The Formation of Amphotericin B Ion Channels in Lipid Bilayers

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ABSTRACT: The ability of amphotericin B (AmB) to form ion-permeable channels in cholesterol containing lipid bilayers was studied by UV/visible absorbance, circular dichroism, and fluorescence spectroscopy. Stable liposomes composed of distearoylphosphatidylcholine, cholesterol, distearoylphosphatidylglycerol, and AmB were prepared so that a wide range of AmB concentrations in the bilayer could be studied. Singular value decomposition analysis (Henry & Hofrichter, 1992) of the circular dichroism spectra of AmB at different AmB/lipid ratios suggests that AmB exists primarily in only two states in the bilayer, a "monomeric" state and an "aggregated" state. The transition from the "monomeric" to the "aggregated" state begins to occur at a critical concentration of 1 AmB per 1000 lipids in the membrane and coincides with the appearance of channel activity. The data support the recent theoretical conclusions of Weakliem et al. (1995) which predict that pore formation in the lipid bilayer will occur when the drug molecule concentration exceeds a critical value. At this critical concentration, it is calculated that a minimum number of 16 AmB molecules per liposome are required to observe channel activity. The results are consistent with the sterol-dependent AmB channel models proposed by de Kruijff and Demel (1974), Andreoli (1974), and Khutorsky (1992). To further elucidate the effects of sterol on AmB-mediated channel formation, liposomes were prepared with varying ratios of cholesterol and AmB. At cholesterol mole percentages greater than 1, channel activity was observed to occur at AmB concentrations just above the critical value. Previous reports show that cholesterol forms "tail-to-tail" dimers at mole percentages greater than 2 (Harris et al., 1995). This suggests that formation of the bilayer-spanning channels by AmB is initiated most efficiently when the tail-to-tail dimer of cholesterol is present. Although the structural nature of the AmB channel could not be unambiguously determined, these experiments provide further evidence in support of the widely held view that AmB's primary mechanism of killing fungal cells occurs by forming ion-permeable channels.

For over thirty years, the polyene antibiotic amphotericin B (AmB)[1] has been one of the most important agents used to combat systemic fungal infections. In spite of severe side effects such as nephrotoxicity (Bolard et al., 1991; Hamilton-Miller, 1973), anemia (MacGregor et al., 1978), and cardiac arrhythmia caused by hyperkalemia upon rapid infusion of the drug (Craven & Gremillion, 1985; Butler et al., 1965), AmB still remains the drug of choice for the treatment of these life-threatening diseases. The observed side effects caused by AmB have been attributed to the interaction of the drug with mammalian cell membranes. Efforts to reduce AmB's toxicity have focused primarily upon formulating the drug in association with a variety of amphiphilic molecules such as lipids and detergents (Lopez-Berestein et al., 1983; Janoff et al., 1988; Brajtburg et al., 1990; Gruda et al., 1988; Szoka et al., 1987; Adler-Moore & Proffitt, 1993). For example, a 2−8-fold reduction of AmB toxicity was observed for a formulation described by Szoka et al. (1987) in which sterols were added to bind the AmB molecules more effectively in the lipid bilayer. Lopez-Berestein and co-workers (1983) developed a liposomal preparation in which AmB was associated with a negatively charged lipid. Although these preparations demonstrated that the association of AmB with lipid greatly reduces the toxicity of the drug, further refinements were necessary in order to improve the stability and efficacy of liposomal AmB. Adler-Moore and colleagues (Adler-Moore & Proffitt, 1993) combined both cholesterol and negatively charged lipids together with phosphatidylcholine to create AmBisome, a highly stable liposomal AmB preparation. Furthermore, while dramatically reducing the toxicity of AmB, AmBisome still retained the potent antifungal activity of AmB, leading to the successful introduction of the first commercially available liposomal therapeutic.

Details of AmB's actions on both fungal and nonfungal cells are not clearly understood, but it is believed that the interaction of AmB with the lipid components of cellular membranes plays a key role in its activity. Numerous studies have investigated the association of AmB with membranes of various compositions (Bolard et al., 1991; de Kruijff & Demel, 1974; Chen & Bittman, 1977; Van Hoogevest & de

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[1] Abbreviations: AmB, amphotericin B; CD, circular dichroism; SVD, singular value decomposition; SSB, sucrose succinate buffer; PBS, phosphate-buffered saline; HSPC, hydrogenated soy phosphatidylcholine; Chol, cholesterol; DSPG, distearoylphosphatidylglycerol; pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid; $R_{A/L}$, AmB to lipid ratio (mole:mole); F, fluorescence without a pH gradient; $F_{\Delta pH}$, fluorescence with a pH gradient; HPLC, high-performance liquid chromatography; SUV, small unilamellar vesicles.

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Kruijff, 1978; Bolard et al., 1980; Jullien et al., 1990), and it has been postulated that preferential destruction of fungal cells by AmB is due partly to its higher affinity for the ergosterol rich fungal membranes than for the cholesterol-containing membranes typically found in mammalian cells (Archer & Gale, 1975). In support of this view, it has been shown by proton efflux measurements that at least ten times greater concentrations of AmB are necessary to obtain equivalent amounts of membrane activity with cholesterol membranes when compared to ergosterol membranes (Vertut-Croquin et al., 1983). In addition, AmB has a higher binding affinity contant for ergosterol ($K_a = 6.9 \times 10^5$ M^{-1}) than for cholesterol ($K_a = 5.2 \times 10^4$ M^{-1}) (Readio & Bittman, 1982). This suggests that the observed therapeutic efficacy of AmB may be related to a differential preference between sterols found in cell membranes.

It has been postulated that AmB's ability to form ion-permeable membrane channels when it associates with membranes is one of the primary causes of its observed cellular toxicity (Holz & Finkelstein, 1970). The exact molecular architecture of an AmB channel is not known, and different models for the formation and structure of AmB channels have been proposed, including sterol-*independent* formation of channels (Hartsel et al., 1991); sterol-*dependent* formation of AmB channels (de Kruijff & Demel, 1974; Bonilla-Marin et al., 1991); and channel formation by a *sequential mechanism* in which AmB first interacts with phospholipids to form nonaqueous pre-pore intermediates that associate with sterols to form complete aqueous channels (Cohen, 1992). In each of these models, the hydroxyl moieties located along one side of the AmB molecule associate with one another to form a hydrophilic pore. The length of the pore corresponds to one-half of the width of a lipid bilayer or to one monolayer leaflet ($\cong$ 2.5 nm). Estimates of the number of AmB molecules required to form a pore have ranged from 4 to 12 with 8 being the generally accepted number (Andreoli, 1974; de Kruijff & Demel, 1974; Bonilla-Marin et al., 1991; Moreno-Bello et al., 1988).

Although the development of AmBisome (Adler-Moore & Proffitt, 1993) was originally directed toward achieving a more effective antifungal therapeutic, we used this novel formulation to study the structure and function of AmB in lipid membranes. In the past, progress in elucidating the molecular architecture of AmB channel(s) in lipid bilayers had been hindered by the lack of a good model system (Hartsel et al., 1993). By incorporating AmB into a stable liposome whose mean size distributions are under 100 nm (in diameter), we have been able to study AmB's ability to form ion-permeable channels in sterol-containing bilayers. Our findings suggest that the channels form when the ratio of the number of AmB molecules relative to the number of lipids reaches a *critical micelle concentration in the bilayer* of 10^{-3} (Weakliem et al., 1995). In addition, the transition from the ion-impermeable to the ion-permeable state occurs concomitantly with significant spectroscopic changes, indicating that two distinct forms of AmB are present.

MATERIALS AND METHODS

Chemicals. Pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid), was obtained from Molecular Probes, Inc. (Eugene, OR). Sodium chloride, sodium phosphate, sodium succinate, and sucrose were purchased from Mallinckrodt. All chemicals were used without further purification.

Composition of Solutions. Sucrose succinate buffer (SSB) was composed of 9% sucrose/10 mM sodium succinate, pH 5.45, and phosphate-buffered saline (PBS) was composed of 100 mM NaCl, 25 mM phosphate, pH 7.4. A stock solution of 10 mM pyranine was prepared in water. This stock solution was used to prepare the pyranine−buffer solution, which is composed of 2.0 mM pyranine in 9% sucrose (w/v) and 10 mM sodium succinate, pH 5.45.

Liposome Preparation. Lipid powders (NeXstar Pharmaceuticals, Inc., San Dimas, CA) containing hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol), distearoylphosphatidylglycerol (DSPG), and amphotericin B (AmB) were dissolved in chloroform to create stock solutions. The relative ratios of AmB to lipid were varied by pipetting different aliquots of the stock solutions into round-bottom glass tubes. The solvent was then evaporated under a stream of nitrogen gas at 65 °C to form a thin, homogeneous film of lipid at the bottom of each tube. To ensure complete removal of solvent, the tubes were placed under vacuum for at least 8 h. Liposomes were prepared by hydration of the lipid films with 5 mL of SSB and incubation of this mixture for 5 min at 65 °C. The lipid suspension was then probe sonicated (Sonics and Materials, Danbury, CT) at 65 °C for 15 min until a transparent solution was obtained. The liposomes were allowed to cool to room temperature before filtration through 0.22 μm pore size membranes (Millipore Corp., Bedford, MA). For samples used in the fluorescence assay, lipid films were hydrated with 2 mM pyranine dye in SSB before sonication. Unencapsulated pyranine was removed by chromatography on a Sephadex G-50-150 column pre-equilibrated with PBS. Concentrations of the lipids and AmB were determined by HPLC. The liposomes were sized by dynamic laser light scattering on a Microtrac ultrafine particle analyzer (UPA; Leeds and Northrup, North Wales, PA). Their size distributions were unimodal with mean diameters ranging from 31 to 81 nm.

Absorption Spectroscopy. Absorbance spectra were taken with a Hitachi U-2000 spectrophotometer (San Jose, CA) at room temperature. The spectroscopic data were recorded at 0.1 nm intervals over the wavelength range 300−440 nm. All samples were diluted to a constant AmB concentration of 0.016 mg/mL with PBS except for the 0.03 mol % AmB samples which were prepared in SSB. The total lipid concentrations were in the range of 11−50 mg/mL. All measurements were taken in 1.0 cm pathlength quartz cuvettes.

Circular Dichroism Spectroscopy. CD spectra were measured using a Jasco J-720 CD spectrometer (Boston, MA) over the range 250−450 nm with 5 mm pathlength cuvettes. The time constant was set at 0.5, and the scan speed was at 100 nm/min. Only one scan was taken of each sample. All samples were diluted in PBS except those samples containing 0.1−0.03 mol % AmB, which were diluted to a constant AmB concentration (0.025 mg/mL) in SSB with the final lipid concentrations in the range of 11−50 mg/mL. Measurements were collected at room temperature.

Fluorometric Analysis. Fluorescence of all samples was monitored with a Hitachi F-4500 fluorescence spectrophotometer (San Jose, CA) at room temperature. The excitation wavelength was set at 454 nm with a slit width setting at 2.5 nm, and the emission wavelength was 513 nm with a slit width set at 5.0 nm. All measurements were taken over a 5 min period. The change in fluorescence without a pH gradient (F) was measured by diluting 0.5 mL of each sample

with 0.5 mL of PBS, pH 7.4, buffer. The fluorescence change in the presence of a pH gradient ($F_{\Delta pH}$) was measured by adding 0.5 mL of PBS at pH 6.8 to 0.5 mL of a liposome sample (total lipid concentration, 12 mg/mL).

For the one-sided addition experiments, liposomes containing 2 mM pyranine in SSB were prepared without AmB. Different AmB stock solutions in DMSO were prepared ranging in concentration from 0.25 to 40 mM. Aliquots of the AmB stock solutions were added to the pyranine containing liposomes to give different $R_{A/L}$. Fluorescence changes were measured at 5 min in the presence (pH 7.4−6.8) and absence of a pH gradient. Liposomes prepared both with and without cholesterol were tested for channel formation upon one-sided addition of AmB.

To study the effect of cholesterol on channel formation, liposomes were prepared with AmB and varying mole percentages of cholesterol (0.5−11%). In these samples, a larger pH gradient (5.5−7.4) was used to increase the sensitivity of the assay. The changes in fluorescence of the samples were measured in the same manner as described previously.

Singular Value Decomposition Analysis. To help ascertain the number of distinct forms of AmB present over a range of conditions, the CD spectral data were evaluated by singular value decomposition analysis (SVD). In this analysis, several CD spectra were obtained over the same m wavelengths ($m = 2001$ wavelengths from 250 to 450 nm). The spectra were grouped into series taken under identical conditions except for the changing of a single variable (the condition variables studied here are the total liposome concentration and concentration of AmB in the liposomal formulation). In the generalized case, the total spectrum consists of a sum of the overlapping spectra of several components. In our case, the component spectra are distinguished by the environment of AmB, leading to aggregate-like or monomer-like spectra. The relative mixture of components can change as the condition variable changes. By employing an analytic method such as SVD to separate the component spectra, the knowledge of the number and nature of the individual components can be extracted.

For analysis, the n spectra from a series of samples were assembled into an m by n matrix, **A**. Note that the matrix **A** contains the entire information content of the spectral series being analyzed. A SVD of this matrix guides the analyst in assessing the minimum number of *basis spectra* which can be recombined linearly in appropriate proportions to best (in a least-squares sense) reconstruct the entire series of spectra, i.e., the matrix **A**. If each spectral species varies in concentration differently as a function of the condition variable, the SVD analysis can separate each distinct chemical species as a distinct basis spectrum. If two or more components vary in identical proportions with the condition variable, the SVD yields one basis spectrum for this combination of components. The singular values of the matrix are unitless values whose magnitude determines (scales) the relative contribution of each basis spectrum. The use of SVD-based data treatment is detailed in Henry and Hofrichter (1992) and Schrager and Hendler (1982). For related treatments, also see Chapados and Trudel (1993) and Chapados et al. (1994). Here we present the details relevant to our application of SVD, which follows most closely Henry and Hofrichter (1992).

SVD analysis was applied to the CD spectral data taken over a range of conditions. The SVD computes $\mathbf{A} = \mathbf{USV}^T$ where **U** is an m by n matrix of basis spectra, **S** is a diagonal matrix of singular values, and $\mathbf{V}^T$ is an n by n matrix of amplitudes. **S** is arranged such that $\mathbf{S} = \mathrm{diag}(s_1, s_2, ..., s_n)$ where $s_1 \geq s_2 \geq ... \geq s_n \geq 0$. The columns of **U** are orthonormal, as are the columns (and rows) of $\mathbf{V}^T$. The ith column of $\mathbf{V}^T$ describes the relative linear contribution of each basis spectrum needed to reproduce the ith original spectrum (i.e., the spectrum corresponding to the condition variable having its ith value in the series). The singular values scale the contribution of each basis spectrum.

If the data were noise free with exactly $k < n$ linearly independent species combining linearly in different proportions to create the spectra for each value of the condition variable, then there would be exactly k non-zero, positive singular values and $n - k$ zero singular values. In the presence of noise an assessment of the number of contributing chemical species can still be made based on the relative magnitudes of the singular values and the autocorrelations of the basis or amplitude vectors (Henry & Hofrichter, 1992; Shrager & Hendler, 1982). Typically, a large gap in the magnitude of the singular values can indicate the transition from valid components to noise components.

The first SVD basis spectrum and its corresponding amplitude vector (corresponding to the largest singular value, s_1) provide the best (in a least-squares sense) single-component approximation to the original data matrix, **A**. That is, if $\mathbf{S}' = \mathrm{diag}(\mathbf{s_1}, 0, ...)$ where $\mathbf{s_1}$ is the first singular value and $\mathbf{S}'$ represents a diagonal matrix with all other singular values set to 0, then $\mathbf{A} \approx \mathbf{A}' = \mathbf{US}'\mathbf{V}^T$. The inclusion of successive basis spectra results in increasingly better approximations to the data. For example, the second singular value and second amplitude vector indicate how the second basis spectrum can be added to the first basis spectrum at each value of the condition variable to improve the fit to the complete data matrix, **A**. If $\mathbf{S}'' = \mathrm{diag}(\mathbf{s_1}, \mathbf{s_2}, 0, ...)$ and $\mathbf{A}'' = \mathbf{US}''\mathbf{V}^T$, then $||\mathbf{A}'' - \mathbf{A}||_2 \leq ||\mathbf{A}' - \mathbf{A}||_2$. The SVD basis spectra are in general, not necessarily the spectra of distinct chemical species, but represent linear combinations of chemical species that give successively better approximations to the complete set of data. For SVD mathematical and computational details, see Golub and vanLoan (1989) and the software of Dongarra (1994).

RESULTS

Stability of AmB in Lipid Bilayers

The amphiphilic nature of AmB makes it sparingly soluble in aqueous solution. For this reason, it is first necessary to demonstrate that AmB is retained in the lipid bilayers of the liposomes over the concentration ranges studied. Since AmB possesses a conjugated heptaene backbone which gives it distinctive spectral properties that are remarkably sensitive to changes in its local environment (Hemenger, 1979; Hemenger et al., 1983; Jullien et al., 1988; Tancrède et al., 1990), the stability of the drug in the lipid bilayer could be monitored by UV/visible and CD spectroscopy. Shown in Figure 1A is a series of UV/visible absorbance spectra of AmB incorporated into liposomes at a constant ratio of AmB to lipid ($R_{A/L}$) and different dilutions. For any particular $R_{A/L}$, the absorbance spectrum does not undergo significant changes except for the overall intensity. To further illustrate this point, measurement of the absorbance of each dilution at 415 nm reveals a monotonic relationship dependent upon the concentration of AmB (Figure 1B). Absorbance spectral

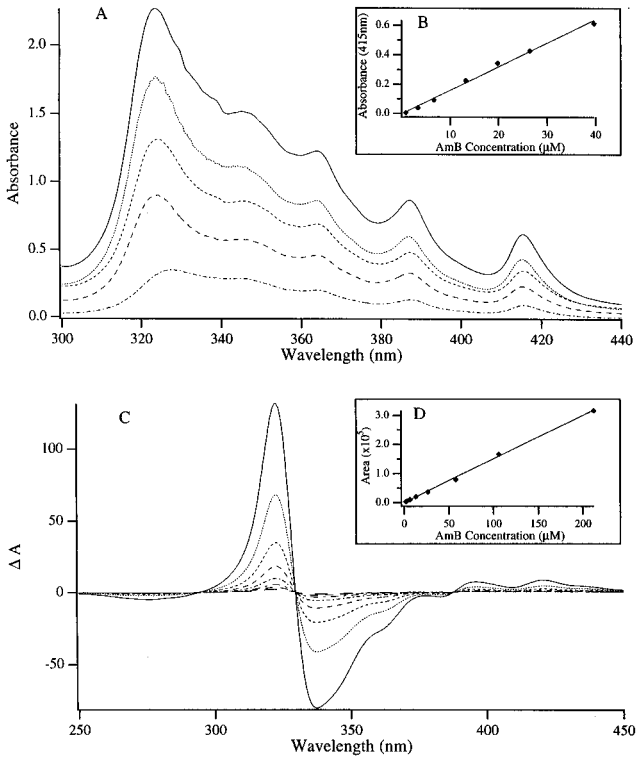

FIGURE 1: Stability of AmB in lipid vesicles. (A) Effect of dilution upon the UV/visible absorbance spectrum of liposomal AmB at constant $R_{A/L}$. Only one dilution series is shown (AmB/lipid = 10^{-1}). The concentrations of AmB (in μM) displayed are 40 (−), 26 (•••), 20 (- - -), 13 (−−−), and 6.6 (−•−). (B) Inset: Plot illustrating the linear relationship between the absorbance changes and the AmB concentration. The absorbance of each sample at 415 nm was measured and plotted against the total AmB concentration. (C) Circular dichroism spectra of liposomal AmB dilutions at a constant $R_{A/L}$ (AmB/lipid = 10^{-1}). This liposomal AmB sample was diluted to the following AmB concentrations (in μM): 200 (—), 100 (•••), 50 (- - -), 26 (−−−), 13 (−•−), 6.6 (-−-), 3.3 (−−−), and 1.6 (−•−). (D) Inset: Plot showing the linear relationship between the area of the dichroic doublet and the AmB concentration.

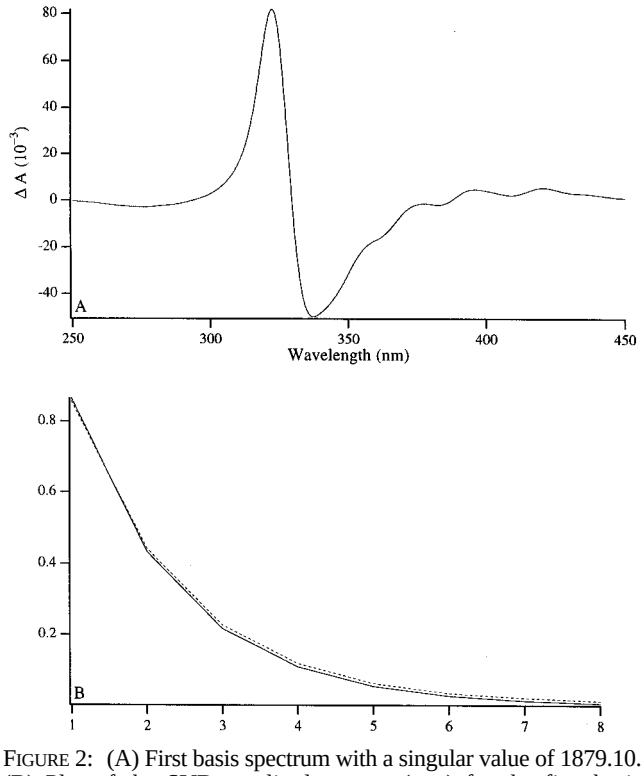

FIGURE 2: (A) First basis spectrum with a singular value of 1879.10. (B) Plot of the SVD amplitude vector (- - -) for the first basis spectrum (corresponding to singular value 1879.10) and a set of normalized concentrations (—).

Table 1: Singular Values Constructed from the 250−450 nm CD Spectra of Liposomal AmB Solutions

AmB concentration (μg/mL)	singular values
196	1879
97.9	25.0
49.0	13.0
24.5	1.88
12.2	1.64
6.12	1.52
3.06	1.39
1.52	1.02

data obtained at other $R_{A/L}$ values (data not shown) exhibit a similar linearity upon dilution of the liposomes. At shorter wavelengths, slight distortions were observed because of the scattering of light from the liposomes. The consistency of the absorbance spectra even at high dilution implies that the incorporation of AmB in the liposomal bilayer is very stable.

Consistent with the absorbance spectra, the circular dichroism spectra of identical samples confirms the stability of AmB in the liposomes (Figure 1C). Dilution of the liposomes over 100 fold does not significantly alter the observed spectra. A plot of the area of the dichroic doublet and its relationship to each dilution (Figure 1D) again shows the linear relationship expected for a stable liposomal preparation of AmB. As an independent confirmation of this interpretation of the fitted peak areas, a SVD analysis of the dilution data was performed. The singular values obtained for the 2001 by 8 matrix constructed from the 250−450 nm CD spectra of liposome solutions with eight concentrations are tabulated in Table 1. Examination of the singular values shows that the first value is significantly larger than all of the other values suggesting that only one species of AmB exists throughout the range of dilutions measured. The first basis spectrum corresponding to the first singular value is shown in Figure 2A. Not surprisingly, the basis spectrum looks to be identical to the observed spectrum. In Figure 2B, a plot of the SVD amplitude vector for the first basis spectrum is plotted along with a set of normalized concentrations. Analysis of the CD spectra by SVD shows that dilution of the liposomes is well described by a single-component spectrum indicating that another species (e.g., aqueous) is not present.

Interaction of AmB within Lipid Bilayers

Since AmB could be stably incorporated into a lipid environment, its structural properties could be further examined as a function of AmB concentration within the liposome. Several samples were prepared with varying ratios of AmB to lipid ($R_{A/L}$). Each sample was diluted to the same total AmB solution concentration of 16 μg/mL (17 μM). The absorbance and CD spectra of each sample were then taken.

Several representative absorbance spectra are presented in Figure 3A. At increasing $R_{A/L}$ ($>10^{-2}$) a large absorbance peak at 325 nm dominates the absorbance spectrum. This peak has been attributed to an "aggregated" state of AmB (Bolard et al., 1991; Ernst et al., 1981). There are also three minor peaks with absorbance maxima at 365, 388, and 415 nm. As the $R_{A/L}$ decreases, the 325 nm peak intensity decreases and the three minor peak intensities, characteristic

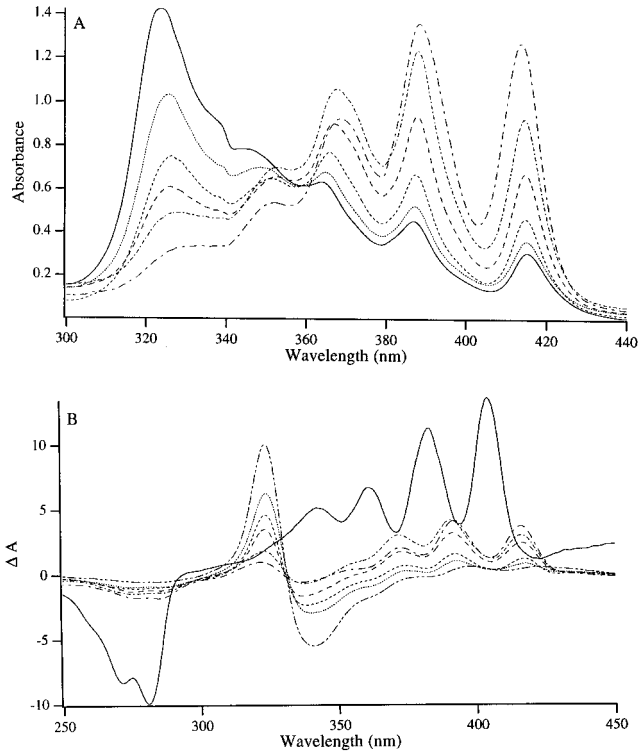

FIGURE 3: Absorbance and circular dichroism spectra of liposomal AmB at different ratios of AmB/lipid. (A) Absorbance spectra. Six representative spectra are displayed at constant AmB concentrations (17 μM) but with the following different relative AmB/lipid ratios (mol/mol): 0.10 (—), 0.05 (•••), 0.03 (- - -), 0.01 (— — —), 0.006 (—•—), and 0.002 (— — —). Samples were prepared by diluting each liposome preparation in PBS to obtain a constant AmB concentration (17 μM) except for the 0.03 mol % AmB sample, which was diluted in SSB to 17 μM. (B) Circular dichroism spectra. The changes in the differential dichroic absorption (ΔA) are displayed for six representative samples at the following $R_{A/L}$ values: 0.10 (—••—), 0.05 (•••), 0.03 (- - -), 0.01 (— — —), 0.006 (—•—), 0.002 (— — —). Also shown is the reference monomeric spectrum of 300 μM AmB in methanol (—). Samples were prepared by dilution of each vesicle preparation in PBS to a constant AmB concentration (34 μM) except for the 0.1−0.03 mol % samples which were diluted in SSB to 34 μM.

of "monomeric" AmB, increase. It should be noted that an isosbestic point was not observed with these data probably because the liposomes scatter more light at the shorter wavelengths.

The CD spectra also undergo significant spectral changes at different $R_{A/L}$. Figure 3B shows the CD spectra of four representative ratios along with the reference spectrum of AmB monomer in methanol. Monomeric AmB exhibits three positive bands at 370, 390, and 415 nm. As the $R_{A/L}$ is increased, the differential dichroic absorbance (ΔA) intensity changes dramatically. The 370 and 390 nm bands disappear to be replaced by several faint bands from 340 to 390 nm while the 415 nm band decreases and shifts to about 423 nm. This is in agreement with results from other studies which have reported positive CD bands at both 415 and 423 nm (Bolard et al., 1980; Ernst et al., 1981). Concurrent with the changes in the monomeric bands, an intense dichroic doublet begins to appear from 320 to 360 nm which is centered at 340 nm. Qualitatively, the appearance of the dichroic doublet is consistent with the formation of an aggregated state of AmB in the bilayer (Jullien et al., 1988; Hemenger et al., 1983). For reference, a spectrum of AmB monomer in methanol has been included with the $R_{A/L}$ data (300 μM). The low $R_{A/L}$ liposome spectra are similar to the monomeric AmB spectrum except that the monomer bands are red-shifted by 10 nm in the lipid environment. Visual inspection of the spectra suggests that the relative amounts of at least two AmB species contribute to the changing shape of the spectra with $R_{A/L}$. In addition, the CD spectra do not appear to be distorted by scattering of light from the liposomes at the lower wavelengths. For this reason, the CD spectra were subjected to analysis by SVD.

Table 2: AmB Concentration in Lipid, Singular Values, and Amplitude Vector Autocorrelations for the SVD of A

trial	$R_{A/L}$ = AmB/lipid (mol:mol)	singular value	amplitude autocorrelation
1	1×10^{-1}	302.86	0.92
2	8×10^{-2}	181.62	0.92
3	5×10^{-2}	44.11	0.58
4	4×10^{-2}	15.35	0.56
5	3×10^{-2}	10.83	0.12
6	3×10^{-2}	7.36	0.65
7	2×10^{-2}	5.14	−0.17
8	1.6×10^{-2}	4.60	−0.06
9	1×10^{-3}	3.68	−0.04
10	6×10^{-3}	3.08	−0.07
11	4×10^{-3}	2.20	0.13
12	3×10^{-3}	1.91	−0.43
13	2×10^{-3}	1.21	−0.72
14	1.6×10^{-3}	1.15	−0.30
15	1.3×10^{-3}	0.98	−0.39
16	1.1×10^{-3}	0.90	−0.44
17	8×10^{-4}	0.80	−0.12
18	5×10^{-4}	0.65	−0.22
19	3×10^{-4}	0.54	−0.78
20		0.43	−0.16

To confirm the qualitative assessment that only two species of AmB are present in the liposome, a SVD analysis of the ratio spectra was performed by assembling the 20 spectra, each with 2001 wavelengths, into a 2001 by 20 matrix, **A**. The results of the analysis are tabulated in Table 2. The first two singular values and autocorrelations calculated by SVD dominate over the remaining values. This result clearly supports the suggestion that AmB exists in two chemically distinguishable environments. Conclusive evidence in this data for more than two states for AmB in these liposomes could not be found. Analysis of the first two basis spectra and their corresponding amplitude vectors shows that changes in the spectra are described by increasing the amount of monomer-like species as $R_{A/L}$ decreases. This can be understood as follows. The first SVD basis spectrum and its corresponding amplitude vector provide the best (in a least-squares sense) single-component approximation to the original data matrix, **A**. The second singular value and second amplitude vector indicate how the second basis spectrum can be added to the first basis spectrum at each $R_{A/L}$ to improve the fit to the complete data matrix. In this case, examination of the second basis spectrum shows that it possesses most of the characteristics of a "monomer" AmB spectrum. Thus, the SVD analysis indicates that the ratio spectra can be described most completely by a base spectrum reminiscent of "aggregated" AmB (U_1) combined in changing proportion with a monomer-like species (U_2; see Figure 4A). The amplitude vectors for these two basis spectra are shown in Figure 4B. Note that the final trial contained no AmB; this is consistent with the near-zero amplitudes for both basis spectra in this trial. The conclusion of two chemical environments for AmB from this data is based on the singular values and amplitude autocorrelations which show a clear break after the second component. See the Discussion for further consideration of this conclusion.

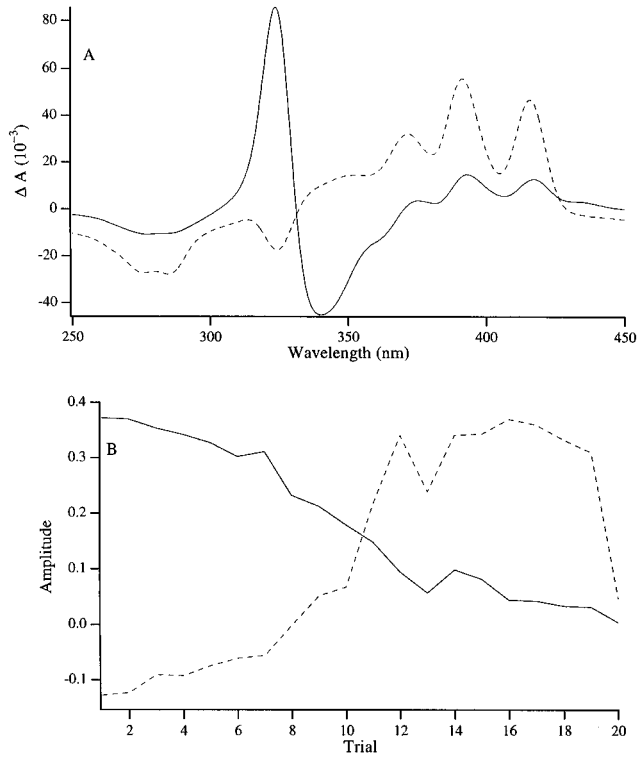

FIGURE 4: (A) Representative basis spectra of "aggregated" (U_1; —) and "monomeric" (U_2; - - -) AmB species. (B) Amplitude vectors for these two basis spectra.

As a measure of similarity between the basis spectra and the reference methanol−monomeric AmB spectrum, the reference spectrum was normalized and its dot product with the basis spectra was taken. The first basis spectrum has an overlap of 0.07 with the reference monomer spectrum. In contrast, the second basis spectrum has a much higher overlap with the reference (0.74). By red shifting the reference spectrum 9.7 nm, the overlap can be improved to 0.91, lending further support to the chemical interpretation of the second SVD spectrum as a red-shifted monomer spectrum.

Ion Channel Formation by AmB

The formation of ion-permeable channels by AmB was monitored by measuring the changes in the fluorescence of the encapsulated pH sensitive probe, pyranine, when a pH gradient was applied across the lipid bilayer. Pyranine possesses several advantageous characteristics including a high fluorescence quantum yield, good water solubility, photostability, and an emission maximum (510 nm) greater than 450 nm which minimizes interference from AmB absorption. The fluorescence intensity of the probe decreases linearly with decreasing pH in the range 5−7 (Straubinger et al., 1990), and, because a pH gradient is created across the liposomal bilayer, the presence of ion channels can be observed by following the changes in fluorescence as the gradient is dissipated (Whyte et al., 1989). The rate of fluorescence quenching is proportional to the number of AmB channels present in each liposome sample in the linear region. Samples containing different $R_{A/L}$ were prepared with a pH gradient across the liposomal bilayer. The change in fluorescence without a pH gradient (F) was measured by diluting 0.5 mL of each AmB liposome sample (pH 7.4) with 0.5 mL of PBS at pH 7.4 and observing the fluorescence after 5 min. The gradient fluorescence ($F_{\Delta pH}$) was obtained

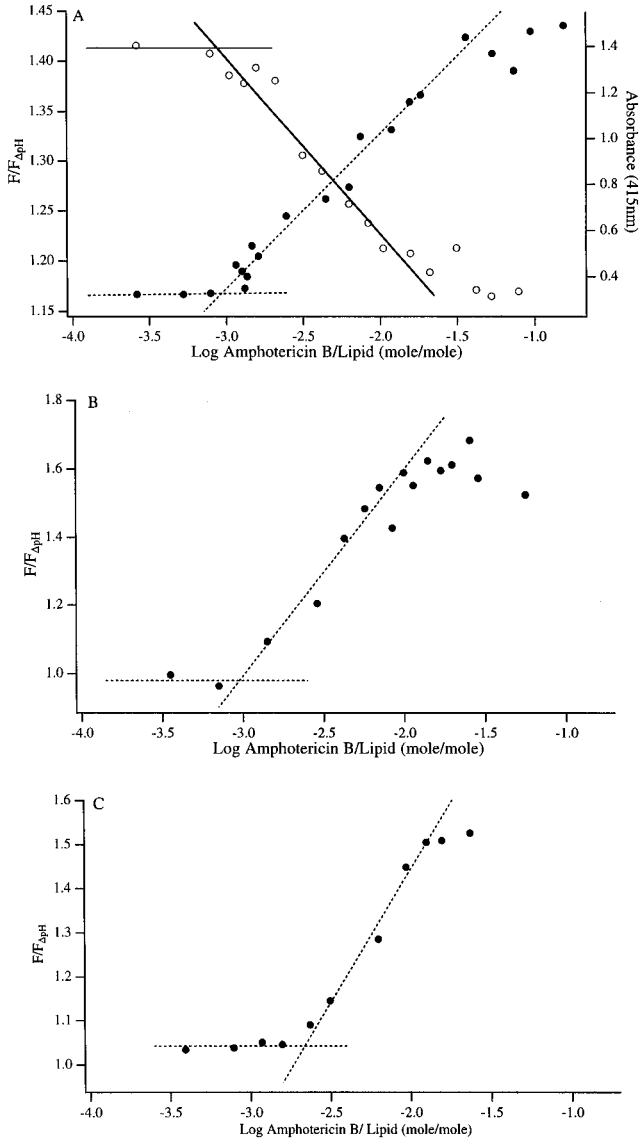

FIGURE 5: Formation and activity of AmB channels in lipid bilayers. Shown are the relative changes in fluorescence of the pH sensitive dye, pyranine for AmB liposomes with different $R_{A/L}$. The liposomes were prepared as described in the Materials and Methods section. Fluorescence changes were measured at 5 min in the presence and absence of a pH gradient. The quantity $F/F_{\Delta pH}$ representing the relative fluorescence change due to channel activity was calculated for each AmB/lipid ratio. The dashed lines highlight extrapolation to the point at which channel activity is first observed. (A) Cholesterol-containing liposomes prepared in the presence of AmB. Channel activity is first observed at 10^{-3} AmB/lipid (solid circles with dashed lines). For comparison, the change from the "monomeric" form to the "aggregated" form is shown by following the absorbance change at 415 nm (open circles with solid lines). Note that the points of inflection for the initial channel activity and the formation of "aggregate" structure occur at very similar $R_{A/L}$. (B) Cholesterol-containing liposomes prepared without AmB. AmB was added to the liposomes as a DMSO solution. Channel activity is first observed at 10^{-3} AmB/lipid. (C) Liposomes prepared without AmB and cholesterol. AmB was added to the liposomes as a DMSO solution. Channel activity is first observed at 2×10^{-3} AmB/lipid.

by adding 0.5 mL of PBS at pH 6.8 to 0.5 mL of the AmB liposome sample and measuring the fluorescence at 5 min. The quantity $F/F_{\Delta pH}$ representing the relative fluorescence change due to channel activity was calculated for each $R_{A/L}$ (Figure 5). The equilibration of the pH across the lipid bilayer provides strong evidence that AmB is forming ion channels in the liposome. It was observed that the changes

Table 3: Summary of the Physical Characterization of Amphotericin B Liposomes by Dynamic Light Scattering[a]

av diameter (nm)[b]	total surface area (Å^2)	av headgroup size (Å^2)[c]	av no. of lipids/liposome
46.3 ± 16.1	1.11×10^6	69	1.61×10^4

[a] The surface areas of the inner and outer leaflets of the bilayer were calculated based upon the mean diameter of 46.3 nm and a 4.5 nm bilayer thickness and assuming spherical lipid vesicles. The average surface area occupied by each lipid molecule was then calculated for 2:1:0.8 HSPC:Chol:DSPG using surface area values for individual lipids adapted from Jain (1988), and the number of lipids in a single liposome was computed. Extrapolation of the data presented in Figure 4 gives the ratio of AmB/lipid at which channel activity first occurs. [b] $n = 16$. [c] Values adapted from Jain (1988).

in fluorescence were extremely rapid at the higher $R_{A/L}$ values ($10^{-1}-10^{-2}$) because the pH gradient was dissipated much faster than the samples near the point where channel activity first occurs ($R_{A/L} = 10^{-3}$). Concurrent with the onset of channel activity, the absorbance at 415 nm begins to decrease, indicating that AmB begins to form an aggregated structure.

Having established that AmB in the presence of lipid can be made to form stable ion-permeable channels, we extrapolated the fluorescence data presented in Figure 5 to determine the minimum number of AmB molecules required to form channels. As shown in Figure 5A, at the point where channel activity is first observed, the $R_{A/L}$ is approximately 1×10^{-3} (1000 lipids/AmB), in agreement with previous reports (Whyte et al., 1989). From the values listed in Table 3 for the average size of the liposomes and the average surface area occupied by each lipid (Jain, 1988) to the total surface area, the number of lipids per liposome was calculated assuming a bilayer thickness of 4.5 nm (Jain, 1988). With the number of lipids per liposome calculated to be approximately 1.6×10^4, the minimum number of AmB molecules per liposome required to observe channel activity is 16.

As a control, AmB was dissolved in DMSO and added at different $R_{A/L}$ to pyranine containing liposomes which did not contain AmB. These liposome samples were prepared with a pH gradient, and the formation of ion channels was monitored by following the changes in fluorescence intensity over a period of 5 min. In comparison to the data obtained for the liposomes prepared *in the presence of AmB*, no significant differences in the onset of channel activity were observed to occur when AmB was added to cholesterol containing liposomes *prepared without AmB* (Figure 5B). However, when AmB was added to liposomes *lacking both cholesterol and AmB*, induction of channel activity did not occur until the $R_{A/L}$ reached a value of 2×10^{-3} (500 lipids/AmB; Figure 5C). This result confirms previous reports by others (Whyte et al., 1989; Wolf & Hartsel, 1995) that AmB will form pores or channels in sterol free lipid bilayers but suggests that in the presence of sterols AmB forms channels more efficiently.

Using the results from the AmB addition studies as a guide, liposomes containing AmB at a $R_{A/L}$ of 2×10^{-3} were prepared with different mole percentages of cholesterol (Figure 6). At this value, minimal channel formation is expected to occur in the absence of sterols, yet channel activity should be observed when the concentration of cholesterol exceeds the critical value necessary for AmB to organize itself into a sterol-dependent channel structure. To

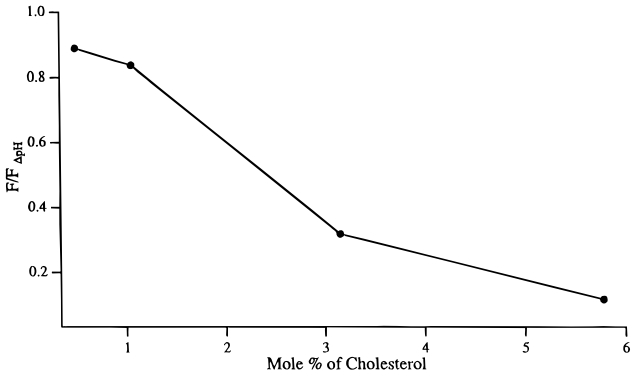

FIGURE 6: Formation and activity of AmB channels in lipid bilayers containing different mole percentages of cholesterol. Pyranine-encapsulated liposomes were prepared containing different mole percentages of cholesterol without AmB. AmB was added as a DMSO solution to the liposomes to give a final AmB/lipid ratio 2×10^{-3}. Fluorescence changes were measured at 5 min in the presence and absence of a pH gradient (pH 5.5−7.4).

increase the sensitivity of the assay, the pH gradient used to monitor channel activity was 5.5−7.4. The results show that for mol % cholesterol amounts less than 1, no channel activity is observed. At values greater than 3 mol % cholesterol, AmB begins to form channels in the liposomes. In addition, the liposomes were also found to be more unstable with less cholesterol; aggregation and possibly, destabilization of the lipid bilayer occurred over a short period of time after preparation. These results suggest that sterols do affect AmB's interactions with lipid bilayers.

DISCUSSION

AmB Stably Associates within Lipid Bilayers Containing Cholesterol and DSPG

AmB has very characteristic spectral properties when solubilized in organic solvents or with detergents (Bolard et al., 1980; Ernst et al., 1981; Brittain, 1994). In the presence of lipid, however, it has been difficult to obtain spectra comparable to the solution spectra (Bolard et al., 1980) because of complex equilibria between the membrane-bound forms of AmB (Vertut-Croquin et al., 1983; Jullien et al., 1988; Witzke & Bittman, 1984; Legrand et al., 1992), self-aggregation of AmB (Bolard et al., 1991; Legrand et al., 1992), and the partial aqueous solubility of the drug at low concentrations (Jullien et al., 1990; Tancrède et al., 1990). By preparing AmB in a stable liposomal form, the changes in the absorbance and circular dichroism (CD) spectra due to the specific interactions of individual AmB molecules with each other and with the surrounding lipids could be reliably monitored.

The stability of AmB incorporated into a cholesterol-containing lipid bilayer was evaluated by measuring the absorbance and CD changes of samples containing different AmB concentrations at constant AmB/lipid ratios ($R_{A/L}$). As shown in Figure 1, absorbance and CD spectra at a $R_{A/L}$ of 10^{-1} do not change appreciably over large concentration ranges. The amplitudes decrease monotonically as the liposomes are diluted, but the spectra retain their shapes. The clear dominance of the first singular value, and the match between the amplitude vector of the primary SVD basis spectrum (Figure 2), and the experimental AmB concentrations indicate that the series of dilution spectra are well-represented by a single chemical system which varies in direct proportion to the total AmB concentration of each

sample. The CD spectrum of AmB in water is very different from that observed in a lipid environment (Jullien et al., 1988). Since an equilibrium between AmB in the liposome and possible aqueous AmB would lead to different ratios of liposomal and aqueous AmB at different total AmB concentrations, such an equilibrium would lead to qualitative changes in the CD spectra. This in turn would lead to two or more dominant basis spectra (large singular values) in the SVD analysis. *Since this is not observed*, we conclude that AmB is stably integrated in the liposomes with respect to the aqueous solvent. Taken together, these results indicate that over a wide range in concentrations AmB does not dissociate from the liposomes in response to an equilibrium with the aqueous environment as previously reported to occur by investigators using other model liposome systems (Bolard et al., 1991; Chen & Bittman, 1977; Witzke & Bittman, 1984; Legrand et al., 1992). Instead, AmB remains firmly associated with the lipid bilayer and hence, any changes in the aggregation state of AmB can be attributed to association/dissociation processes, i.e., "*micellization*" within the lipid bilayer (Weakliem et al., 1995).

AmB Associates in Lipid Bilayers to Form Ion-Permeable Channels

In spite of ambiguities associated with much of the reported data regarding the structure of the AmB channel and its relevance to the mechanism of action, several models have emerged based upon AmB's observed interactions with lipid bilayers [reviewed in Hartsel et al. (1993)]. The most popular model is the classic sterol-*dependent* pore model proposed by several groups (Holz & Finkelstein, 1970; Andreoli, 1974; de Kruijff & Demel, 1974). In this model, sterols play a central role in AmB channel formation by acting as spacers between AmB molecules. Association of a number (8−12) of AmB−cholesterol complexes such that the hydrophilic regions assemble into a cylindrical pore long enough to span one leaflet of the bilayer (i.e., one monolayer) form the basic unit necessary for channel activity. While some results indicate that a single pore can span the bilayer because of "compression" of the lipids down to the width of a monolayer by interdigitating their alkyl chains (Marty & Finkelstein, 1975; Janoff et al., 1988), other evidence suggests that two pores can "dimerize" in the bilayer to form a full-length channel (Andreoli, 1974; de Kruijff & Demel, 1974). Although these models provide a plausible explanation for AmB's interactions with lipid bilayers, there have been enough conflicting results reported to warrant consideration of other possibilities for the structure of the AmB channel. For example, sterol-*independent* channel structures have been proposed by several groups (Dennis et al., 1970; Whyte et al., 1989; Wolf & Hartsel, 1995) while others suggest that AmB may do nothing more than create defects in the membrane (Hartsel et al., 1991). Some studies suggest that the aggregation state of AmB *before it interacts* with the bilayer may be important (Bolard et al., 1991) or that AmB can undergo rearrangements from one type of channel structure to a presumably more stable one once it is in the bilayer (Cohen, 1992). It thus seems likely that AmB, depending upon the specific conditions, possesses several distinct mechanisms of channel activity.

To better understand the nature of AmB's interactions with membranes and the parameters involved in the formation and structure of ion channels, liposomal AmB samples containing cholesterol were prepared and their channel activity followed by encapsulating a pH sensitive fluorescent dye. When a pH gradient is imposed across the bilayer, AmB channels are detected as changes in fluorescence. Rapid equilibration of the pH gradient provides clear evidence that ions are passing freely in and out of the liposomes. The equilibration of the pH was measured as a function of $R_{A/L}$, and it was found that discernible channel activity begins to occur at $R_{A/L}$ values of approximately 1×10^{-3}. As shown in Figure 5, channel formation proceeds from $R_{A/L} = 1 \times 10^{-3}$ up to values of 1×10^{-2} at which point the equilibration of the pH gradient begins to occur in less than a second (unpublished observations). Extrapolation of the data to the point where channel activity first occurs yields a minimum number of 16 AmB molecules required for any liposome to contain a channel. It is interesting to note that the data support previously proposed models for the AmB channel (de Kruiff & Demel, 1974) in which eight AmB molecules associate with eight sterols to form a pore, and two pores further associate to form a bilayer-spanning channel. However, addition of AmB in DMSO to cholesterol-containing liposomes without drug (i.e., no AmB in the bilayer), exhibits essentially identical changes in fluorescence (Figure 5), indicating that the calculated number of AmB molecules required to form a channel may be coincidentally close to the number predicted by the proposed model. It is thus difficult to make any specific conclusions regarding the exact molecular architecture of the AmB channel in the presence of sterols. Although a specific structure of an AmB channel cannot be established from this study, further investigation using this system should provide some interesting insights into the structural nature of AmB's interactions within lipid bilayers.

Sterols have been purported to play a role in AmB's potent antifungal effect as well as its extremely toxic profile (Brajtburg et al., 1990; Bolard et al., 1991). These effects have been attributed primarily to AmB's ability to associate with sterols in membranes to form ion channels. To test the dependence of AmB channel formation on the presence of sterols, AmB was added to SUVs prepared with or without cholesterol. It was found that AmB promoted channel activity in the absence of cholesterol, confirming previous observations that cholesterol is not absolutely necessary for channel formation (Hartsel et al., 1991) and may be related to the observation that the highly curved bilayer found in SUVs is more susceptible to insertion of an amphiphilic molecule like AmB (Whyte et al., 1989; Wolf & Hartsel, 1995). However, AmB-mediated channel activity in cholesterol-containing SUVs was observed to occur at lower ratios of AmB to lipid, suggesting that AmB will preferentially interact with sterols in lipid bilayers. These results suggest a simple solution to the controversy over the role of sterols in AmB's interactions with lipid bilayers: in the absence of sterols, AmB behaves like any other amphiphilic molecule in destabilizing lipid bilayers but, in the presence of sterols, AmB's structure allows it to assemble more effectively in lipid bilayers to form ion channels.

The effect of cholesterol on channel activity was evaluated by preparing liposomes containing AmB at a $R_{A/L}$ value of 2×10^{-3} with different mol % of cholesterol. At up to 1 mol % cholesterol, no channel activity was observed. At values greater than 1 mol % cholesterol, channels begin to appear. Under these conditions, it is estimated that 10 times as many cholesterol molecules as AmB molecules are present before assembly into ion channels occurs. Although this

result does not provide clear evidence in favor of a direct association of AmB and sterol in a 1:1 mole ratio, it does provide further confirmation that AmB does have a preferential affinity for sterols in the bilayer. The dependence of AmB channel activity on cholesterol concentration could also be related to the formation of microdomains of cholesterol in the lipid bilayer (Harris et al., 1995). As reported by Harris et al. (1995), at greater than 2 mol %, cholesterol begins to form "tail-to-tail" dimers which span the bilayer. These tail-to-tail dimers of cholesterol could possibly be important in facilitating the organization of AmB into bilayer-spanning channel structures similar to those proposed previously by Andreoli (1974) and de Kruijff and Demel (1974).

Relationship of Spectral Changes to the Interactions of AmB in Lipid Bilayers

AmB possesses a conjugated heptaene backbone which gives it distinctive spectral properties. By following the spectral changes caused by the relative orientation of the transition dipoles of the heptaene in a lipid environment and correlating those changes to observed channel activity, information about the structural nature of the channel may be obtained. When AmB is incorporated into liposomes at different $R_{A/L}$, dramatic shifts in the spectra become apparent. However, it is difficult to make accurate structural interpretations based on the absorbance increases at 325 nm and the appearance of the dichroic doublet. Although these spectral features almost certainly indicate the presence of aggregated AmB (Ernst et al., 1981; Hemenger et al., 1983; Chapados et al., 1994) there are at least three ways that AmB could exist in the liposome structure consistent with the appearance of the 325 nm absorbance peak and the dichroic doublet in the CD spectrum. Each requires close interaction of the conjugated backbones of AmB: (1) *intra-channel* AmB−AmB interactions resulting from the proximity of parallel-stacked AmB molecules in a *single* channel structure; (2) *inter-channel* interactions resulting from close contact of a pair of AmB molecules belonging to *different* channel structures and; (3) *non-channel* residual AmB aggregated in the membrane.

The SVD analysis predicts that AmB exists in only two states in the bilayer, but it does not clearly differentiate between the three possibilities for aggregation. A strong driving force toward the formation of the intra-channel aggregated structure is consistent with the amphiphilic nature of the AmB molecule, whose hydrophilic edges would have a tendency to form "reverse micelles" in the lipid bilayer above a specific critical micelle concentration (Weakliem et al., 1995). However, Ernst et al. (1981) have estimated that distances of less than 6 Å between the backbones of adjacent AmB molecules are necessary to obtain the "aggregated" absorption spectra. The cholesterol−AmB pore model of de Kruiff and Demel (1974) and Andreoli (1974) leads to an approximate distance of 10−12 Å between adjacent AmB polyene backbones due to the cholesterol spacers. Thus, the AmB molecules in a cholesterol containing pore should still display spectra characteristic of a "monomer." Since increased channel activity apparently correlates with increased AmB aggregation in the liposome, one explanation for the observed spectral changes might be that the channel structures do not contain cholesterol spacers. Although it has been demonstrated that AmB can self-associate to generate spectral changes similar to those observed in these studies, and evidence for channel structures in the presence of lipids without sterols has been reported (Janoff et al., 1992; Hartsel et al., 1993), our results suggest that sterols play an important role in channel formation. Alternatively, assuming that the pores contain lipid or cholesterol spacers, then the 325 nm "aggregate" AmB peak may arise from individual channel structures in the bilayer interacting with each other. The membrane may even undergo a phase separation in which tightly packed domains consisting of AmB channels form in the lipid bilayer. The formation of microdomains and separate lipid phases has been well documented (Harris et al., 1995; Lehtonen et al., 1996; Huang et al., 1993) and lends support to the possibility that AmB could behave in this manner. Such an arrangement would lead to the parallel stacking of the AmB heptaene backbones from *different* channels in close proximity (i.e., the inter-channel structure), and would remain consistent with the cholesterol-containing pore model. The "monomeric" characteristics of the spectra would then be generated by the intra-channel interactions and the "aggregated" spectra would appear as more channels associate with each other. Further study of the AmB−liposome system under different conditions will be needed in order to make more quantitative conclusions.

It should also be noted that the current experiments were performed by bringing the total AmB concentration to a constant value. As a result, the liposome concentrations used at the larger $R_{A/L}$ values may lead to artifacts caused by light scattering. To test this possibility, SVD analysis of the first 10 spectra at the lower $R_{A/L}$ values was performed and the conclusion of two AmB environments is more convincing for this set. The singular values for the first three basis spectra are 296.42, 31.23, and 9.18 with amplitude autocorrelations of 0.90, 0.67, and 0.06. The first two basis spectra are qualitatively indistinguishable from the first two basis spectra in the SVD of all 20 ratio trials.

CONCLUSIONS

For several decades, AmB has been used extensively to combat serious fungal infections, yet its mechanism of action has not been clearly elucidated. The most widely accepted view holds that the primary cause of AmB's fungicidal effects is dependent upon its interaction with the ergosterol in the fungal cell membrane. Unfortunately, AmB's affinity for ergosterol is not so specific that it will not bind to other sterols such as cholesterol (Readio & Bittman, 1982) or to other lipids. The nonselective binding affinity of AmB for lipids and its disruptive effects on membranes are considered to be the main causes of its well-known toxic side effects. Early reports on AmB's interactions with lipid bilayers suggested that its mechanism of action as well as its toxicity was related to the formation of ion channels (Dennis et al., 1970; Marty & Finkelstein, 1975). While many studies have been directed toward characterizing pore formation by AmB, it has been difficult to obtain a definitive answer because of the complex interactions of the drug with itself and with the lipid and aqueous environment. Thus, results from several investigators have led to various models describing the channel [reviewed in Hartsel et al. (1993)]. In this study, we have demonstrated that AmB can be stabilized in lipid bilayer membranes thus providing a model system to monitor the interactions of the drug with lipids.

By using a series of liposome formulations which retain AmB exclusively in the bilayer, we investigated some of

the details underlying the physical and structural properties of AmB in sterol containing membranes. As shown by SVD analysis of the CD spectra, AmB exists in two and only two states over a wide range of AmB/lipid ratios from 10^{-1} to 10^{-4}. As judged by the spectral data, the two states of AmB in lipid bilayers most closely resemble a "monomeric" form (red-shifted by approximately 10 nm) and an "aggregated" form of AmB. Concurrent with the appearance of the aggregated form ($R_{A/L} = 10^{-3}$) channel activity begins to occur as detected by changes in pyranine fluorescence. Increasing values of $R_{A/L}$ (up to 10^{-1}) produces a greater proportion of aggregated AmB and faster quenching of fluorescence (i.e., more pores are formed). Our results further suggest that AmB does interact with lipid bilayers in the absence of sterols but that it is more effective at forming ion channels when sterols are available. In addition, channel formation promoted by the presence of sterol requires sterol concentrations (in the case of cholesterol) in excess of 1 mol % which may be related to the formation of tail-to-tail dimers of cholesterol in the bilayer. It is interesting to note that the results presented here are not inconsistent with the octameric, double-pore model proposed by de Kruijff and Demel (1974) and Andreoli (1974) more than 20 years ago.

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