical modification studies suggest that both His and Cys residues could be involved in catalysis.

*Site-directed Mutagenesis of Conserved Histidine and Cysteine Residues.* Although chemical modification is a simple and effective method to investigate the role particular residues may play in enzymatic activity, it is often limited by the introduction of steric hindrance and/or the presence of nonspecific modification. Incorrect conclusions may then be drawn from data based solely upon such experiments. In order to ascertain the importance of the conserved His and Cys residues for activity towards SM, a site-directed mutagenesis strategy was employed to mutate His 136, His 138, His 151, His 272, Cys 16, Cys 83, Cys 176, Cys 188, Cys 189, Cys 221, Cys 252 and Cys 342 to Ala. The His residues chosen include residues conserved between prokaryotic and eukaryotic sequences and those shared only by eukaryotic sequences (Fig. 1). Cys residues conserved between mouse and human sequences were mutated since the activity of the human enzyme is also redox sensitive and inhibited by sulphydryl reagents (data not shown), as described for the mouse enzyme (8).

Each single mutant was constructed by three step PCR using mutated primers (Table IA), expressed in *E. coli* as GST fusion proteins and purified using glutathione beads. SDS-PAGE analysis revealed that the WT and His mutants exhibited similar expression levels and identical mobilities (Fig. 3A). All recombinant proteins were detected by the anti-GST antibody (Fig. 3B). The same results were obtained with the Cys mutants (data not shown). The ability of WT and mutant proteins to hydrolyze radiolabeled SM was investigated. Specific activity was determined under the same conditions for each mutant and expressed as a percentage of specific activity relative to that of the WT. As shown in Table II, the specific activity of His136Ala and His272Ala mutants were 0.5% that of WT enzyme, while the His138Ala and His151Ala mutants were 47% and 11%, respectively. In contrast to the His mutants, the specific activity of each Cys mutant was similar to the WT enzyme with specific activities ranging from 100% to 115%. These results showed that the two highly conserved residues His136 and His272, and to a lesser extent His151, are important for the enzyme activity. However, the eight conserved Cys residues
have no catalytic role since their replacement by alanines did not impair the catalytic activity. Given that the mouse enzyme contains 17 Cys residues, it is likely that inhibition of the enzyme by sulfhydryl reagents is due to steric hindrance resulting from the alkylation of Cys residues close to the active site.

Further Characterization of Histidine Residues. In addition to the analysis of levels of expression and mobility (Fig 3A), a comparison of the fluorescent spectrum of the WT enzyme and His mutants (Fig. 3C) showed that replacements by Ala did not cause detectable conformational changes to these proteins. These data rule out that mutagenesis-induced gross structural changes could underlie inactivation of the His mutant enzymes.

To confirm that essential catalytic His residues are within the active site of the mouse enzyme, the protein was preincubated with DEPC in the presence of increasing concentrations of SM, PC or MgCl\(_2\). In the presence of SM substrate protection of the enzyme against DEPC-inactivation was observed (Fig. 4). Under the same conditions, no protection was obtained with PC (Fig. 4), which is not a substrate for this enzyme (8), or with MgCl\(_2\) (data not shown). These results demonstrated that essential DEPC-sensitive His residues are present in the active site. This data also suggested that these His residues were not involved in the chelation of the magnesium cation.

We also performed steady-state kinetics and characterization of His138Ala and His151Ala mutants that still possessed significant enzyme activity. Steady-state kinetics parameters (V\(_{\text{m}}\), K\(_{\text{m}}\), k\(_{\text{cat}}\) and k\(_{\text{cat}}\)/K\(_{\text{m}}\)) were determined from Linewear-Burk plots of standard Michaelis-Menten kinetics assays. The kinetics parameters are shown in Table III. The K\(_{\text{m}}\) values for the His138Ala and His151Ala showed only a 1.2 fold increase and a 1.9 fold decrease, respectively, compared to the WT enzyme. Therefore, the substrate binding efficiency was not greatly altered in these His mutants. On the contrary, bigger changes of k\(_{\text{cat}}\) values for the two His mutants were observed. The k\(_{\text{cat}}\) values for His138Ala and His151Ala mutants are respectively 4.5 and 31 times lower (21.5 % and 3% of the WT enzyme k\(_{\text{cat}}\) respectively) than the k\(_{\text{cat}}\) value of the WT enzyme. The catalytic efficiency, k\(_{\text{cat}}\)/K\(_{\text{m}}\), is therefore mainly altered by k\(_{\text{cat}}\) values, with the His138Ala and His151Ala mutants being 5.5 and 19 times less efficient.
enzymes than the WT NSM. These results indicate that, in addition to the essential His136 and His272 residues, His151, and to a lesser extent His138, are important and may be involved in catalysis. Inhibition studies of the His138Ala and His151Ala mutants (Fig. 5) show that despite their reduced activity compared to the WT enzyme, these two mutants can be totally inhibited by DEPC. These data demonstrate that full inhibition of these mutants requires the inactivation of the other essential His residues in the active site.

As described earlier, in addition to SM, the WT enzyme was previously shown to also hydrolyze lysophosphospholipids with the choline headgroup, such as lysoPC (9). We analyzed the hydrolysis of lysoPC by the WT enzyme, His272Ala mutant and B. cereus NSM. The assay was performed in the presence of 0.1% Triton, which was absolutely required for hydrolysis of SM but only marginally stimulated hydrolysis of lysoPC. The WT enzyme was more efficient in lysoPC hydrolysis (up to 50% of SM hydrolysis) than B. cereus enzyme (up to 5% of SM hydrolysis). When unlabeled SM or lysoPC were added to NSM competition assay, under conditions when 75% of the activity could be inhibited by SM, the same concentration of lysoPC (1.25 mM) resulted in 31% inhibition of the cloned mouse enzyme and 7% inhibition of the B. cereus enzyme. The His272Ala mutant was inactive with lysoPC as a substrate.

Possible Regulation of the Enzyme Activity by Reversible Disulfide-Bond Breakage/Formation. Our results show, that conserved Cys residues are unlikely to have a catalytic role for mouse NSM/LysoPLC since their replacement by alanines does not impair the catalytic activity. Since the enzyme activity is redox sensitive, we analyzed whether the enzymatic activity could be redox regulated through reversible breakage/formation of disulfide bonds as shown for several redox-regulated enzymes (21,25-27). In our experiments, mouse polyhistidine-tagged NSM/LysoPLC (polyHis-NSM/LysoPLC) purified from E. coli, in the absence of reducing agent, was analysed for NSM activity and also in a “disulfide-sensitive mobility shift” assay used in similar studies (21,27,28). As shown in Fig. 6A, the electrophoretic mobility of the mouse polyHis-NSM/LysoPLC differs under reducing and non-reducing conditions. This shift is consistent with the presence of one or several intramolecular disulfide bonds in the molecule. We have previously shown that the mouse enzyme is stimulated by increasing concentrations of reducing
agents, such as DTT or β-mercaptoethanol, with the enzymatic activity reaching a maximum at concentrations of reducing agents equal or above 1 mM (8). The activation of NSM/LysoPLC by DTT (concentrations increasing from 0-20 mM) was monitored in parallel with the electrophoretic mobility of the enzyme (Fig. 6B). At 1 mM DTT the enzyme was highly active and the mobility fully shifted (left panel). This mobility of the enzyme is identical to the mobility obtained by boiling the enzyme in the presence of 100 mM DTT suggesting that the protein is fully reduced. However, the presence of disulfide bonds whose reduction does not affect the electrophoretic mobility but could affect the activity could account for the differences in the range 0-1 mM DTT (right panel) and for the 20% difference in activity observed with 1 and 20 mM DTT (left panel).

We next analyzed whether the reversible inhibition of the mouse NSM/LysoPLC by reactive oxygen species was due to the formation of disulfide bonds. In the presence of 1 mM H₂O₂, the activity towards SM was inhibited (60% of the control) (Fig. 6C, bar H₂O₂) while in the presence of 1 mM DTT the enzyme was fully activated (670% of the control) (Fig. 6C, bar DTT). In parallel, a disulfide-sensitive shift was detected between the fully reduced enzyme (Fig. 6C, lane DTT) and the control enzyme (Fig. 6C, lane Control), but not between the H₂O₂-oxidised enzyme (Fig. 6C, lane H₂O₂) and the control enzyme (Fig. 6C, lane Control), suggesting that the inhibition of activity by H₂O₂ could be due to the formation of disulfide bonds which are not detectable by the mobility shift assay. When the fully reduced and active NSM/LysoPLC is incubated with H₂O₂ (Fig. 6C, bar and lane DTT/ H₂O₂) the activity is 60% inhibited and the mobility of the enzyme is shifted to that obtained with the control enzyme and the H₂O₂-oxidised enzyme. Conversely, when the H₂O₂-oxidised protein is incubated with DTT (Fig. 6C, bar and lane H₂O₂ /DTT) its activity towards sphingomyelin is stimulated (600% of the control) and its mobility is shifted to that of the fully reduced enzyme. These results show that there is a strong correlation between the reversible activation/inhibition of the enzyme by reducing/oxidising agents and the reversible breakage/formation of disulfide bonds.

When NSM activity and mobility of single Cys mutants (Cys16Ala, Cys83Ala, Cys176Ala, Cys188Ala, Cys189Ala, Cys221Ala, Cys252Ala and Cys342Ala) was analysed, the effects of reducing/oxidising agents were not substantially different from the changes described
for the WT. This has suggested that formation of several disulfide bonds and multiple Cys residues could be involved. When a mutant protein incorporating all eight Cys to Ala replacements was constructed, its analyses demonstrated a loss of redox-dependent changes. As shown in Fig. 6D, the mutated protein migrated under oxydising conditions as a reduced WT enzyme while under reducing conditions migration of the mutant and the WT proteins was similar.

*Mapping of determinants for the ER localization* - Although cloned mammalian enzymes do not have recognized ER retention signals (29), they were found in this compartment (8,10). Therefore, to determine the region of mouse NSM/LysoPLC required for the ER localization, a series of deletion mutants from the N- and C-terminus were made (Fig. 7A). The expression of all proteins, containing the GFP tag, was confirmed by Western blotting (Fig. 7B). The wild type and all of the mutants, except those consisting solely of the transmembrane domains, were assessed for NSM activity. While the expression of the wild type protein resulted in 100-150 fold increase of the enzyme activity in COS cell extracts, the activity of all mutants was identical to background levels (data not shown). Even the mutant that lacks the two transmembrane domains but includes entire region of similarity with the bacterial enzyme (residues 1-287) had no detectable enzyme activity.

When the localization of the mutants was examined, it was found that deletions from the N-terminus and from the C-terminus leaving the first transmembrane region (TM1) intact (e.g. protein containing residues 110-350) had the same localization to the ER as the wild type. The removal of both TM regions, however, in the 1-287 deletion mutant resulted in a loss of the ER localization (Fig. 7C). The importance of the TM1 region for the ER localization was further demonstrated by the study of GFP-fusion protein incorporating only residues within the TM1 region (residues 320-346). As shown in Fig. 7C, this region was sufficient for localization to the ER.
Discussion

The isolation of mammalian proteins with sequence similarity to bacterial NSM (6) opened a possibility that these proteins could be involved in the regulated generation of ceramide known to be important for a number of cellular functions (1-3). However, related bacterial and mammalian lipid-hydrolyzing enzymes (e.g. PI-PLC (7)) could have a number of different properties including critical catalytic residues, substrate specificities, regulatory mechanisms and determinants of cellular localization. Characterization of these properties is important for the understanding of their cellular functions.

The comparison of eukaryotic sequences with bacterial NSM, together with the secondary structure prediction, suggested that the eukaryotic enzymes contain a domain involved in catalysis (adopting the DNAse I-like structure) and a unique transmembrane domain incorporating two membrane spanning regions (Fig. 1). Based on this comparison, it is also likely that all enzymes share the same catalytic mechanism i.e. general acid/base mechanism involving two histidine residues (4). Chemical modification of histidine residues and the substrate protection experiments demonstrated that the murine enzyme contains essential histidines which are present within the active site (Fig. 2 and 4). Subsequent site-directed mutagenesis (Table II) has shown that mutations His136Ala and His272Ala at positions corresponding to general base and acid in other phosphodiesterases, resulted in a great reduction of the enzyme activity consistent with their proposed function. Mutational analysis of the corresponding residues in bacterial NSM and DNAse I had a similar impact on activity of these enzymes (4). It has also been reported (10) that the His272Asn mutation in the human enzyme resulted in a loss of NSM activity, as determined in transiently transfected HEK 293 cells. However, in those experiments effects on folding and stability could not be ruled out. In our studies, purified protein was used and the analysis of fluorescence spectra excluded the possibility of large conformational changes. In addition to these two histidines, the replacement of two other His residues (His138 and His151), present only in eukaryotic sequences, had somewhat smaller effects on the enzyme activity. Although their role is not clear, kinetic analysis and fluorescence spectra of purified proteins suggest that the mutations affected the catalytic rate rather than substrate binding or overall protein folding (Fig. 3, Tables II and III).
Both bacterial and mammalian enzymes have a clear preference for SM when compared to membrane phospholipids (8,22). However, hydrolysis of lysophospholipids such as lysoPC at a much lower rate (0.5-5%) compared to SM, has been reported for bacterial NSMs (22,30). Lysophospholipids are also hydrolyzed by mammalian ASM (31) and the cloned mammalian enzymes similar to bacterial NSM (9) consequently designated as NSM/lysoPLC. As described in Results, we have shown that the mammalian enzyme had a lower ratio of SM/lysoPC hydrolysis when compared directly to the \emph{B. cereus} NSM. Furthermore, in cells stably expressing the human clone, accumulation of the product of lysoPAF and not of SM hydrolysis have been detected (9). These data support the possibility that in cells lysoPAF rather than SM could be used as a substrate.

Data described in our previous studies (8) and the data presented here, demonstrate that both mammalian sequences (mouse and human) contain highly reactive Cys residues and that the reduced state is essential for the activity. This is further supported by the chemical modifications of these residues (Fig. 2). However, mutational analysis of Cys residues conserved between the mouse and human enzyme (Table II) excluded a possibility that any of the single cysteine residues are essential for activity and it is therefore unlikely that they participate in the catalytic reaction. Using electrophoretic shift analysis, it is shown that under oxidizing conditions cysteine residues participate in the formation of intramolecular S-S bridges leading to reversible inactivation of the enzyme (Fig. 6). While for this enzyme this could be only an \emph{in vitro} phenomenon, a number of examples illustrate a regulatory role for modification of Cys residues in cells. For example, modification of the catalytic Cys in caspase-3 could underlay redox regulation of this enzyme (32). Regulation of protein activity by reversible formation/breakage of intramolecular S-S bonds has also been demonstrated for a number of proteins from bacteria, plants and mammalian cells (21,25-27). In neurogranin, a PKC substrate which functions through an interaction with calmodulin, the formation of S-S bridges in the presence of NO donors (or other oxidants) results in the loss of the interaction with the regulatory proteins (21). Since NSM/lysoPLC resides in the ER (8,10), it is difficult to consider the redox environment and its possible changes without knowing the membrane topology of the enzyme. Even if the catalytic part is luminally oriented and exposed to the oxidizing
environment, the protein could still be kept in the reduced state as described, for example, for the cholera toxin (33).

Originally, finding that the mammalian enzymes localize to the ER (8,10) was surprising since they were considered as candidates for signalling NSM and expected to be present at the plasma membrane where agonist-induced SM hydrolysis had been suggested to take place (34). Recent findings (35,36), demonstrating that the ER has a separate signalling machinery responding to signals known to induce stress in this organelle, do not preclude a signalling role for these enzymes. However, very low abundance of SM in the ER (37) may imply that other substrates (e.g. LysoPAF) could be hydrolyzed in this compartment. Our studies have demonstrated (Fig. 7) that the cloned enzyme requires only one of the two transmembrane domains for the ER localization and that this region was sufficient for this specific interaction. Thus, the localization is not determined by specific retention sequences such as KDEL or di-lysine/di-arginine motifs found in some ER proteins (29) and absent in NSM/lysoPLC, but is likely to be related to the properties of the TM regions. Analysis of different ER proteins suggested that both, the length and composition (within and in the proximity) of the TM regions could be involved in determining the ER localization (38-40). In addition, and in agreement with a recent study (10), we found that removal of transmembrane helices resulted in a loss of the NSM activity. It is therefore possible that interaction surfaces are formed between the catalytic part and the C-terminal part containing the TM regions, which could be important for the formation of the functional protein.

In summary, our studies provide further insights into the properties of the mammalian enzymes and their relationship with the bacterial NSMs. These related phosphodiesterases are likely to share a common catalytic mechanism but could have overlapping substrate specificity; in addition, the mammalian enzymes have unique properties related to possible regulatory mechanisms and the cellular localization. Our data also suggest mutations that could generate potential dominant negative (removal of catalytic histidine residues) or constitutively active (removal of cysteine residues) molecules that could help further studies of defining the role for these enzymes in mammalian cells.
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Footnote: *The database searches and alignments were performed using BLAST 2 and CLUSTAL W 1.8
Figure legends

Figure 1. Alignment of amino acid sequences with similarity to bacterial NSM from different species.

The following sequences (with accession number) were used for the alignments: mouse (CAA10994), human (NP003071), C. elegans (CAB04885), S. cerevisiae (CAB39367), S. pombe (CAB39367), and B. cereus (CAA31333). Alignments were done with CLUSTAL W1.8, Multialin and Dialign 2.1. These three algorithms gave similar results. The CLUSTAL W1.8 alignment is shown in this figure. Highly conserved regions containing conserved histidine residues are shaded. Cysteine residues conserved between mouse, human and C.elegans or between mouse and human only are shadowed and underlined. Residues conserved in all sequences are in uppercase and less conserved residues are in lowercase. Residues mutated in this study are indicated by an asterisk. For B. cereus sequence, the first 27 N-terminal amino acids (present only in the proenzyme) were not taken into account. Secondary structure elements predicted for the mouse sequence by PHDsec are shown: helix, strand and coil regions are represented by boxes, arrows and lines, respectively.

Figure 2. Effect of chemical modification of histidine and cysteine residues on the enzyme activity.

A. Purified GST-NSM/lysoPLC enzyme (100 µg/ml) was preincubated with freshly diluted DEPC (at specified final concentrations) for 15 min at room temperature and remaining NSM activity determined. A control was prepared without DEPC. (Inset) Purified enzyme was incubated in the presence of 500 µM DEPC at room temperature and the specific modification of histidine residues was determined spectrophotometrically, after 5 and 15 min incubation. Blanks were determined with DEPC alone and with enzyme alone.

B. As above, purified protein was preincubated without (control) or with specified final concentrations of different sulphydryl-specific reagents for 15 min at room temperature.

Figure 3. Analysis of the wild type and proteins with single histidine mutations.

A. Purified GST-NSM/lysoPLC enzymes (~3 µg each) were loaded onto a 12% gel and
subjected to electrophoresis under denaturing and reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250.

B. Aliquots (10 µl) of each purified enzyme were analyzed by Western-blotting using monoclonal anti-GST antibody.

C. The intrinsic tryptophan fluorescence of the purified WT and His mutant enzymes (2 µM) was determined using a thermoregulated spectrofluorimeter. Excitation wavelength was 295 nm and emission spectra were recorded between 300 and 400 nm. Corrections were made for values obtained with the buffer alone.

Figure 4. **Substrate protection against inactivation by DEPC.**

Purified GST-NSM/lysoPLC was preincubated with increasing concentrations of SM or PC for 10 min at room temperature before treatment with 200 µM DEPC for 15 min and the remaining NSM activity determined.

Figure 5. **Effect of DEPC on histidine mutants.**

Equal amounts of the WT NSM/lysoPLC, H138A and H151A mutants were preincubated with 200 µM DEPC for 15 min at room temperature and the remaining NSM activity determined.

Figure 6. **Analysis of the reversible disulfide-bond breakage/formation.**

A. Polyhistidine-tagged NSM/lysoPLC, purified from bacteria in the absence of reducing agents, was analysed under non-reducing and reducing conditions by SDS-PAGE and Western-Blotting using a monoclonal anti-polyhistidine antibody.

B. Left Panel. The enzymatic activity of purified protein (200 µg/ml) was determined in the presence of increasing concentrations of DTT (1-20 mM). In parallel, polyHis-NSM/lysoPLC was incubated with the same concentrations of DTT for 15 min at room temperature and subjected to SDS-PAGE under non-reducing conditions and Western-blotting with anti-polyHis antibody. A control with the fully reduced enzyme (FR lane) was obtained by boiling the enzyme in the sample buffer containing 100 mM DTT.

Right panel. Same experiment as in Left panel with concentrations of DTT ranging from 0 to 1
C. The NSM activity of purified enzyme (control bar), H$_2$O$_2$-oxidised enzyme (H$_2$O$_2$ bar) and DTT-reduced protein (DTT bar) was assayed in the absence of reducing agents in the assay buffer. The H$_2$O$_2$-oxidised enzyme was also assayed in the presence of DTT (H$_2$O$_2$/DTT bar) while the DTT-reduced enzyme was assayed in the presence of H$_2$O$_2$ (DTTred/H$_2$O$_2$ bar). In parallel, purified polyHis-NSM/lysoPLC (control lane), H$_2$O$_2$-oxidised (H$_2$O$_2$ lane) and DTT-reduced enzyme (DTT lane) were subjected to SDS-PAGE and Western-blotting with anti-polyHis antibody. H$_2$O$_2$-oxidised enzyme preincubated with 1 mM DTT (H$_2$O$_2$/DTT lane) and DTT-reduced enzyme preincubated with 1 mM H$_2$O$_2$ (DTT/H$_2$O$_2$ lane) were also analysed as above.

D. The myc-NSM/LysoPLC (WT) and myc-NSM-LysoPLC mutant containing replacements of the 8 conserved Cys residues (Smut) were H$_2$O$_2$-oxidised (1 mM) or DTT-reduced (1 mM), subjected to SDS-PAGE and Western-blotting with anti-myc antibody.

Figure 7. Localization studies using deletion mutants with the GFP-tag.

A. Schematic representation of protein sequences present in different deletion mutants where gray squares represent the transmembrane domains. The constructs of the wild type and deletion mutants subcloned into pEGFP-C1 vector, were used for transfection of COS cells and analyzed after 24 hrs.

B. The PNS was prepared from harvested cells and 50 µg of protein from each condition subjected to SDS-PAGE and Western blotting using anti-GFP antibody.

C. Transfected COS cells were fixed and examined using confocal microscopy. Although all constructs were analyzed, only the wild type (left), mutant containing residues 320-346 (middle) and mutant incorporating residues 1-287 (right) are shown. A summary of ER localization for all constructs is shown in A.
### Table IA. Synthetic oligonucleotides used for site-directed mutagenesis

| Mutant (Forward) | Primer Sequence (5’-3’) |
|------------------|-------------------------|
| His136A          | GTGACTGCTCTACATGCT      |
| His138A          | GACTCATCTAGCTGCTGAG     |
| His151A          | CTTTGCAAGCGTGTTGACC     |
| His272A          | CTCTGATGCGAGGCCTTC      |
| Cys16A           | CAATCTCAAGCGCTGGGACATC  |
| Cys83A           | AGGCAGTGCCCTCGCTGTCG    |
| Cys176A          | GTTCTATTGGCTGGAAGACC    |
| Cys188A          | CCTGGGCGCCCTGCTGC       |
| Cys189A          | GGGCTGCGCCCTGCTGAAAG    |
| Cys221A          | CCCAAGAACGCTACGTCAGC    |
| Cys252A          | CACGTCTGCGCTAGACTCTG    |
| Cys342A          | GTTGCTGCTGCGCTCCTGCG    |
| Wild type (Forward) | GACGCTCTTGTAGTGTTGTTT  |
| Cys16A/83A/176A and |                       |
| His136A/138A/151A | GTACAGCCGACAGAAGGA     |
| Cys188A/189A     | GATGCTTTCGTTGAGACTTG    |
| Cys221A/252A/342A and |                       |
| His272A          | GTTGCGGATGAACTTGG       |
| Wild type (Backward) | CGGAATTCTTAAGCTCTGTCCTCCCCTCC |
| Cys16A           | GTTGGTGGATGAACTTGG      |
| Cys83A/176A/188A and |                       |
| His136A/138A/151A | CTGTGAGGGTCACAGCCCTG    |
| Cys221A/252A/342A and |                       |
| His272A          | CGGAATTCTTAAGCTCTGTCCTCCCCTCC |
| Mutant  | Forward Primer                      | Reverse Primer                      |
|---------|-------------------------------------|-------------------------------------|
| 75-419  | CGGGATCCAGCGGGATGATAGGCAG          | CGGAATTCTTAAGCTCAGTCGCCCTCC         |
| 110-419 | CGGGATCCGGAGACTGGTTCTGTGGG         | CGGAATTCTTAAGCTCAGTCGCCCTCC         |
| 1-350   | CGGGATCCATGAAGCTCAACTTTTCTCTAC     | CGGAATTCTTACCTGGCTCTTCTCTCTG        |
| 110-350 | CGGGATCCGGAGACTGGTTCTGTGGG         | CGGAATTCTTACCTGGCTCTTCTCTCTG        |
| 1-380   | CGGGATCCATGAAGCTCAACTTTTCTCTAC     | CGGAATTCTTACTGGCTCCTGCTGCTG         |
| 1-287   | CGGGATCCATGAAGCTCAACTTTTCTCTAC     | CGGAATTCTTACTGAGGGGCTGTGC           |
| 320-380 | CGGGATCCGCTCGCTGTGGGCTGC           | CGGAATTCTTACCTGGCTCTGCTGCTG         |
| 288-346 | CGGGATCCGAAGACCCCTGTACTGCTCTG     | CGGAATTCTTATGCAGGCAGGACACACAG       |
| 320-346 | CGGGATCCGCTCGCTGTGGGCTGC           | CGGAATTCTTATGCAGGCAGGACACACAG       |
Table II. NSM activity of purified wild type and mutant enzymes

| Enzyme    | Relative Percent Specific Activity* |
|-----------|------------------------------------|
| Wild type | 100                                |
| His136A   | 0.5                                |
| His138A   | 47                                 |
| His151A   | 11                                 |
| His272A   | 0.5                                |
| Cys16A    | 110                                |
| Cys83A    | 115                                |
| Cys176A   | 110                                |
| Cys188A   | 100                                |
| Cys189A   | 115                                |
| Cys221A   | 100                                |
| Cys252A   | 103                                |
| Cys342A   | 105                                |

*Relative specific activity expressed as percentage of specific activity relative to that of the WT enzyme.
Table III. Comparison of kinetic parameters for the wild type and mutant enzymes

| Enzyme    | Km  | Vm    | kcat | kcat/Km |
|-----------|-----|-------|------|---------|
|           | µM  | nmole/min | min⁻¹ | min⁻¹,µM⁻¹ |
| Wild type | 180 | 0.01   | 0.70 | 3888    |
| His138A   | 215 | 0.003  | 0.15 | 698     |
| His 151A  | 110 | 0.0004 | 0.023| 209     |
A

Not Reduced

Reduced

45 kDa
B

Graph showing NSM Activity (% control) with DTT Final Concentration (mM) in the x-axis and values ranging from 0 to 600 in the y-axis. The graph compares Reduced and Oxidised states.

Graph showing NSM Activity (% control) with DTT Final Concentration (µM) in the x-axis and values ranging from 0 to 600 in the y-axis. The graph compares Reduced and Oxidised states.

Images of gel electrophoresis with arrows indicating 45 kDa.
A

|            | Wild Type | 75-419 | 110-419 | 1-350 | 110-350 | 1-380 | 1-287 | 320-380 | 288-346 | 320-346 |
|------------|-----------|--------|---------|-------|---------|-------|-------|---------|---------|---------|
| ER Localisation | ✓        | ✓      | ✓       | ✓     | ✓       | ✓     | ✓     | ✓       | ✓       | ✓       |

B

C

Wild Type  320-346  1-287
Structural Requirements for Catalysis and Membrane Targeting of Mammalian Enzymes with Neutral Sphingomyelinase and Lysophospholipid Phospholipase C Activities
Fernando Rodrigues-Lima, Amanda C. Fensome, Michelle Josephs, Joe Evans, Robert J. Veldman and Matilda Katan

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