Structural Basis of the Sphingomyelin Phosphodiesterase Activity in Neutral Sphingomyelinase from Bacillus cereus*

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Sphingomyelinase (SMase, EC 3.1.4.12) from Bacillus cereus (Bc-SMase) hydrolyzes sphingomyelin to phosphocholine and ceramide in a divalent metal ion-dependent manner. Bc-SMase is a homologue of mammalian neutral SMase (nSMase) and mimics the actions of the endogenous mammalian nSMase in causing differentiation, development, aging, and apoptosis. Thus Bc-SMase may be a good model for the poorly characterized mammalian nSMase. The metal ion activation of sphingomyelinase activity of Bc-SMase was in the order Co2+ ≥ Mn2+ ≥ Mg2+ > Ca2+ > Sr2+. The first crystal structures of Bc-SMase bound to Co2+, Mg2+, or Ca2+ were determined. The water-bridged double divalent metal ions at the center of the cleft in both the Co2+- and Mg2+-bound forms were concluded to be the catalytic architecture required for sphingomyelinase activity. In contrast, the architecture of Ca2+-binding at the site showed only one binding site. A further single metal-binding site exists at one side edge of the cleft. Based on the highly conserved nature of the residues of the binding sites, the crystal structure of Bc-SMase with bound Mg2+ or Ca2+ may provide a common structural framework applicable to phosphohydrolases belonging to the DNase 1-like folding superfamilly. In addition, the structural features and site-directed mutagenesis suggest that the specific β-hairpin with the aromatic amino acid residues participates in binding to the membrane-bound sphingomyelin substrate.

The catalytic mechanism of the sphingomyelin hydrolytic activity remains to be elucidated in atomic detail, as there are no crystal structures of SMase in complex with the essential divalent metal ions. The sphingomyelin hydrolytic activity of Bc-SMase is believed to proceed in the manner of acid base catalysis, in which His-296 is proposed to generate an activated water and the essential Mg2+ ion at Glu-53 is suggested to stabilize a negatively charged transition state. The proposed catalytic mechanism of Bc-SMase is similar to that of bovine DNase I. In fact, Bc-SMase and bovine DNase I are homologous proteins and share a common architecture of conserved putative catalytic amino acid residues (7). However, the proposed catalytic mechanism does not fully explain the role of the essential divalent metal ion, i.e. the divalent metal ion type dependence for hydrolytic catalysis, because of the lack of the bound essential metal ions in all the currently available structures.

Bc-SMase and neutral sphingomyelinase (nSMase) in mammalian cells share similar metal ion dependence and considerable amino acid sequence identity (20%), including conserved residues involved in divalent metal ion binding, and are thus believed to have a similar hydrolytic mechanism. SMases in mammalian cells are classified into the following three groups according to the optimum pH of the SM hydrolytic activity: neutral SMase, acid SMase, and alkaline SMase (8). The detailed mechanism of the sphingomyelin hydrolysis activity of Bc-SMase may provide insight into sphingolipid metabolism in mammalian cells.

The only available structure of SMase is from the bacterium, Listeria ivanovii, reported recently (7). The bacterial SMase was confirmed to be a member of the DNase I-like folding superfamily (12–14), and the putative active site amino acid residues of the bacterial SMase were found to be geometrically identical to the corresponding amino acid residues of enzymes in the DNase I-like folding superfamilly. The bacterial SMase differs, however, in having a unique hydrophobic β-hairpin structure.

The L. ivanovii SMase structure allowed elucidation of a number of key features of the catalytic mechanism of bacterial SMases. However, structural information on the role of the essential divalent metal ions in catalysis is still lacking, and the hydrolytic molecular mechanism remains elusive.

Bacterial SMases have been reported to bind to the cell surface when catalyzing hemolysis. The crystal structure of SMase from L. ivanovii indicated that interaction with the membrane would occur at the unique hydrophobic β-hairpin region (7). This was inferred based on two indirect observations. First, there are two solvent-exposed aromatic amino acid residues at the top of the β-hairpin. Second, the corresponding region of structurally similar enzymes is a short loop. The role of the β-hairpin remains to be confirmed experimentally.

In this study we propose that the water-bridged double divalent metal ions are essential in the hydrolytic activity of Bc-SMase based on the
high resolution crystal structures of Bc-SMase with the metal ions Co$$^{2+}$$, Mg$$^{2+}$$, and Ca$$^{2+}$$, respectively. It is the divalent metal ion bound to His-296 that activates the water molecule as the nucleophile in sphingomyelin hydrolysis. Simplified assays using very low concentrations of substrate indicated a divalent metal ion-type dependence for the hydrolytic activity of Bc-SMase compatible with the divalent metal ion architecture. In addition, we confirmed experimentally that the β-hairpin, the unique feature of hemolytic SMases, participates in not only the binding of Bc-SMase to the cell membrane and SM liposomes but the hydrolytic activity of Bc-SMase.

EXPERIMENTAL PROCEDURES

Expression and Purification—Bc-SMase was overexpressed in Bacillus subtilis ISW1214 transformed with the plasmid vector, pFY300PLK, carrying cDNA of Bc-SMase cloned from B. cereus IAM 1029. The Bc-SMase was secreted into the culture medium. The 80% (v/v) ammonium sulfate fraction of the harvested culture medium was sequentially purified through a Cu$$^{2+}$$ column and a DEAE-Sepharose column. The purity of samples was verified using SDS-PAGE stained with Coomassie Brilliant Blue. The Bc-SMase was observed as a single band at 35 kDa.

Site-directed Mutagenesis—The transforming site-directed mutagenesis kit (BD Biosciences) was used with the primers W284A, 5′-TGCAACTGTACATCAGCGTTCAAAAATAT-3′, and F285A, 5′-ACTGTTACATCTGCGCCGCAAATATTACG-3′, to prepare the modified plasmids. The genetic sequence of Bc-SMase in each plasmid was confirmed with an ABI 310 PRISM™ genetic analyzer (Applied Biosystems).

Preparation of Liposomes—Multilamellar liposomes composed of phospholipid and cholesterol were prepared according to previous reports (15, 16). A mixture of phospholipid (0.5 μmol) and chloroform was evaporated under reduced pressure to form a lipid film on the wall of a conical bottomed flask. After drying under reduced pressure for 1 h, the lipid film was hydrated by vortexing at 45–50 °C or above the phase transition temperature of the SM used, in 100 μl of carboxyl fluorescein and 0.9% (w/v) NaCl. The liposome suspensions were centrifuged at 22,000 × g for 15 min at 4 °C to remove the non-encapsulated marker and washed three times by centrifugation in 20 mM Tris-HCl buffer (pH 7.5) containing 0.9% (w/v) NaCl (Tris-buffered saline). The resulting liposomes were suspended in 200 μl of Tris-buffered saline.

SMase Activity Assay—The hemolytic activity of the enzyme was determined by the amount of hemoglobin released from sheep erythrocytes using a method described previously (17). The reaction mixture containing the enzyme at various concentrations, 3% (w/v) sheep erythrocytes, 20 mM Tris-HCl buffer (pH 7.5), 3 mM MgCl$$\text{2}$$, and 0.9% (w/v) NaCl, was incubated for 30 min at 37 °C and then centrifuged at 500 × g for 3 min in order to prepare the test aliquot. Lysis was determined spectrophotometrically at A$$^{550}$$. The SM liposome disruption activity was determined by the amount of carboxyl fluorescein in the test aliquot. The SM liposome solution containing 20 mM Tris-HCl (pH 7.5), 1 mM MgCl$$\text{2}$$, and 0.9% (w/v) NaCl was incubated with Bc-SMase for 30 min at 37 °C. The wavelengths for excitation and measurement were 490 and 530 nm, respectively.

All hydrolytic activities of the enzymes were measured using the 14C-labeled SM, [N-methyl-14C]SM (Amersham Biosciences), as substrate (18). The concentration of the labeled SM was well below the critical micelle concentration of SM. The enzyme was dialyzed against a solution containing 20 mM Tris-HCl (pH 7.5), 0.1% (v/v) Triton X-100, and 1 mM EDTA to remove the contaminant metal ions. The reaction mixture containing the dialyzed enzyme (50 ng/ml) and an additional divalent metal ion (5 mM) was preincubated at 37 °C for 30 min, and then the reaction mixture was incubated with 1.25 μM [N-methyl-14C] SM at 37 °C for various periods. The reaction was terminated by the addition of the stop solution (CHCl$$\text{3}$$/MeOH, 2:1), and the radioactivity of the labeled product in the chloroform phase was measured by the liquid scintillation counter LSC-6100 (Aloka).

Membrane Binding Assay of Bc-SMase—The membrane binding assay was performed by surface plasmon resonance (SPR) analysis using a Biacore3000 system and the associated analysis software package (Biacore). The enzyme solutions at concentrations of 10, 5, and 0 μg of enzyme/ml were applied to the L1 sensor chips coated with or without SM liposome at a flow rate of 10 μl/min in a running buffer (20 mM Tris-HCl (pH 7.5), 3 mM CaCl$$\text{2}$$, and 0.9% (v/v) NaCl) at 25 °C. Dissociation was monitored for 100 s at least in a constant flow of running buffer without enzyme.

The binding assay using the 125I-labeled enzyme was performed according to Bolton and Hunter (19). SM liposomes in a buffer (20 mM Tris-HCl (pH 7.5), 0.9% (v/v) NaCl, 3 mM CaCl$$\text{2}$$) were incubated with 10 ng/ml wild-type or mutant enzymes at 37 °C for 30 min. After incubation, the SM liposomes were collected by centrifugation at 20,000 × g for 20 min. The treated liposomes were washed in buffer and then treated with 10% (v/v) trichloroacetic acid, followed by centrifugation at 20,000 × g for 10 min. The pellet was then dissolved in buffer supplemented with 2% (w/v) SDS, boiled for 3 min, and subjected to SDS-PAGE followed by autoradiography using a FLA2000 (Fuji Film).

Crystalization—Crystals of Bc-SMase were obtained by the hanging drop vapor diffusion method at 10 °C. The crystallization drop for the calcium acetate-bound form was prepared by mixing equal volumes of protein solution (10 mg/ml Bc-SMase in 20 mM Tris-HCl (pH 7.0)) and reservoir solution (18% (w/v) polyethylene glycol 8000, 0.2 M Ca(O-OOC$$\text{2}$$)$$\text{2}$$, and 0.1 M sodium cacodylate (pH 6.5)). For the calcium and cobalt chloride-bound crystals, calcium acetate was replaced with calcium chloride or cobalt chloride at a concentration of 0.2 M. In both cases the sodium cacodylate buffer was replaced with 0.1 M MES (pH 6.5). The crystals grew to maximum dimensions of 0.2 × 0.2 × 0.1 mm within a few weeks. The crystals of the magnesium-bound form were obtained in 16% (w/v) polyethylene glycol 8000, 0.2 M MgSO$$\text{4}$$, 0.1 mM CaCl$$\text{2}$$, and 0.1 M MES (pH 6.5).

The crystals of the calcium acetate-bound form were soaked for 12 h in the reservoir solution containing 5 mM samarium acetate to prepare the heavy metal derivative crystals.

X-ray Data Collection and Structure Determination—The diffraction images of the calcium acetate-bound form and the samarium-derivative crystals were collected at 100 K by a DIP2030 detector/MXC-18 (MacScience) using the monochromatized CuK$$\alpha$$ x-ray beam. The crystals were doped in paratone-N (Hampton Research) to prevent ice formation at cryogenic temperatures. All diffraction images were processed by MOSFLM (20) and SCALA (20) to prepare diffraction intensity data sets. The intensity data sets reduced using the apparent higher symmetry $\bar{C}$, $\alpha = 77.7$ Å, $\beta = 65.4$ Å, $c = 59.9$ Å, $\beta = 101.2^\circ$, were prepared to obtain initial phases by the single isomorphous replacement method taking account of the anomalous effect, whereas the true space group of the crystals was $P1$. The diffraction images of the crystal of the calcium chloride form up to 1.4 Å resolution were measured using RAXIS-V (RIGAKU) detector at BL45XU, SPring-8, and processed by MOSFLM (20) and SCALA (20) in the true space group P1 ($\alpha = 65.4$ Å, $\beta = 72.7$ Å, $c = 77.9$ Å, $\alpha = 112.2^\circ$, $\beta = 90.0^\circ$, $\gamma = 116.8^\circ$). The diffraction images of the cobalt chloride form up to 1.77 Å resolution were collected using RAXIS-VII on a FR-E x-ray generator (Rigaku) and processed by HKL2000 (21) in the true space group P1 ($\alpha = 65.1$ Å, $\beta = 72.7$ Å, $c = 78.1$ Å, $\alpha = 112.0^\circ$, $\beta = 90.1^\circ$, $\gamma = 116.6^\circ$). The diffraction images of the crystal of the magnesium-bound form to 1.80 Å resolution were col-
Crystal Structure of Bc-SMase Complexed with Metal Ions

**RESULTS**

**Overall Structure**—In this study, the crystal structures of Bc-SMase complexed to the functional metal ions Mg$^{2+}$, Co$^{2+}$, or Ca$^{2+}$ were determined at 1.8, 1.8, and 1.4 Å resolution, respectively. The overall structure of Bc-SMase consisted of a β-sandwich architecture made up of α/β motifs (Fig. 1A) and is basically the same independent of the bound metal ion. There are three distinct metal ion-binding sites in a long horizontal cleft across the Bc-SMase molecule (Fig. 1B). A double

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|----------------------------------------------------------|
| **TABLE 1** Statistics of diffraction data and refinement |

| Data set for phasing* |
|-----------------------|
| Generator/detector: | MXC18/DIP2030 |
| Space group: | P1 |
| Crystal: | Ca$^{2+}$-1 |
| Cell dimensions: | a = 77.7 Å, b = 65.4 Å, c = 59.9 Å, β = 101.15° |
| Resolution (Å): | 20.0-1.40 |
| No. of reflections measured/unique: | 434,852/213,905 |
| Completeness (all/shell): | 93.5/92.3 |
| I/σ(I) (all/shell): | 8.5/1.4 |
| Rmerge (all/shell): | 9.2/42.0 |
| r.m.s.d. (all/shell): | 2.4/42.0 |

| Data set for refinement |
|-------------------------|
| Space group: | P1 |
| Cell dimensions: | Ca$^{2+}$-1 |
| Resolution (Å): | 20.0-1.40 |
| No. of reflections measured/unique: | 434,852/213,905 |
| Completeness (all/shell): | 93.5/92.3 |
| I/σ(I) (all/shell): | 8.5/1.4 |
| Rmerge (all/shell): | 9.2/42.0 |
| r.m.s.d. (all/shell): | 2.4/42.0 |

* The diffraction data sets for the initial phasing were prepared using the apparent higher crystallographic symmetry C2.

* The diffraction data set from calcium 2 was used to calculate the anomalous difference Fourier map using the longer x-ray wavelength.

* r.m.s.d. indicates root mean square deviation.

**Overall Structure**—In this study, the crystal structures of Bc-SMase complexed to the functional metal ions Mg$^{2+}$, Co$^{2+}$, or Ca$^{2+}$ were determined at 1.8, 1.8, and 1.4 Å resolution, respectively. The overall structure of Bc-SMase consisted of a β-sandwich architecture made up of α/β motifs (Fig. 1A) and is basically the same independent of the bound metal ion. There are three distinct metal ion-binding sites in a long horizontal cleft across the Bc-SMase molecule (Fig. 1B). A double

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Co$^{2+}$ site was identified in the central cleft as the active site located next to the C-terminal end of β-strand 2 (site A) and the loop from Asn-291 to Val-298 (site B). The third Co$^{2+}$-binding site is located in the edge of the cleft at the N-terminal end of α-helix 2 (Fig. 1C). The number of the bound metal ions in the central site is dependent on the divalent metal ion. Two Co$^{2+}$ ions were bound to the active site compared with only one Ca$^{2+}$ ion. In contrast, in every case only one metal ion was bound to the edge site (Fig. 2).

Central Metal-binding Site as the Catalytic Site—We investigated the relationship between Bc-SMase hydrolytic activity and the essential divalent metal ions, Co$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Sr$^{2+}$. The hydrolytic activity of Bc-SMase was measured using [N-methyl-14C]SM as substrate maintained well below the critical micelle concentration to allow calculation of simple kinetics without involvement of the micelle. The values of $V_{\text{max}}$ (μmol min$^{-1}$ mg protein$^{-1}$) and $K_m$ (μM) for Co$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Sr$^{2+}$ were 14.2:14.2, 11.5:14.9, 10.8:16.1, 1.0:19.1 and 0.2:18.0, respectively (Fig. 3). These results show Bc-SMase with bound Co$^{2+}$, Mn$^{2+}$, or Mg$^{2+}$ has high catalytic activity, but Bc-SMase with bound Ca$^{2+}$ or Sr$^{2+}$ exhibits much lower catalytic activity under the experimental conditions.

![Crystal Structure of Bc-SMase Complexed with Metal Ions](image-url)
Comparison of the binding modes of Co$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$ at the central cleft in the Bc-SMase crystal structures revealed water-bridged double Co$^{2+}$ ions separated by 3.5 Å (Fig. 4A). The bond distance between the bridging water and Co$^{2+}$ in site A of Glu-53 and site B of His-296 is 2.0 Å in each case. The bond angle between the two Co$^{2+}$ ions via the bridging water is ~120°. The two Co$^{2+}$-centered octahedral bi-pyramids tilt at the bridge water by about 66° as the angle between the bridging water and Co$^{2+}$ ions separated by 3.5 Å (Fig. 4B). The well defined bound waters form hydrogen bonds with the side chains of the amino acid residues Asn-16 and Asp-295. The average coordinating bond length for the Co$^{2+}$ and Mg$^{2+}$ bound to site A was 2.2 Å in both cases. The architecture of the central metal-binding site B of the Mg$^{2+}$-bound form was also identical to that of the Co$^{2+}$-bound form; however, there was no defined Mg$^{2+}$ ion in the electron density map at the site B (Fig. 4B). As seen for the Co$^{2+}$ ions, the peak heights of the Mg$^{2+}$ ion at Glu-53 (site A) was much higher than that at His-296 (site B). In contrast to the double-linked octahedral metal coordination in the Co$^{2+}$- and Mg$^{2+}$-bound forms, there was only one Ca$^{2+}$ at the carboxylate oxygen of Glu-53 with five defined bound waters in a hepta-coordinate system in the Ca$^{2+}$-bound form (Fig. 4C). The average coordination distance of the Ca$^{2+}$ ion (2.5 Å) was longer by 0.3 Å than those of the Co$^{2+}$ and Mg$^{2+}$. The bound waters coordinated to the Ca$^{2+}$ form hydrogen bonds with Asn-16 and Asp-295.

The amino acid residues involved in divalent metal ion binding at the active site of Bc-SMase are Asn-16, Glu-53, Asp-195, Asn-197, Asp-295, and His-296 (Fig. 4A). These six amino acid residues are superimposable on the corresponding conserved amino acid residues of enzymes in the DNase 1-like folding superfamily (DNA repair exonuclease III from Escherichia coli (30), DNA repair endonuclease HAP1 from human (31), inositol polyphosphate-5-phosphatase of synaptotagmin from Schizosaccharomyces pombe (32), the subunit B of cytolethal distending toxin from Haemophilus ducreyi (33), endonuclease of human Line-1 Orf2P (34) and endonuclease from the telomere-specific long interspersed nuclear element (35)). Almost all these residues are conserved in other bacterial and mammalian nSMases. The exception is Asn-16, which is substituted by Val in some mammalian enzymes (Fig. 5A). This suggests that the mode of metal binding found in Bc-SMase is a common feature among not only nSMases but also members of the DNase 1-like folding superfamily (Fig. 5B).

**Exposed Hydrophobic Amino Acid Residues—** A unique feature of the Bc-SMase structure is the β-hairpin (Trp-279 to Tyr-290) (Fig. 1, A and C) not found in the structure of DNase I. The β-hairpin structure protrudes into the solvent with Trp-284 and Phe-285 located at the apex of the β-hairpin directly exposed to the bulk solvent. The β-hairpin structure of Bc-SMase is similar to that of the SMase from L. ivanovii (7). In order to investigate the role of Trp-284 and Phe-285 in the β-hairpin of Bc-SMase, these residues were replaced with Ala by site-directed mutagenesis. The structure of the variant enzymes was almost identical to...
Crystal Structure of Bc-SMase Complexed with Metal Ions

A.

FIGURE 4. The central metal-binding site. A, the central metal-binding site of the Co\(^{2+}\)-bound form. The bound Co\(^{2+}\) ions and the coordinated waters are shown by blue and red spheres, respectively. Dashed lines represent the hydrogen bonds between ligand water molecules and amino acid residues. The amino acid ligands of the bound Co\(^{2+}\) ions are the carboxyl oxygen of Glu-53 (site A) and the imidazole nitrogen of His-296 (site B). B, superimposition of the central metal-binding sites of the Co\(^{2+}\)-bound form and the Mg\(^{2+}\)-bound form. The amino acids of the Co\(^{2+}\)-bound form and the Mg\(^{2+}\)-bound form are shown by cyan and green carbons, respectively. The bound Co\(^{2+}\) ion and associated ligand waters are represented by the blue and gray spheres, respectively, and the bound Mg\(^{2+}\) ion and associated ligand waters are represented by the orange and red spheres, respectively. The violet and blue cage models are the \(F_{o}-F_{c}\) residual electron density map of the Mg\(^{2+}\)-bound form contoured at 5.5σ and the anomalous difference Fourier map of the Co\(^{2+}\)-bound form contoured at 7σ, respectively. C, the central metal-binding site of the Ca\(^{2+}\)-bound form is superimposed on that of Co\(^{2+}\)-bound form. The amino acids with green and cyan carbons are those of the Co\(^{2+}\)-bound form and the Ca\(^{2+}\)-bound form, respectively. The bound Ca\(^{2+}\) and Co\(^{2+}\) ligand waters and the bound Ca\(^{2+}\) and Co\(^{2+}\) ligand waters are colored in violet, red, blue, and gray, respectively. The blue cage model is the anomalous difference Fourier map of the Ca\(^{2+}\)-bound form contoured at 4σ.

the wild-type Bc-SMase as assessed by CD spectroscopic analysis (data not shown). However the liposome disruption activity of W284A and F285A mutants was a thousand- and a hundredfold lower than that of the wild-type enzyme, respectively (Fig. 6A). In addition, the hemolytic activity of both mutants was a thousandfold lower than that of the wild-type enzyme (Fig. 6B). We examined whether the mutated enzymes hydrolyze solubilized [N-methyl\(^{14}\)C]SM at a significantly lower concentration than the critical micelle concentration of SM. The catalytic activity of the variant enzymes was below the detection limit of the assay (greater than a 10-thousand-fold reduction compared with wild-type Bc-SMase).

Binding of W284A and F285A to SM Liposome—We determined whether Trp-284 and Phe-285 play an important role in binding to the membrane. SPR analysis showed that the Bc-SMase response rapidly reached a plateau and returned quickly to base line after the end of the injection of the SM liposome (Fig. 6C). The SPR response of W284A and F285A to SM liposomes showed one-tenth- and one-fifth-fold attenuation, respectively, compared with the wild type (Fig. 6, C, D, and E). 125I-Bc-SMase or the 125I-mutant enzymes were incubated with SM liposomes in the presence of 3 mM Ca\(^{2+}\). Binding of W284A and F285A mutants to SM liposomes was a thousand- and a hundredfold lower than the wild type, respectively (Fig. 6F). It is therefore apparent that the affinities of W284A and F285A mutants to SM liposomes are much weaker than the wild-type enzyme.

DISCUSSION

We have determined the crystal structures of Bc-SMase in complex with the divalent metal ions Co\(^{2+}\), Mg\(^{2+}\), or Ca\(^{2+}\). The metal complex structures contained two Co\(^{2+}\), one Mg\(^{2+}\), or one Ca\(^{2+}\) in the central cleft (Fig. 4) and in all cases one other metal ion at the side edge of the cleft (Fig. 2). The divalent metal ion bound at Glu-53 is concluded to be essential for hydrolytic activity. The central cleft is likely to be the active site, because Glu-53, Asp-195, and His-296 in the central cleft have been confirmed to be involved in the hydrolytic activity of Bc-SMase (5, 36).

Glu-53 was reported previously as the binding site of the hydrotically essential Mg\(^{2+}\) (5), whereas mutation of Asp-195 or His-296 abolishes hydrolytic activity (36).

The binding of both Co\(^{2+}\) and Mg\(^{2+}\) to the central cleft catalytic center showed a double hexacoordinated architecture with double octahedral bi-pyramids but that of Ca\(^{2+}\) was a hepta-coordinated architecture (Fig. 4). It appears that the binding mode differences between Co\(^{2+}\)/Mg\(^{2+}\) and Ca\(^{2+}\) in the central cleft play a crucial role in the activity of Bc-SMase.

The activities of Bc-SMase bound to various metal ions were in the following order, Co\(^{2+}\) ≥ Mn\(^{2+}\) ≥ Mg\(^{2+}\) ≥ Ca\(^{2+}\) ≥ Sr\(^{2+}\), compatible to the order of the average Lewis acid strengths of these divalent metal ions (37). The average Lewis acid strength of metal ions represents the empirical acid strength, because the average Lewis acid strength is the value of the oxidation number divided by the average coordination number in many experimentally determined atomic structures (37). The average Lewis acid strengths of Co\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), and Sr\(^{2+}\) are reported to be 0.351, 0.344, 0.334, 0.274, and 0.233 valence units, respectively (37) (Fig. 3), with Co\(^{2+}\) and Sr\(^{2+}\) the strongest and weakest Lewis acids of those tested.

The coordination structures of the bound Co\(^{2+}\), Mg\(^{2+}\), and Ca\(^{2+}\) at the active site of Bc-SMase support the fact that the actual Lewis acid strengths of the ions in the active site of Bc-SMase are similar to the reported average observed Lewis acid strengths (37). In the catalytic site of Bc-SMase, Mg\(^{2+}\) and Co\(^{2+}\) exhibit a hexacoordinate structure, whereas Ca\(^{2+}\) exhibits a hepta-coordinate structure (Fig. 4) consistent with the average observed coordination numbers of Co\(^{2+}\) (5.70), Mg\(^{2+}\) (5.98), and Ca\(^{2+}\) (7.31) (37).

The bound divalent metal ion as the Lewis acid at the active site of Bc-SMase would be directly involved in substrate binding. The differences in \(K_{m}\) of Co\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), and Sr\(^{2+}\) are small, in contrast to the \(V_{max}\) values that differ widely (Fig. 3). The difference in the Lewis acid strength of Co\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), and Sr\(^{2+}\) is relatively similar to the observed differences in \(K_{m}\) values suggesting that the divalent metal ions acting as the Lewis acid interact with the negatively polarized atoms of the substrate directly, with the result that the \(K_{m}\) value changes gradually according to the Lewis acid strength of the metal ion (Fig. 3).

The metal ions examined can be divided into two classes with respect to the efficiency of the Bc-SMase hydrolytic activity. Metal ions effective
in hydrolysis are Co$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$, whereas metal ions ineffective in hydrolysis are Ca$^{2+}$ and Sr$^{2+}$ (Fig. 3). There are also two clear modes of metal ion binding at the catalytic site. The Mg$^{2+}$ and Co$^{2+}$ show a water-bridged metal ion-binding mode, whereas the Ca$^{2+}$ ion exhibits a single metal ion-binding mode. The architecture of the Mg$^{2+}$-bound form in site A is the same as that of the Co$^{2+}$-bound form. The Ca$^{2+}$-
bound form revealed that the Glu-53 and His-296 coordinate Co^{2+} at site A and site B of the central cleft, respectively (Fig. 4A). In the Mg^{2+}-bound form, Glu-53 coordinated Mg^{2+} at site A as proposed (5), but Mg^{2+} ion was not defined on His-296 at site B (Fig. 4B). However, the average distances between Co^{2+} and the water molecules at site A and the effects of Co^{2+} on SMase activity were similar to those of Mg^{2+} (Fig. 3), indicating that the mode of Mg^{2+} binding in the central cleft in the propagation of the hydrolytic reaction could be the same as that of Co^{2+}. In fact, the architecture of the water molecules and amino acid residues (Asp-195, Asn-197, and His-296) in site B of the Mg^{2+}-bound form are coincident with those at site B of the Mg^{2+}-bound form. However, the peak height of Co^{2+} bound to Glu-53 in site A (28 μg/ml) was much higher than the Co^{2+} bound to His-296 in site B (9 μg/ml) in the anomalous difference Fourier map (Fig. 4B), suggesting that site B has a lower affinity for metal ions than site A. In the 2|F_o| − |F_c| electron density map of the Mg^{2+}-bound form, the height of electron density peak of the Mg^{2+} bound to the Glu-53 at site A was not very high (6.0σ), although this is the higher affinity site. We conclude His-296 binds Mg^{2+} at site B, although the electron density of Mg^{2+} was not defined in site B of Bc-SMase complex structure of Mg^{2+} at the pH 6.5 of the crystallization condition. The crystallization pH value is comparable with the pK_a of histidine, and a metal ion associated with His-296 is expected to bind with higher occupancy at higher pH values because of less competition with proton ions. Thus, it is highly plausible that Bc-SMase binds Mg^{2+} at site B of the central cleft as observed in the Co^{2+}-bound form (Fig. 4B). In contrast, bound Ca^{2+} exhibits a hepta-coordination pattern at site A in the central cleft (Fig. 4C), a markedly different pattern from the double hexa-coordination, double octahedral bi-pyramids, seen in the Co^{2+}- and Mg^{2+}-bound forms. The average distance between Ca^{2+} and the bound water molecules is significantly longer than that observed in the Co^{2+}- and Mg^{2+}-bound forms as shown in Fig. 4C. The differences in coordinate distance are consistent with the ionic radii of hexa-coordinated Mg^{2+} (0.72 Å) and Co^{2+} (0.745 Å), compared with hepta-coordinated Ca^{2+} (1.06 Å) (38). It is likely that the binding of a second Ca^{2+} at site B in the central cleft of Bc-SMase is restricted by the geometry of the Ca^{2+}-coordinated water molecules at site A. Taken together, these results indicate that the water-bridged double divalent ions in the central cleft is the essential architecture for SMase activity.
The catalytic mechanism of Bc-SMase is proposed as follows based on the SM docking model (Fig. 7). Two metal ions bound to Glu-53 and His-296 at sites A and B of the central cleft of Bc-SMase, respectively, serve as a foothold for binding of SM in the proper orientation to the active site. The divalent cation linked to His-296 provides the general base water. The phosphate moiety of SM binds to the central cleft of Bc-SMase at the site of the water-bridged double metal ions in the model (Fig. 7A). The divalent metal ion at Glu-53 binds to the SM by direct interactions with the amido oxygen and the ester oxygen O-4 between C-1 and the phosphorus of SM (Fig. 7B). The water-bridged double divalent cations and the side chain of Asn-197 bind to the oxygen atoms of the phosphate moiety of SM. The bonds delocalize a negative charge on the phosphate group, resulting in a positively polarized phosphorus. The divalent metal ion at His-296 and the side chains of Asp-195 and Asn-197 lower the pKₐ value of the bound water molecule (Fig. 7B, Wat1), resulting in an activated water molecule. This activated water molecule attacks the positively polarized phosphorus of SM to form a pentavalent phosphorus. The growing negative charge on the oxygens of the pentavalent phosphorus, the reaction transition state, is delocalized by the double divalent metal ions, resulting in formation of ceramide and phosphocholine.

The model shows that the catalytic site containing the water-bridged metal ions accommodates various transition states with pentavalent phosphorus, as seen in other crystal structures of DNase I-like folding superfamily members (Fig. 7, C–E), and stabilizes each of the negative charges on the transition states. The key residues in binding the water-bridged double Ca²⁺ ions to the active site of SMase are conserved in inositol polyphosphate-5-phosphatase domain of synaptojanin from S. pombe, and deoxyribonuclease I from bovine are shown in C–E, respectively. The water-bridged double cation architecture accommodates various reactions in the transition states with a pentavalent phosphate. The bound Co⁴⁺ ions and the water molecules are shown by the blue and the red spheres, respectively. D, the positively charged amino acid residues (yellow carbons) are close to the negatively charged phosphate group.

The catalytic site of Bc-SMase possesses an aromatic β-hairpin (Trp-279 to Tyr-290) conserved in hemolytic SMases from S. aureus (25) and from L. ivanovii (7) (Fig. 5A). This region is not found in other structurally characterized members of the DNase I-like folding superfamily. Replacement of Trp-284 and Phe-285 with alanine in the β-hairpin, W284A and F285A mutants, reduced binding to SM liposomes and disruption of SM liposomes and sheep erythrocytes (Fig. 6, A and B). SPR analysis confirmed the dramatic reduction in membrane binding of these mutants compared with the wild-type enzyme (Fig. 6, C, D, and E). These observations show that Trp-284 and Phe-285 are required for relocation of Bc-SMase to the cell membrane (Fig. 6F). In the crystal structure of the Mg²⁺-bound form, a
molecule of MES was bound to the aromatic cluster, including Tyr-25, Trp-28, Tyr-242, Trp-284, Tyr-288, and Tyr-290 (Fig. 8A). The top of the β-hairpin was twisted in order to hold the bound MES (Fig. 8B). The positive head group of the MES molecule is a structural mimic of the phosphocholine moiety of the substrate, SM (Fig. 8C). Thus the phosphocholine moiety of the substrate may bind to the hydrophobic patch in the same manner as the MES molecule seen in the crystal structure. It can be postulated that the exposed aromatic amino acid residues, Trp-284 and Phe-285, initially interact with the positively charged phosphocholine moiety. Thus the exposed aromatic amino acid residues participating in the edge metal-binding site and the exposed aromatic amino acid residues of Trp-284 and Phe-285 are shown by the stick models with yellow carbons. The bound metal ion at the edge metal-binding site is colored in blue. The bound SMs are shown as stick models with cyan carbons.

In the amino acid sequence alignments of the Bc-SMase and mammalian SMases, the solvent-exposed loop (Fig. 5A, blue characters) from Asn-92 to Pro-98 is one of the fingerprint regions for bacterial Bc-SMase. This solvent-exposed loop is located next to Glu-99 and Asp-100, both of which form part of the edge metal-binding site of Bc-SMase. Furthermore, it is linked to another loop containing Phe-55 and Asn-57, which form the remaining part of the edge metal-binding site as seen in the Ca2+-bound crystal structure (Figs. 1C and 2). This suggests that the solvent-exposed loop may be regulated by Ca2+ binding to the side edge site. Bc-SMase adsors to the erythrocyte in a Ca2+-dependent manner during hemolysis (2). In fact, Obama et al. (5) demonstrated that the Ca2+-binds to Asp-100 of Bc-SMase. This implies that the solvent-exposed loop may be involved in the Ca2+-dependent membrane binding (Fig. 8D). The solvent-exposed loop is also conserved in bacterial SMases from S. aureus (25) and L. ivanovii (7), both of which have hemolytic activity, and asparagines corresponding to Asp-100 of Bc-SMase are also conserved (Fig. 5).

In conclusion, we have solved the structure of Bc-SMase complexed with metal ions (Co2+, Mg2+, and Ca2+) revealing the presence of a double central ion-binding site. The suggested mechanism of action of the enzyme may be applicable to several members of the DNase I-like folding superfamily. A hydrophobic β-hairpin containing Trp-284 and Phe-285 and the solvent-exposed region from Asn-99 and Pro-98 are involved in binding of SMase to the cell membrane.

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