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Biochimica et Biophysica Acta 1238 (1995) 156–162

BBA Biochimica et Biophysica Acta

Osmotic stress sensitizes sterol-free phospholipid bilayers to the action of Amphotericin B

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Received 26 January 1995; revised 4 April 1995; accepted 20 April 1995

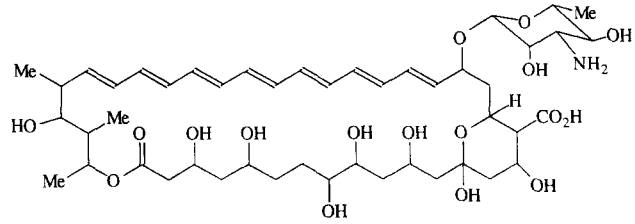
Abstract

We have tested the ability of Amphotericin B to form ion channels/defects in osmotically stressed large unilamellar vesicles (LUV) using pyranine fluorescence detected ion/H^+ exchange. We found that sterol-free LUV exhibit greatly increased sensitivity to AmB channel formation in the soluble oligomer state (> 0.5 μM) under modestly hypoosmotic conditions (< 100 ΔmosM). These vesicles are completely insensitive under isoosmotic conditions. The related antibiotics, Amphotericin B methyl ester and Nystatin showed almost no activity under hypoosmotic conditions in the absence of sterol. This difference may be attributable to differences in solution oligomeric states. Experiments with KCl and $CaCl_2$ internal buffers demonstrate that these sterol-free AmB membrane disruptions are highly selective for monovalent cations (K^+) over anions (Cl^-), ruling out massive lysis or unselective membrane defects caused by osmotic pressure. Thus, AmB seems to be acting as a 'molecular harpoon', an expression coined to describe substances which can selectively target osmotically stressed, strained or highly curved membranes. These results may provide a rationale for AmB's reported anti-HIV activity and reported activity against sterol-free small unilamellar vesicles (highly curved membranes) as well as the reduced activity of liposomal drug delivery systems toward cholesterol-containing and sterol-free membranes (fewer soluble oligomers).

Keywords: LUV; Membrane-active antibiotic; Pyranine; Ion channel; Osmotic stress; Amphotericin B

1. Introduction

Amphotericin B (AmB) is a very effective membrane-active antibiotic used to fight systemic fungal infections. Unfortunately, AmB has a low therapeutic index due to toxic side effects of current preparations (see [1] and references therein). Despite this, AmB still remains the drug of choice against most serious systemic fungal infections. The selectivity toward fungi presumably rests in the selective interaction of AmB with ergosterol in fungi versus cholesterol in mammalian cell membranes.



Amphotericin B

Research has been conducted to determine the mode of action and the active species of AmB ([2,3] and references within) with the goal of increasing its therapeutic index and to better understand the nature of the channel forming complex. Recent work suggests that AmB is especially toxic toward cholesterol-containing vesicles and mammalian cells when in a soluble oligomeric state which has

Abbreviations: AmB, Amphotericin B; LUV, large unilamellar vesicle(s); SUV, small unilamellar vesicle(s); Mops, 3-(*N*-morpholino)propanesulfonic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; EPC, egg phosphatidylcholine; AmE, amphotericin B-methyl ester; Ny, Nystatin.

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SSDI 0005-2736(95)00122-0

a characteristic excitonic CD spectrum. This structure may also attack membranes in a sterol independent manner [4–6]. Several less toxic AmB liposomal drug delivery mixtures have been developed and tested and these seem to work by reducing the amount of this free oligomeric AmB in solution though the precise structure of this active oligomer is still unknown [7–9].

Related work suggests that some membrane-active drugs and newly synthesized amphiphilic agents may be acting as 'molecular harpoons'. This term was introduced by Regen and co-workers to describe their synthetic amphiphilic molecules which recognize osmotic stress in lipid bilayers because of their hydrophobic wedge-shaped tips. These molecules insert disruptively when the lipid packing density of the outer monolayer is reduced by internal swelling caused by osmotic pressure [10–15]. The structure and selectivity of these defects is unknown but they can cause channel-like leakage rather than total lysis. Like AmB, these substances' activities are strongly dependent upon their oligomerization state and the presence of sterols in the membrane.

These similarities suggest that it is possible that AmB also acts in this manner [13]. If AmB or its oligomers can indeed act as molecular harpoons, sterol free LUV might be expected to experience an increased sensitivity to AmB under hypoosmotic conditions due to pressure-induced splaying of the outer monolayer. A similar increase in activity could help explain the ability of AmB to induce channels in the absence of sterol in egg phosphatidylcholine (EPC) small sonicated unilamellar vesicles (SUV, $r \sim 150$ Å) since such vesicles have a small radius of curvature and experience a 'looseness' in the external bilayer leaflet similar to that produced by internal osmotic pressure [16]. Activity against sterol-free large unilamellar vesicles (LUV, $r > 500$ Å) is not observed even at very high AmB concentrations [17,18]. In this report we test the hypothesis that AmB can act as a molecular harpoon and that its activity depends upon its oligomerization state and the magnitude of the osmotic gradient.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) was isolated from fresh egg yolks by using the Singleton procedure [19]. Purified Amphotericin B and Nystatin were generous gifts from Bristol-Myers Squibb Pharmaceuticals (Princeton, NJ). Amphotericin B-methyl ester was a generous gift from Professor Jacques Bolard. FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone) was obtained from Sigma (St. Louis, MO). Valinomycin, a K^+ ionophore and 4-bromo-A23187, a Ca^{2+} ionophore, were purchased from CalBiochem (La Jolla, CA). Laser-grade pyranine (1,3,6-pyrenetrisulfonic acid) was purchased from Eastman-Kodak (Rochester, NY).

2.2. Preparation and assay of lipid vesicle samples

Lipids in chloroform were dried by an argon stream and vacuum desiccated for approx. 1 h to remove chloroform. The lipids were dispersed in 25 mM Mops buffer, pH 7.20 with either 100 mM K_2SO_4, 100 mM KCl or 50 mM $CaCl_2$ solution with 2.2 mM pyranine (internal buffers) except where indicated. Freeze-thawed lipid dispersions were extruded 10x through a 1000 Å Nucleopore filter using the Extruder (Lipex, Vancouver BC). External pyranine was removed by gel filtration through an internal buffer equilibrated column of Sephadex G-25. Following separation, 5 μM FCCP was added to vesicles to allow for free exchange of H^+ ions. An instantaneous salt (KCl, K_2SO_4, $CaCl_2$) and osmolality gradient was created by diluting the vesicles with variable ratio stopped flow syringes to a 6:1 ratio. Osmotic gradients were established by mixing the isoosmotic sucrose 25 mM Mops, pH 7.20 buffer with an identical buffer without sucrose and determining osmolarity. The osmolarity of the buffer/vesicle solutions was monitored using an automatic osmometer (μOsmette, Precision Systems, Natick, MA). AmB and AmE and Ny were introduced to the sucrose/Mops diluting buffer from DMSO stock solutions made up to 1.3 mM, 1.4 mM and 1.3 mM, respectively. The *initial* osmotic difference (see Discussion) between the vesicle lumen and external medium is expressed as $\pm\Delta$mosM, where a positive sign indicates inside hyperosmotic.

2.3. Measurement of K^+, Cl^-, and net ion currents in LUV

Ion currents were measured with an On Line Instrument Systems (OLIS, Jefferson, GA) converted Durrum D-110 stopped-flow spectrophotometer in the fluorescence mode. Stopped-flow techniques for measuring ion currents using the pH sensitive dye pyranine as a reporter molecule have been developed over the past few years and the concentrations, instrumentation, data handling and interpretation and other experimental conditions were identical to those in Hartsel et al. [18] with the main difference being that 1000 Å diameter extruded LUV were used in vesicle studies instead of sonicated vesicles. For the current studies, 25 mM Mops pH 7.20 with 100 mM K_2SO_4 or KCl or 50 mM $CaCl_2$ was rapidly diluted with a hypoosmotic sucrose solution/25 mM Mops, pH 7.20 external buffer in a temperature controlled stopped flow fluorimeter (22° ± 2°C). The final total lipid concentration was 1–3 mM. Our assay measures pH changes inside the LUV that result from the AmB induced K^+ currents when sulfate is the counter ion (sulfate is not permeant) and net current when Cl^- is the counterion since Cl^- may be permeant to a lesser degree depending upon the conditions [20]. With the incorporation of the protonophore FCCP into the LUV bilayers, H^+ can equilibrate rapidly across the membrane.

Hence, the imposed salt gradients coupled with the ionophoric action of AmB cause a H^+ for K^+ (or Cl^-) exchange that is limited by the rate of AmB induced K^+ or Cl^- efflux. The pyranine molecules entrapped in the target LUV provide a sensitive fluorescence assay for detecting interior vesicular pH changes. The initial rate of pH change induced by AmB was used for comparisons of AmB's channel activity under different osmotic conditions and is expressed in ΔpH s^{-1} [6,18,21,22]. The points represent the average initial slope of three trials ± 1 S.D.

3. Results

The pyranine fluorescence detected ion current method employed here has proven useful in detecting AmB-induced ion currents under isoosmotic salt gradient conditions [6,18,21]. However, interpreting electrogenic K^+ or Cl^- currents by fluorescence changes under hypoosmotic conditions might pose some problems due to the possible significant osmotic swelling, lysis and/or internal dilution of LUV due to water influx under these conditions [23].

As a test for the reliability of this method we created KCl gradients in *opposite directions* under hypoosmotic pressure gradients and treated the sterol free EPC LUV with valinomycin. If this method works qualitatively under these conditions, we would expect opposite fluorescence changes. In fact Fig. 1 (a + g) shows good qualitative and quantitative agreement for valinomycin activity although not surprisingly, due to differences in ionic strength, the

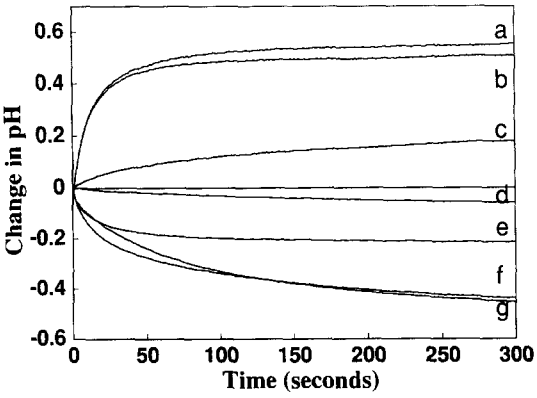

Fig. 1. Time-course plot of the change in pH resulting from electrogenic K^+ ion current from sterol-free EPC vesicles with oppositely oriented KCl gradients. Leakage induced by (a) 0.025 μM valinomycin, (b) 0.025 μM valinomycin and 10.0 μM AmB, and (c) 5.0 μM AmB in EPC vesicles containing 350 mM sucrose, 22.5 mM KCl, and 25 mM Mops, pH 7.20 diluted with 100 mM KCl to produce an osmotic gradient of +186 ΔmosM. Traces of (d) 0.5 μM AmB, (e) 5.0 μM AmB, (f) 5.0 μM AmB and 0.05 μM valinomycin, and (g) 0.05 μM valinomycin were added to EPC vesicles with 100 mM KCl diluted by 40 mM sucrose to produce a +113 ΔmosM gradient. The direction of the fluorescence change in each case shows that $K^+ > Cl^-$ selective channels are formed by AmB in the presence of an osmotic gradient and also demonstrates that treated vesicles can maintain a potential, thus ruling out significant LUV lysis.

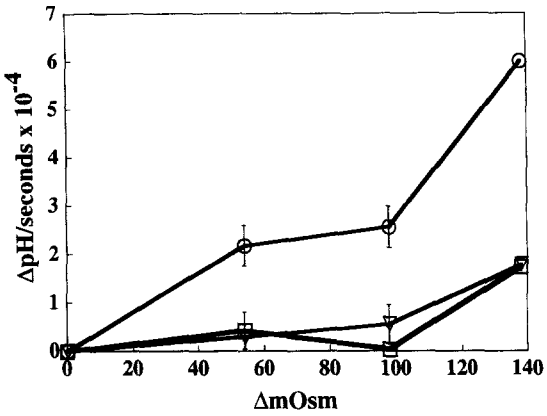

Fig. 2. Comparison of the initial pH change induced by 5.0 μM AmB (○), 5.0 μM AmE (▽), 5.0 μM Nystatin (□) on sterol-free LUV containing 100 mM K_2SO_4 under various initial osmolarity gradients. The zero point which had an equivalent ionic, but no osmotic, gradient was indistinguishable from the baseline.

kinetics are different. In addition, Fig. 1 demonstrates that at these ΔmosM the vesicles are sensitive to AmB in the absence of sterol, and again, show opposite changes in fluorescence indicating $K^+ > Cl^-$ selectivity. This study (see Fig. 2) as well as past studies have shown that AmB under isoosmotic conditions shows no membrane activity on sterol free LUV under these concentrations [17,18].

In all other experiments, the K^+ ionophore valinomycin was added with the highest [AmB] as a control to ensure that significant vesicle osmotic lysis and/or pyranine loss was not occurring, i.e., to determine whether the vesicles could maintain a stable diffusion potential. In no case was catastrophic rupture of the vesicles observed. In addition, if vesicle lysis were occurring, pyranine would be released into the external solution. As an additional control, ~2 mg/ml vesicle dispersions at high +ΔmosM were exposed to 10 μM AmB for 10 min and run again through a Sephadex G-25 gel column. Qualitatively, there were no signs of the highly fluorescent external pyranine retained on the column (data not shown); i.e., there was complete retention of pyranine associated with the vesicle fraction.

Since AmB in aqueous buffer solution can adopt at least three types of aggregate structures (see Discussion), we tried to determine whether it was a soluble species or insoluble aggregate (an inactive species against red blood cells) which was the major contributor to observed ion currents. A 2.0 μM (final diluted concentration) AmB dilution buffer was made from either a 0.1 mM AmB DMF or a 1.3 mM DMSO stock solution. The final solution of AmB from DMF is expected to be virtually free of the insoluble aggregate [4] whereas that made from the DMSO stock should have an appreciable insoluble aggregate component (est. 20–30%; Ref. [4]). Using these solutions, an initial +148 ΔosM gradient was imposed upon sterol free LUV with 100 mM K_2SO_4 inside. Within the standard error of these measurements, identical currents were generated by these two preparations, indicating that the insolu-

ble aggregate form is not a necessary species for osmotic recognition ($(-2.9 \pm 0.9) \cdot 10^{-4}$ ΔpH/s for DMSO and $(-2.7 \pm 1.0) \cdot 10^{-4}$ ΔpH/s for DMF). It is surprising that these currents are so similar in that the buffer made from the DMF stock should have relatively more of the soluble oligomer form. However, considering the difference in buffer composition and temperature (22°C vs. 37°C) from Ref. [4], perhaps the solution from DMSO stock has a greater soluble oligomer and lesser monomer component (see also temperature dependence in [6]). These data combined with the observed 0.5 μM threshold for non-sterol dependent activity strongly suggests that the soluble aggregate/oligomer form is the species which recognizes osmotic stress.

Fig. 2 illustrates the effect of increasing osmotic gradients on the activity of 5.0 μM AmB, AmE, and Nystatin on sterol-free LUV in K_2SO_4 buffer. There is an increase in the initial rate of K^+ current as the internal vesicle pressure increases at constant AmB concentration as probed by fluorescence detected H^+/K^+ exchange. A substantial increase in K^+ permeability is not observed for AmE and Ny until higher internal pressures are attained and still the rate is far less than that of AmB. In the absence of an osmotic differential, the fluorescence trace is indistinguishable from the baseline.

The pH detected initial rate of K^+ leakage increases as the AmB concentration increases at constant osmotic pressure difference with a constant K_2SO_4 gradient (Fig. 3) in the presence of LUV containing 10 mol% cholesterol or no sterol. Notice, however, that there is approx. a 30-fold higher leakage rate at a given [AmB] for the cholesterol-containing EPC vesicles relative to sterol free.

In the presence of KCl gradients AmB always produced a K^+ selective response. Figs. 1 and 4 demonstrate the K^+ over Cl^- selectivity of the osmotic pressure enhanced AmB channels. In Fig. 4 a decrease in fluorescence occurred in all cases, indicating the predominance of the K^+ current with the equilibrium ΔpH reaching nearly the same value as the highly selective K^+ ionophore valinomycin (not shown). In these experiments the initial currents observed in the presence of cholesterol were similar in magnitude to those in sterol-free vesicles unlike the induced currents in LUV made in K_2SO_4 as shown in Fig. 3 in which the K^+ currents were considerably greater in the presence of cholesterol (~ 30-fold). This may be explained by a lower K^+ vs. Cl^- selectivity (a less selective channel) with cholesterol as compared to no sterol giving an apparent reduction in the initial K^+ current due to simultaneous flux of Cl^-.

The lack of Cl^- permeance in AmB-induced channels in sterol-free stressed LUV was confirmed by experiments where $CaCl_2$ was entrapped within sterol free LUV (Fig. 5). Only a very small Cl^- current was detected over 10 minutes and Ca^{2+} was essentially impermeant, again suggesting strong monovalent cation selectivity. In contrast, cholesterol containing LUV show significant AmB in-

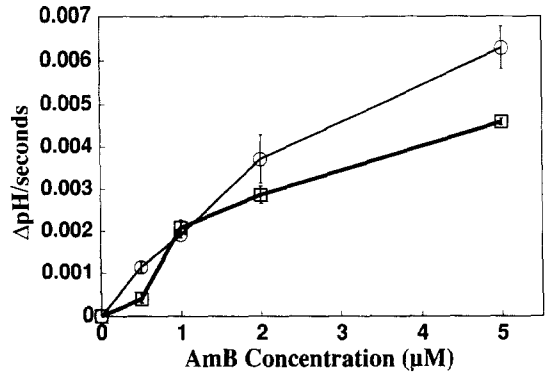

Fig. 4. Initial currents in vesicles containing 100 mM KCl internal buffer. The initial osmolarity gradient was at +103 ΔmosM while AmB concentration was increased. Comparison was made between EPC vesicles containing 10% cholesterol (○) and those containing no sterol (□).

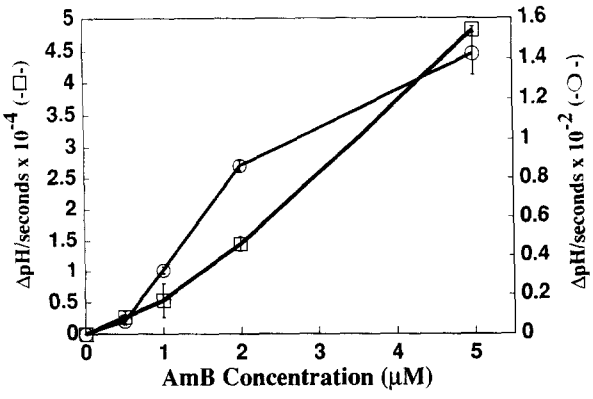

Fig. 3. Illustration of the effect of cholesterol and AmB concentration on K^+ ion leakage vesicles containing 100 mM K_2SO_4 represented by initial pH change in the presence of the 10 mol% cholesterol/EPC (○) versus EPC vesicles without sterol (□). The initial osmolality gradient was +136 ΔmosM. Note the higher scale used for cholesterol experiments.

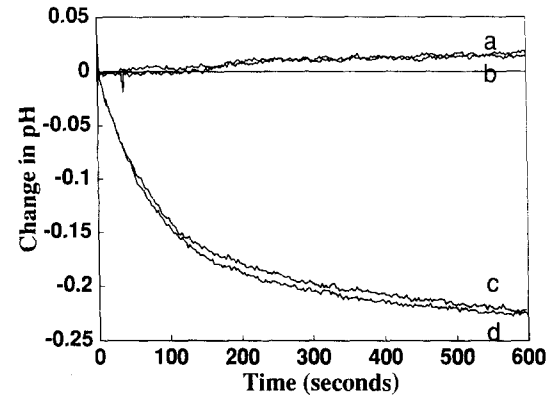

Fig. 5. Traces of the pH change due to the permeance of Cl^- or Ca^{2+} with the addition of (a) 2.0 μM, (b) 5.0 μM AmB, (c) 5.0 μM calcium ionophore 4-bromo-A23187 and 5.0 μM AmB, and (d) 5.0 μM 4-bromo-A23187 alone. The vesicles were extruded with 50 mM $CaCl_2$ inside and the osmotic gradient was +76 ΔmosM.

duced permeability for Cl^- not only under isoosmotic conditions but under osmotic stress (Fig. 4 and [18,20]).

4. Discussion

Based on ultrastructure and trapped volume measurements Mui et al. [23] showed that extruded 1:1 cholesterol/egg phosphatidylcholine vesicles may be ellipsoidal rather than spherical and may swell up to +50% more trapped volume under slight osmotic gradients. It is not known whether this 'rounding up' process adds to osmotic strain on the bilayer or whether the results are applicable to pure egg phosphatidylcholine or 10% cholesterol LUV used in most of the present experiments with AmB. Other studies on osmotically active LUV vesicles composed of pure phospholipid (dioleoylphosphatidylglycerol) showed that osmotic stress and strain increased with applied osmotic gradients and could be satisfactorily modeled by assuming polydispersity and a maximal swelling of only 6% [24,25]. Other studies on extruded LUV of similar size and composition to ours have show that these vesicles are osmotically active and swell up *with* accompanying osmotic membrane stress up to an osmotic gradient of about $\Delta mosM \cong +200$ [12]. After this point, size stays relatively the same while residual osmotic pressure continually increases to a point where solute leakage/rupture occurs. A common feature of all of these studies was that significant osmotic lysis/leakage of untreated membrane vesicles as measured by carboxyfluorescein leakage did not occur before about +400–500 ΔmosM, well above the modest gradients in the current measurements.

The most serious concern to the current study with reference to these prior results is the possibility that osmotically induced rounding up could cause a significant dilution of the LUV lumen osmolality without accompanying increases in membrane osmotic stress or strain [23]. For our experiments, we calculated that even if the maximal swelling reported in [23] occurred (+50%) *without* any added stress, all of our measurements, with the exception of the lowest (+54 ΔmosM points) in Fig. 2, would still have significant residual osmotic gradients (from 30–55% of predicted) and hence osmotic strain. The fact that the +54 ΔmosM point does show measurable sensitivity to AmB probably indicates that some osmotic strain does indeed occur upon rounding up or that these vesicles are more nearly spherical. In any case, due to this unsettled question of the swelling factor in LUV, all osmotic gradients are reported as *initial* gradients.

There has been much interest recently in the effect of the association state, structure and physical nature of the bilayer target on the membrane activity of drugs, peptides and synthetic surfactants. Regen and co-workers has shown that certain amphipathic compounds with a wedge or harpoon shape can selectively insert (as monomers generally) into osmotically stressed membranes and cause leakage but not necessarily rupture [11,12]. In these cases, cholesterol seems to relatively enhance osmotic stress sensitivity [12]. In addition, the non-ionic detergent, Triton X-100 was shown to cause a slow leakage of trapped solutes in LUV when introduced in the monomeric state (< cmc) and massive catastrophic lysis when introduced from the micellar state against gel phase vesicles while leakage pathways dominate in fluid vesicles [13]. The membrane activity of various classes of membrane active agents in these and other studies (cf. [26,27]) was measured by the extent of the leakage of the self quenching dye carboxyfluorescein, which does not offer insight into the microscopic nature and organization of these channel/defects. The current studies have the advantage of being able to gain more specific information as to the molecular nature of channels induced in osmotically stressed environments by looking at the ionic selectivity.

At least three distinct forms of AmB can coexist in aqueous preparations: the monomeric, soluble oligomer, and insoluble aggregate. The relative occurrence of these forms is complicated depending upon [AmB], time, temperature, stock solvent and stock concentration, and buffer composition [4–6]. It has been suggested that only the soluble oligomeric form of AmB is active against cholesterol containing and sterol free membranes. Under the conditions of these experiments and stocks, the soluble oligomeric form of AmB begins to predominate at about 0.5 μM [6]. This is also the concentration at which the osmotically stressed sterol-free vesicles become sensitive. Nystatin, a topical antifungal drug with a similar structure to AmB, does not aggregate below a 10 μM concentration [5] and accordingly it shows almost no activity in our experiments (Fig. 2). Thus, it seems that the soluble oligomer and/or insoluble aggregate is the principal active species recognizing osmotic stress. Since our experiments using different stock solvents suggests no major effect from the insoluble aggregate species, it is highly likely that the soluble oligomer is the main osmotically active species. This conclusion is in excellent agreement with results on a tethered oligomeric surfactant vs. its monomeric counterpart synthesized by Regen and co-workers [11]. In this case, the tethered oligomer showed a significant stress induced carboxyfluorescein leakage yet had almost no effect on isoosmotic LUV. In contrast the monomer showed only slightly enhanced activity in response to osmotic stress. It is interesting that AME also shows little activity in light of the fact that it should be self-associated at the concentrations used in Fig. 2 [5]. However, the much higher water solubility of the cationic AME as compared to zwitterionic AmB and Ny may explain this lack of effect. Perhaps this is also related to the relatively lower acute toxicity of AME toward mammalian cells.

In agreement with studies on molecular harpoon surfactants [12], the presence of cholesterol enhanced the relative sensitivity of osmotically stressed vesicles to AmB as shown in Fig. 3, but unlike these harpoons, cholesterol also

enhances AmB sensitivity under isoosmotic conditions. It is likely that the specific association and interactions of AmB in both monomeric and oligomeric states with cholesterol complicates interpretation of molecular harpoon action under these circumstances.

In the present study, we have shown that AmB can act as an exquisitely sensitive molecular harpoon. But what then is the active oligomeric structure and why does it act as a molecular harpoon? Based on CD, theoretical and NMR interpretation it has been speculated that the antiparallel dimer may be the fundamental unit of aggregation for AmB in solutions [5,28–31]. The calculated/experimental antiparallel dimer turns out to be very similar in structure to the X-ray diffraction unit cell of the iodo-AmB derivative [32]. It has been recently noted that the crystal structure dimer of AmB has a remarkably polar and apolar face like the membrane disruptive amphiphilic 'rafts' proposed for some membrane active helical peptides (see Fig. 2 in Refs. [2,33]). Using Quanta generated CPK models we have estimated that the hydrophobic surface of the antiparallel dimer is large and rectangular with an estimated 11 Å × 24 Å span and a shallow triangular cross-section with the polyene at the apex. In contrast, the monomer could conceivably bind to a membrane by insertion of the heptaene chain, presenting a hydrophobic surface only 3.1 Å wide which would not be nearly so disruptive. Based on this model it would not be surprising that an oligomer with such a large hydrophobic surface should selectively target ('harpoon') osmotically stressed membranes with their more accessible hydrophobic acyl chain interiors.

These results also help explain the previously puzzling results on the strong activity of AmB on sterol free SUV and the total lack of activity of Ny on similar vesicles [16,21]. SUV produced by sonication have a small radius ($r = 150$ Å) creating a similar outer monolayer stress condition as that of vesicles under osmotic stress where the hydrophobic region of the phospholipid becomes more exposed. This is also a reasonable explanation for the increased binding affinity and higher binding rate of AmB and derivatives for sterol free SUV vs. sterol free LUV [17,34]. We would expect that other surfactants demonstrating selective activity for osmotically stressed membranes would also show enhanced activity against SUV. Experiments are currently underway to determine whether osmotic stress enhanced AmB binding to LUV is the mechanism of this molecular harpoon type activity. A natural situation where such a small radius of curvature like an SUV is observed is the outer lipid membrane of various envelope viruses such as HIV. The reported anti-HIV activity of AmB and some of its derivatives may be a consequence of this curvature [35,36].

One of the most remarkable findings of this study is that, contrary to expectations that rather nonselective membrane disruption would be observed, the AmB channels induced by osmotic stress are highly monovalent cation selective even in the absence of sterol. While it is clear that sterols, especially ergosterol, can interact directly with AmB in the channel formation process (see [3,4] and references therein), *sterols are not necessary to produce highly cation selective AmB channels*. Thus, it seems that osmotic stress induced changes in the lipid bilayer may actually 'catalyze' the formation and assembly of AmB channel structures within the vesicle membrane. It will be interesting to assess whether other osmotically sensitive surfactants may also produce more defined channel structures than previously thought.

Acknowledgements

This work was supported by the NSF (MCB-9204564) and the Petroleum Research Fund (#25249-B3) and the Research Corporation Summer Scholar award (to B.D.W.).

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