Membrane lateral phase separations and chlortetracycline transport by Bacillus megaterium

(fluorescence/spin label partitioning/N-phenyl-1-naphthylamine/2,2-dimethyl-1-butyl-4-pentyl-N-oxyloxazolidine)

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ABSTRACT Chlortetracycline, a fluorescent probe of its own active transport, has been used to study lateral phase separations of membrane lipid in Bacillus megaterium cells. Arrhenius plots of initial accumulation rates are triphasic, with transitions or characteristic temperatures of 29° and 8.5°. At the higher temperature, the mobility of the chlortetracycline, as measured by fluorescence polarization, is markedly altered. Chlortetracycline transport exhibits saturation kinetics, and fluorescence energy transfer from protein to bound antibiotic can be observed. N-Phenyl-1-naphthylamine, a lipophilic fluorescent probe, responds to changes in the hydrophobic regions of the membrane that are distinct from membrane protein. The fluorescent properties of N-phenyl-naphthylamine in partitioning and polarization experiments are altered most significantly at the lower characteristic temperature. No fluorescence energy transfer between N-phenyl-naphthylamine and membrane protein or bound tetracycline can be detected. In correlation electron spin resonance experiments on the partitioning of a lipid-soluble spin label, the same characteristic temperatures detected in the fluorescence studies were measured. These data suggest that different probes may respond to either or both of the characteristic temperatures describing the lateral phase separation. Between these characteristic temperatures the chlortetracycline transport system is most intimately associated with relatively immobile lipids that are surrounded by a more mobile lipid phase.

Tetracyclines are broad-spectrum antibiotics that bind to the bacterial ribosome, where they specifically inhibit protein biosynthesis (1, 2). The antibiotic is most likely accumulated within bacteria by an active transport system, which may account for the specificity of the drug (3). In several recent papers, we have shown that tetracyclines can be used as fluorescent probes of their own transport through bacterial membranes (4–7). Qualities that make them ideal for transport studies are the specificity of binding of the probe to the transport site in the bacterial membrane and the large increase in the quantum yield of fluorescence when tetracyclines bind to these apolar regions of the membrane through a divalent cation complex. The latter property results in great sensitivity for detection of the probe without perturbation of membrane integrity. Monitoring the tetracycline fluorescence allows one to follow antibiotic accumulation continuously. We have previously used the fluorescence to demonstrate saturation of the tetracycline transport system in Staphylococcus aureus and to examine the role of the membrane lipid in this transport system (4, 5). The temperature dependence of the transport rate, as measured by fluorescence enhancement, and tetracycline mobility changes, as measured by fluorescence polarization, were used in these studies (5).

Changes in the physical state of the membrane lipids of bacterial and mammalian cells may affect membrane function at two characteristic temperatures (8–10). It has been proposed that the higher of these characteristic temperatures t5 represents the point above which all of the membrane lipid is fluid. Below the lower characteristic temperature t1 all of the membrane lipid is in a solid or gel phase. Between t5 and t1 lateral phase separations exist that result in discrete patches of solid and fluid phases (11). In this paper we present evidence for lateral phase separations in membranes of the Gram-positive bacterium Bacillus megaterium. We have used chlortetracycline as a fluorescence probe of its own transport system. The data presented here suggest that the chlortetracycline active transport system in B. megaterium is sensitive to both t5 and t1 of the lateral phase separation. Experiments are also described that allow the assignment of t5 to events at or near the chlortetracycline transport proteins of the membrane and t1 to the pure hydrocarbon regions of the membrane.

MATERIALS AND METHODS

Growth and Properties of Bacillus megaterium. Cultures of a wild-type strain of B. megaterium were the gift of the Department of Bacteriology and Public Health, Washington State University. These cells were sensitive to tetracycline and chlortetracycline, with the minimal inhibitory concentration of the latter being 0.05 μg/ml as assayed in Difco Penassay Broth. Cells were grown in minimal media broth (12) at 37° C to late logarithmic phase of growth and collected by centrifugation at 10,000 × g. The bacteria were then washed twice in 20 vol of 10 mM Tris-HCl buffer, pH 7.0, containing 0.2% D-glucose. In all fluorescence studies the whole cells were suspended in the above buffer to an OD600 nm = 0.10 as measured in a 1-cm cuvette. Membrane vesicles of the B. megaterium cells were prepared by the lysozyme/EDTA method of Kaback (13). After purification, these vesicles were suspended in the above buffer to a protein concentration of 0.2 mg/ml. This procedure yielded optically clear solutions. Lipid extraction of the whole cells was carried out by the method of Bligh and Dyer (14). Transmethylation of the fatty acids with boron trifluoride/methanol followed by gas chromatography on a diethylene glycol succinate column at 170° C showed that the major fatty acids of these cells were the branched-chain fatty acids, anteiso and iso-C15 (1.3%), C14 (1.0%), C15 (9.7%), C16 (8.3%), C17 (2.6%), and the normal fatty acids C14 (4.2%), C15 (1.6%), C16 (27.4%), C17 (1.2%), and C18 (41.6%).

Chemicals. Chlortetracycline-hydrochloride (chlortetracycline) was purchased from Calbiochem and determined to

Abbreviations: doxylecane, 2,2-dimethyl-1-butyl-4-pentyl-N-oxyloxazolidine; ESR, electron spin resonance.

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be greater than 98% pure as measured by paper chromatography (15). The N-phenyl-1-naphthylamine was purchased from Eastman Organic Chemicals and was twice recrystallized from acetone before use. Fresh solutions of aqueous chlortetracycline and 1 mM N-phenylnaphthylamine in methanol stock solutions were prepared daily. The spin label 2,2-dimethyl-4-butyl-4-pentyl-N-oxyloxazolidine (doxyldecane) was a gift of A. D. Keith, Pennsylvania State University.

Fluorimetry. The kinetic measurement of fluorescence enhancement and fluorescence polarization studies were carried out on a Hitachi Perkin–Elmer MPF-3L spectrofluorimeter fitted with a thermostated cell compartment and polarizing attachment as described (5). In all studies the bacterial suspensions were continuously stirred while the temperature was monitored with a thermocouple probe calibrated to an accuracy of ±0.1°. In the polarization studies, corrections to the polarization values, \( P = (I_\parallel - I_\perp)/(I_\parallel + I_\perp) \), were made according to the method of Azumi and McGlynn (16). Under these conditions 1 \( \mu \)M N-phenylnaphthylamine in glycerol had a limiting polarization value of 0.29 at 25° with excitation at 350 nm and emission measured at 420 nm. In the N-phenylnaphthylamine polarization studies, B. megaterium vesicle suspensions were used in place of whole cells to limit scattering effects on the polarization measurements. As demonstrated previously with other bacteria, the time-dependent fluorescence enhancement of the chlortetracycline/B. megaterium cell suspension after the addition of the antibiotic directly monitors its accumulation (5, 6). Fluorescence measurements are corrected for temperature quenching effects where noted. Chlortetracycline was used in these studies because its quantum yield is greater in an apolar environment than that of tetracycline and, thus, it is a more sensitive fluorescence probe. Fluorescence excitation spectra were run on the Hitachi Perkin–Elmer MPF-3L spectrofluorimeter at 25° with both excitation and emission bandwidths set at 4 nm. The recorded excitation spectra were then corrected for spectral dispersion of the excitation energy and for the spectral characteristics of the detection system.

Electron Spin Resonance (ESR) Spectroscopy. To 20 mg of membranes (weight), prepared by the method of Kaback (13) and dispersed in 1 ml of 0.1 M potassium phosphate buffer, pH 6.6, was added 2 \( \mu \)l of a 10 mM doxyldecane chloroform solution. Following mild agitation for 15 sec on a Vortex mixer, the membranes were pelleted by centrifugation and the pellets were resuspended in 0.1 ml of buffer. ESR spectra of membranes in an aqueous sample cell were obtained with a Varian E-9 spectrometer equipped with variable temperature control. Temperatures were also measured directly with a thermistor inserted into the sample cell. Signals were stored in a Fabri-tek 1072 time averager. To obtain the intensity of the immobilized component, the spectrum of the mobile form was removed by subtraction of a spectrum of free doxyldecane stored in another part of the memory. Double integration of the remaining immobilized component gave the relative intensity. All spectrometer settings were held constant during an experiment.

**RESULTS**

When chlortetracycline is added to a suspension of respiring B. megaterium cells, there is a time-dependent fluorescence enhancement. This enhancement has been attributed to the binding of the chlortetracycline molecules to the membrane binding sites of the chlortetracycline active transport system and the subsequent transport of the antibiotic (4–7). The initial rates of fluorescence enhancement correspond to the initial rates of chlortetracycline transport. If these initial rates of fluorescence enhancement are measured as a function of temperature in B. megaterium cells and the appropriate Arrhenius plot is made, three distinct phases for the transport process over the temperature range of 0°–40° are seen (Fig. 1). The two characteristic temperatures occur at 20° \( (t_0) \) and 9.5° \( (t_1) \). These temperatures are consistently reproducible to ±0.0° from one sample culture to the next. Furthermore, the phenomena measured at the characteristic temperatures are totally reversible. Taking the cell suspension temperature above either characteristic temperature and then lowering it again below the characteristic temperature does not alter the transport rate observed for a particular temperature. This indicates that simple heat denaturation or an irreversible rearrangement of the membrane components is not occurring in these phase processes.

Polarization studies of chlortetracycline can be used to obtain information about its mobility. We have previously demonstrated that polarization values obtained at steady state accumulation levels of chlortetracycline give transitions correlating directly with those obtained in Arrhenius plots (5). A decrease in polarization values at a specific temperature indicates a relative increase in the mobility of the fluorescent probe. Figure 2A shows that at steady-state accumulation levels, a change in mobility indicating increased rotational freedom of the probe is observed at 20° \( (t_0) \). Qualitatively it appears likely that the chlortetracycline mobility changes most at the higher characteristic temperature, because no change in polarization is observed at \( t_1 \). The experiments reported below were, therefore, designed as an attempt to elucidate the properties associated with the two characteristic temperatures.

The transport site to which chlortetracycline binds on the membrane is most likely to be protein in nature. Fluorescence energy transfer between bacterial membrane protein and chlortetracycline might, therefore, be observable. Energy transfer is shown in the excitation spectrum in Fig. 3. In addition, an enhancement of chlortetracycline fluorescence at 520 nm upon excitation at 290 nm is seen when fluorescence of B. megaterium cells with antibiotic is compared to a methanol solution of chlortetracycline. When excited at 290 nm, the
protein emission at 330 nm is markedly reduced if chlortetracycline has been accumulated by the bacteria.

Because of poor spectral overlap between bacterial protein fluorescence emission and chlortetracycline absorbance, energy transfer can occur only when chlortetracycline is bound very close to protein. We have not yet conclusively proven that all observed fluorescence comes from tetracycline at the transport site. Some fluorescence could arise from tetracycline in the cytoplasmic space. Several lines of evidence, however, indicate that fluorescence is from the membrane transport site. Initial rate studies with biphasic (5) and triphasic Arrhenius plots (Fig. 1) correlate with mobility and membrane changes at the chlortetracycline temperatures as detected by fluorescence polarization at steady state. Escherichia coli vesicles (13) will pump tetracycline, and the fluorescence can be used to follow accumulation of antibiotic (E. V. Lindley and J. A. Magnuson, unpublished results). We have not succeeded in preparing vesicles of B. megaterium that will accumulate tetracycline. Caswell (17) has failed to detect any energy transfer from mitochondrial protein to chlortetracycline and has concluded that the fluorescing sites are far from protein. Because no specific transport proteins would be present in mitochondria, this is the expected result. With bacteria, however, energy transfer is observed. With the above reservations, we have carried out the following calculations using the theory of singlet resonance energy transfer as developed by Förster (18). In this treatment, the distance \( R_0 \) between chromophores, which corresponds to a singlet–singlet transfer of 50 percent efficiency, can be calculated from the equation of Brand and Witholt (19). When this calculation is done for the tryptophan–chlortetracycline donor–acceptor pair, using an overlap integral and a 0, 0 wave number for the emission of the donor calculated from spectral data, \( R_0 \) is found to be 23.7 Å. Approximations for tryptophan lifetime of 2.0 nsec and for refractive index of 1.6 were based on earlier work by Stryer (20). The orientation factor was taken as the square root of 2/3 (19). Förster’s theory further predicts that the rate constant for the singlet–singlet transfer is proportional to \( r^{-6} \), in which \( r \) is the distance between the donor and acceptor. The efficiency \( E \) of transfer at a defined distance can then be described as

\[
E = r^{-6} / (r^{-6} + R_0^{-6})
\]

If we assume that 10% efficiency of energy transfer is the minimum needed to be detected in our system, then the chlortetracycline must be bound closer than 34.2 Å from membrane tryptophan. This is a reasonable assumption, because only a fraction of the membrane protein will have chlortetracycline bound to it.

The lower characteristic temperature in the Arrhenius plot is believed to reflect the point at which all of the membrane lipids enter a crystalline state. To study this possibility, a highly lipophilic fluorescence probe, N-phenylnaphthylamine, was used. With 2 \( \mu \)M N-phenylnaphthylamine in suspensions of B. megaterium cells at 25°, there is a dramatic increase in fluorescence over that of an aqueous suspension of the probe. This is indicative of N-phenylnaphthylamine partitioning into the lipophilic hydrocarbon regions of the membrane (21, 22). There is excellent evidence that the membrane-bound N-phenylnaphthylamine is located in regions distinct from the chlortetracycline transport sites and other membrane protein in B. megaterium. Ballard and coworkers (23) have previously reported the lack of fluorescence energy transfer between bound N-phenylnaphthylamine and membrane protein in B. megaterium cells, protoplasts, and vesicles, although the spectral overlap between these two fluorescent moieties is excellent. Our results substantiate this report even at saturating concentration of the probe. In addition, there is no observable fluorescence energy transfer from N-phenylnaphthylamine to chlortetracycline in B. megaterium suspensions, even though there is good spectral overlap between these two fluorescent molecules. This result suggests that the bound N-phenylnaphthylamine is located in lipid regions distinct from the chlortetracycline transport site or other membrane proteins.

\( R_0 \) values calculated for the protein–N-phenylnaphthylamine and N-phenylnaphthylamine–chlortetracycline fluorescence pairs are 27.4 Å and 34.3 Å, respectively. Because no energy transfer was observed between these probes, it is likely that the N-phenylnaphthylamine is sequestered at sites greater than about 50 Å from the membrane protein or chlortetracycline binding site. This possibility is valid if it is assumed that we detect a 5% efficiency of transfer.

Ordered-to-fluid phase transitions at the N-phenylnaphthylamine binding sites may be detected by an increase in fluorescence intensity due to the greater partitioning of the aqueous probe into the hydrocarbon phase of the membrane (22). When the fluorescence intensity of N-phenylnaphthylamine is monitored as a function of temperature in the presence of intact B. megaterium cells (Fig. 4), the transition temperature is centered at 8°, extending from 6° to 10°. The same
characteristic temperature is seen in *B. megaterium* vesicles as measured by this N-phenylnaphthylamine fluorescence enhancement technique. A slight hysteresis effect of about 2° is seen between increasing and decreasing temperature runs. This temperature correlates well with *t*₁ of the Arrhenius plot for chlorotetracycline transport.

Phase transitions at the N-phenylnaphthylamine sites may also be detected by polarization studies similar to those described for chlorotetracycline. When these polarization measurements are carried out as a function of temperature on *B. megaterium* vesicles (Figure 2B) it can be seen that the N-phenylnaphthylamine undergoes a decrease in mobility at temperatures below approximately 7°–9°. This temperature, again, corresponds to *t*₁ as measured by Arrhenius plots of chlorotetracycline transport and to the phase transition measured by N-phenylnaphthylamine fluorescence enhancement.

Partitioning of spin labels into fluid lipid phases of membranes has been utilized most commonly to detect lateral phase separations (24–27). The spin-label probe doxyldecane was used in this work to detect changes in membranes of *B. megaterium*. With freshly prepared membranes one broad spectrum and a relatively sharp component were observed. Spectra in Fig. 5 show results at a variety of temperatures. At low temperatures the mobile component clearly makes up a relatively large percentage of the total signal. The intensity of the immobile component is plotted as a function of temperature in Fig. 6. The amount of immobilized spectra increases from approximately 10° to 20°, which correlates closely with the characteristic temperatures determined above. Below 5° and above 21° the spectrum was unchanged. The process was entirely reversible over the range 2° to 30°.

Two interesting points should be noted about the ESR work. Above 33° an irreversible loss of signal occurred within minutes. A reduction process, similar to that reported by Baldassare and coworkers (28) may be taking place. If the membranes with spin label were stored at 2° for four weeks, no loss of signal was observed. The second point concerns the stability of the membranes. ESR spectra of freshly prepared membranes with doxyldecane showed the two-component spectra. The ratio of these two components changed with temperature. After aging for several weeks a third immobile component was observed. This may be due to an irreversible denaturation of membrane structure. The amount of the very broad third component was not temperature dependent. After six weeks of storing at 2° this very broad component and the sharp component were the only two visible in the spectra. Thus, in the work presented in Fig. 6, some of the integration value reported is due to the very broad, temperature-independent component. This is inconsequential to our results, however, because the data do follow changes in amount of mobile signal. At 20° and above, most of the signal is from immobilized doxyldecane. Below 20°, a mobile component is present that is subtracted from the total signal, giving the immobilized value presented in Fig. 6.

**DISCUSSION**

Spin label studies have detected two characteristic temperatures in bacterial cells of complex lipid composition, while pure phospholipid dispersions showed only one phase transition (8, 11, 25, 26). In all of these studies it was concluded that these upper and lower boundaries (*t*₂ and *t*₁) represented the beginning and the end of the lateral phase separation of membrane lipids. Because biological membranes are complex mixtures of lipids, it was suggested that *t*₂, *t*₁, or both of these characteristic temperatures might influence particular membrane functions. Early reports of this type came from studies on the transport of β-glucosides and β-galactosides in *E. coli* fatty acid auxotrophs (8, 9).
In this report we have utilized a sensitive fluorescence technique to follow bacterial accumulation of chlorotetracycline and to show that the transport process is sensitive to two characteristic temperatures of a lateral phase separation. For the B. megaterium cells described, the temperatures are 10° apart. The results indicate that the higher temperature $t_1$ corresponds to events most intimately associated with the transport site. The lower temperature $t_2$ reflects order–disorder changes in the lipid hydrocarbon regions of the membrane where N-phenylnaphthylamine is sequestered. The results from both fluorescence and ESR studies are consistent with this explanation.

Freeze-fracture electron microscopic studies show protein aggregation at the onset of the lateral phase separation for E. coli cells (11, 29). For Bacillus species, however, no particle aggregation is observed in electron microscopic studies conducted below the phase transition temperature identified by differential scanning calorimetry or enzymatic studies of membrane-bound functions (29). The lack of particle aggregation has been attributed to the presence of branched-chain fatty acids in these bacteria. The data presented here suggest that even where gross particle aggregation may not be seen by electron microscopy, the onset of the lateral phase separation occurs around the protein regions of the membrane.

This work also demonstrates that different spectroscopic techniques designed to measure membrane phase transitions may actually report $t_2$, $t_1$, or both, depending on the sensitivity of the technique and the probe’s specific binding site. Our results indicate that N-phenylnaphthylamine polarization and fluorescence intensity studies reflect $t_2$. Chlorotetracycline mobility detected by fluorescence polarization is sensitive to $t_2$, while transport rates are responsive to both $t_2$ and $t_1$. In our earlier work with S. aureus, the transition temperatures reported most probably correspond to $t_2$, with $t_1$ being below 0° (5). The spin label doxyldecane partitions over the whole range from $t_3$ to $t_1$, as reported by others (10). Further investigations are necessary to determine if other membrane func-

tions give the same characteristic temperatures for this system.

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