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Rapid Report

Temperature effects on the aggregation state and activity of Amphotericin B

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Amphotericin B (AmB) in aqueous solution becomes less aggregated as temperature is increased as measured by CD spectroscopy. Concomitantly, AmB's ability to induce K^+ leakage from cholesterol-containing large unilamellar vesicles (LUV) decreases 4-fold per 10°C increase. In contrast, ergosterol-containing vesicles show no clear trend in their temperature sensitivity to AmB action. These data strongly support a model of AmB action where drug aggregates and not monomers are the major channel-inducing species against cholesterol- but not ergosterol-containing membranes.

After 35 years of use, Amphotericin B (AmB) is still the drug of choice for treating systemic fungal infections, despite the battery of unpleasant side effects. The rapid increase in systemic mycoses associated with immunodeficient patients has made this drug more important than ever before (for reviews, see Refs. 1–3). Liposomal AmB preparations, which greatly reduce the acute and chronic toxicity of AmB, will soon be available for general use, though the means by which they achieve reduced toxicity is not entirely understood [4]. An abundance of research over the past 10 years in design and testing of these liposomal preparations has led to increased interest in the effects that different AmB supramolecular aggregates have on both efficacy and toxicity and has led to a reexamination of its mode of action [5].

The most widely accepted model for the anticellular activity of this drug involves the formation of 1:1 Amphotericin/sterol aggregates in cell membranes which subsequently associate into a transmembrane barrel with a large -OH lined aqueous pore down the middle [1]. These pores allow efflux and influx of ions and other solutes, eventually causing cell death. The stronger association of Amphotericin with ergosterol versus cholesterol explains the higher toxicity towards fungi. However, conflicting membrane permeability data concerning AmB channel ion selectivity, sterol requirements and mode of delivery has accumulated over the past 15 years and suggests there exist a multiplicity of AmB channel structures and modes of action. Some of these novel structures and mechanisms of action may rival the widely-accepted sterol/pore model in functional significance [5].

Attention has focused recently on the effect of different self-associated AmB species or monomers against fungal and mammalian cells [6,7]. One of the simplest ways of altering the aggregation state in a given AmB solution is by changing the temperature. At higher temperatures, CD spectra indicate that AmB shifts from a more associated to a more monomeric state. In this study, we show that temperature-dependent aggregation as monitored by CD, has a profound and differential effect on AmB's ability to cause release of K^+ from large unilamellar vesicles composed of egg phosphatidylcholine with incorporated cholesterol or ergosterol.

Egg phosphatidylcholine (EPC) was isolated from fresh eggs using the Singleton procedure [8]. Ergosterol and cholesterol were obtained from Sigma (St. Louis, MO, USA) and recrystallized 3-times before use. Purified Amphotericin B was a gift from the Squibb Institute for Medical Research (Princeton, NJ, USA). FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was obtained from Sigma. Laser grade pyranine (1,3,6-pyrenetrisulfonic acid) was purchased from Eastman-Kodak (Rochester, NY, USA).

For pyranine pH-detected membrane potential fluorescence studies, freeze-thawed lipid dispersions were extruded 10-times through a 1000-Å Nucleopore filter

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using the Extruder (Lipex, Vancouver, BC, Canada) in 25 mM Mops buffer at pH 7.20, 2.2 mM pyranine and 100 mM K_2SO_4. The external pyranine was exchanged for a buffer identical to the internal buffer (except for pyranine) on a Sephadex G-25 column. All preparations contained 10 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrozone) to ensure rapid proton equilibration. AmB was introduced to the diluting buffer from a 10 mM DMSO stock solution.

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 [9–11] and the concentrations, instrumentation and other experimental conditions were identical to those in Hartsel et al. [11] with the main difference being that 1000 Å diameter extruded LUV were used in sterol-containing vesicle studies instead of sonicated vesicles. For the current studies, 100 mM K_2SO_4 was rapidly diluted (6:1) with an osmotically balanced sucrose (6.2%)/Mops external buffer in a temperature-controlled OLIS modified stopped-flow fluorimeter. 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 (sulfate is not permeant, [11]). With the incorporation of the protonophore FCCP into the LUV bilayers, H^+ can equilibrate rapidly across the membrane. Hence, the imposed K^+ gradients coupled with the ionophoric action of AmB cause a H^+ for K^+ exchange that is limited by the rate of AmB induced K^+ 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 adjusted for temperature was used for comparisons of AmB's channel activity at different temperatures. The points represent averages of two stopped-flow experiments.

Temperature-controlled circular dichroism (CD) spectra were recorded with a Jobin Yvon Mark V Dichrograph. $\Delta\epsilon$ is the molar dichroic absorption coefficient of the peak-to-trough (approx. 335 nm–355 nm) span of the intense dichroic doublet characteristic of the soluble and insoluble self-associated form of AmB [1,12,13]. The AmB was added from a 10 mM DMSO solution to the same osmolar sucrose/Mops dilution buffer used above.

Fig. 1 demonstrates the influence of both temperature and concentration on the aggregation state of AmB. The three sets of measurements represent the relative amounts of the self-associated species for 0.2 μM, 0.5 μM and 1.0 μM. At room temperature (approx. 25°C) this spans the range in which AmB goes from a largely monomeric (or possibly an optically inactive dimer) to a highly-associated optically-active form. The trend in Fig. 1 clearly shows a significant decrease in the amount of self associated AmB with

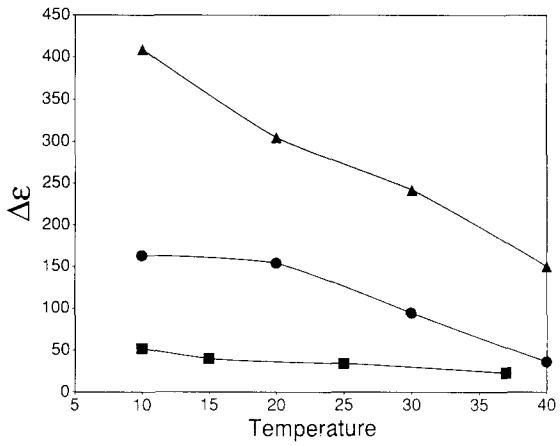

Fig. 1. Comparison of the intensity of the CD doublet at various temperatures for 0.2 μM AmB (■), 0.5 μM AmB (●) and 1.0 μM AmB (▲).

increasing temperature, especially for the 0.5 μM and 1.0 μM solutions.

The results of the temperature-dependent AmB-induced K^+ permeability in LUV with 10 mol% cholesterol or ergosterol are shown in Figs. 2 and 3. At 0.5 μM AmB, the cholesterol-containing vesicles (Fig. 2) shows a striking exponential drop in the initial pH detected K^+ efflux rate with increasing temperature. The temperature dependence is well-fitted by a straight line in a logarithmic scale (Fig. 2, inset) with an apparent Q_{10} of 0.25, that is, there is a 4-fold decline in initial K^+ current per decade increase in temperature.

The temperature dependence of AmB's effect on the ergosterol LUV permeability is not so clear-cut. As expected, at all concentrations and temperatures, initial currents are greater against ergosterol containing vesicles than against cholesterol. However, at both 0.5

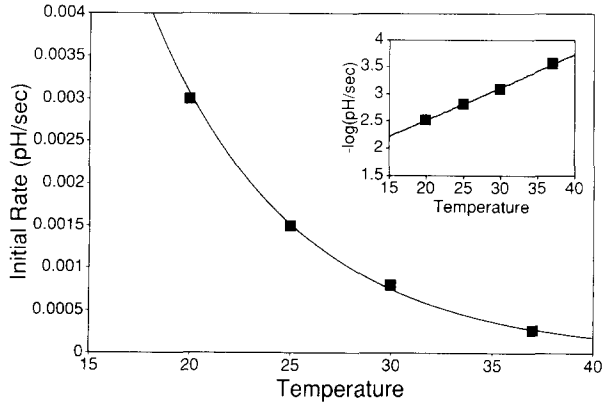

Fig. 2. Temperature dependence of the initial rate of pH change in 10 mol% cholesterol/EPC LUV. The pH change was caused by electrogenic K^+ efflux produced by 0.5 μM AmB. The curve was fitted to an exponential function $f(x) = 5.11 \cdot 10^{-2} \cdot \exp(-0.141 \cdot x)$, $r = 0.998$. The inset shows the same data plotted on a log scale.

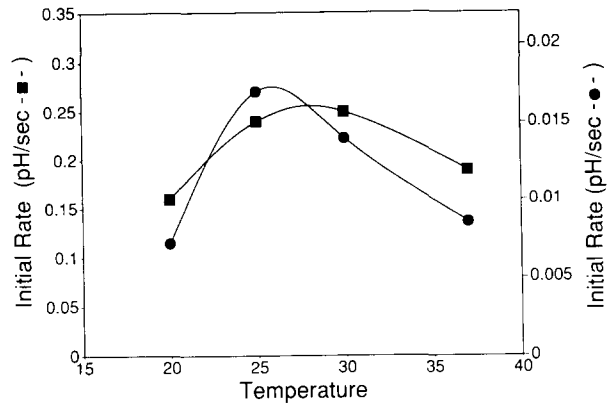

Fig. 3. Temperature dependence of the initial rate of pH change in 10 mol% ergosterol/EPC LUV. AmB concentrations were 0.5 μM (■) and 1.0 μM (●). The lines are drawn simply to guide the eye and do not imply a theoretical fit. Note the different scales used for the two different concentrations.

μM and 1.0 μM there seems to be a slight activity maximum at 25–30°C with a lower permeability induced at both higher and lower temperatures rather than a clear trend towards greater or lesser membrane activity. This more complicated behavior probably represents a convergence of several opposing forces. Firstly, these ergosterol vesicles are probably sensitive to both the self-associated and monomeric species and may be forming different types of channels with different permeability characteristics. Secondly, the rates for both channel formation and destruction are undoubtedly affected by temperature and also influenced by the changing solution supramolecular structures. Furthermore, even long-lived aqueous channel permeation rates will be affected by the increase in ionic mobility with temperature corresponding to a Q_{10} of about 1.3 [14].

An interesting temperature dependence for Nystatin (a polyene closely related to AmB) induced conductance was observed in early studies involving cholesterol-containing planar bilayers [15]. In this system, ion conductance decreased precipitously by as much as 10^4 per 10°C increase in temperature for double-sided addition protocols. Other researchers observed similar trends in biological systems (lobster axons and erythrocytes), but with much smaller temperature effects [15,16]. To our knowledge analogous planar bilayer experiments have not been carried out with ergosterol in place of cholesterol. The relatively simple temperature dependence for cholesterol-containing LUV observed in the present study may be fortuitous, or it may reveal an underlying simplicity in the mode of action of AmB towards cholesterol containing membranes. Specifically, it would conform to the model of AmB action which proposes that cholesterol containing membranes are primarily sensitive to AmB aggregates, whereas ergosterol-containing membranes would also be sensitive to monomers. The recent observations of clear difference in the ion selectivities of channels formed in the presence of these two sterols provides additional support for this hypothesis [17].

The recent comprehensive model of AmB's mode of action form Bolard's group suggests only the soluble aggregated form of AmB is effective against cholesterol or sterol free membranes, whereas the monomeric solution form of AmB would be most effective on ergosterol-containing membranes [6]. A third species, the insoluble aggregate form, would be less effective on both systems. The current CD experiments cannot distinguish the soluble aggregate from the insoluble aggregate, and so we cannot speculate as to the relative activities of these supramolecular aggregates. A key piece of evidence supporting the Bolard model came from temperature-dependent effects of AmB on fungal cells and human erythrocytes [6]. It was shown that higher temperatures decreased AmB-induced erythrocyte K^+ permeability but increased yeast cell K^+ permeability. Unfortunately, the presence of intrinsic transport systems and the overall increase in intrinsic permeability with higher temperature of yeast cells without AmB complicated the interpretation of these results. The present study, by virtue of the use of model membrane vesicles, shows very clearly that the differences in temperature-dependent permeability are related only to the sterol present and the supramolecular structure of AmB. Another recent study [7] concerning the solvent dependence of AmB activity against red blood cells (RBC) and fungal cells confirms that only the water-soluble optically-active form (dimer minimum) of AmB was active against RBC. The monomeric and insoluble aggregate forms were not active against RBC, but the monomeric form remained quite effective against fungal cells.

Our current observations coupled with these may help reconcile the seemingly paradoxical results in many previous studies where the concentration of AmB stock solutions, the identity of the stock solvent and temperature were considered to be insignificant factors in determining the activity of AmB. These results also explain concisely why lowering the chemical potential of AmB to below the critical micelle concentration (e.g., by association with lipids/liposomes) can reduce mammalian AmB toxicity while maintaining efficacy against pathogenic fungi. Alternatively, one would predict that more water-soluble, and hence less aggregated, AmB derivatives should also show a higher therapeutic index than AmB. In many studies, this is indeed observed although the chemical nature of the AmB 'head group' region also has a profound influence on antifungal activity [18–20].

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