Amphotericin B and Cholesterol in Monolayers and Bilayers

Jennifer Sykora,† Solomon Yilma,‡ William C. Neely,† and Vitaly Vodyanoy*,‡

Department of Chemistry and Department of Anatomy, Physiology, and Pharmacology, Auburn University, Auburn, Alabama 36849

Received July 24, 2002. In Final Form: November 1, 2002

The antimycotic activity of amphotericin B depends on its ability to make complexes with cell membrane sterols. Surface pressure (π) as a function of surface area (A) and $\pi-A$ hysteresis were measured for monolayers of amphotericin B/cholesterol mixtures on the water/air interface. Specific area per molecule of amphotericin B and free energy of mixing were calculated as a function of concentration of amphotericin B. When chloroform/methanol was used as a spreading solvent, the $\pi-A$ isotherms of the mixed monolayers exhibited characteristic transitions from the gas to liquid-expanded, then liquid-condensed, and finally the solid state. The mean molecular area of the mixed monolayers was significantly higher than the calculated sum of the molecular areas of the pure components. This expanding effect was accompanied by a large $\pi-A$ hysteresis and a positive excess of free energy of mixing at high π. In contrast, when 2-propanol/water was used as spreading solvent, the mixed monolayers at 20 °C exhibited $\pi-A$ isotherms with no visible transitions, low hysteresis, a condensing effect, and a negative free energy of mixing. The most stable monolayers were produced from molecules of amphotericin B and cholesterol with a 2:1 stoichiometry. At this ratio, amphotericin B and cholesterol form ion channels in lipid bilayers with conductance of 4−400 pS. These results provide a better understanding of the biological activity of amphotericin B. Artificial amphotericin B/cholesterol ion channels having large conductance could be useful in nanotechnology.

Introduction

Amphotericin B is a polyene macrolide antibiotic that is widely used for its antifungal activity, despite its undesirable side effects.[1] In biological systems, amphotericin B acts by forming ion channels that induce ion leakage across lipid membranes.[2,3] The formation of these channels is highly dependent on the presence of sterols (cholesterol or ergosterol) in the membrane.[4] The sterol molecules facilitate the assembly of the channel by acting as a "glue" that links the amphotericin B (AmB) monomers together. It has been initially suggested that the channels are composed of AmB−sterol complexes made of superimposed antibiotic and sterol molecules, whose polar groups face toward the inside of the channel, where they interact with water molecules, and whose hydrophobic groups interact with the aliphatic chains of membrane phospholipids.[5] Stoichiometries for AmB−sterol complexes ranging from 1:4 to 1:0.7 have been reported.[6]

The electrophysiological properties of AmB channels in lipid bilayer membranes have been extensively studied in the past in order to reveal the mechanism of action of AmB. However, the results of these studies, which were performed using various AmB:cholesterol ratios, have primarily revealed channels with conductances less than 70 pS.[7,8−11]

The monolayer technique has been employed to study the interaction of AmB with sterols.[6,12−16] Isotherms of AmB from these studies revealed curves that were biphasic and contained a long transitional plateau region. Surface properties of AmB monolayers appeared to be strikingly different, depending on the spreading solvents.[6,14,15] Circular dichroism (CD) and proton nuclear magnetic resonance (NMR) studies on the aggregation of AmB in solution have demonstrated that the aggregation state of AmB depends on the polarity of the solvents.[17]

In this work, we have studied surface properties of monolayers of amphotericin B/cholesterol mixtures on the water/air interface using two different spreading solvents: 2:1 v/v chloroform/methanol and 3:1 v/v 2-propanol/water. The specific area per molecule of AmB and the free energy of mixing were calculated as a function of the concentration of AmB. From these studies, we have shown the importance of spreading solvent for the stability of

* Corresponding author. Address: Department of Anatomy, Physiology and Pharmacology, 109 Greene Hall, Auburn University, AL 36849. Tel.: +1-334-844-5405. Fax:+1-334-844-5388. E-mail: vodyavi@vetmed.auburn.edu.

† Department of Chemistry.

‡ Department of Anatomy, Physiology, and Pharmacology.

(1) Clark, A. In *New Approaches for Antifungal Drugs*; Fernandes, P., Ed.; Birkhauser: Boston, pp 1−19.
(2) Bolard, J. *Biochim. Biophys. Acta* **1986**, *864*, 257.
(3) Holz, R.; Finkelstein, A. *J. Gen. Physiol.* **1970**, *56*, 125.
(4) Kerridge, D.; Whelan, W. L. In *Mode of action of anti-fungal agents*; Trinci, A. P. J., Ryley, J. F., Eds.; Cambridge University Press: Cambridge, 1983; pp 343−375.
(5) de Kruijj, B. Demel, R. A. *Biochim. Biophys. Acta* **1974**, *339*, 57.
(6) Seoane, R.; Minones, J.; Conde, O.; Casas, M.; Iribarnegaray, E. *Biochim. Biophys. Acta* **1998**, *1375*, 73.
(7) Ermishkin, L. N.; Kasumov, K. M.; Potzeluev, V. M. *Nature* **1976**, *262*, 698.
(8) Borisova, M. P.; Ermishkin, L. N.; Silberstein, A. Y. A. *Biochim. Biophys. Acta* **1979**, *553*, 450.
(9) Ermishkin, L. N.; Kasumov, Kh. M.; Potzeluev, V. M. *Biochim. Biophys. Acta* **1977**, *470*, 357.
(10) Brutyan, R.; McPhie, P. J. *Gen. Physiol.* **1996**, *107*, 69.
(11) Cotero, B. V.; Rebolledo-Antunez, S.; Ortega-Blake, I. *Biochim. Biophys. Acta* **1998**, *1375*, 43.
(12) Seoane, R.; Minones, J.; Conde, O.; Iribarnegaray, E.; Casas, M. *Langmuir* **1999**, *15*, 5567.
(13) Seoane, R.; Minones, J.; Conde, O.; Casas, M.; Iribarnegaray, E. *Langmuir* **1999**, *15*, 3570.
(14) Wojtowicz, K.; Gruszecki, W. I.; Walicka, M.; Barwicz, J. *Biochim. Biophys. Acta* **1998**, *1373*, 220.
(15) Saint-Pierre-Chazalet, M.; Thomas, C.; Dupeyrat, M.; Gary-Bobo, C. M. *Biochim. Biophys. Acta* **1988**, *944*, 477.
(16) Barwicz, J.; Tancrede, P. *Chem. Phys. Lipids* **1997**, *85*, 145.
(17) Balakrishnan, A.; Easwaran, K. *Biochim. Biophys. Acta* **1993**, *1148*, 269.

10.1021/la0206716 CCC: $25.00
Published on Web 12/31/2002

monolayers. We have shown that AmB and cholesterol form a complex with a stoichiometry of 2:1 that can produce highly conductive ion channels in phospholipid bilayers.

Experimental Section

Materials.

Amphotericin B was purchased from Sigma Co. (St. Louis, MO). Cholesterol and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL). Both were used without further purification. Chloroform, methanol, and 2-propanol were purchased from Aldrich Chemical Co. (Milwaukee, WI) with a purity of 99+%. We used anhydrous hexane, 95+% from Aldrich, packaged under nitrogen.

Experiments Using 2:1 v/v Chloroform/Methanol Spreading Solvent.

A series of mixed cholesterol/AmB spreading solutions was prepared with 2:1 v/v chloroform/methanol at molar ratios of AmB (N_A) ranging from 0 to 1. The stability of the spreading solutions was controlled by measurements of the electronic absorption spectra measured by a Shimadzu UV-3101PC spectrophotometer over the range of 300−450 nm. The monomeric species of AmB were determined at 365, 385, and 408 nm, while the aggregate form of AmB was resolved at a maximum absorption peak of 340 nm.[18] The solutions were spread on a subphase solution of 55 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM NaCl, and 2 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) made with deionized doubly distilled water (Milli-Q water purification system, Millipore Corp., Bedford, MA) and adjusted to pH 7.4 using KOH. A spreading time of 15 min was allowed for equilibration of the monolayer before compression. Surface pressure−area isotherms were measured on a KSV 2200 LB Langmuir−Blodgett film balance. The system contains a Wilhelmy type surface balance (0−100 mN/m; sensitivity, 0.05 mN/m) and a Teflon trough (45 × 15 cm^2). The temperature of the subphase was controlled (±0.1 °C) by water circulating through a quartz coil located on the bottom of the trough. Temperature was measured by a thermistor located just below the water surface. The compression rate was 30 mm/min (45 cm^2/min).

Experiments Using 3:1 v/v 2-Propanol/Water Spreading Solvent.

Cholesterol was dissolved in hexane in an atmosphere of dry nitrogen. AmB was dissolved in a 3:1 v/v mixture of 2-propanol and water. The AmB solution was centrifuged at 15 000 g at 4 °C for 1 h to remove any material that did not dissolve. The amount of the AmB in the solution was determined by evaporating a 5 mL sample of the solutions and weighing the residue. The AmB concentration was additionally controlled by monitoring the absorption at 408 nm (1.3×10^5 M^{-1} cm^{-1} extinction coefficient[14]). A concentration of 0.14 ± 0.011 mg/mL AmB was the highest achieved. Both spreading solutions were stored in the dark in a −20 °C freezer.

Amphotericin B/Cholesterol Mixed Monolayers.

A series of mixed cholesterol and AmB films, ranging from 0 to 1 mole fraction of AmB, was made on the same subphase solution described above. The cholesterol solution was spread first. After an evaporation time of 10 min, the AmB solution was spread. For measurements made at 20 °C, 40 min was allowed to elapse after spreading of the AmB solution. For measurements at 10 °C, cholesterol and AmB were spread on a subphase at 20 °C. After waiting for 20 min, the subphase was cooled to 10 °C before compression. This was necessary for complete removal of the 3:1 (v/v) 2-propanol/water solvent. Surface pressure−area isotherms were measured as described above.

The specific molecular area of AmB (A_A) in the AmB/cholesterol mixed monolayers was estimated by the equation[19]

$$A_A = (A_{AC} - A_C N_C)/N_A \quad (1)$$

where A_{AC} and A_C are extrapolated "zero-pressure" areas[20] for the mixed monolayer and the cholesterol monolayer, respectively, and N_A and N_C are mole fractions of AmB and cholesterol, respectively.

Thermodynamic properties obtained from the surface area−pressure isotherms for monolayers of AmB, cholesterol, and its mixtures were calculated by using the following equations[21−23]

$$\Delta G_M^E = \int_0^\pi (A_{AC} - N_A A_A - N_C A_C)\mathrm{d}\pi \quad (2)$$

$$\Delta G_M^I = RTN_A \ln N_A + RTN_C \ln N_C \quad (3)$$

$$\Delta G_M = \Delta G_M^E + \Delta G_M^I \quad (4)$$

where ΔG_M^E is the excess free energy of mixing, ΔG_M is the free energy of mixing, ΔG_M^I is the free energy of mixing for an ideal system, π is the surface pressure, R is the universal gas constant, and T is the absolute temperature.

Reconstitution of Amphotericin B/Cholesterol Channels in Lipid Bilayers.

Lipid bilayers containing pure 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine were formed on the tip of patch pipets using the tip-dip technique previously described.[24,25] The bilayers were formed in asymmetric saline condition by the successive transfer of two lipid monolayers on the tip of the patch pipet. The extracellular solution (bathing the cis-side of the membrane) contained 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, and 5 mM Tris-HCl at pH 7.4, while the intracellular solution (inside the patch pipet) contained 110 mM KCl, 4 mM NaCl, 2 mM NaH_2CO_3, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 mM MOPS at pH 7.4. A 2:1 (mol/mol) mixture of 0.6 μg AmB and 0.12 μg cholesterol dissolved in a chloroform/methanol (2:1 v/v) solvent was sonicated together with 10 μg phospholipid (in hexane) and 10 μL of pseudoextracellular solution in order to form liposomes. The emulsion was then carefully transferred to the surface (air−water interface) of the pseudoextracellular solution bathing the cis-side of the membrane. Incorporation of AmB into the membrane was achieved by dipping the tip of the patch pipet into the emulsion. The membrane-incorporated AmB molecules were subjected to electrophysiological studies at room temperature (~22 °C). The resulting single channel current fluctuations were filtered at 5 kHz and recorded on VHS tapes for later analysis.

Data Analysis.

Single channel data segments of 5−120 s lengths were digitized at 100-μs intervals and transferred to a computer as data files. The data were later subjected to statistical analysis using the Fetchan module of the pCLAMP data analysis program (Axon Instruments) as well as the Microcal Origin data analysis and technical graphics program.

Results

Absorption spectra indicate that, at the concentrations used in this work (10^{-4}−10^{-6} M), AmB was mostly in a monomeric form in methanol, 2-propanol, (3:1) v/v 2-propanol/water, and (2:1) v/v chloroform/methanol while mostly aggregated in chloroform (data not shown). Thus, both spreading solutions contain AmB molecules in the monomeric state.

Figure 1 depicts the π−A isotherms at 20 and 10 °C for mixtures of cholesterol and AmB using chloroform/methanol (Figure 1A,B) and propanol/water (Figure 1C,D) spreading solutions. Pure cholesterol formed a typical condensed monolayer. Extrapolation of the linear portion of the curve to zero pressure yielded a molecular area of about 38 ± 0.604 (SE) A^2/molecule. When using the

(18) Romanini, D.; Avalle, G.; Nerli, B.; Pico, G. *Biophys. Chem.* **1999**, *77*, 69.
(19) Barnes, G. T. *J. Colloid Interface Sci.* **1991**, *144*, 299.
(20) Gaines, G. L., Jr. *Insoluble Monolayers at Liquid−Gas Interfaces*; Interscience: New York, 1966.
(21) Goodrich, F. C. *Proc. 2nd Int. Congr. Surf. Act.* **1957**, *1*, 85.
(22) Vilallonga, F.; Altschul, R.; Fernandez, M. S. *Biochim. Biophys. Acta* **1967**, *135*, 406.
(23) Pathirana, S; Neely, W. C.; Myers, L. J.; Vodyanoy, V. *Langmuir* **1992**, *8*, 1984.
(24) Suppiramaniam, V.; Bahr, B A.; Sinnarajah, S.; Owens, K.; Rogers, G.; Yilma, S.; Vodyanoy, V. *Synapse* **2001**, *40*, 154.
(25) Vodyanoy, V.; Bahr, B. A.; Suppiramaniam, V.; Hall, R. A.; Baudry, M.; Lynch, G. *Neurosci Lett* **1993**, *50*, 80.

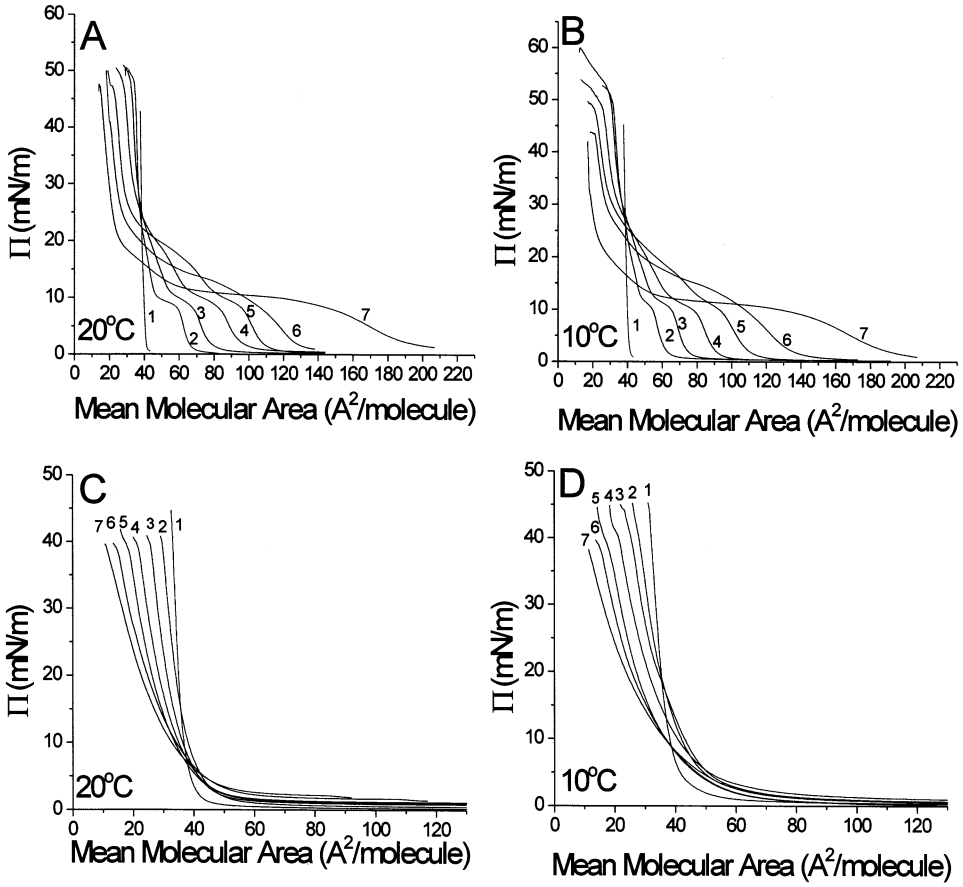

Figure 1. Surface pressure−area isotherms of mixtures of AmB and cholesterol using different spreading solvents at 10 and 20 °C. Panels A and B are those obtained from using (2:1) chloroform/methanol spreading solvent at 20 and 10 °C, respectively. Panels C and D are from (3:1) 2-propanol/water spreading solvent at 20 and 10 °C, respectively. Numbers 1−7 correspond to mole fractions of AmB: 0, 0.2, 0.334, 0.5, 0.667, 0.8, and 1.0, respectively. Isotherms are an average of two to four runs. Standard deviations of the surface pressure and area were determined at 11 equally spaced points along the curves at each mole fraction of AmB. Mean standard deviations for each series of isotherms are as follows: (A) 0.021 Å^2/molecule, 0.51 mN/m; (B) 0.053 Å^2/molecule, 0.25 mN/m; (C) 0.07 Å^2/molecule, 0.43 mN/m; (D) 0.021 Å^2/molecule, 0.11 mN/m.

chloroform/methanol spreading solvent, the $\pi-A$ isotherms of pure AmB monolayer came through the liquid-expanded state (LE), the LE + liquid-condensed state (LC) (plateau at about 10 mN/m), and finally LC and the solid state S. The extrapolated zero-pressure area of AmB in the LE region was found to be ∼200 Å^2/molecule, which agrees well with the area estimated from molecular models for AmB assumed to lie in the plane of the interface.[15] The molecular area estimated from the S (condensed solid) region of the isotherm was only 28 ± 1.058 (SE) Å^2/molecule. $\pi-A$ isotherms of mixtures containing N_A of 0.2, 0.334, and 0.8 exhibited two transient regions separated by a plateau around 10 mN/m. Isotherms of mixtures containing N_A of 0.5 and 0.667 exhibited different behavior. When first compressed, the monolayer showed the characteristic LE to LC transition at about 10 mN/m, but on further compression, a plateau region was reached at ∼16 mN/m, followed by a further rise in surface pressure before the monolayer collapsed at ∼50 mN/m.

The $\pi-A$ isotherms of pure AmB and mixed AmB/cholesterol monolayers at 20 and 10 °C using (3:1) v/v 2-propanol/water as AmB spreading solvent had a very different behavior (Figure 1C,D) compared to those obtained using the chloroform/methanol solvent. They all had features of LE monolayers without a visible transition to the S state. The extrapolated zero-pressure area of AmB in this solvent was around 35 ± 0.433 (SE) Å^2/molecule.

The mean molecular areas of the AmB/cholesterol mixtures were dependent on composition, temperature, surface pressure, and the spreading solvent used (Figure 2). Figure 2A illustrates the results for extrapolated areas obtained using the chloroform/methanol spreading solvent. Straight lines in Figure 2 represent the ideal concentration dependence of molecular area calculated by the equation $A_{ideal} = N_A A_A + N_C A_C$. The mean molecular area of the mixed monolayers was significantly higher than the calculated sum of the molecular areas of the pure components for the entire concentration range at both temperatures (expanding effect). The mean molecular areas for all mixtures were slightly higher at 10 °C than that of 20 °C. The condensing effect in mixed monolayers spread from the chloroform/methanol solutions was observed only for low surface pressures with a maximal departure from the additivity line at an AmB to cholesterol ratio of 2:1. Figure 2C illustrates results for extrapolated areas obtained when the propanol/water solvent was employed. At 20 °C, positive deviations from additivity were observed at all mixtures, except where the mole fraction of AmB was 0.667. Deviation from additivity was negative (condensing effect) at this composition, which corresponds to an AmB to cholesterol ratio of 2:1. At 10 °C, the extrapolated areas at all compositions were slightly larger than those at 20 °C. Positive deviations from additivity were experienced at mole fractions of AmB below 0.5.

Figure 3 shows compression−expansion $\pi-A$ isotherms of (2:1) AmB/cholesterol monolayer using different spreading solvents. Figure 3B demonstrates two consecutive

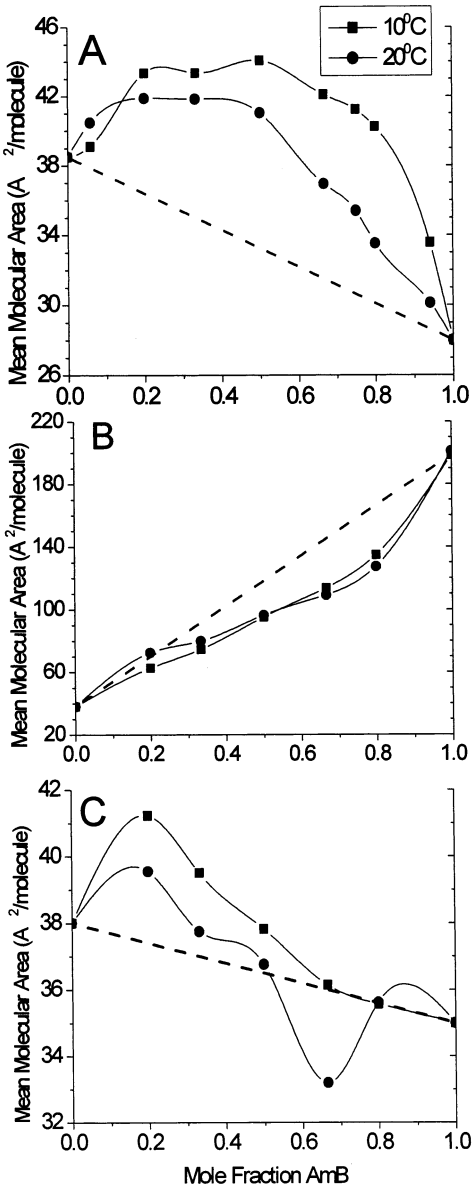

Figure 2. Mean molecular areas of mixed AmB and cholesterol monolayers as a function of AmB concentration. Points are experimental values obtained from isotherms. Panels A and B are from (2:1) chloroform/methanol as a spreading solvent. Panel A is the area extrapolated from the steep linear portion just prior to collapse. Panel B is the area extrapolated from the steep linear portion just prior to the plateau. Panel C is the extrapolated areas from the isotherms obtained using (3:1) 2-propanol/water as a spreading solvent. Squares are for measurements made at 10 °C and circles are for 20 °C. Mean standard deviations for the extrapolated areas are: (A) (20 °C) 0.920 Å^2/molecule, (10 °C) 0.674 Å^2/molecule; (B) (20 °C) 1.690 Å^2/molecule, (10 °C) 1.956 Å^2/molecule; (C) (20 °C) 0.341 Å^2/molecule, (10 °C) 0.463 A^2/molecule.

compression−expansion cycles for monolayers spread from the propanol/water solution. When first compressed, the monolayer showed that the LE to LC transition started at ∼10 mN/m and 90 Å^2/molecule. On further compression, another transition was reached at ∼16 mN/m and 68 Å^2/molecule, followed by a further rise in pressure before the expansion began. When the monolayer was reexpanded and a second compression carried out, the surface pressure−area curve was shifted to lower areas, either because fewer molecules of AmB were present at the interface or because they occupied a smaller area due to aggregation. The main shift occurred at low pressures, when the monolayer was in the LE state; i.e., it occurred before all the LE monolayer in the LE−LC two-phase region had been converted to LC state. The first and second compression curves were very similar at high surface pressure (above 20 mN/m), indicating that the number of molecules and their state may be the same. During the expansion the surface pressure dropped almost vertically from ∼45 to 16 mN/m and the isotherm is drawn almost parallel to the first compression line, making a large hysteresis. The hysteresis estimated for the first and second compression−expansion cycles were large and equal to 993 and 910 cal/mol, respectively. In contrast, the two consecutive compression−expansion isotherms of monolayers spread from the propanol/water solvent (Figure 3B) had a much smaller hysteresis, equal to 304 and 233 cal/mol, respectively. The hysteresis of five consecutive compression−expansion cycles measured for these monolayers was found steady at the level of 256 ± 12 (SE) cal/mol (data not shown). Additionally, the monolayer had a high respreading capability; thus, the isotherms of the second cycle are practically superimposed on that of the first cycle.

Figure 4 depicts the free energy of mixing (ΔG_M) calculated from pressure−area isotherms and plotted against the mole fraction of AmB along with the free energy of mixing for the ideal system (ΔG_M^I) calculated with eq 4 (Figure 4). Free energy values were dependent on composition, spreading solvent, and temperature. The significant negative departure of ΔG_M from ideality for monolayers spread from the chloroform/methanol solutions were observed only at low surface pressure (Figure 4A,B, lower curves). At higher surface pressures, a significant positive departure was observed for the same monolayers (Figure 4A,B, upper curves). Very different results were obtained using the propanol/water solvent (Figure 4C,D). For all surface pressures, the free energy of mixing revealed a minimum at $N_A = 0.667$ (AmB to cholesterol ratio of 2:1). At 20 °C, the negative departure from ideality held for monolayers of all compositions. As the surface pressure was increased, the free energy values became increasingly negative at all compositions. At 10 °C, the values of ΔG_M at compositions corresponding to $N_A = 0.2$, 0.334, and 0.5 were above that for an ideal mixture at all surface pressures. As surface pressure increased, the values decreased. At the same temperature, for mixtures corresponding to $N_A = 0.667$ and 0.8, the free energy values were very close to those for the ideal system.

When AmB and cholesterol mixed at 2:1 ratio were incorporated into patch tip-dip phospholipid bilayers and subjected to electrophysiological studies, ion channel current fluctuations with distinct open and closed states were observed (Figure 5A). Each state revealed a distinct subpopulation of current levels that were statistically significant (Figure 5B). The amplitude of the channel currents revealed that the channels had diverse conductances of up to 400 pS. No current fluctuations were detected for AmB incorporated in patch phospholipid bilayers devoid of cholesterol (Figure 5A).

Discussion

The mean molecular area obtained for pure cholesterol [38 ± 0.604 (SE) A^2/molecule] is in agreement with values published by other authors.[12,26,27] The isotherms of the mixed monolayers using the chloroform/methanol solvent are similar to those already in the literature published by Saint-Pierre-Chazalet et al.[15] and Seoane et al.[12] Mono-

(26) Muller-Landau, F.; Cadenhead, D. A. *Chem. Phys. Lipids* **1979**, *49*, 31.
(27) Ries, H. E., Jr.; Swift, H. *Colloids Surf.* **1989**, *40*, 145.

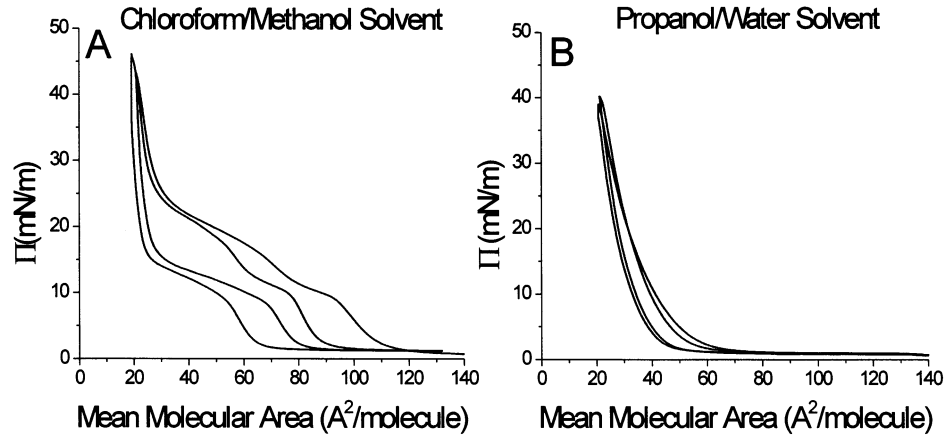

Figure 3. Compression−expansion isotherms of a mixture of (2:1) AmB/cholesterol at 20 °C using (2:1) chloroform/methanol (A) and (3:1) 2-propanol/water (B) as spreading solvents. Rates of compression and expansion were 30 mm/min.

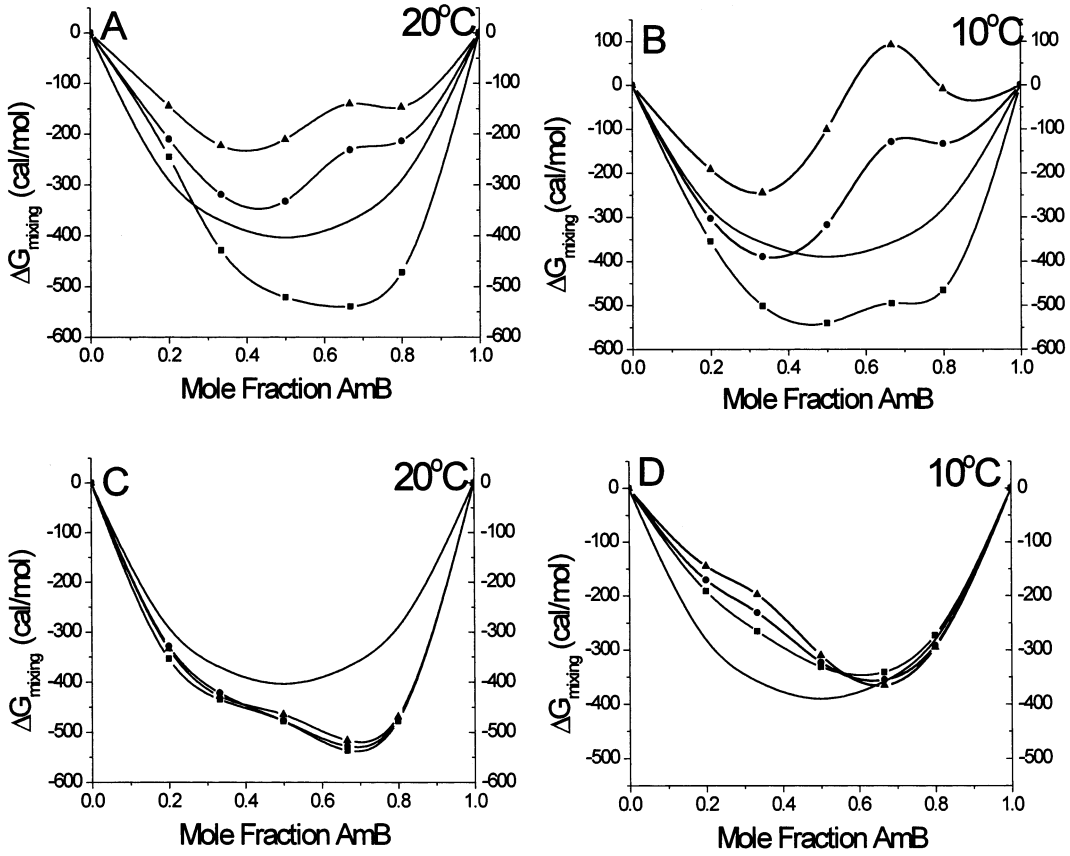

Figure 4. Free energy of mixing (ΔG_m) as a function of mole fraction AmB. Panels A and B are data from experiments using (2:1) chloroform/methanol spreading solvent at 20 and 10 °C, respectively. Panels C and D are from experiments using (2:1) 2-propanol/water spreading solvent at 20 and 10 °C, respectively. The solid line is for that of an ideal mixture. Symbols represent the following compressions: 0−10 mN/m (squares), 0−20 mN/m (circles), and 0−30 mN/m (triangles).

layer studies of AmB using solvents such as dimethylformamide (DMF) have yielded isotherms that had two phases separated by a long plateau around 10 mN/m. When mixed with sterols such as cholesterol or ergosterol, the length of the plateau was proportional to the amount of AmB in the mixture.[6] We have made similar observations when using the chloroform/methanol solvent. The plateau has been attributed to a change in orientation of the AmB molecule from a horizontal to vertical orientation and to desorption of the molecule into the subphase.[12,15] When using this solvent, the extrapolated molecular area of AmB was found to be 28 ± 1.058 (SE) Å^2/molecule at 20 °C. This compares with a reported value of 25 Å^2/molecule[12,15] using a different spreading solvent. The difference could be due to differences in compression rate, spreading solvent, subphase composition, and pH, and/or differences in purity of AmB sample. The molecular area of 28 Å^2/molecule estimated from the condensed region of the $\pi-A$ isotherm is lower than the theoretical 55 Å^2/molecule, estimated from the theoretical space-filling model[15] for the AmB molecule positioned vertically to the interface. The difference between the theoretical and experimental molecular areas of the AmB molecule in the condensed region can be explained by the loss of molecules from the monolayer. The AmB molecules desorb from the monolayer[12,15] or perhaps some of the molecules may be "squeezed out" of the monolayer.[28,29] Bangham[28] has suggested that overcompression of surfactant monolayers

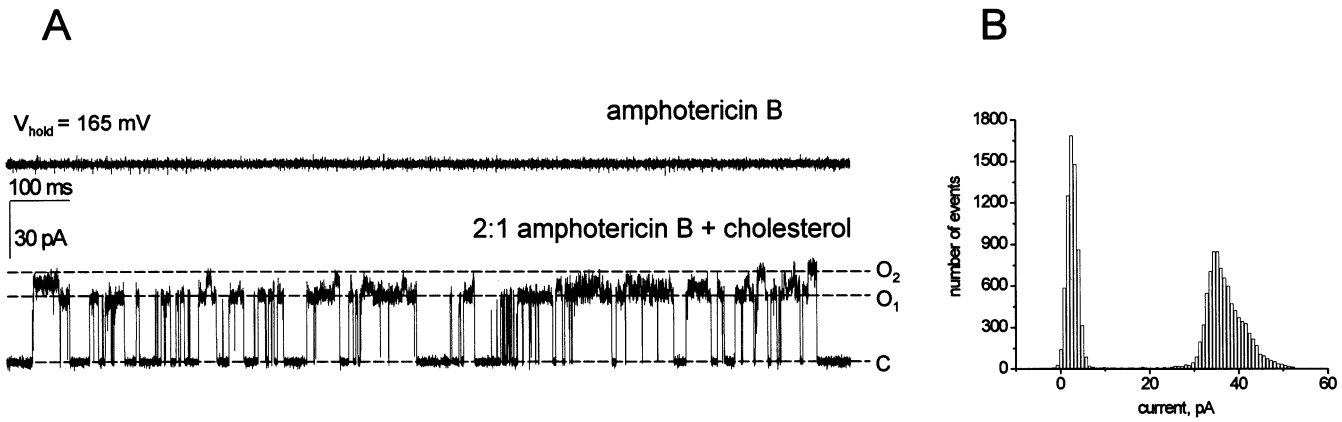

Figure 5. Properties of amphotericin B/cholesterol channels. (A) Voltage clamp records of lipid bilayers containing AmB were carried out in the absence (top) and presence (bottom) of cholesterol. Voltage was clamped at 165 mV. Traces were filtered at 5 kHz and sampled at 100-μs intervals. Openings are upward. (B) Amplitude distribution histograms of single channel data observed in the presence of cholesterol (lower trace in A) revealed a distinct closed level and multiple open levels.

leads to a loss of surface molecules either into a reversible, dry multilamellar phase or to an irreversible, collapsed phase.

When using the propanol/water solvent, the molecular area of AmB was found to be around 35 ± 0.433 (SE) Å^2/molecule at 20 °C. This compares with a reported value of 36 Å^2/molecule reported by Wojtowicz[14] et al., who also used (3:1) v/v 2-propanol/water as a spreading solvent at 25 °C. We speculate that these monolayers can tolerate compression with a rapid, reversible loss of molecules from the surface,[29] providing for better respreading during the process of expansion. This explains the observation of smaller than theoretical molecular area and small hysteresis of the monolayers spread from the propanol/water solvent.

The thermodynamic analysis of a binary system shows that the most stable state of a system is the state for which the free energy is a minimum at given temperature and pressure.[30,31] If a binary system is stable, then certain criteria of the system properties must be fulfilled. These criteria are as follows:[23,31] (1) Solutions are stable only if their ΔG_M values are more negative than the ΔG_M values of a heterogeneous mixture of the pure components. (2) The ΔG_M value of a stable solution must be more negative than the ΔG_M values of all possible two-phase mixtures. For our system, criterion 2 for the existence of stable solution could be defined as the requirement for the ΔG_M curve to be concave toward the horizontal axis.[31] Using these criteria we can identify the regions of stability in AmB/cholesterol mixed monolayers.

Criterion 1 is not fulfilled for the monolayers spread from the chloroform/methanol solvent with the observed positive departure from ideality of the free energy of mixing (ΔG_M) at high surface pressure (Figure 4A,B upper curves). This indicates the unstable state of the monolayer under these conditions. The observed positive departure from ideality of the free energy of mixing (ΔG_M) at high surface pressure is accompanied by the expanding effect observed in monolayers spread from chloroform/methanol solvent (Figure 2A). Only at low pressures, below 10 mN/m, do these monolayers get stabilized by what is manifested by the condensing effect (Figure 2B) and by the negative departure of ΔG_M from that of the ideal system (Figure 4A,B lower curves). At 20 °C, criteria 1 and 2 are both met for these monolayers at low pressures and mole fractions of AmB between ~0.3 and 1 (Figure 4A, lower curve), indicating the stability of monolayers in this region. At low surface pressures at 20 °C, the most stable state of the monolayer is achieved at $N_A = 0.667$. When the system is cooled to 10 °C, criterion 2 is not fulfilled for monolayers between 0.5 and 0.8 mole fractions of AmB (Figure 4B, lower curve), because ΔG_M values are more negative for $N_A = 0.5$ and 0.8 than those along the line $N_A = 0.5$, 0.667, 0.8 (where this portion of the curve is not concave toward the horizontal axis). These results lead us to conclude that at these conditions there is a gap in stability of mixed monolayers between 0.5 and 0.8 mole fractions of AmB. The expanding effect in mixed AmB/cholesterol monolayers and values of free energy of mixing agree well with the reported results.[12,15]

Applying the same criteria of stability to the ΔG_M of the AmB/cholesterol mixed monolayers spread from the propanol/water solvent at 20 °C (Figure 4C), we can conclude that mixed monolayers at these conditions have two stable regions of solution along the line at $N_A = 0$, 0.2, 0.334 and 0.667, 0.8, 1, with compositions determined by two minima in the curve of the free energy of mixing, with the deepest minimum at $N_A = 0.667$. At 10 °C, the system is unstable between $N_A = 0$ and 0.667, but it behaves as an ideal mixture of AmB and cholesterol between $N_A = 0.667$ and 1 with a minimum at 0.667. Our data indicate that the mean molecular areas of AmB/cholesterol mixed monolayers spread from the propanol/water solvent are dependent upon concentration and have also a minimum at $N_A = 0.667$. This finding suggests that the most favorable interactions between AmB and cholesterol occur at this mole fraction. The minima in mean molecular area coincide with the minima in free energy, indicating the formation of a 2:1 AmB/cholesterol complex.

The differences in the monolayers formed from the different spreading solvents may be attributed to the fact that AmB can be in a monomeric or aggregated state and that the aggregated state of AmB is dependent on solvent polarity and on AmB concentration.[17,18,32,33] Although absorption spectra indicate that AmB in both spreading solutions is mostly in a monomeric form, the AmB in chloroform is primarily in the aggregated state. We can

(28) (19) Bangham, A. D. *Lung* **1987**, *165*, 17.
(29) Vodyanoy, V.; Bluestone, G. L.; Longmuir, K. J. *Biochim. Biophys. Acta* **1990**, *1047*, 284.
(30) Birdi, K. S. *Lipid and biopolymer monolayers at liquid interfaces*; Plenum Press: New York, 1989.
(31) Wagner, K. *Thermodynamics of alloys*; Addison-Wesley Press: Cambridge, MA, 1952.
(32) Shervani, Z.; Etori, H.; Taga, K.; Tadayoshi, Y.; Okabayashi, H. *Colloids Surf. B* **1996**, *7*, 31.
(33) Shervani, Z. *Colloids Surf B* **1993**, *70*, 213.

speculate then that the (2:1) chloroform/methanol solution of AmB dissolves methanol very quickly into the subphase, leaving a concentrated solution of AmB in chloroform at the interface. This can lead to aggregation of AmB. This conclusion is consistent with results showing that circular dichroism spectra revealed highly aggregated AmB in chloroform.[17]

Similar effects of the spreading solvent on monolayers of another antibiotic, valinomycin, have been reported.[34] The effects were attributed to the highly polar internal ring structure of valinomycin in contrast to the relatively small terminal polar group of a typical lipid, which shows no significant solvent effect.

The physical state of AmB in monolayers and membranes is important for an understanding of its antibiotic activity, toxicity, and formation of ion channels. Toxicity studies on mice have shown that monomeric AmB was less than half as toxic after 24 h and about six times less toxic after 1 week than the corresponding solutions of fungizone, in which AmB is almost totally in an aggregated form.[35] Amphotericin B is one of the rare drugs that affects the course of prion diseases and modifies the kinetics of abnormal prion protein accumulation in the central nervous system.[36] The exact mechanism of its action is unknown; however, there is evidence that AmB inhibits PrP(Sc) generation in scrapie-infected cells.[37] We speculate that misfolding and aggregation of the prion protein is modulated by the AmB/sterol complexes. We use the 2:1 amphotericin B:cholesterol ratio because the free energy of mixing is minimal and the stability of the system is maximized. AmB ion channels observed in this work are of unusually high conductance, reaching up to 400 pS. These channels were composed of AmB and cholesterol molecules assembled with 2:1 stoichiometry. The conductance observed in our experiments is very high compared to the AmB channel conductance previously observed in 0.5 and 0.25 mm diameter black lipid membranes made from *n*-hexane[7] and *n*-heptane[8] solutions of brain extract (2−8 pS), in 0.1−0.3 mm diameter solvent-free synthetic phospholipid/cholesterol membranes[10] (4−23 pS), and in tip-dip patch pipet bilayers made of a synthetic phospholipid with and without cholesterol[11] (2−70 pS). We attribute our high conductance to one or more factors that contribute to the assembly and stabilizing of the channels of high conductance. One factor could be the 2:1 ratio of the AmB and cholesterol mixture used in our lipid bilayer experiments. The results from our monolayer studies have shown that the AmB/cholesterol complex is most stable at 20 °C when the two are mixed at a 2:1 ratio. Therefore, it goes to reason that the AmB/cholesterol complex that is assembling to form the ion channels in lipid bilayers will also be the most stable at this ratio. This can work to stabilize channels of higher conductance and extend their lifetime. Another factor could be the prefabrication in liposomes of AmB/cholesterol channels at 2:1 stoichiometry followed by their reconstitution in bilayers similar to the method used in reconstitution of AMPA channels.[25] We believe this technique helped to further stabilize the channels by allowing the AmB/cholesterol complex to preassemble into larger pore structures while still in the small area of the lipid bilayer membrane of the liposomes.

Acknowledgment. We thank Randy O. Boddie for technical assistance. This work was supported in part by grants from DARPA MDA972-00-1-0011, FAA 01-G-022, US Army N66001-1099-0072, and by the Auburn University Peaks of Excellence Program in conjunction in the Auburn University Detection and Food Safety Center.

LA0206716

(34) Ries, H. E., Jr. *Langmuir* **1990**, *6*, 883.
(35) Barwicz, J.; Christian, S.; Gruda, I. *Antimicrob. Agents Chemother.* **1992**, *36*, 2310.
(36) Adjou, K. T.; Deslys, J.-P.; Demaimay, R.; Dormont, D. *Trends Microbiol.* **1997**, *5*, 27−31.
(37) Mange, A.; Milhavet, O.; McMahon, H. E.; Casanova, D.; Lehmann, S. *J. Neurochem.* **2000**, *74*, 754−762.