Activation of Human Acid Sphingomyelinase through Modification or Deletion of C-terminal Cysteine*

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Acid sphingomyelinase (ASM) is the lysosomal phosphodiesterase that hydrolyzes sphingomyelin to ceramide and phosphocholine. Deiciencies in the activity of this enzyme result in type A and B Niemann-Pick disease, an autosomal recessive lipid storage disorder accompanied by accumulation of sphingomyelin in lysosomes, in contrast to type C Niemann-Pick disease which is caused by defective cholesterol transport. The human form of ASM has been purified from various sources including placenta, brain, and urine (3–5). ASM purified from human urine was shown to be a 70-kDa glycoprotein with an acidic pH optimum (5). A cDNA for human ASM has also been isolated and characterized (6, 7), leading to production of recombinant human ASM protein from Chinese hamster ovary (CHO) cells (8) and insect cells (9).

One form of Niemann-Pick disease is caused by a deficiency in the enzymatic activity of acid sphingomyelinase. During efforts to develop an enzyme replacement therapy based on a recombinant form of human acid sphingomyelinase (rhASM), purified preparations of the recombinant enzyme were found to have substantially increased specific activity if cell harvest media were stored for several weeks at −20 °C prior to purification. This increase in activity was found to correlate with the loss of the single free thiol on rhASM, suggesting the involvement of a cysteine residue. It was demonstrated that a variety of chemical modifications of the free cysteine on rhASM all result in substantial activation of the enzyme, and the modified cysteine responsible for this activation was shown to be the C-terminal residue (Cys629). Activation was also achieved by copper-promoted dimerization of rhASM (via cysteine) and by C-terminal truncation using carboxypeptidase Y. The role of the C-terminal cysteine in activation was confirmed by creating mutant forms of rhASM in which this residue was either deleted or replaced by a serine, with both forms having substantially higher specific activity than wild-type rhASM. These results indicate that purified rhASM can be activated in vitro by loss of the free thiol on the C-terminal cysteine via chemical modification, dimerization, or deletion of this amino acid residue. This method of activation is similar to the cysteine switch mechanism described previously for matrix metalloproteinases and could represent a means of post-translational regulation of ASM activity in vivo.

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‡ The abbreviations used are: ASM, acid sphingomyelinase; rhASM, recombinant human acid sphingomyelinase; MMTS, methyl methane thiosulfonate; DTT, dithiothreitol; DTNB, dithionitrobenzoic acid; CPY, carboxypeptidase Y; OGM, Oregon Green® 488 maleimide; MALDI-TOF, matrix-assisted laser desorption time of flight-mass spectrometer; BSA, bovine serum albumin; CHO, Chinese hamster ovary; HPLC, high pressure liquid chromatography.
two forms was shown to be different because of proteolytic processing of the lysosomal form. Whether differences exist in the C termini of the two forms has not yet been determined; however, C-terminal processing has been reported for several other lysosomal enzymes including acid α-glucosidase (17) and cathepsin D (18, 19).

It has been proposed that histidine and glutamate residues may be involved in metal-binding sites within ASM, and comparison of the ASM primary sequence with known zinc metalloproteins suggests as many as seven potential zinc-binding sites (13). The actual stoichiometry of zinc binding and the specific amino acids responsible for coordination of metal ion within ASM remain to be determined. There are 17 cysteines in the ASM sequence. Recently, Lansmann et al. (20) assigned the disulfide bond pattern of rhASM purified from a baculovirus expression system. Based on a process of elimination, their work shows that the C-terminal cysteine (Cys629) is the unbridged free cysteine. It has been shown that dithiothreitol (DTT), but not reduced glutathione, inhibits the enzymatic activity of ASM in a concentration-dependent manner (21). However, the mechanism of this inactivation is not as yet understood. The inactivation may not be simply due to disulfide reduction, as effects of DTT on protein activity unrelated to disulfide reduction have been reported (22). It has also been shown that ASM can be activated in terms of its enzymatic activity, such as by lysosomal lipids and the sphingolipid activator protein SAP-C (23).

Enzyme replacement therapy has been proven to be an effective means of treating lysosomal storage diseases (24, 25). With respect to ASM, it has been shown that a recombinant form of the enzyme, expressed in CHO cells, has characteristics similar to that of the enzyme expressed in CHO cells, has characteristics similar to that of the enzyme expressed in normal human ASM (26). However, the activity of recombinant, CHO cell-derived rhASM increased when the cell harvests were stored frozen at 20 °C for several weeks prior to purification. This paper describes an investigation of this unexpected activation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Unless specified otherwise, all reagents were purchased from Sigma. Other reagents and supplies were obtained from sources as indicated under “Methods.”

**Methods**

**rhASM Production**—Recombinant human ASM protein (rhASM) was produced using CHO cells transfected with a vector containing a full-length human ASM cDNA, obtained from the IMAGE consortium (GenBank™ accession number AI558078). In the case of the mutant forms of rhASM, the open reading frame was amplified by PCR utilizing reverse primers in which the terminal cysteine codon, TGC, was either deleted or mutated to TCC (serine). PCR products were cloned into mammalian expression vectors containing a dihydrofolate reductase-selectable marker. Plasmids were sequenced through the entire open reading frame to ensure fidelity of the intended sequence. Dihydrofolate reductase-deficient CHO-DXB11 cells were transfected using Lipo- fectAMINE (Invitrogen), and selection was carried out in nucleotide-deficient media supplemented with 10% dialyzed fetal bovine serum and 0.2 mM methotrexate (Calbiochem). rhASM expression levels were further boosted by subjecting the uncloned pooled cells to incremental increases in methotrexate concentration. This process generated several uncloned pools that maintained high level rhASM expression upon the withdrawal of methotrexate. The protein was purified from the CHO-conditioned media by conventional orthogonal chromatographic procedures, including hydrophobic interaction and ion exchange steps. The purified rhASM protein was shown to start with His-Pro-Leu-Ser-Pro at the N terminus, corresponding to a 570-amino acid mature protein with signal peptide cleaved between Ala20 and His24 of the published human ASM sequence (6). This N-terminal sequence is the same as that of the recombinant form of ASM protein produced from insect SF21 cells (9) and CHO cells (12).

**Standard in Vitro Activity Assay**—Samples containing rhASM were incubated at 37 °C with the small molecular weight synthetic substrate 2-(N-hexadecanoylamino)-4-nitrophenylphosphorylcholine (Calbiochem) at 12.5 mM in 250 mM sodium acetate, pH 5.5, containing 0.1 mM zinc acetate, 0.25 mg/ml BSA, and 0.15% Tween 20. After a 30-min incubation at 37 °C, 0.2 mM glycine-NaOH containing 50% ethanol was added to stop the reaction. Activity was determined by monitoring the release of 2-(N-hexadecanoylamino)-4-nitrophenolate produced during the reaction (ε max = 15.0 mM−1 cm−1) and expressed in units/mg protein, where 1 unit is 1 μmol of product formed per min at 37 °C. In the activity assays designed to determine metal ion dependence, rhASM was diluted in the same buffer but with no metal ions present. Various amounts of divalent metal ions were then added to the samples for a 30-min preincubation at 37 °C prior to the addition of 2-(N-hexadecanoylamino)-4-nitrophenylphosphorylcholine substrate, which was prepared in the same buffer as described above but containing no metal ions.

**Natural Substrate in Vitro Activity Assay**—An Amplex Red sphingomyelinase assay (Molecular Probes) was used to determine activity with respect to cleavage of the natural sphingomyelinase substrate phosphatidylcholine. The assay was performed as suggested by the manufacturer. A coupled reaction involving two additional enzymatic steps is used to measure the hydrolysis of sphingomyelin. Briefly, ASM hydrolyzes sphingomyelin to ceramide and phosphorylcholine, which is then hydrolyzed by alkaline phosphatase to phosphate and choline. Choline, in turn, is oxidized by choline oxidase to betaine and H2O2. H2O2 then reacts with the Amplex Red reagent to produce highly fluorescent resorufin, which is detected by fluorescence emission at 590 nm. The resulting fluorescence is compared with a resorufin standard curve. ASM activity is expressed in units/mg protein, where 1 unit is defined as the amount of rhASM that generates 1 μmol of resorufin product per min at 37 °C.

**ASM Incubation in Conditioned and Feed Media**—ASM-producing CHO cells were cultured in media containing no serum or other sources of additional protein (proprietary commercial media from Invitrogen). The conditioned media containing secreted ASM protein was collected and stored for up to 160 days at 4, −20, and −80 °C. Samples were stored at 4, −20, and −80 °C for up to 4 weeks. The same preparation of purified rhASM was also diluted in 20 mM Hepes buffer, pH 8.5, as a buffer control. The incubated samples were removed at various time points and subjected to the standard in vitro activity assay.

**Kinetic Analysis**—Activities were determined at 415 nm using varying concentrations of substrate, 2-(N-hexadecanoylamino)-4-nitrophosphorylcholine, in 250 mM sodium acetate, pH 5.5, containing 0.1 mM zinc acetate, 0.25 mg/ml BSA, and 0.15% Tween 20. Kinetic parameters were determined from Edie-Hofstee plots of the resulting data.

**DTNB Assay**—rhASM samples were first concentrated in 20 mM sodium citrate, 150 mM NaCl, pH 6.0. Free thiol content was measured at 412 nm using 0.8–1.2 mg of rhASM and 0.5 mM DTNB in native and denaturing buffers and incubated for 10 min at room temperature before reading absorbance values. Results were compared with a cysteine standard curve (Molecular Probes). Cu2+/DTNB—Copper-promoted Dimer Formation—rhASM protein was dialyzed and diluted in 20 mM Tris-HCl, 150 mM NaCl, pH 7.0, to a final concentration of 0.5 mg/ml CuSO4 (0.1 mM unless specified otherwise) was added, and the mixture was incubated at 37 °C for 30–60 min. The samples were analyzed by gel electrophoresis on a 4–20% pre-cast gel (NOVEX) with Coomassie Blue stain. In experiments designed to study disruption of the dimer, DTNB or EDTA was added to a final concentration of 100 and 20 mM, respectively, just prior to loading the samples on the gel.

**Chemical Modification and Fluorescence Labeling**—Methyl methionine disulfonate (MMTS) and Oregon Green® 488 maleimide (OGM, from Molecular Probes) were used to label the free thiol groups of rhASM. The protein was first diluted in assay dilution buffer to 1.5–3.0 μg/ml, and various amounts of MMTS (50 nM to 50 mM) or OGM (0.2 pm to 20 μM) were added to the protein. The modification was carried out at room temperature for 60 min and then was analyzed for ASM...
activity. Preparative scale OGM modification was carried out by mixing 350 \(\mu\)l of 1.6 mg/ml rhASM with a 100-fold excess OGM, followed by incubation at room temperature for 2 h. The excess OGM dye was removed by dialysis.

**Peptide Mapping and Mass Spectrometry**—rhASM was labeled via thiol-specific maleimide chemistry using OGM (from Molecular Probes). The labeled protein was denatured in 6 \(\mu\)g guanidine HCl, 100 mM Tris-HCl pH 8.6, reduced with 40 mM \(\beta\)-mercaptoethanol, alkylated using a slight excess of 4-vinylpyridine (Sigma), and digested overnight with trypsin using an enzyme to protein ratio of 1:20. The digested peptides were fractionated on a C4 Vydac reverse-phase HPLC column using a 10-100 HPLC (Agilent Technologies). Absorbance at 215 and 280 nm was used to monitor the peptide peaks, and absorbance at 495 nm was used to monitor the elution of the OGM-labeled peptide. The labeled peptide was collected, and its molecular weight was analyzed on a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker BIFLEX).

**Carboxypeptidase Y Treatment**—Carboxypeptidase Y (CPY, obtained from Roche Applied Science) was used to cleave amino acids from the C terminus of the rhASM protein. Various ratios of CPY to rhASM (1:1 to 1:260,000) were combined in 20 mM citrate, 200 mM NaCl, pH 6.0, buffer and incubated on ice for 8 h. The samples were then subjected to activity assay and fluorescent labeling by OGM to measure the remaining free cysteine. Labeled rhASM was analyzed by SDS-PAGE on a 4–20% Tris-glycine gel (NOVEX) and visualized on a fluorescence reader (FACE imager from Glyko) to quantify the intensity of the bands. The CPY reaction was also carried out using immobilized CPY beads (Pierce) to confirm the results from the solution digest. Briefly, aliquots of 1 mg/ml rhASM (0.1 ml) were mixed with 10 \(\mu\)l of the CPY beads on top of a 0.45 \(\mu\)m spin filter (Ultrafree-MC from Millipore). The digested protein was spun through the filter to remove the immobilized CPY at various time points, and the filtrate was then assayed for ASM activity.

**RESULTS**

**Increased rhASM Activity with Storage of Cell Harvests at \(-20^\circ\text{C}\)**—rhASM was expressed in a stably transfected recombinant CHO cell line. After harvest of the secreted protein, it was observed that the rhASM activity in the conditioned media increased under certain conditions of storage. A systematic study was carried out to monitor the activity change by incubation at three storage temperatures (Fig. 1A). It was found that rhASM activity is stable if the harvest media is stored at \(-80^\circ\text{C}\), with no change seen over a 160-day period. However, a large increase in ASM activity was seen upon storage of the harvest media at \(-20^\circ\text{C}\) (\(-10\)-fold by 160 days). There was only a slight increase in activity when the media were stored at \(4^\circ\text{C}\).

To determine whether the increase in activity at \(-20^\circ\text{C}\) was due to a change in the specific activity of rhASM itself or whether it might be due to interaction with other molecules (e.g. association with an activator or loss of an inhibitor), rhASM was purified to homogeneity from harvest media. One preparation was made from fresh harvest media (designated the “low activity form” of rhASM in this study) and a second preparation was made from media that had been stored at \(-20^\circ\text{C}\) for 3 months (designated the “high activity form” of rhASM in this study). Activity measurements of the purified rhASM from these two preparations indicated a substantial difference in specific activity for the two as follows: 17.3 units/mg for the preparation from fresh harvest versus 80.2 units/mg for the preparation from harvest stored at \(-20^\circ\text{C}\). Thus, purified enzyme from harvests stored at \(-20^\circ\text{C}\) has \(-5\)-fold higher specific activity than enzyme purified from fresh harvests. Neither purified preparation contained significant breakdown products, indicating that activation was not a result of degradation during storage (see Fig. 2).

A kinetic analysis of the two forms of rhASM was performed using varying concentrations of a synthetic substrate, 2-[(N-hexadecanoylamino)-4-nitrophenylphosphoryl]choline. Kinetic parameters were determined from Eadie-Hofstee plots of the resulting data (Table I). The two enzyme preparations bind the substrate with comparable \(K_m\) values; however, the overall reaction turnover (\(V_{\max}\)) is increased about 4–5-fold for the higher activity lot. This suggests that the activity difference is due to differences in their catalytic effectiveness. It should be
noted that the \( K_m \) values reported here for the synthetic substrate

\[
2-(N\text{-hexadecanoylamino})-4\text{-nitrophosphorylcholine},
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in 250 mM sodium acetate, pH 5.5, containing 0.1 mM zinc acetate, 0.25 mg/ml BSA, and 0.15% Tween 20. Kinetic parameters were determined from Eadie-Hofstee plots of the resulting data.

| rhASM protein          | \( K_m \) (mM) | \( V_{\text{max}} \) (units/mg) |
|------------------------|---------------|---------------------------------|
| Low activity form      | 1.7           | 21.1                            |
| High activity form     | 1.9           | 92.8                            |

The purified high and low activity forms were further analyzed in a number of different biochemical and biophysical assays including SDS-PAGE, reverse-phase HPLC purity, circular dichroism (far-UV), and oligosaccharide profiling. No significant differences between the two forms were observed (data not shown). Both forms were inhibited by DTT (\(~50\%\) inhibition at 1 mM DTT), consistent with prior reports of the effect of DTT on ASM activity (21). These results indicated no major structural or obvious biochemical differences between the two forms of rhASM protein.

Because the harvest media present during the \(-20^\circ\)C activation contained not only media components but also secreted proteins and other compounds from the CHO cells, an experiment was performed to determine whether cellular factors are required for the activation. Samples of the purified low activity form of rhASM were incubated with fresh feed media at 4, \(-20^\circ\)C, and \(-80^\circ\)C, and the activity was monitored over a period of several weeks (Fig. 1B). The results of this incubation were similar to what was found for the incubation of the cell harvest. Again, rhASM was substantially activated upon incubation at \(-20^\circ\)C but not at 4 or \(-80^\circ\)C. The activation was not observed in the incubation in Hepes buffer control for all three temperatures. Because there was no protein or serum present in this feed media, it is clear that one or more of the small molecular weight components in the media cause rhASM activation via a process that is most favored at \(-20^\circ\)C.

Free Thiol Content of the Two Forms of rhASM—The free thiol content in the two forms of rhASM protein was assessed with a DTNB assay. Approximately 0.96 mol of thiol/mol of protein was measured in the low activity form of rhASM, when the assay was carried out in 4 mM guanidine HCl, whereas the high activity form had 0.02 mol of thiol/mol of protein. It appears that the single free thiol group in the low activity form of rhASM is lost during the \(-20^\circ\)C incubation and thus is undetectable in the high activity form. Free cysteine could also be detected in the low activity form in the absence of guanidine HCl but with a lower number during the standard 10-min DTNB incubation time (0.4 mol of thiol/mol of protein). Increasing the incubation time with DTNB from 10 to 60 min resulted in an increased thiol number (1.0 mol of thiol/mol). This suggests that the cysteine is only partially solvent-accessible or that it is constrained and some conformational perturbation is necessary for its reactivity with DTNB. This result also suggests that the rhASM protein is highly structured, as would be expected because 16 of the 17 cysteines in rhASM are paired (20).

| Metal Dependence of rhASM Activity—The secretory form of rhASM purified from CHO cell harvest is reported to require zinc for activity enhancement and stabilization (8). In the standard activity assay used in this study, 0.1 mM zinc acetate was routinely included in the assay buffer. Activity assays were performed in the absence of added zinc and with varying amounts of different metal ions to study the metal ion dependence of the high and low activity forms of rhASM. Pure preparations of the two forms of rhASM protein were incubated with increasing concentrations of metal ions before mixing with assay substrate made in the same buffer but containing no metal ions. In the absence of metal ions, both forms have rather low activity (Table II). Both forms displayed zinc-dependent increases in activity, with maximum activity at \(~0.1\) mM Zn\(^{2+}\). This result is consistent with the zinc requirement for ASM protein previously reported. Other divalent metal ions (Mg\(^{2+}\), Mn\(^{2+}\), and Ca\(^{2+}\)) were tested in a similar manner and found to have little effect on the activity of either form of rhASM (data not shown). However, when the effect of copper ion was tested using copper sulfate, a striking difference between the two forms was observed (Table II). At a copper ion concentration of 0.1 mM the activity of the low activity form was increased 6-fold, whereas the high activity form was unaffected. It is important to point out that the "low" and "high" activity forms were defined in this study under the standard assay condition, which contains 0.1 mM zinc. Their relative activity is actually reversed in the presence of 0.1 mM copper ions. Although copper activation had been reported previously (9) for recombinant ASM secreted from insect SF21 cells, the reason behind it is poorly understood. Copper ions can affect protein structure and function in many ways, including metal ion competition, metal ion-promoted dimerization, etc. Because a difference in free thiol was observed in the high and low activity forms of rhASM protein, we investigated the possibility of copper-promoted dimer formation (see below).

| Copper-promoted rhASM Dimer Formation—To understand the copper-dependent increase in activity, the two forms of the rhASM protein were incubated with 0.1 mM copper sulfate and examined on a 4–20% SDS-PAGE gel under non-reducing conditions (Fig. 2). Copper incubation of the low activity form resulted in the appearance of a higher molecular weight band corresponding in size to a dimer of rhASM (130–140 kDa). Conversion of monomer to dimer did not occur in the copper-incubated high activity form. Therefore, copper enhances dimer formation in the low but not the high activity form. The dimer formed in this manner is sensitive to DTT as it was not present when DTT was included in the loading buffer. This suggests the involvement of a disulfide bond in the dimer and that copper promotes the formation of this intermolecular disulfide bond. This is consistent with the common practice in protein refolding experiments where copper ions are added to promote intra-molecular disulfide bond formation.

Zinc ions, on the other hand, are inert in terms of oxidation/reduction reactivity and thus are not expected to have any effect on disulfide formation. A copper and zinc titration experiment was carried out side by side using the low activity form.
of rhASM to define further the nature of the dimer. The result, as shown in Fig. 3, indicates that the dimer does not form as a result of zinc incubation.

It was reported previously (27) that metal ions can coordinate with amino acid side chains from two protein monomers, resulting in non-covalent dimer formation. To examine whether this is the case for the rhASM dimer, EDTA was included in the loading buffer, and the samples were examined by SDS-PAGE (middle panel of Fig. 3). There was only a slight decrease in the intensity of the copper-enhanced dimer band, which completely disappeared when DTT was used in place of EDTA (bottom panel of Fig. 3). This result supports the proposal that the dimer is formed via a copper-induced intermolecular disulfide bond. This is consistent with the result from the DTNB assay indicating there is a free cysteine in the low activity form but not in the high activity form. The result also suggests that the disulfide bond-linked rhASM dimer may be responsible for the copper-dependent activation effect (Table II).

Chemical Modification of rhASM Free Thiol—The loss of the free cysteine appeared to correlate with increased activity, based on the specific activity of the high and low activity preparations and the copper-enhanced dimer formation. Experiments were designed to test whether rhASM activation could be achieved by chemically modifying the thiol group of the free cysteine residue in the low activity form. The small molecular weight thiol modifier MMTS was used to test this possibility. MMTS modifies cysteine to form a mixed disulfide bond (S–S–CH₃). Due to its small size, it is among the least perturbing cysteine modification reagents available for protein structure/function studies. The low activity form of rhASM was modified by MMTS, and its activity was measured using the standard activity assay (Fig. 4). As indicated in Fig. 4, increasing concentrations of MMTS resulted in progressively greater activation of rhASM, with a maximal activation of ~5-fold. This result supports the proposal that modifying the cysteine residue causes rhASM activation.

It was noted that if copper ions are used in place of zinc ions in the activity assay, the media-activated high activity form of rhASM has lower activity (see Table II). To test whether the MMTS-activated form of rhASM has similar characteristics to the media-activated form, this effect of copper on activity was examined. When the zinc ions were replaced by increasing copper concentration in the preincubation phase of the activity assay with MMTS-modified rhASM, a higher activity was first observed, followed by a clear pattern of inhibition (data not shown). Although the reason behind the inhibition is not well understood, it does suggest that both MMTS modification and incubation at −20 °C in media lead to rhASM activation via loss of a free thiol group.

OGM was also used to modify free cysteine on the low activity form of rhASM. OGM represents a larger molecular weight modifier that, unlike MMTS modification, is irreversible. It also acts as a fluorescent tag to allow tracking of the modification. OGM modification resulted in activation of rhASM in a standard activity assay, very similar to that observed with MMTS modification (data not shown). This indicates significant flexibility in the nature of the modification and the size of the modifier that will allow for activation. It appears that as long as the free cysteine is blocked or occupied, rhASM activation is achieved.

Determination of the Modified Cysteine Residue—To identify the position of the free cysteine in rhASM that is involved in this activation, 0.5 mg of OGM-modified rhASM was prepared for peptide mapping and characterization. The rationale was to take advantage of the absorbance/fluorescence properties of Oregon Green® 488 to identify cysteine-labeled peptide(s) within the peptide maps. Peptides from a trypsin digestion were separated on a C4 reverse-phase HPLC column, and the elution of OGM-labeled peptides was monitored by absorbance at 495 nm. Only one major peak was detected in the peptide map at this wavelength, consistent with the DTNB result indicating only one free cysteine in the protein. This peak was collected and analyzed by MALDI-TOF mass spectrometry (Fig. 5). The labeled peptide has a molecular mass of 2875.4 daltons, which corresponds to the calculated mass of the C-terminal tryptic peptide of rhASM (HLMPDGSLPEAQSLWPRPLFC = 2394.2 daltons) coupled to OGM dye (+463.4 daltons) plus 18 daltons (total = 2875.6 daltons). This additional 18 daltons is not unexpected, because opening of the maleimide ring in OGM by hydrolysis at elevated pH has been reported previously (28). To confirm further that it is the C-terminal peptide that was modified, post-source decay fragmentation...
was carried out on the MALDI-TOF target with the labeled peptide peak. As shown in Fig. 5B, the fragmentation process did break the sulfur-carbon linkage of the coupling bond and produced the mass of the C-terminal peptide free of dye (2394.3 daltons versus the theoretical mass of 2394.2 daltons). This result clearly identifies the C-terminal cysteine as the free cysteine that is modified by OGM, and its modification is presumably responsible for the increase in activity. The identification of this residue as the single free cysteine is consistent with the recent data of Lansmann et al. (20) in which they determined the disulfide bonding pattern of ASM.

Carboxypeptidase Y Treatment of rhASM—CPY cleaves amino acids sequentially from the C-terminal end of proteins. Because the labeling results described above indicated that it is the C-terminal free cysteine that is responsible for activation of rhASM, CPY treatment was performed to see whether removal the C-terminal cysteine also results in activation of rhASM. The low activity form of rhASM was incubated with CPY at different enzyme-to-protein ratios, and the activity was monitored for each reaction. The loss of C-terminal cysteine was monitored in this experiment by the susceptibility of the protein to OGM labeling. CPY-digested samples were incubated with OGM before loading onto a 4–20% SDS-PAGE to determine the degree of OGM labeling. The intensity of the fluorescence band for each reaction was quantitated as described under “Experimental Procedures” (Fig. 6A). Clearly, rhASM gradually loses the ability to be labeled by OGM as the CPY concentration is increased, reflecting the loss of the C-terminal free cysteine. Activity measurements showed that rhASM activity increases as more CPY is used in the incubation (Fig. 6B). In parallel with this digestion using soluble CPY, an incubation time course using immobilized CPY was also performed, and a similar pattern of rhASM activation was observed (data not shown). These results indicate that enzymatic deletion of the C-terminal cysteine results in activation of rhASM, consistent with the conclusion from the thiol modification data.

Site-directed Mutagenesis of the C-terminal Cysteine—From the data described above, it appears that either modification or deletion of the C-terminal cysteine leads to a substantial increase in the activity of rhASM. To confirm the role of the C-terminal cysteine in this activation, site-directed mutagenesis was carried out to generate rhASM mutants in which the C-terminal cysteine (Cys\(^{629}\)) is either eliminated by substituting with a stop codon (Cys\(^{629}\)del) or mutated to serine (Cys\(^{629}\)Ser). The mutant proteins were overexpressed in stably transfected CHO cells and purified. The specific activity of the purified mutant forms was measured and compared with the wild-type full-length rhASM protein (Table III). Both mutants displayed an increase of ~5-fold in specific activity compared with the full-length wild-type form. These results confirm the conclusion that modification or deletion of the thiol group on the C-terminal cysteine leads to activation of rhASM.

**Fig. 5. Identification of the modified free cysteine by mass spectrometry.** OGM-labeled rhASM was digested with trypsin and fractionated by reverse-phase HPLC. The major fluorescently labeled peptide was analyzed by MALDI-TOF mass spectrometry (A). Fragmentation of this sample by post-source decay was used to unambiguously identify this as the labeled peptide (B).

**Fig. 6. rhASM activation by CPY treatment.** rhASM was treated with various concentrations of CPY, and loss of C-terminal cysteine was assessed by the ability to label with the fluorescent thiol modifier OGM as described under “Experimental Procedures” (A). The activity of the CPY-treated samples was measured using the standard rhASM activity assay (B). The x axis represents the ratio of the molar concentration of CPY to rhASM in both panels.
To check whether the results obtained using the synthetic substrate also apply to the natural substrate for ASM, the activities of the wild-type and the mutant forms of rhASM were measured in an assay that uses sphingomyelin as the substrate. This assay measures the hydrolysis of sphingomyelin in a coupled reaction that ultimately produces the highly fluorescent compound resorufin and has been used previously (26) to detect activation of acid sphingomyelinase associated with ultraviolet radiation-induced apoptosis. By using this natural substrate assay, the activity of the two mutant forms was found to be 5-fold higher than the wild-type rhASM (Table III). These results confirm the conclusion that modification or deletion of the thiol group on the C-terminal cysteine leads to activation of rhASM.

**DISCUSSION**

The results presented in this work suggest a critical role for the C-terminal cysteine in the enzymatic activity of rhASM. It appears that any change that causes a loss of the free sulfhydryl group on this amino acid also results in activation of the enzyme. It was demonstrated that this activation can be achieved in various ways: 1) copper-promoted dimerization of rhASM via the C-terminal cysteine; 2) thiol-specific chemical modification of this cysteine to form a mixed disulfide bond or a sulfur-carbon linkage; 3) deletion of this cysteine by carboxypeptidase or recombinant DNA technology; and 4) site-specific mutation to change the cysteine to a serine residue. Therefore, we can attribute the activation effect to the loss of the free thiol group of the C-terminal cysteine.

The low and high activity forms of rhASM were shown to be indistinguishable in a number of biochemical and biophysical assays, including circular dichroism, suggesting that the activation is not accompanied by a gross structural change in the protein. Preliminary kinetic characterization of the two forms has indicated that although their $V_{max}$ values are quite different (~5-fold), their $K_m$ values are about the same (Table I). This suggests that the activity difference is due to differences in their catalytic effectiveness rather than in their ability to bind substrate.

There are many ways that a free thiol group can affect the structure and/or activity of a protein. First, it can stabilize a particular conformation by forming hydrogen bonds. This is unlikely to be the case here because the serine mutant, although theoretically capable of forming such bonds, leads to a large change of activity. A free thiol can also be directly involved in the active site of an enzyme, as in the case of the cysteine protease family. However, as the blockage of the free thiol in rhASM causes a gain of activity rather than a loss of activity, it is unlikely that this is the mechanism. A third way for a free cysteine to be involved is to serve as a metal ligand in a metalloprotein. This has been found in structural motifs (e.g., zinc finger proteins) and in the active sites of certain enzymes, for example mammalian liver alcohol dehydrogenase (29).

It is well established that zinc is required for the activity of both the lysosomal and secretory forms of ASM (13). Several potential zinc-binding sites have been proposed for ASM based on sequence comparison with known zinc metalloenzymes. However, actual zinc-binding site(s), the stoichiometry of zinc binding, and the relationship of bound zinc to the active site of ASM are unknown. Based on the mechanism found for other phosphodiesterase metalloenzymes, it is reasonable to propose for rhASM that a zinc-coordinated water molecule initiates the nucleophilic attack required for sphingomyelin hydrolysis. Assuming this is the case, we propose the following model to explain the effect of C-terminal cysteine modification (Fig. 7).

In the low activity form, the free C-terminal cysteine is involved in the active site zinc coordination, either by competing with a water molecule for coordination with zinc as shown in Fig. 7 or by forming a non-optimal five-ligand coordination structure. This decreases the ability of zinc to ionize water for the nucleophilic attack and leads to decreasing enzymatic activity. As thiol is a better zinc ligand than water, the non-optimal structure may be energetically favorable as long as the cysteine is freely available. In the high activity form of rhASM, however, the free cysteine is lost by either chemical modification or deletion and is no longer available for coordination. As a result, zinc coordinates with a water molecule, resulting in an optimal structure for catalysis. This model is essentially identical to the "cysteine switch" activation mechanism described previously (27, 28) for the matrix metalloproteinase family. In that case a conserved Cys residue in the proenzyme coordinates with zinc and prevents the proenzyme from becoming active. The cleavage of an N-terminal peptide containing the Cys removes this coordination and switches the enzyme from a non-catalytic to a catalytic form (30). The same group also demonstrated activation via in vitro modification of Cys (31). To our knowledge, the work reported here is the first evidence of a cysteine switch mechanism of activation outside of the matrix metalloproteinase family and suggests that this method of enzyme activation may be more widespread.

The nature of the cysteine modification that caused activation of rhASM in the media harvest is not clear; however, it could involve one of several possibilities: oxidation, deletion, or other modifications. It is interesting that this modification is favored at −20 °C but not at 4 or −80 °C. Preliminary data from incubation of rhASM at −20 °C with feed media in the presence of 50% glycerol suggests that it is the formation of ice that promotes activation at −20 °C. Because there were no protein or serum factors present in the feed media, we postulate that one or more of the small molecular weight components in the feed media cause rhASM activation via a process that is most favored at −20 °C. It is possible that this is a result of a

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**TABLE III**

Activity of wild-type (WT) and mutant rhASM proteins

| rhASM protein | Activity pNP assay | Fold activation | Activity Amplex red assay | Fold activation |
|---------------|--------------------|----------------|---------------------------|---------------|
| WT (low activity form) | 17.3 | 1.0 | 1.75 | 1.0 |
| WT (high activity form) | 80.2 | 4.6 | ND<sup>a</sup> | ND<sup>a</sup> |
| Cys<sup>629</sup> → del | 85.3 | 4.9 | 9.42 | 5.4 |
| Cys<sup>629</sup> → Ser | 91.1 | 5.3 | 9.28 | 5.3 |

<sup>a</sup> ND, not determined.

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**Fig. 7.** Proposed model for rhASM activation through availability of the C-terminal cysteine residue.
considering the recent report (42) of a link between H2O2-induced by copper (39) and that deletion or oxidation of free mitogenic activity (38). It was shown that this dimerization can or C-terminal modification may play a role in this regulation. In particular, C-terminal trimming has been reported for the lysosomal proteinase, cathepsin D. Either two or six amino acids are cleaved from the C terminus of the light chain as part of the proteolytic processing that converts the inactive single chain cathepsin D propeptide to the active double chain form (18, 19). In another context, Wisselaar et al. (17) have shown that C-terminal processing plays a role in the maturation of acid α-glucosidase and that a thiol protease may be involved in this process. More recently, Arunachalam et al. (32) showed that as many as 18 residues are cleaved from the C terminus of a lysosomal enzyme, γ-interferon-inducible lysosomal thiol reductase, to generate the mature form of the enzyme. In the case of ASM, multiple processed forms have been reported previously in normal and I-cell fibroblasts (10) and COS-1 cells transfected with human ASM cDNA (11). The secreted protein from CHO cells (8) and insect cells (9) clearly have its C-terminal biochemistry (Harris, R. E., ed) Vol. 2, pp. 2625–2639, McGraw-Hill, Inc., New York.

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