Fluorescence Studies on the Molecular Action of Amphotericin B on Susceptible and Resistant Fungal Cells†

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ABSTRACT: The molecular action of amphotericin B (AmB) on the cell membranes of both AmB-susceptible and AmB-resistant fungal cells was investigated through the use of the fluorescent membrane probe trimethylammonium diphenylhexatriene (TMA-DPH). AmB, the most effective drug for the treatment of systemic fungal infections, is known to interact specifically with membrane sterols, especially ergosterol (the major sterol in fungal cells). Treatment of AmB-susceptible *Candida albicans* and *Cryptococcus neoformans* cells with AmB induced a novel biphasic change in TMA-DPH fluorescence intensity over time. The initial decrease in fluorescence intensity results from energy transfer between AmB and TMA-DPH when AmB binds to the fungal cell membrane. The second phase of increasing fluorescence intensity is interpreted in terms of a combination of probe repartitioning and probe segregation as a result of the formation of membrane pores via the aggregation of AmB−ergosterol complexes. An AmB-resistant strain of *C. neoformans*, containing 94% of aberrant δ-8 double-bonded ergosterol precursors and only 4% of ergosterol (71% ergosterol in wild-type cells), exhibited the first phase of AmB binding but not the second phase of increasing fluorescence intensity. This result suggests that AmB's antifungal activity lies in its ability to form membrane pores due to aggregation of AmB−ergosterol complexes. The AmB-induced biphasic fluorescence intensity profile may lead to further elucidation of the molecular action of AmB on fungal cells and may provide a sensitive method for screening the development of drug resistance in fungal cells.

Amphotericin B (AmB),[1] a polyene antifungal agent isolated from *Streptomyces nodosus*, has been the most effective drug for the treatment of systemic fungal infections [reviewed in Bolard (1986)]. Much experimental evidence has suggested that, in order to kill various species of fungi, AmB must first bind to ergosterol, the main sterol in fungal cell membranes. As AmB binds, it displaces the sterol from its normal phospholipid interaction to form a sterol/AmB complex (van den Bossche et al., 1987). Aggregation of sterol/AmB complexes eventually forms membrane pores (Bolard, 1986; van den Bossche et al., 1987; Brajtburg et al., 1990; Bolard et al., 1991; Warnok, 1991; Balakrishman et al., 1993) each of which contains eight alternating AmB molecules and eight sterol molecules (Finkelstein & Holtz, 1973; Kleinburg & Finkelstein, 1984). The inside of the pore is hydrophilic due to the hydroxyl groups of AmB. The outside of the pore is hydrophobic due to the interdigitating hydrocarbon portions of the AmB molecules and sterol molecules. Molecular modeling and permeability studies have estimated the pore size to be 4 Å in diameter with a higher cation rather than anion affinity (De Kruiff & Demel, 1974; Bolard et al., 1991; Khutorsky, 1992; Langlet et al. 1994). Some studies suggest that the formation of membrane pores leads to enhanced membrane permeabilities (Bolard et al., 1991) and ultimately to cell lysis and death (Medoff & Kobayashi, 1980; van den Bossche et al., 1987; Odds, 1988; Brajtburg et al., 1990). Although the physiochemical damage of the cell membrane by AmB has not been proven to be the primary mode of action of AmB's lethal effects, it has been shown to correlate with AmB's lethal action (Beggs, 1994) and to be a crucial step that always precedes cell death (Bolard et al., 1991).

However, the usage of AmB as an effective antifungal agent has encountered some limitations. For example, AmB also has an affinity for cholesterol, the main sterol in mammalian cell membranes (van den Bossche et al., 1987). Due to this affinity for cholesterol-containing membranes, AmB is toxic to mammalian cells and thus cannot be given in high enough concentrations to completely eradicate the fungal infection. Furthermore, a strain of *Cryptococcus neoformans* recently isolated from an AIDS patient was found to be resistant to AmB (Kelly et al., 1994). This resistance arose from a defect in sterol δ-8⇒7 isomerase, which resulted in the accumulation of aberrant δ-8 double-bonded ergosterol precursors and the depletion of the normal amount of ergosterol in the membrane (Kelly et al., 1994). Apparently, the physical nature of the interaction between AmB and membrane sterols plays a crucial role in the pharmacological effect of AmB on both fungal and mammalian cells. Thus, it is of great importance to determine the quantitative differences in the molecular events involved

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[1] Abbreviations: AmB, amphotericin B; NaDOC, sodium deoxycholate; TMA-DPH, 1-[4-(trimethylammonia)phenyl]-6-phenylhexa-1,3,5-triene; DMF, dimethylformamide.

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in the membrane alterations caused by the complexing of AmB with different membrane sterols.

Quantitative measurement of AmB binding to cell membranes has proven to be technically difficult. Separation of the bound form of AmB from free AmB by standard centrifugation methods is not practical due to the formation of sedimentable aggregates of AmB (Henry-Toulme et al., 1989). Recently, the binding of AmB to various cell types has been quantitatively measured via fluorescence quenching (Bolard, 1986; Henry-Toulme et al., 1989; Joly et al., 1992). 1-[4-(Trimethylammonia) phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), a fluorescent membrane probe, can rapidly incorporate into the plasma membrane. The positively charged TMA group is at the lipid−water interfacial region and the DPH portion intercalated in the fatty acyl chains. Thus TMA-DPH allows for probing of the less deep region of phospholipid bilayer (Pendergast et al., 1991) where the lipids are in direct contact with membrane sterols (Huang & Mason, 1982; Straume & Litman, 1987; Lentz, 1989). Moreover, TMA-DPH possesses an emission spectrum that partially overlays the absorption spectrum of AmB. When TMA-DPH and AmB are in close proximity, resonance energy transfer between TMA-DPH and AmB would occur, leading to a decrease in TMA-DPH fluorescence. This quenching of TMA-DPH fluorescence has been used to quantitatively measure the binding of AmB to murine thymocytes and renal tubular cells (Henry-Toulme et al., 1989; Joly et al., 1992).

In the present study we have utilized the fluorescence properties of TMA-DPH to not only monitor the binding of AmB to fungal cells but also to follow the time course of membrane alterations occurring upon the binding of AmB. Treatment of medically important yeasts, *C. neoformans* and *Candida albicans*, with AmB decreases the molecular order of cell membranes as revealed by the steady-state anisotropy of TMA-DPH fluorescence. More interestingly, AmB induces a novel biphasic change in TMA-DPH fluorescence intensity over time. We have studied the physical origin of this biphasic change and examined the response of the biphasic change to temperature and AmB concentration. It was found that the biphasic behavior correlates with the AmB-induced membrane events previously proposed by others [see Bolard (1986) for review], with the first phase of the intensity change corresponding to the binding of AmB to cell membranes and the second phase related to the formation of aggregates of AmB−ergosterol complexes. Using this biphasic variation of fluorescence intensity as an index, we have explored the difference in the action of AmB on membranes between normal fungal cells and an AmB-resistant strain. This level of understanding will be useful in designing methods to reduce the cytotoxicity of AmB and to enhance its antifungal activity.

MATERIALS AND METHODS

Materials. AmB was purchased as fungizone [45% (w/w) AmB, 35% sodium deoxycholate, 20% phosphate buffer] from Sigma (St. Louis, MO). A stock solution of AmB was prepared at a concentration of 2 mg/mL (note: the AmB concentrations reported are AmB concentrations only, not fungizone concentrations) in sterile water and kept at 4 °C in the dark. Working solutions were diluted from this stock with sterile water. The fluorescent probe TMA-DPH was purchased from Molecular Probes (Eugene, OR). Stock solutions of TMA-DPH were prepared in 100% ethanol and kept at 4 °C. The concentration of TMA-DPH was determined using an extinction coefficient at 355 nm equal to 74×10^3 M^{-1} cm^{-1}. Zylamolase 20 T was purchased from ICN Biomedicals Inc. (Costa Mesa, CA).

Cell Culture. Fungal cell strains used were *C. albicans* B311 (ATCC 32354), *C. neoformans* (ATCC 76484), and an AmB-resistant strain of *C. neoformans* [1-40-B (CN-3)], which was a generous gift of Dr. William Powderly at Washington University, St. Louis, MO. All fungal cell cultures were maintained on SAB plates (2% glucose, 1% bactopeptone, 1% agar). Stock cultures were kept in sterile water at 4 °C. Yeast cell suspensions for each experiment were grown overnight in a chemically defined liquid synthetic media for *Candida* or *Cryptococcus* (SMC) (Lee et al., 1975a,b) at 28 °C in a shaking water bath. The cell suspensions were then harvested by centrifugation (1000*g* for 15 min), washed and resuspended in phosphate buffered saline (PBS).

Cell Labeling and Fluorescence Measurements. Yeast cell suspensions ($\sim 1 \times 10^6$ cells/mL as counted by hemacytometer) were added to a cuvette in the sample chamber of either an SLM DMX 1000 fluorometer (Urbana, IL) for fluorescence intensity measurements or an ISS K-2 fluorometer (Champaign, IL) for steady-state fluorescence anisotropy measurements. The cells were incubated at the desired temperature controlled by a bath circulator. TMA-DPH dissolved in 25% ethanol was dispersed into the sample, making the final concentration of probe ~ 2 μM. The final concentration of ethanol in the sample was 0.025% which had previously been found to be without a significant effect on cell viability. The quantum yield of this probe in an aqueous solution is virtually zero (Pendergast et al., 1981), yet as the probe is incorporated into the hydrophobic environment of the cell membrane the fluorescence intensity increases dramatically. The fluorescence intensity levels off after ~ 2 min as the incorporation of the probe approaches equilibrium. After the increase in fluorescence intensity had reached a steady-state level, the indicated concentrations of AmB were added and fluorescence measurements were taken. All fluorescence intensity measurements were made at an excitation wavelength of 365 nm and an emission wavelength of 427 nm; this corresponds to the emission maximum of TMA-DPH emission spectra in phospholipid membranes (Pendergast et al., 1981; Henry-Toulme et al., 1989). Relative fluorescence intensity was standardized to a F/F_o value. F_o was defined as the steady level of fluorescecence intensity after the addition of TMA-DPH. F was the fluorescence intensity at each time point after the addition of AmB. The ratio of F/F_o was plotted as a function of time. For steady-state fluorescence anisotropy experiments, the excitation wavelength was 365 nm and the emission was observed through a Schott KV418 filter. Blank readings from cell suspensions without probe were subtracted from the sample readings. For all fluorescence measurements, a narrow slit width was used for excitation and photobleaching was not detected. For the preincubation experiments, *C. neoformans* cells were incubated with the indicated concentrations of AmB at 37 °C for 15 min in the sample chamber of the SLM DMX 1000 fluorometer. After the preincubation period, 2 μM TMA-DPH was added as

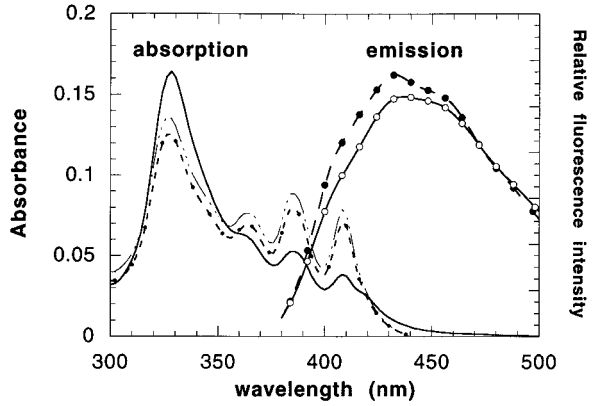

FIGURE 1: Absorption spectrum of 2 μM AmB in presence of *C. neoformans* cells recorded at $t = 0$ s (—), $t = 5$ min (—●—), and $t = 10$ min (— - - - —) after addition of AmB. TMA-DPH fluorescence spectrum in the presence of *C. neoformans* cells before (- - ● - -) and after the addition of 5 μM AmB (— ○—). Excitation wavelength was 365 nm.

previously indicated and fluorescence measurements were taken.

Preparation of Spheroplasts. Spheroplasts of *C. albicans* were prepared from an overnight culture grown in SMC at 28 °C in a shaking water bath. The cells were harvested by centrifugation [1000*g* for 10 min at room temperature (~25 °C)], washed twice with 0.1 M Tris/0.5 M $MgSO_4$ buffer, pH 6.8, and resuspended in 0.1 M Tris/0.5 M $MgSO_4$ buffer. β-Mercaptoethanol was added to the cell suspension to a final concentration of 100 mM, and the cells were incubated at room temperature for 45 min on a gyratory shaker. Zymolase 20T was added to the cells to a final concentration of 1 mg/mL, and the cells were incubated for an additional 2 h at room temperature on a gyratory shaker. Spheroplasts were harvested by centrifugation (300*g* for 15 min), washed with 0.1 M Tris/0.5 M $MgSO_4$ buffer, and resuspended in 0.1 M Tris/0.5 M $MgSO_4$ buffer stabilized with 0.6M KCl (Elorza et al., 1983; Gopal et al., 1984).

Absorption Measurements. The absorption spectrum of AmB was measured in the presence of *C. albicans* cells and *C. neoformans* cells on a Hewlett Packard 8452A diode array spectrophotometer (Wilmington, DE) at 37 °C. A background spectrum of the yeast suspension was taken and recorded as the blank. Two micromolar AmB (as fungizone) was added to each sample at $t = 0$ s, and the changes in AmB's absorption spectrum induced by binding to the cells were recorded over time.

RESULTS AND DISCUSSION

Time Course for the AmB-Induced Changes in TMA-DPH Fluorescence Intensity. TMA-DPH solubilized in ethanol was found to readily incorporate into fungal cell membranes as evidenced by the significant increase in fluorescence intensity upon addition of the probe. This increase in intensity is characteristic of TMA-DPH embedded in a lipid environment since the quantum yield of TMA-DPH in the membrane is much higher than that in water (Pendergast et al., 1981). Figure 1 depicts the typical fluorescence emission spectrum of TMA-DPH when incorporated into the fungal cells before and after the addition of AmB. Figure 1 also illustrates a significant overlap between the absorption spectrum of AmB and the emission spectrum of TMA-DPH in fungal cells.

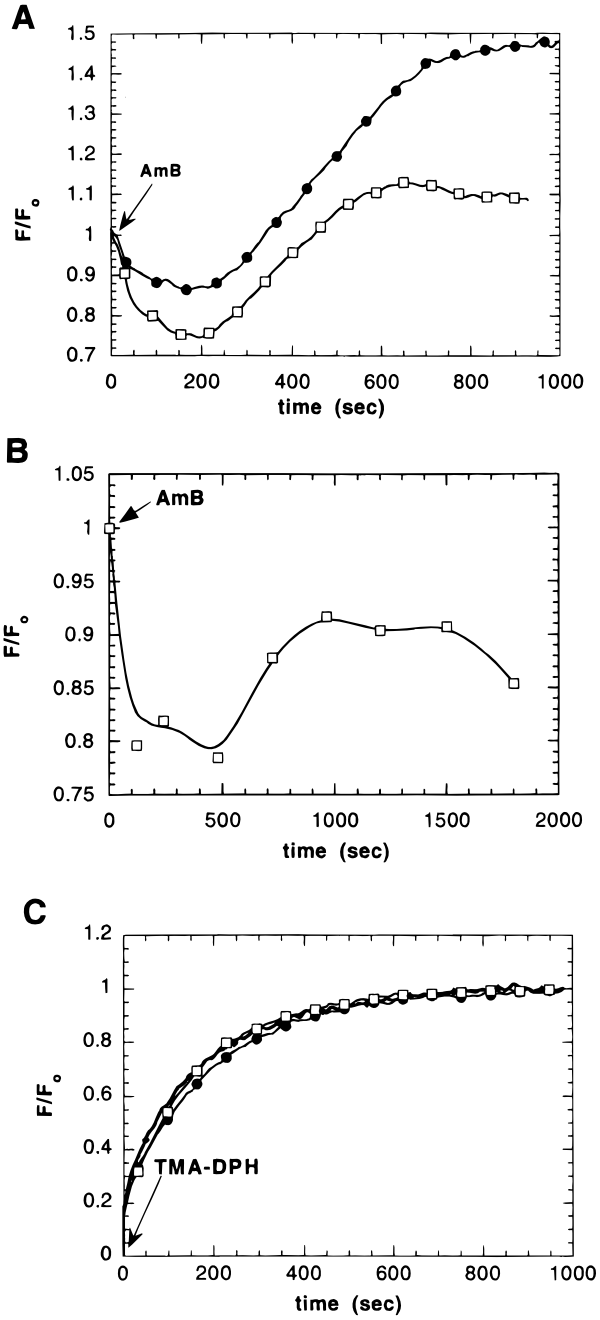

FIGURE 2: (A) AmB-induced changes in TMA-DPH fluorescence intensity in *C. albicans* over time. This is a representative graph of six experiments. Approximately 1.5×10^6 cells/mL were labeled with 2 μM TMA-DPH. Temperature was 37 °C. Data points were collected every 2 s. Symbols are included for line differentiation only. Addition of 2 μM AmB (●) and 5 μM AmB (□). (B) Fluorescence intensity changes induced by AmB in spheroplasts of *C. albicans*. AmB (5 μM) (□) was added at $t = 0$ s. Each symbol represents a data point. Cells were resuspended manually before each time point to prevent cell aggregation. This is a representative graph of three experiments. (C) Fluorescence intensity of TMA-DPH in *C. neoformans* preincubated with 2 μM AmB (□), 5 μM AmB (○), and 10 μM AmB (△) for 15 min prior to the addition of TMA-DPH. Temperature was 37 °C. Data points were collected every 2 s. Symbols are included for line differentiation only.

After fluorescence intensity of TMA-DPH had reached a steady-state level (~30 s), AmB was added. This addition of AmB caused the fluorescence intensity of TMA-DPH in *C. albicans* to initially decrease with time (Figure 2A). This was followed by an increase in fluorescence intensity which eventually reached a saturation point.

The initial decrease of fluorescence intensity is not surprising. Similar results were observed previously in murine thymocytes by Henry-Toulme et al. (1989) and in renal tubular cells by Joly et al. (1992). This decrease of TMA-DPH fluorescence was proposed by these authors to result from the resonance energy transfer between TMA-DPH (donor) and AmB (acceptor). The extent of quenching of TMA-DPH fluorescence by AmB has been used by these investigators to quantitatively measure the binding of AmB to cells. The surprising result is the increase of fluorescence intensity appearing after ~200 s (Figure 2A). Since DPH fluorescence is highly sensitive to the immediate environment [reviewed in Toptygin and Brand (1995) and Gratton and Parasassi (1995)], the biphasic change in TMA-DPH fluorescence in fungal cells (Figure 2A) may reflect the membrane alterations caused by AmB. To ensure that this is the case, we have conducted the following control experiments.

Control Experiments: (i) Can the Biphasic Behavior of TMA-DPH Fluorescence Be Attributed to Cell Wall, Detergent, or Optical Artifacts? In fungal cells, TMA-DPH must penetrate the cell wall before interacting with the plasma membrane. To determine if the biphasic nature of TMA-DPH fluorescence intensity is related to the cell wall, spheroplasts of *C. albicans* cells (fungal cells whose cell wall has been enzymatically removed by Zymolase 20T) were assayed instead of native cells. Cells were resuspended prior to each intensity measurement due to the formation of spheroplast aggregates. Figure 2B shows that a similar biphasic fluorescence intensity profile is seen in the case of spheroplasts. Although one cannot rule out the possibility that TMA-DPH interacts with cell wall, Figure 2B indicates that most likely the biphasic change does not originate from the cell wall.

The biphasic profile is not due to the presence of the detergent sodium deoxycholate (NaDOC) in the commercial AmB preparation. When NaDOC alone (without AmB) was added to the fungal cells in the presence of TMA-DPH, the biphasic change in fluorescence intensity was not seen (data not shown). Also, AmB solubilized in dimethylformamide (DMF), not NaDOC, did produce the biphasic fluorescence intensity profile (data not shown). The biphasic change in fluorescence intensity is not due to cell aggregation either, since no significant changes in scattered light were detected (data not shown). The scattered light was measured at 570 nm at a right angle with respect to the excitation (550 nm) using the SLM DMX-1000 fluorometer.

There is the possibility that the increase in fluorescence intensity in the second phase could result from a change in the absorption spectrum of AmB occurring when AmB incorporates into the fungal cell membrane and forms an AmB/ergosterol complex as previously proposed by many investigators [see Bolard (1986) and Brajtburg et al. (1990) for review]. If this change in the absorption spectrum resulted in a blue-shift, then the spectral overlap between the absorption spectrum of AmB and the emission spectrum of TMA-DPH would be reduced. This reduction in overlap could result in diminished resonance energy transfer between AmB and TMA-DPH, thus releasing AmB's ability to quench TMA-DPH fluorescence resulting in an increase in fluorescence intensity.

To test this possibility, the changes in AmB's absorption spectrum induced by incorporation of AmB into *C. albicans* and *C. neoformans* cells were monitored over time. There was a time-dependent change in the absorption spectrum of AmB in the fungal cells (Figure 1). Similar results were obtained in *C. albicans* cells (data not shown). In both cell types, the absorbance of low energy bands increases significantly within 5 min after the addition of AmB. These changes may arise from the appearance of the aggregated state of AmB−sterol complexes (Gruda & Drussault, 1988). Nevertheless, these changes in AmB's absorbance produced an increase in the overlap region between the absorption spectrum of AmB and the emission spectrum of TMA-DPH. This would increase the incidence of resonance energy transfer, increasing AmB's quenching of TMA-DPH fluorescence. Therefore, the increase in fluorescence intensity in the second phase of the biphasic profile (Figure 2A) cannot be explained by changes in the absorption spectrum of AmB in fungal cells.

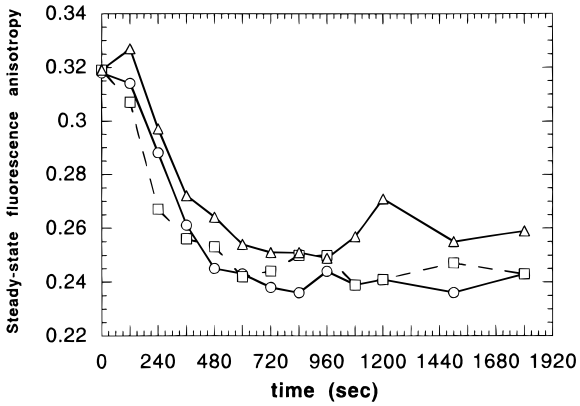

FIGURE 3: AmB-induced changes in steady-state fluorescence anisotropy of 2 μM TMA-DPH in *C. albicans* cells over time. Temperature was 37 °C. Each symbol represents a data point. AmB (1 μM) (○), 2 μM AmB (□), and 5 μM AmB (△). The typical standard deviation was 0.013, $n = 14$.

(ii) Can the Second Phase of the Biphasic Behavior of TMA-DPH Fluorescence Be Attributed to an AmB-Induced Tighter Packing of the Lipid Bilayer? Since the fluorescence intensity and lifetime of DPH are inversely proportional to the dielectric constant of the environment adjacent to the probe (Gratton & Parasassi, 1995), the increase in TMA-DPH fluorescence in the second phase of the biphasic profile (Figure 2A) may be due to the exclusion of water from the membrane as a result of AmB-induced tighter packing in the bilayer. However, fluorescence anisotropy measurements (Figure 3) show this is not the case. The addition of AmB to fungal cells resulted in a sharp decrease in TMA-DPH anisotropy (Figure 3) in the first 250 s and then leveled off. Assuming that the fluorescence lifetime of TMA-DPH remains unchanged within this time period, the anisotropy data would imply that AmB causes a decrease, rather than an increase, in the molecular order of lipid acyl chains (Jahnig et al., 1979; Heyn et al., 1979). In fact, within the first 250 s the fluorescence lifetime of TMA-DPH is most likely to decrease with time as a result of energy transfer. After the correction for lifetime contributions, the trend of decreasing the molecular order of lipid acyl chains by AmB is likely to persist. Hence, our present result is consistent with the previously proposed mechanism of action of AmB in that the binding of AmB induces membrane destabilization (Bolard, 1986; van den Bossche et al., 1987; Henry-Toulme et al., 1989; Brajtburg et al., 1990; Bolard et al., 1991; Warnok, 1991).

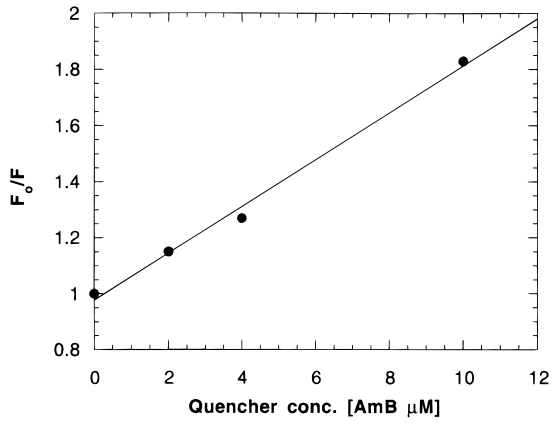

FIGURE 4: Stern−Volmer plot of TMA-DPH fluorescence in DMF versus AmB concentration. TMA-DPH (2 μM) was dissolved in dimethyl formamide (DMF) in the sample chamber of a SLM DMX 1000 fluorometer at 37 °C. Increasing concentrations of AmB were added, and the change in fluorescence intensity at 427 nm was monitored. Excitation was 365 nm.

Plausible Explanations of the Biphasic Change in TMA-DPH Fluorescence Intensity. It can be assumed that the binding of AmB to the fungal cell membrane is a spontaneous and relatively fast process. Once inside the membrane, AmB will interact with the embedded TMA-DPH. As discussed earlier, the first phase of the biphasic intensity profile can be interpreted by virtue of resonance energy transfer (a long-range interaction) between membrane-bound AmB (acceptor) and membrane-bound TMA-DPH (donor) (Henry-Toulme et al., 1989; Joly et al., 1992). Resonance energy transfer is believed to mainly occur because of an overlap between the absorption spectrum of AmB and the emission spectrum of TMA-DPH (Henry-Toulme et al., 1989). Henry-Toulme et al. (1989) calculated a Föster distance, R_o, for AmB and TMA-DPH to be ~40 Å and found that this value is almost invariant with AmB concentration and environment. However, the Stern−Volmer plot of TMA-DPH fluorescence in DMF versus AmB concentration is linear (Figure 4), giving a Stern−Volmer constant of 0.08 μM^{-1}. Assuming that the lifetime of TMA-DPH fluorescence is ~10 ns, a dynamic quenching rate constant is calculated to be 8×10^8 M^{-1} s^{-1}. This value does not exceed the diffusion limit, suggesting that in fungal cell membranes, AmB may also quench the fluorescence of TMA-DPH via a short-range, collisional mechanism. In any case, the decrease in fluorescence intensity in the first phase can be attributed to energy transfer, but whether the energy transfer is a long-range or a short-range interaction remains to be answered.

The second phase of the biphasic intensity profile may also be related to the AmB-induced membrane alterations. It can be postulated that, once inside the membrane, AmB not only interacts with TMA-DPH but also forms a complex with ergosterol via van der Waals interactions (depicted in Figure 5), a suggestion previously made by others (Finkelstein & Holtz, 1973; Kleinburg & Finkelstein, 1984; Bolard, 1986; van den Bossche et al., 1987; Brajtburg et al., 1990; Bolard et al., 1991; Warnok, 1991; Balakrishman et al., 1993). The formation of AmB−ergosterol complexes may then be followed by a relatively slow lateral diffusion and association of complexes to form aggregates (Figure 5). It has been suggested that these aggregates eventually comprise a membrane pore and the membrane pore confers higher

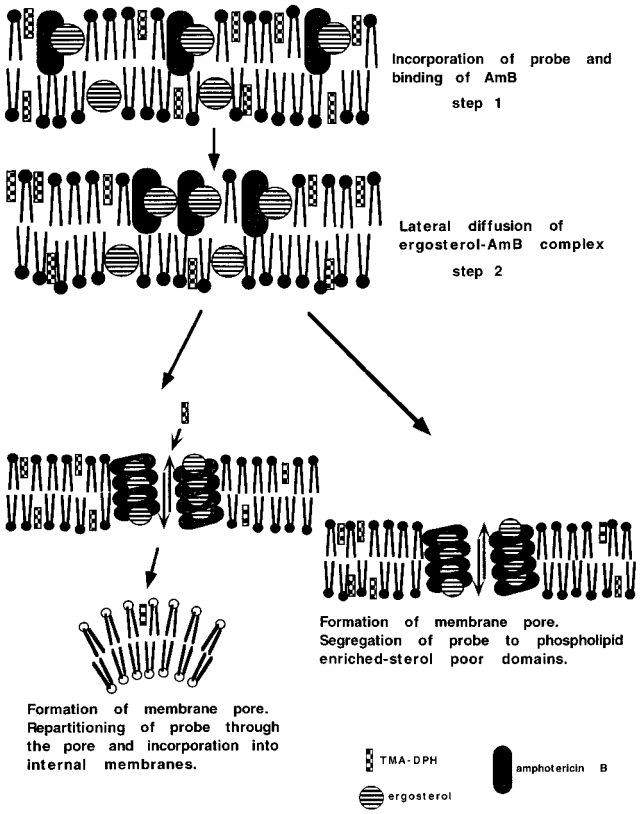

FIGURE 5: Proposed mechanisms to explain the novel AmB-induced biphasic change in TMA-DPH fluorescence intensity.

permeability to ions, water, and nonelectrolytes. At this point, two mechanisms can be proposed to explain the second phase of the biphasic fluorescence intensity profile.

According to the first proposed mechanism, the second phase of increasing fluorescence intensity is a result of an increased distance between TMA-DPH and AmB in the membrane. This increase in distance arises as ergosterol complexes with AmB, the AmB−ergosterol complexes aggregate, and AmB is displaced from the phospholipid domain (Bolard et al., 1991). Consequently, the probe molecules laterally segregate from the AmB-ergosterol aggregates to phospholipid-enriched, sterol-poor domains. Since resonance energy transfer efficiency is inversely proportional to the sixth power of the distance between donor and acceptor, this segregation decreases the energy transfer between AmB and TMA-DPH, thereby increasing fluorescence intensity.

The second mechanism proposes that the increase in TMA-DPH fluorescence at ~200 s is the result of a decreased efficiency of collisional dynamic quenching due to the changes in quenchers. Initially, membrane-bound AmB is the primary quencher of TMA-DPH fluorescence. As time elapses, the amount of AmB−ergosterol complex increases and if these complexes are less efficient quenchers of TMA-DPH fluorescence than the membrane-bound AmB, then as the ratio of the AmB−ergosterol complex to the membrane-bound AmB is elevated, the extent of quenching will be decreased (Figure 2A). Both the decrease in resonance energy transfer (a long-range interaction) due to probe segregation and the decrease in dynamic quenching (a short-range interaction) due to changes in the quencher species can be considered generically as a decrease in energy transfer.

However, neither of these mechanisms can explain why at certain conditions the F/F_o value in the second phase

becomes greater than one. This problem can be reconciled in terms of probe repartitioning through the AmB-induced membrane pore. Once formed, the pore would allow those TMA-DPH molecules originally in the aqueous phase to partition into intracellular membranes or additional probe molecules may be incorporated into the plasma membrane. The anisotropy data (Figure 3) suggest that AmB "loosened" the packing order of membrane lipids over time. This type of disordering effect has been demonstrated to facilitate solute partitioning into membranes (DeYoung & Dill, 1990). The increase of incorporation of probe molecules into a lipid environment of either the plasma or intracellular membranes would increase fluorescence intensity since the fluorescence quantum yield of TMA-DPH in a membrane is approximately 1000 times greater than in water. This repartitioning not only explains the appearance of the second phase but also explains why the F/F_o value can exceed the original value (i.e., >1).

The repartitioning mechanism and the release of energy transfer are not mutually exclusive. These two mechanisms may actually occur at the same time. Both mechanisms relate the formation of AmB−ergosterol aggregates (or membrane pore) to the second phase of the biphasic profile presented in Figure 2A. It is interesting that the release of potassium ions reaches a saturation point at ~2 min after the addition of 1.7 μM AmB to *C. albicans* cells (Beggs, 1994). In comparison, our data (Figure 2A) showed that the biphasic behavior of TMA-DPH fluorescence occurs at ~2.5 min after the addition of 2.0 μM AmB to *C. albicans* cells. Also, the changes in the absorption spectrum of AmB (Figure 1) correlate in a time-dependent manner with the biphasic change in TMA-DPH fluorescence. The maximum change in the absorption spectrum of AmB occurs at >10 min (Figure 1), approximately the same time that the TMA-DPH fluorescence intensity reaches saturation (~16 min). According to our hypothesis (Figure 5), the intensity saturation point is the time when the formation of membrane pore is nearly complete. The time scale for the maximal potassium permeability (Beggs, 1994) and absorption change (Figure 1) agrees with the time where the biphasic change of fluorescence intensity appears (Figure 2A). *C. neoformans* cells preincubated with 2, 5, or 10 μM AmB for 15 min did not demonstrate a biphasic change in TMA-DPH fluorescence (Figure 2C). This period of preincubation is consistent with the proposed time frame needed for membrane pore formation to reach completion. Since pore formation was complete, there is no change in the distance between donors and acceptors nor any change in the quencher species. Thus, the biphasic profile is abolished. These data further suggest that the AmB-induced biphasic change in TMA-DPH fluorescence is a reflection of the reorganization of the membrane which occurs upon the formation of membrane pores.

Effects of AmB Concentration and Temperature on the Biphasic Profile of TMA-DPH Fluorescence. TMA-DPH fluorescence intensity varies with AmB concentration in all regions of the biphasic profile (Figure 6A). Notably, the fluorescence intensity of TMA-DPH in the second phase reaches a saturation point at an earlier time when the concentration of AmB becomes higher. It is possible that increased amounts of AmB in the membrane facilitate completion of membrane pores, as a result of aggregation of AmB−ergosterol complexes. Furthermore, increased

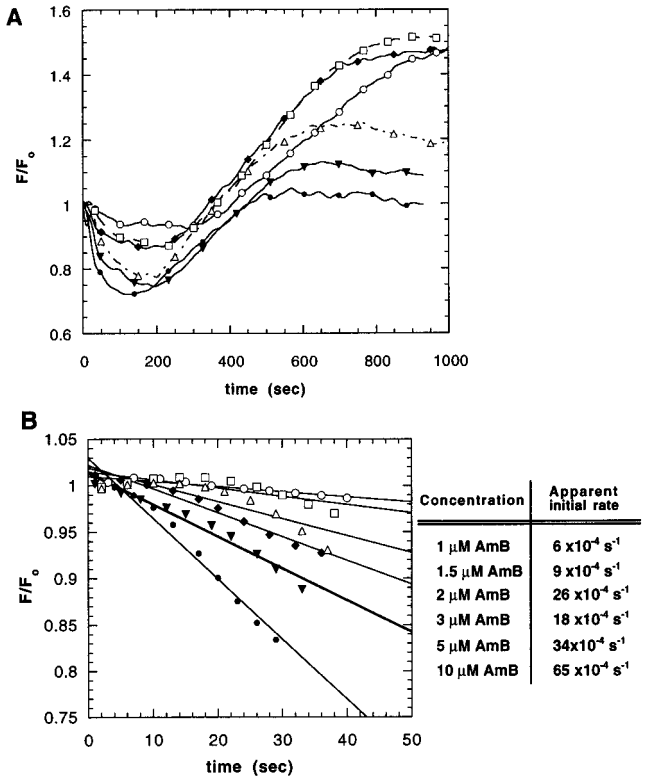

FIGURE 6: (A) Concentration dependence of the AmB-induced fluorescence intensity profile in *C. albicans* cells. Various concentrations of AmB were added at $t = 0$ s, and the fluorescence intensity changes were monitored over time. Data were collected every 2 s. Symbols are included for line differentiation only. AmB (1 μM) (○), 1.5 μM AmB (□), 2 μM AmB (◆) 3 μM AmB (△), 5 μM AmB (▼), 10 μM AmB (●). (B) Apparent initial rates of decrease in TMA-DPH fluorescence resulting from quenching by increasing concentrations of AmB as a function of AmB concentration. This is a representative graph of six experiments.

amounts of AmB in the membrane may also create more membrane pores as each pore may consist of a finite number of AmB molecules [eight AmB molecules per channel (Khutorsky, 1992)]. Assuming that these membrane pores are homogeneously distributed in the membrane, AmB molecules would be separated to a lesser extent at high concentrations and thus only decrease the energy transfer a finite amount. This explains why the fluorescence intensity increase in the second phase decreases with increasing AmB concentration (Figure 6A).

Figure 6B shows that the initial rate of TMA-DPH fluorescence quenching increases with increasing concentrations of AmB. A decrease in the average distance between the donor TMA-DPH and the acceptor AmB molecule would occur when the amount of AmB in the membrane increases. Since resonance energy transfer is dependent upon the distance between donor and acceptor, a decrease in the average distance would increase the efficiency of energy transfer, thus increasing the quenching rate of TMA-DPH fluorescence intensity.

Figures 7A and 8A show the effect of temperature on the fluorescence intensity of TMA-DPH in *C. neoformans* and *C. albicans*, respectively, in the presence of AmB. It appears that temperature increases the initial rate of TMA-DPH fluorescence quenching (Figures 7B and 8B). This result is expected because temperature is known to disorder membrane lipid packing which facilitates solute partitioning into membranes (DeYoung & Dill, 1990). Thus, it is likely that

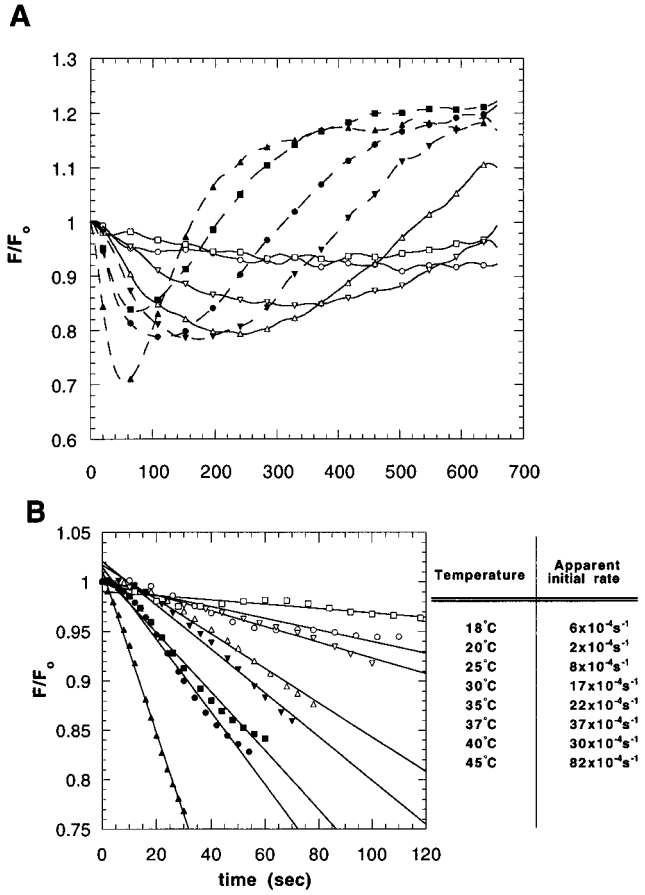

FIGURE 7: (A) Changes in the TMA-DPH fluorescence intensity profile in *C. neoformans* cells at 18 °C (○), 20 °C (□), 25 °C (▽), 30 °C (△), 35 °C (▼), 37 °C (●), 40 °C (■), and 45 °C (▲). Cells were incubated at the indicated temperatures for at least 5 min prior to the addition of TMA-DPH. At $t = 0$ s 5 μM AmB was added and the changes in fluorescence intensity were monitored over time. Data were collected every 2 s. Symbols are included for line differentiation only. (B) Initial rates of decrease in TMA-DPH fluorescence resulting from quenching by AmB as a function of temperature. This figure is an enlargement of the region from $t =$ 0 s to $t = \sim$80 s of panel A. This is a representative graph of six experiments.

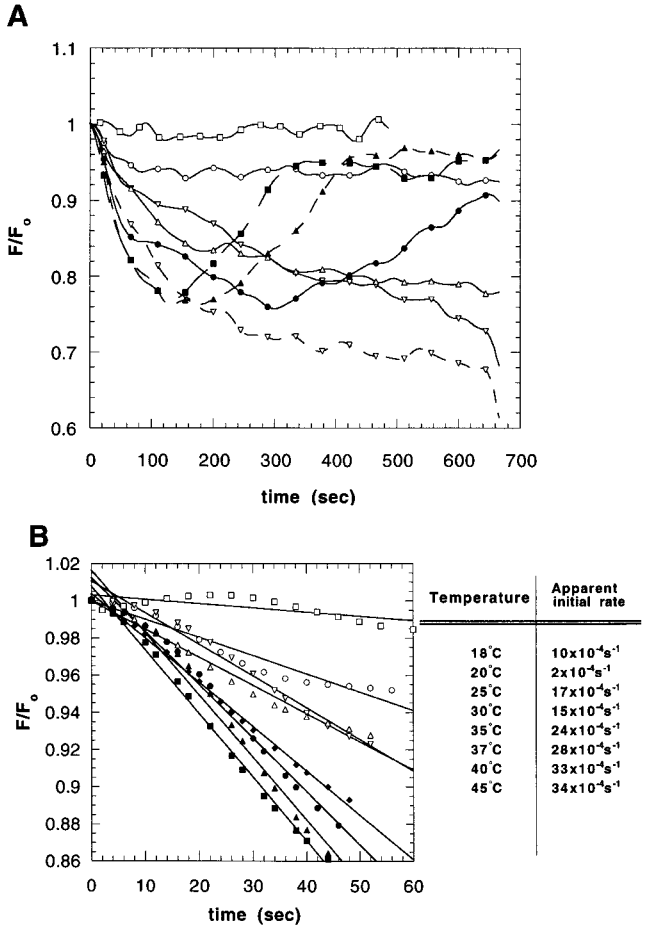

FIGURE 8: (A) Changes in the TMA-DPH fluorescence intensity profile in *C. albicans* cells at 18 °C (○), 20 °C (□), 25 °C (▽), 30 °C (△), 35°C (- - -▽- - -), 37 °C (●), 40 °C (■), and 45 °C (▲). Cells were incubated at the indicated temperatures for at least 5 min prior to the addition of TMA-DPH. At $t = 0$ s 5 μM AmB was added, and the changes in fluorescence intensity were monitored over time. Data were collected every 2 s. Symbols are included for line differentiation only. (B) Initial rates of decrease in TMA-DPH fluorescence resulting from quenching by AmB as a function of temperature. This figure is an enlargement of the region from $t = 0$ s to $t = \sim$60 s of panel A. This is a representative graph of six experiments.

at higher temperatures more AmB and TMA-DPH molecules reside in the membrane, causing more effective energy transfer between them.

The second phase of the biphasic profile also varies with temperature (Figures 7A and 8A). The biphasic pattern is not discernible at low temperatures (18−25 °C) but becomes detectable at 30 °C (Figure 7A) to 37 °C (Figure 8A) and above. As explained earlier, the second phase of the biphasic profile is related to the formation of aggregates of AmB−ergosterol complexes (i.e., membrane pores). This process must be governed by the lateral mobility of the AmB−ergosterol complexes in the membrane which depends on the physical state of the lipid matrix and the temperature and pressure, with higher temperature, lower pressure, and the liquid-crystalline state favoring the lateral motion. Although the physical state of membrane lipids in *C. neoformans* and *C. albicans* is not characterized, increased temperature alone should facilitate the lateral motion of AmB−ergosterol complexes in the membrane, producing aggregates of AmB−ergosterol complexes (or membrane pores). At lower temperatures, this process may take a much longer time, which explains why the biphasic profile appears only at higher temperatures. It is important to note that the optimum growth temperature of *C. albicans* and *C. neoformans* is 28 °C. There are previous examples showing that cell membranes become significantly more fluid at temperatures near or higher than the growth temperature [e.g., Kao et al. (1992)]. This concept can also explain why the second phase of intensity increase levels off at ∼300 s after the addition of AmB at 45 °C, while, at 37 °C, the intensity continues to rise at this time (Figures 7A and 8A). It appears that membrane pores are formed and completed at an earlier time due to a more fluid environment caused by a higher temperature. In support of this hypothesis, K^+ permeability, which has been proposed to reflect the appearance of membrane pores, has been shown to increase with a concomitant increase in temperature (Bolard et al., 1991).

It is evident that the fluorescence intensity profile in both *C. neoformans* and *C. albicans* is greatly affected by changes in temperature. However, it should be noted that there is a difference in the intensity profile obtained in each yeast species, namely, the observation that the fluorescence intensity in *C. neoformans* exceeds a F_o/F value of 1 whereas in *C. albicans* this is not the case (Figure 7A and 8A). We

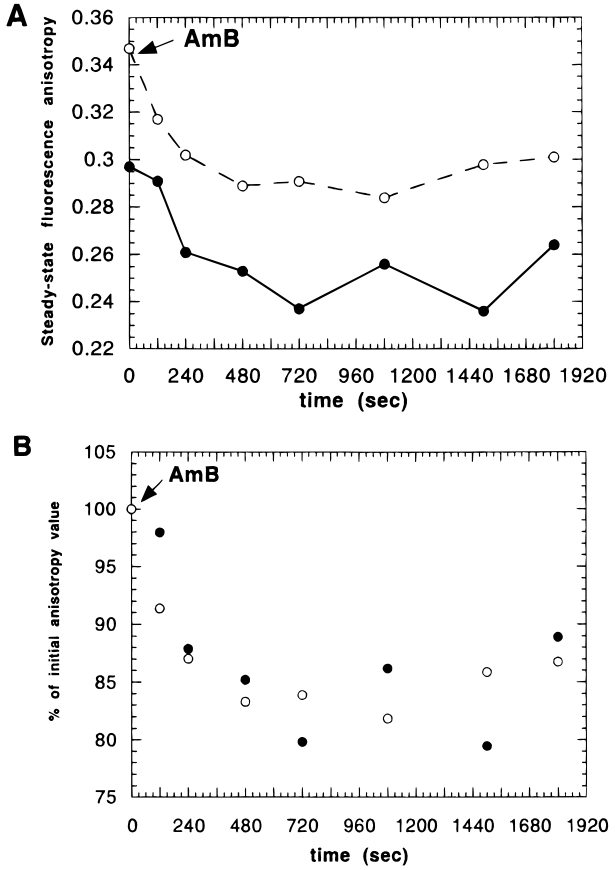

FIGURE 9: (A) AmB-induced changes in steady-state fluorescence anisotropy of TMA-DPH in AmB-susceptible (●) and AmB-resistant (○) *C. neoformans* cells. Temperature was 35 °C. Each symbol represents a data point. The typical standard deviation was 0.013, $n = 11$. (B) Depiction of the percent of changes in steady-state fluorescence anisotropy induced by AmB in AmB-susceptible (●) and resistant (○) *C. neoformans* cells.

have previously hypothesized how the F_o/F value could surpass its original value of 1.

Comparison of AmB-Induced Changes of TMA-DPH Fluorescence in Normal Fungal Cells with Those in an AmB-Resistant Strain. Recently, a strain of *C. neoformans* isolated from an AIDS patient was found to be resistant to AmB (Kelly et al., 1994). This resistance arose from a defect in a sterol δ-8⇒7 isomerase, which results in the accumulation of aberrant δ-8 double-bonded ergosterol precursors and the depletion of normal amounts of ergosterol in the membrane. Steady-state fluorescence anisotropy data (Figure 9A) suggest that the AmB resistant strain of *C. neoformans* has a higher membrane lipid order than the AmB susceptible strain as suggested by the initially higher steady-state anisotropy value of the resistant strain as compared to the susceptible strain (assuming that the fluorescence lifetimes of TMA-DPH in both strains are virtually identical). However, the binding of AmB to both strains yielded the same percent decrease in anisotropy values over time (Figure 9B), indicating that AmB can increase membrane fluidity of both strains.

More interestingly, the AmB resistant strain did not demonstrate a distinct biphasic fluorescence intensity profile at 37 °C (Figure 10A−C). The reported difference between the resistant and susceptible strains is their membrane sterol content, in which the major sterols in this resistant strain were ergosta-5,8,22-dienol (35.6%), ergosta-8,22-dienol (8.5%), ergosta-8,24(28)-dienol (25.7%), and ergosta-8-enol (24.3%)

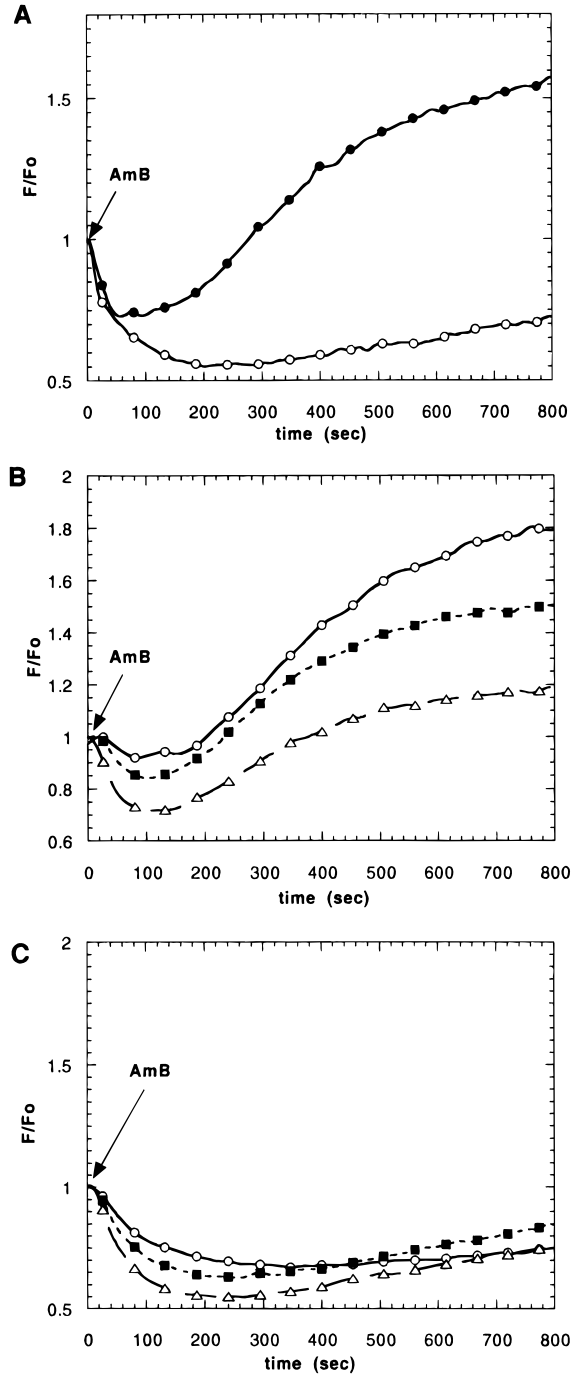

FIGURE 10: Differences in the fluorescence intensity profile induced by AmB in AmB-susceptible and AmB resistant strains of *C. neoformans*. Temperature was 37 °C. Data were collected every 2 s. Symbols are included for line differentiation only. (A) addition of 5 μM to both AmB-susceptible (●) and AmB-resistant *C. neoformans* (○). (B) Addition of 2 μM AmB (○), 5 μM AmB (■), and 10 μM AmB (△) to AmB-susceptible *C. neoformans*. (C) Addition of 2 μM AmB (○), 5 μM (■), and 10 μM AmB (△) to AmB-resistant *C. neoformans*. This is a representative graph of six experiments.

(Kelly et al., 1994). The AmB-susceptible strain contained 71% ergosterol, whereas the AmB-resistant strain had only 4% ergosterol. Thus, our result implies that AmB's ability to induce a biphasic change in fluorescence intensity is dependent upon the specific sterols present in the fungal cell membrane.

It is also important to note that this resistant strain does contain a cell wall. The observation that this resistant strain

did not give rise to the biphasic intensity profile when incubated with AmB further supports our supposition (the conclusion derived from the results of Figure 2B) that the appearance of the second phase of increasing fluorescence intensity is not a result of the TMA-DPH probe molecules interacting with the cell wall.

There is sterol selectivity for AmB binding. Bittman et al. (1974) and Chen and Bittman (1977) demonstrated that at 36 °C AmB binds faster to ergosterol than cholesterol in dimyristoylphosphatidylcholine (DMPC) small unilamellar vesicles and that the δ-7 double bond is of critical importance whereas the δ-5 double bond is not essential. The majority of the sterols in the AmB-resistant *C. neoformans* strain were lacking this critical δ-7 double bond and contained an aberrant δ-8 double bond instead (Kelly et al., 1994). It was previously demonstrated that AmB binds more tightly to ergosterol than cholesterol, desmosterol, lanosterol, β-sitosterol, and stigmasterol (van den Bossche et al., 1987). This is thought to be the reason for fungal cells having a higher sensitivity to AmB than mammalian cells since ergosterol is the dominant sterol in fungal cells. Bolard et al. (1991) also proposed that the binding of AmB to sterols other than ergosterol is weak or nonexistent due to conformational changes in non-ergosterol sterols that lead to smaller van der Waals interactions with AmB (Langlet et al., 1994). Due to the relatively weak interactions between AmB and non-ergosterol sterols, Bolard et al. (1991) have proposed that AmB and cholesterol do not form a membrane pore. Only ergosterol-containing cells form AmB-sterol membrane pores.

Recently, other investigators have discovered a human T cell line that is resistant to AmB (Buttke & Folks, 1992). This particular T cell line was found to be defective in lanosterol demethylation, and, as a result, all membrane cholesterol was replaced with 4,4′,14-trimethyl sterols. It appeared that this cell line's resistance to AmB results from the inability to form pores since these 4,4′,14-trimethyl sterols do not readily associate with AmB.

In our experiments the AmB-resistant *C. neoformans* strain was not able to produce the AmB-induced biphasic fluorescence intensity profile under the same conditions that the AmB-susceptible strain clearly demonstrated a biphasic profile. The fact that the resistant strain did not exhibit the biphasic fluorescence intensity profile suggests that the resistant strain is not able to form this transmembrane pore due to the possible incapacity of the altered sterols (δ-8 double-bonded ergosterol precursors) present in the membrane to form a complex with AmB. This finding is consistent with the proposal of other investigators, as discussed previously, that sterols other than ergosterol do not complex very efficiently with AmB and therefore do not create a transmembrane pore.

Our hypothesis for the novel AmB-induced biphasic fluorescence intensity profile is summarized in Figure 11. The initial decrease in fluorescence intensity (region I) is due to energy transfer between AmB and TMA-DPH. Two mechanisms are proposed to explain the second phase of increasing fluorescence intensity. The increase in fluorescence intensity in region IIa is either due to repartitioning of TMA-DPH probes from the aqueous phase to membranes, possibly through a membrane pore, or due to probe segregation from AmB-ergosterol aggregates, or both. The leveling off of fluorescence intensity seen in Region IIb is due to the completion of the membrane pore via the aggregation of AmB−ergosterol complexes. The extent of energy transfer is sensitive to the concentration of AmB added and to the temperature at which the profile was taken. The second phase of increasing fluorescence intensity is also dependent upon the temperature, AmB concentration, and, interestingly, the sterol composition of the fungal cell membrane.

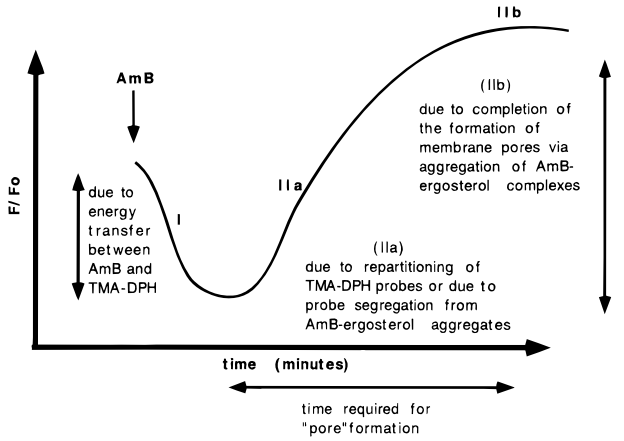

FIGURE 11: Summary of the interpretation of the AmB-induced changes in TMA-DPH fluorescence intensity profile.

This work demonstrates a new application for TMA-DPH fluorescence. Utilization of this fluorescent technique provides a mechanism to view the molecular action of AmB on fungal cell membranes. The heightened level of understanding of AmB's mode of action may help design methods to reduce the cytotoxcity of AmB and to enhance its antifungal activity. For example, the observation that the AmB-resistant strain of *C. neoformans* did not demonstrate this biphasic profile, indicating inefficient sterol complexation with AmB, suggests that this method may be useful in detecting other yeast cell strains presenting this mechanism of resistance to AmB. Since fluorescence is a highly sensitive technique, this method could also provide a mode to study the molecular action of other membrane acting antifungal agents. For example, previously, a dramatic synergism has been shown between AmB and alkyl glycerol ethers in inhibiting the growth of a number of species in two genera of yeast *Candida* and *Cryptococcus* (Haynes et al., 1994). Since both of these drugs are thought to be membrane acting antifungal agents, analyzing changes in the AmB-induced fluorescence intensity profile produced by alkyl glycerol ethers could be utilized to further investigate the synergism between these two antifungal agents.

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