Ergosterol Increases the Intermolecular Distance of Amphotericin B in the Membrane-Bound Assembly As Evidenced by Solid-State NMR[†]

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ABSTRACT: Amphotericin B (AmB) exerts its antifungal activity by forming ion-permeable assemblies across lipid bilayers. To investigate AmB−AmB bimolecular interactions in the assembly, we carried out $^{13}C\{^{19}F\}$REDOR experiments using 14-^{19}F- and ^{13}C41-labeled AmBs in sterol-containing and sterol-free palmitoyloleoylphosphatidylcholine (POPC) membranes and measured the average distance between the labeled sites of AmBs in membrane-bound forms. The REDOR results suggested that the intermolecular distance of AmB molecules is significantly increased in the ergosterol membrane as compared with the cholesterol membrane. This sterol-dependent change was supported by the UV spectra of AmB in lipid bilayers, in which the excitonic absorption band arising from the aggregated state of AmB shifted to longer wavelength in ergosterol-containing POPC membrane. The REDOR experiments also disclosed that the head-to-head orientation of AmB is predominant in both of the sterol-containing membranes and AmB−POPC interaction was detected only in the ergosterol membrane. Ergosterol significantly influences the interactions between AmB molecules as well as those between AmB and POPC, which may facilitate formation of ion-permeable channels in ergosterol-containing membrane.

Amphotericin B (AmB)[1] is a polyene macrolide antibiotic isolated from *Streptomyces nodosus* and used for treatments of systemic fungal infections (*1, 2*). It is known that AmB forms ion-permeable channels in cell membranes in a sterol-dependent manner, where higher affinity to ergosterol than to cholesterol is thought to be responsible for selective toxicity to fungi. The architectures of this AmB ion channel have been extensively studied so far, which include the famous "barrel-stave" model (*3*), where the polyhydroxy part of AmB comes close together to form a hydrophilic pore and the hydrophobic polyene region faces outside to interact with sterol and phospholipid. Based on this channel model, a number of *in silico* studies have been carried out to provide more plausible channel architectures; e.g., Baginski et al. have performed computer simulations for AmB assemblies (*4*) and deduced that the amino group of AmB interacts with the carboxylic acid of a neighboring AmB molecule, which leads to the "head-to-head" interaction in the assembly. However, details of the intermolecular interactions remain largely unknown chiefly due to the luck of proper experimental methodologies to examine the molecular recognitions among membrane-bound entities.

Solid-state NMR spectroscopy provides a versatile tool for the structural elucidation of biological molecules in membranes (*5*−*7*). The rotational echo double resonance (REDOR) experiment is one of the important techniques frequently used for measurement of interatomic distance (*8, 9*). In particular, $^{13}C\{^{19}F\}$REDOR makes it possible to measure long-range $^{13}C-^{19}F$ distances over 10 Å (*10*) since the ^{19}F nucleus possesses favorable features such as high gyromagnetic ratio, 100% natural abundance, and low background signals. Application of this technique is, however, mostly limited to small peptides mainly due to the feasibility of preparing ^{13}C- and ^{19}F-labeled compounds. Therefore, it is still challenging to explore the applicability of REDOR techniques for nonpeptidic compounds such as AmB.

In the previous studies, we examined the AmB−phospholipid (*11, 12*) and AmB−sterol (*13*) interactions using solid-state NMR and demonstrated the utility of this method for the structural elucidation of membrane-bound assemblies at the atomic level. In those reports we proposed that AmB forms a single-length channel, which is surrounded by ergosterol molecules. However, the AmB−AmB interaction remains unanswered. In this study we report the first direct observation of the bimolecular interaction of AmB in membrane-bound forms using $^{13}C\{^{19}F\}$REDOR techniques.

MATERIALS AND METHODS

Materials. AmB and cholesterol were purchased from Nacalai Tesque (Kyoto, Japan). Ergosterol was from Tokyo Kasei (Tokyo, Japan), and palmitoyloleoylphosphatidylcholine (POPC) was from Avanti Polar Lipid Inc. (Alabaster, AL). [tri-^{13}C]AmB (**1**) was prepared as reported previously (*11*). Briefly, the AmB-producing species *S. nodosus* was

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[1] Abbreviations: AmB, amphotericin B; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MAS, magic angle sample spinning; REDOR, rotational echo double resonance.

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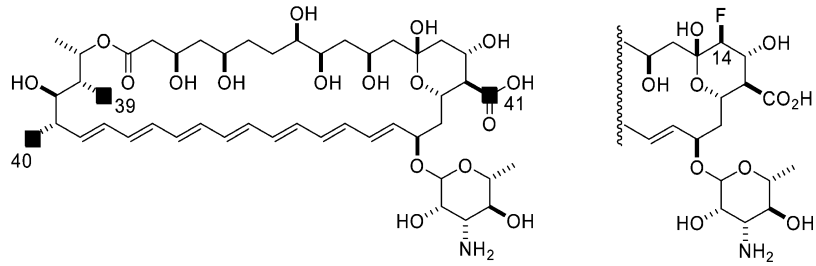

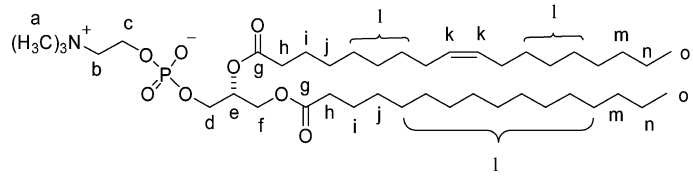

FIGURE 1: Structures of labeled AmBs and phospholipid: **1**, [tri-^{13}C]AmB that is ^{13}C-labeled at the positions indicated by squares; **2**, 14-F AmB; and POPC, palmitoyloleoylphosphatidylcholine.

obtained from American Type Culture Collection (ATCC 14899) and cultured in FCA medium containing sodium [3-^{13}C]propionate to produce site-specifically 39-, 40-, 41-labeled AmB. About 20 mg of **1** with average 15% ^{13}C enrichment at C39, C40, and C41 was obtained from 50 mL of the culture. 14-F AmB (**2**) was chemically derived from AmB as reported previously (*14*). 14-F AmB was purified by silica gel open column chromatography, and its structural identity was confirmed by mass spectrometry and ^{1}H NMR.

Solid-State NMR Measurements. The labeled AmBs **1** (1.8 μmol), **2** (1.8 μmol) and POPC (36.8 μmol) were dissolved in $CHCl_3$/MeOH (10% of POPC was replaced with ergosterol or cholesterol for sterol-containing samples), and the solvent was evaporated to afford a thin film. After drying in vacuo for 8 h, the membrane was hydrated with 31.4 μL of 10 mM HEPES buffer and 1 mL of H_2O and dispersed by vortexing and sonication. Then, the lipid suspension was freeze−thawed five times to produce large vesicles. The suspension was lyophilized, rehydrated with D_2O (31.4 μL), and packed into a glass tube. The glass tube was sealed with epoxy glue and inserted into a ϕ 5 mm MAS rotor. ^{13}C{^{19}F}REDOR spectra were recorded at 75.315 MHz for ^{13}C with ^{19}F irradiation at 281.743 MHz on a CMX300 (Varian/Chemagnetics) spectrometer with the MAS frequency of 5000 ± 2 Hz. The rotor temperature was maintained at 30 ± 1 °C with a temperature controller. The spectral width was 30 kHz. Typically, the π/2 pulse width for ^{1}H NMR was 4 μs, and the π pulse widths for ^{13}C and ^{19}F were 10 and 12 μs, respectively. The contact time for cross-polarization transfer was set to be 1.5 ms. The REDOR spectra were acquired with a recycle delay of 2 s under TPPM ^{1}H-decoupling with field strength of 71 kHz (*15*). The dephasing times of the REDOR measurements were 4.8, 8, 12.8, 16, 24, and 32 ms, and xy-8 phase cycling was used for ^{19}F irradiation (*16*).

UV Spectral Measurements. AmB (15 nmol) and POPC (1.5 μmol) were dissolved in $CHCl_3$/MeOH (10% of POPC was replaced with corresponding sterols for sterol-containing samples), and the solvent was evaporated to afford a thin film. After drying in vacuo for 8 h, the lipid film was suspended to 8% sucrose buffer (1 mL) by vortexing and sonication. Then the lipid suspension was freeze−thawed four times to produce large vesicles. An aliquot of the resultant suspension (170 μL) was diluted with 1360 μL of 8% sucrose buffer, and UV spectra were recorded on a UV-2500 spectrophotometer (Shimadzu, Kyoto, Japan) with a 1.0 cm path length quartz cell at ambient temperature.

RESULTS AND DISCUSSION

Solid-State NMR Measurements. We have recently carried out the kinetic analysis (*17*) for interactions between AmB and sterol-free or sterol-containing membrane using surface plasmon resonance techniques and found that AmB has significantly higher affinity to sterol-containing palmitoyloleoylphosphatidylcholine (POPC) membrane than sterol-free POPC membrane. Therefore, in order to pick up minute differences of AmB−AmB interactions possibly induced by sterols, we adopted POPC as a membrane constituent. For the REDOR experiments, a 1:1 mixture of **1** and **2** (Figure 1) was incorporated into POPC membranes at a **1/2**/POPC molar ratio of 1/1/20 and into sterol-containing POPC membranes at a **1/2**/sterol/POPC molar ratio of 1/1/2/18. Since the REDOR dephasing effects depend mainly on ^{13}C−^{19}F distance, its magnitude should reflect the intermolecular distance between AmBs in membrane-bound forms.

Figure 2 shows the full-echo (S_0) and difference (ΔS) spectra of the ^{13}C{^{19}F}REDOR which were recorded in sterol-free POPC bilayers at a dephasing time of 12.8 ms. Signals appearing at 181, 22, and 13 ppm correspond to C41, C40, and C39 of **1**, respectively (*11*). In the ΔS spectrum, significant dephasing effects were observed for the C41 and C40 positions. A peak reduction of the C41 signal indicates that headgroups of **1** and **2** are close to each other to undergo the "head-to-head" interaction. On the other hand, the REDOR dephasing at C40 indicated that the "head-to-tail" interaction also occurs under these conditions, suggesting that two or more different types of bimolecular interactions are involved in the sterol-free POPC bilayers. Based on the barrel-stave model reported by Baginski et al. (*4*) an AmB assembly consists of "head-to-head" interactions, and therefore, the head-to-tail orientation may arise from AmB

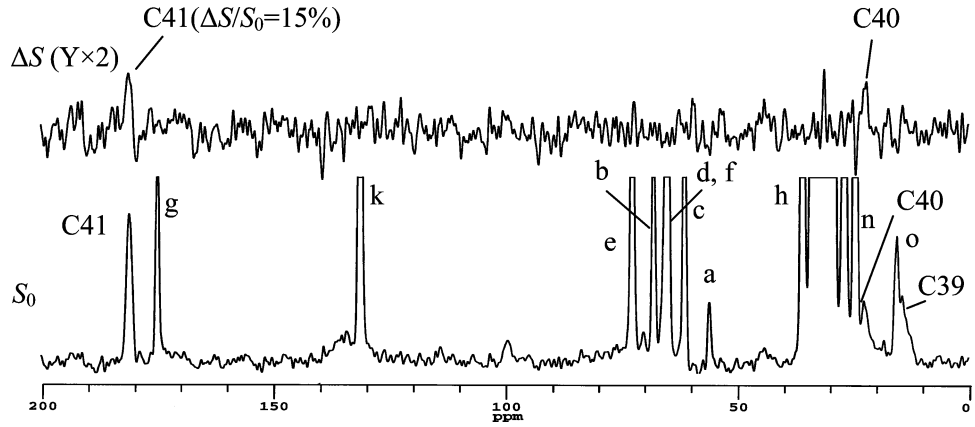

FIGURE 2: $^{13}C\{^{19}F\}$REDOR spectra of the sterol-free POPC membrane in the presence of ^{13}C- and ^{19}F-labeled AmBs **1** and **2** at 30 °C. The labeled AmBs were present at the ratio of **1**/**2**/POPC (1:1:20) in 10 mM HEPES/D_2O (50% wt) at pH 7.0. ΔS is a difference spectrum and S_0 is a full echo spectrum. Small alphabets are ^{13}C signals of POPC. These spectra were obtained after 64 rotor cycles of ^{19}F dephasing (12.8 ms). The number of scans was 62336.

molecular aggregates different from the channel structure. In fact, the crystal packing of *N*-iodoacetyl-AmB predominantly follows the head-to-tail orientation (*18*). We carried out the REDOR experiments with a dry powder sample of a 1:1 mixture of ^{13}C- and ^{19}F-AmB without phospholipid and found that both head-to-head and head-to-tail orientations occurred; the REDOR dephasing with 40 rotor cycles (5.7 ms) was observed for C41 at 21% and for C40 at 16% (see Supporting Information for spectra). AmB in the powder state is, therefore, supposed to form randomly oriented aggregates rather than channel-like assemblies. Similar aggregates to those in the powder state may partly remain in the sterol-free membrane, consequently giving rise to the head-to-tail interaction. As described later, the UV spectra of AmB in sterol-free membrane (Figure 5) further imply the presence of AmB weakly interacting with the membrane constituents. The bands at 408, 386, and 365 nm are characteristic of monomeric AmB in an aqueous medium (*19*), which provides no signal in the REDOR spectrum due to its high mobility. These seemingly contradicting results may be accounted for by the multiple states of AmB molecules in the POPC preparation, which may comprise AmBs in large aggregates, those dissolved in an aqueous medium, and probably those bound to membrane.

$^{13}C\{^{19}F\}$REDOR spectra of **1** and **2** in cholesterol-containing POPC membrane are shown in Figure 3a. In contrast to the sterol-free membrane, REDOR dephasing was observed for C41 but not for C40. The result indicates that "head-to-head" interaction between AmBs is dominant in cholesterol-containing membrane. Meanwhile, the REDOR dephasing ratio at C41 is comparable with that of sterol-free membrane (Figures 2 and 3a), which reveals that cholesterol hardly affects the intermolecular distance between AmBs in the "head-to-head" orientation.

We next recorded the REDOR spectra of **1** and **2** in the ergosterol-containing membrane (Figure 3b). The dephasing effect was also observed at C41 as in the case of the cholesterol membrane although the dephasing ratio was significantly decreased (Figure 3). The attenuated REDOR effect may be caused by the higher mobility and/or larger intermolecular distance of AmBs in the ergosterol membrane. To examine the possibility of molecule motion, we carried out the same REDOR experiments at −20 °C (see Supporting Information) and found that the obtained dephasing ratio for C41 was virtually same as that at 30 °C. Therefore, the reduced dephasing ratio for C41 in the presence of ergosterol is largely ascribable to increased intermolecular distance between AmBs. Additionally, the dephasing effects were also observed for the terminus of POPC acyl chains (signals m and n) in the ergosterol-containing membrane (Figure 3b); similar effects for the acyl-chain signals were detected in the different dephasing times of 24 and 32 ms. These effects were detected in neither choresterol-only nor PC-only membrane, suggesting that interaction between AmB and POPC is enhanced by ergosterol. Interestingly, ^{13}C atoms dephased by ^{19}F-AmB were located at the tail portions of POPC acyl chains. This may indicate that POPC interacting with AmB molecules forms an interdigitated bilayer to adjust its hydrophobic length to the molecular of AmB; the length of hydrophobic side is approximately 22 Å (*18*). The result is consistent with the report by Nguyen et al. that the introduction of AmB induces bilayer interdigitation (*20*). We have also shown the importance of hydrophobic length of PC in formation of ion channels by AmB using covalently linked AmB−PC conjugates (*21*). Moreover, it is reported (*11*) that the C39/C40 methyl groups of AmB come close to the polar head of PC. These results imply that interactions between the tail part of AmB and the polar head of PC (and vice versa) play an important role in forming a stable assembly in the membrane.

Intermolecular Distance Calculation. The REDOR results revealed that the average distance between neighboring AmBs in the membrane is significantly increased by ergosterol. In the next step, we aimed to determine the intermolecular distance between 14-F and C41 from the REDOR data. Since the ^{13}C enrichment in C41 of **1** is approximately 15%, the *intra*molecular interaction derived from natural abundance ^{13}C (1.1%) in **2** is not negligible. Therefore, the observed dephasing ratios at C41 should be treated as the sum of intramolecular and intermolecular contributions. For estimating the intramolecular effect, we used the 14-F/C41 distance of 4.9 Å that was derived from X-ray crystallography (*18*). For determining the intermolecular 14-F/C41 distance from the REDOR dephasing, we assumed that a neighboring pair of ^{13}C- and ^{19}F-labeled AmBs largely induced REDOR dephasing, and long-range AmB−AmB interactions beyond the closest AmB molecules were negligible since, judging from computer simulation results (*4*),

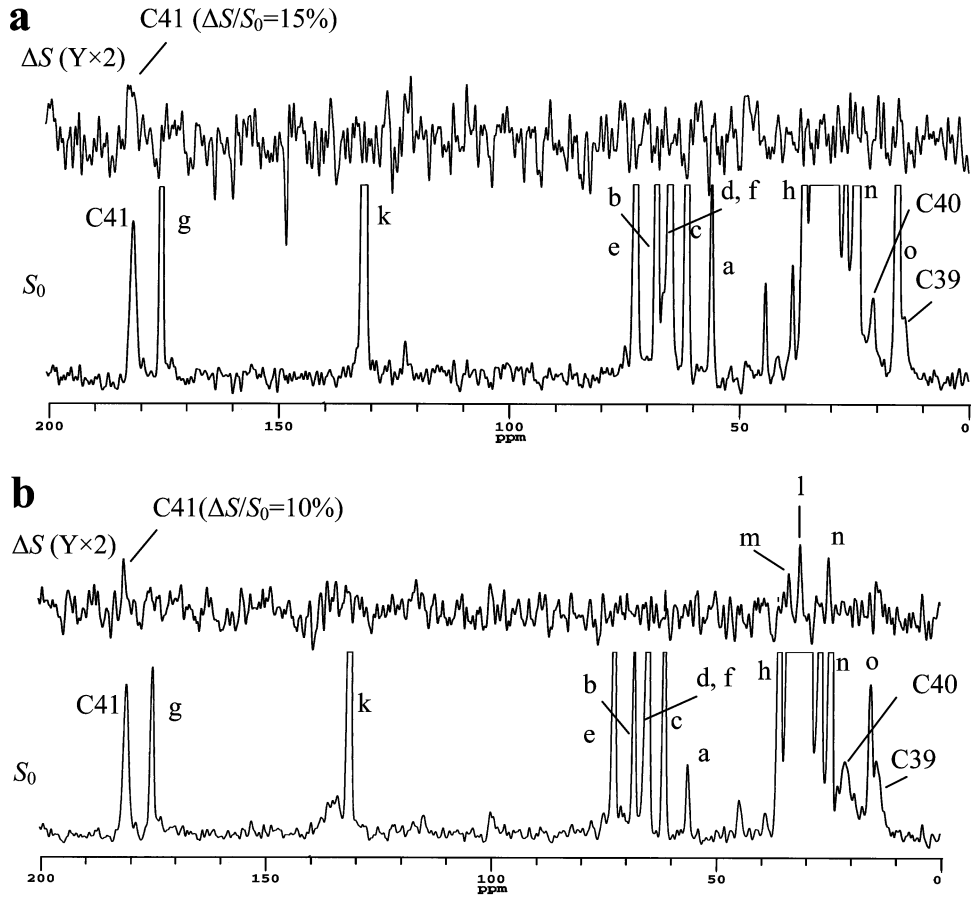

FIGURE 3: $^{13}C\{^{19}F\}$REDOR spectra of cholesterol-containing (a) and ergosterol-containing (b) POPC membranes in the presence of ^{13}C- and ^{19}F-labeled AmBs **1** and **2** at 30 °C. The labeled AmBs were present at the ratio of **1/2**/sterol/POPC (1:1:2:18) in 10 mM HEPES/D_2O (50% wt) at pH 7.0. ΔS is a difference spectrum and S_0 is a full echo spectrum. Small alphabets are ^{13}C signals of POPC. These spectra were obtained after 64 rotor cycles of ^{19}F dephasing (12.8 ms). The number of scans of each experiment was 61440.

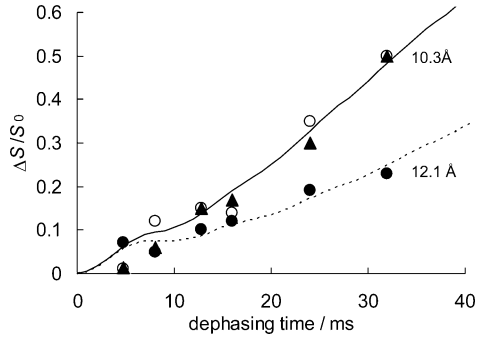

FIGURE 4: Experimental $^{13}C\{^{19}F\}$REDOR dephasing values ($\Delta S/S_0$) of C41 in sterol-free POPC (▲), cholesterol-containing POPC (○), and an ergosterol-containing sample (●). Solid and dashed lines are best fit curves of $^{13}C-^{19}F$ distance calculated in two spin systems for 10.3 and 12.1 Å, respectively. When the S/N ratios of the REDOR spectra (Figure 3) are taken into account, these distances should include standard errors of 10.3 ± 0.5 and 12.1 ± 1.0 Å.

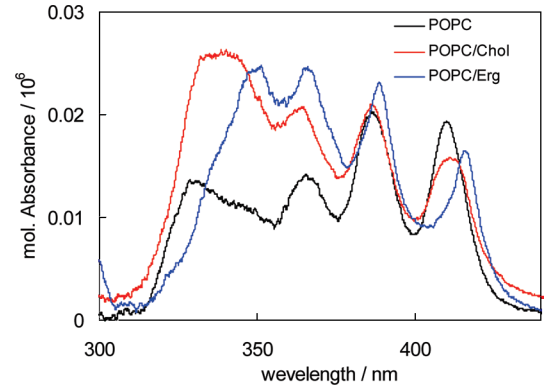

FIGURE 5: UV spectra of AmB in POPC membranes at the AmB/lipid molar ratio of 0.01. The molar ratio of POPC/sterol was 9:1. The concentration of AmB was kept at 1.67 μM in each sample.

the distance in such cases was estimated to exceed 15 Å. We made another assumption that ^{13}C- and ^{19}F-AmB molecules are randomly arranged in the assemblies, judging from the similarities between 14-F AmB **2** and AmB in biological activities and spectroscopic properties (*14*). Therefore, the maximum intermolecular dephasing ratio was set at 0.75 (the effects of $^{19}F-^{13}C-^{19}F$ spin systems are discussed below). On the basis of these assumptions, we calculated theoretical dephasing curves as shown in Figure 4 using a Bessel function expression (*22*). Consequently, the average distance between intermolecular 14-F and C41 was estimated to be about 10 Å for the sterol-free and cholesterol-containing membrane. On the other hand, in the ergosterol-containing membrane, the distance was increased to 12 Å, implying that the dense aggregation of AmB molecules is prevented by ergosterol. The short AmB−AmB distance in the sterol-free or cholesterol-containing membranes and the long distance in the ergosterol membrane support the previous results that AmB has no direct interaction with cholesterol in the membrane (*19, 23*), whereas possessing high affinity with ergosterol (*13, 17*).

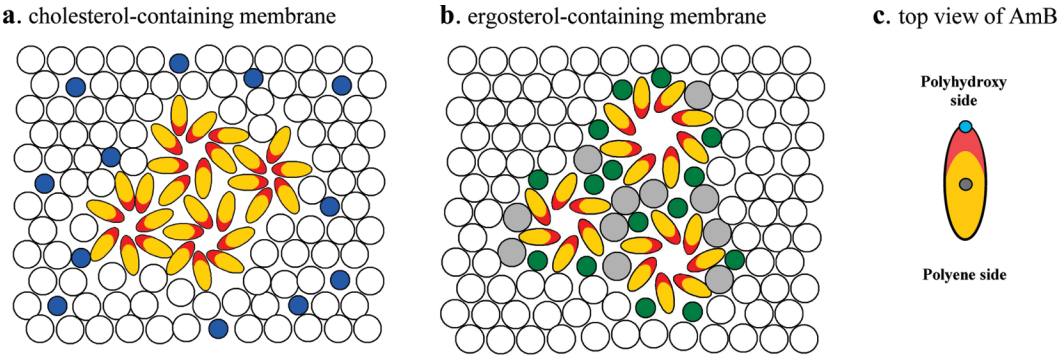

FIGURE 6: Schematic representation of AmB/sterol/POPC interactions inside POPC membranes. (a) In cholesterol-containing membrane, AmB forms large aggregates which are phase-separated from lipid bilayers; yellow ovals with a red tip, AmB; blue cycles, cholesterol; and open cycles, POPC. (b) In ergosterol-containing membrane, the AmB−sterol−POPC complex is formed, which may correspond to an ion conductive "active" channel; yellow ovals with a red tip, AmB; green cycles, ergosterol; gray cycles, POPC closely interacting with AmB; and open cycles, other POPC. (c) Top view of AmB, where blue and gray circles denote the position of ^{19}F-labeled C14 and ^{13}C-labeled C41, respectively (see Supporting Information). Although six, seven, or eight molecules of AmB per channel are depicted according previous reports (*3, 4*), relevant experimental data were not obtained in the present study.

There can be two intermolecular distances between 14-F and ^{13}C41 in a point-symmetric assembly such as the barrel-stave model, where the distance between the same positions in neighboring AmBs gives rise to a single value but the distance between the different positions such as the present case is not same. The labeled sites of **1** and **2**, both of which are positioned on the polyhydroxyl-polyene axis (Figure 6c), should make these two distances relatively close in a radially oriented assembly. We assume that AmB takes a radially arranged or slightly tilted configuration in the assembly as shown in Figure 6. In such cases, the smaller of the two distances should be close to those estimated from REDOR with a deviation less than +0.7 Å (detailed descriptions of the REDOR curves for these systems are given in Supporting Information).

These discussions are based on a two-spin system of ^{13}C and ^{19}F. In the AmB assemblies, however, three spin systems of ^{19}F−^{13}C−^{19}F should be partly involved, where REDOR dephasing effects become dependent on ^{19}F−^{13}C−^{19}F angles. According to computer simulation experiments for membrane-associated assemblies of AmB (*4*), the angles possibly range from 30° to 130°. Provided ca. 25% of ^{13}C-AmB molecules are contacted with two ^{19}F-AmB molecules, the ^{13}C−^{19}F distance estimated from the REDOR results by SIMPSON (*24*) should be 12.1−12.5 and 10.3−10.7 Å for ergosterol- and cholesterol-containing membranes, respectively (detailed accounts for three spin systems with possible ^{19}F−^{13}C−^{19}F angles are given in Supporting Information). The contribution of three spin systems to the estimated ^{13}C−^{19}F distance is thus relatively small, and the difference in the distances between the two sterol membranes does not heavily depend on the spin systems. Therefore, for further discussion, we will use the distance based upon two spin systems.

UV Spectral Measurements. To further examine the interactions between AmBs in POPC membranes, we recorded the UV spectra of large multilamellar vesicles. It is known that intense absorption maxima of AmB at 408, 384, and 370 nm are due to the monomeric state while hypsochromically shifted absorption bands at 317−350 nm are ascribable to AmB in the oligomeric state (*25*). Figure 5 showed the UV spectra of AmB in sterol-free POPC and in 10% sterol-containing POPC membranes at an AmB/lipid molar ratio of 0.01. The small red shift at 408 nm in the ergosterol membrane is thought to be caused by increased hydrophobicity around the heptaene group of AmB (*25*), where the higher affinity to ergosterol is supposed to stabilize the binding of AmB to the membrane. In the sterol-free membrane, AmB showed a hypsochromically shifted band at 329 nm. A similar absorption band was observed for the cholesterol-containing membrane, where AmB gave rise to a stronger absorption in the 330−345 nm range. On the other hand, in the ergosterol-containing membrane, a different absorption band was observed, which was significantly shifted to a longer wavelength centered at 350 nm. Gagoœ et al. have reported that the hypsochromic shift in the aggregate form of AmB can be accounted for by the exciton splitting theory (*26*), according to which the wavelength of the excitonic absorption band depends on the distance between the nearest neighboring heptaene chromophores of AmBs. The shorter distance leads to a more hypsochromic shift in case that neighboring chromophores are parallel to each other. Therefore, the hypsochromic absorptions around 330 nm in sterol-free and cholesterol-containing membranes suggest the dense aggregation of AmB is formed, where the distance between the heptaene groups of the neighboring AmB molecules is relatively small. Meanwhile, the absorbance at 350 nm observed with the ergosterol-containing membrane indicates that the distance between AmB chromophores is larger than those in sterol-free and cholesterol-containing membranes. These results are quite consistent with the REDOR experiments.

Figure 5 further shows that the absorbance at 329 nm in the sterol-free membrane is significantly low as compared with those at 330−345 and 350 nm for respective cholesterol- and ergosterol-containing membranes. The intensity ratio at 329 and 410 nm represents the relative proportion between the monomeric and aggregated forms (*26*), thus indicating lower aggregation level in the sterol-free membrane. A possible explanation for this result is that a significant proportion of AmB molecules escaped from the sterol-free membrane and stayed in an aqueous phase. In fact, AmB as a monomeric form in an aqueous suspension gave rise to a similar triplet absorption band in the longer wavelength region (Supporting Information), which closely resembles that of the sterol-free membrane in Figure 5.

In view of these observations, we attempt to propose a model for AmB−AmB interactions in POPC membranes (Figure 6). Since 14-F is located in the polyhydroxy side of the AmB molecule, the fluorine atom should be segregated from the other assemblies when the AmBs form a barrel-stave assembly (Figure 6). If this type of assembly is composed of AmB molecules with the same orientation as suggested by previous reports (*4, 27*), the REDOR effects from 14-F should be derived largely from head-to-head interaction in an *intra*assembly manner but not from head-to-tail one in an *inter*assembly manner. In cholesterol-containing membrane, the REDOR experiments revealed that AmB forms dense aggregates predominantly with "head-to-head" orientations. Since AmB−POPC interactions were not observed in the cholesterol-containing membrane, the AmB assemblies may be largely phase-separated from surrounding lipid bilayers (Figure 6a). In the ergosterol-containing membrane (Figure 6b), since the distance between AmB molecules was significantly increased and the interaction between AmB and the fatty acyl chains was detected, POPC and ergosterol are plausibly incorporated into AmB assemblies and consequently increase the average distance among AmB molecules. Recently, an IR study disclosed the close vicinity of ergosterol to AmB in the DPPC membrane (*28*). The mixed assembly formed in the ergosterol-containing membrane may possibly act as an ion-permeable channel (Figure 6b).

In conclusion, we examined AmB−AmB bimolecular interactions in bilayer membranes by solid-state NMR using ^{13}C- and ^{19}F-labeled AmB. In cholesterol- or ergosterol-containing POPC membranes, REDOR dephasing effects were observed between the headgroups of AmB molecules, indicating the dominant "head-to-head" interactions in membrane assemblies. The REDOR dephasing effect was significantly reduced in the ergosterol-containing membrane. Additionally, AmB−POPC interactions were detected in the ergosterol-containing membrane. These results suggest that ergosterol increases the distances between AmBs by directly interacting with AmB and facilitates formation of an AmB−sterol−PC assembly, which may correspond to "an active ion channel". To examine the AmB−ergosterol interactions in more detail, solid-state NMR experiments using ^{19}F-labeled AmB and ^{13}C-labeled ergosterol (and vice versa) are currently underway.

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SUPPORTING INFORMATION AVAILABLE

REDOR spectra of labeled AmBs **1** and **2** in the powder state at 30 °C and in ergosterol-containing POPC at −20 °C, REDOR dephasing curves for F−C−F spin systems and for two difference C−F distances, and UV spectrum of AmB in 8% sucrose buffer. This material is available free of charge via the Internet at http://pubs.acs.org.

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