Molecular Packing of Cord Factor and Its Interaction with Phosphatidylinositol in Mixed Monolayers

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ABSTRACT Cord factor (trehalose 6,6′-dimycolate, CF) is a glycolipid located in the outer mycobacterial cell wall that is implicated in the pathogenesis of mycobacteria. Furthermore, CF is a convenient model for studying mycolic acid residues, the major lipid constituents of the mycobacterial cell wall that are believed to form a barrier against drug penetration. The surface properties of CF and its interactions with phosphatidylinositol (PI) have been investigated using the monolayer technique. During compression/expansion/recompression cycles, CF monolayers switch from a loosely packed to a more tightly packed structure. The change in surface properties suggests a molecular rearrangement, perhaps involving interdigitation of long and short chains of the CF molecules. In CF-PI monolayers, maximal lateral packing density occurs between 0.5 and 0.7 mole fraction CF, which is close to the relative composition of mycolic acid residues and shorter-chain lipids in the mycobacterial cell wall. Low concentrations of CF increase the order in PI monolayers, consistent with CF toxicity involving rigidification of cell membranes.

INTRODUCTION

The currently accepted structural model for the mycobacterial cell wall postulates a highly asymmetric lipid bilayer (Minnikin, 1982; Nikaido et al., 1993; Brennan and Nikaido, 1995). The inner leaflet is a monolayer of mycolic acid residues covalently bound to arabinogalactan polymers. Mycolic acids are high molecular weight α-alkyl, β-hydroxy fatty acids; the long chain in *Mycobacterium tuberculosis* consists of 50 to 60 carbons and the α-branch is typically 24 carbons long. The mycolic acid residues are present primarily as groups of four on the pentaarabinoside cell-wall units (McNeil and Brennan, 1991) and are thought to be arranged with their chains parallel and oriented perpendicular to the plane of the cell wall (Nikaido et al., 1993). The outer leaflet of the mycobacterial cell wall bilayer is composed of extractable, loosely bound lipids such as trehalose dimycolate, glycopeptidolipid, phospholipid, and sulfolipid, which would render the outer surface relatively hydrophilic and negatively charged (Minnikin, 1982; Nikaido et al., 1993). It is proposed that acyl chains of the loosely bound lipids interact with the covalently bound mycolic acid chains, although the nature of these interactions is not clear.

Mycolic acid residues are present in the loosely bound glycolipid, trehalose-6,6′-dimycolate, commonly called cord factor (CF). The symmetrically dimycolated disaccharide CF contains a heterogeneous population of mycolic acid residues with the long acyl chains differing in structure and chain length. In virulent human strains of *M. tuberculosis*, the long acyl chains contain dicyclopropane and methoxy moieties (Minnikin, 1982). CF has been implicated in mycobacterial pathogenesis (Durand et al., 1979a; Behling et al., 1993; Laneelle and Daffe, 1991; Rastogi and David, 1988). Apparently, CF diffuses into host cell membranes and damages their function (Woodbury and Barrow, 1989). It has been reported that CF structurally alters mitochondrial membranes, resulting in loss of respiratory control, deranged electron transport, and inhibition of oxidative phosphorylation (Asselineau and Asselineau, 1978; Durand et al., 1979a). Studies with CF monolayers supported on solid hydrophobic beads indicated that monolayers of CF molecules are more effective than CF multilayers and micelles in inducing inflammatory and immunological responses involving the glycolipid (Behling et al., 1993; Retzinger et al., 1981). A better understanding of the physicochemical properties of CF and its interactions with various phospholipids is needed to determine the mechanism of CF toxicity to biological membranes.

The mycolic acid residues in the mycobacterial cell wall are believed to be largely responsible for the low permeability and fluidity of the cell wall (Nikaido et al., 1993). Furthermore, the molecular organization of these residues may play a key role in mycobacterial resistance to a wide range of drugs and in nutrient uptake (Nikaido et al., 1993; Brennan and Nikaido, 1995). Although CF represents a relatively small fraction of the total mycolic acid residues in the mycobacterial cell wall, its mycolic acid composition is probably the same as that of the arabinogalactan-bound residues (Goren, 1990; Brennan, 1988). Thus, CF is a convenient model for studying the packing, orientation, and organization of mycolic acid residues. Detailed information

Received for publication 10 July 1996 and in final form 24 September 1996.

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Abbreviations used: CF, cord factor; L-E, liquid-expanded; L-C, liquid condensed; S-C, solid-condensed; SCM, surface compressional modulus; DPPA, dipalmitoylphosphatidic acid; A_{cp}, close-packed area occupied by one molecule; A_{lo}, lift-off area.

0006-3495/96/12/3311/09 $2.00

on the physical structure of the lipid layer would be very useful for understanding the drug permeation process and might help in designing antimicrobial drugs able to penetrate the cell wall of pathogenic mycobacteria.

Historically, monolayer studies have played a pivotal role in the understanding of membrane structure (Gains, 1966). More recently, monolayer studies have been used to illustrate the interaction of bacteria with fatty acids and phospholipids (Birdi, 1989), to mimic the structure of the archaebacterial cell envelope (Pum and Sleytr, 1994), and to model the insertion of mycobacterial glycopeptidolipids into a bilayer membrane (Lopez-Martin et al., 1994). In this study, we use the monolayer technique to study the molecular packing and orientation of CF as well as its interactions with a negatively charged phospholipid, phosphatidylinositol (PI). PI is present along with other negatively charged lipids in the outer leaflet of mycobacterial cell walls (Nikaido, 1993; Goren, 1990) and is a common phospholipid in host cell membranes (e.g., Tzagoloff, 1982).

MATERIALS AND METHODS

Materials

Two fractions of CF were generously provided by M. Goren (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO): a highly pure synthetic CF (mol wt = 2900) made from mycolic acid residues isolated from *M. tuberculosis* H37Rv (Liav and Goren, 1980, 1984) and a natural cord factor isolated from *M. tuberculosis* Peurois (mol wt = 2750), originally obtained from E. Lederer. Both CFs contain several subclasses of mycolic acid residues, e.g., methoxy mycolic acid (Fig. 1) in CF from the Peurois strain (Adam et al., 1967; Polonsky et al., 1978) and dicyclopropanoyl mycolic acid (not shown) in CF from the H37Rv strain (Kaneda et al., 1988). The two CFs yielded almost identical surface pressure-area (π-A) isotherms. Results reported here for pure CF are from experiments done with both CFs, whereas the mixed monolayer study was done with the synthetic CF. PI (soybean) and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Dipalmitoylphosphatidic acid (DPPA) was obtained from Avanti Polar Lipids (Alabaster, AL). All chemicals were analytical grade. The water was double distilled or deionized (18 MΩ-cm resistivity), obtained with a Milli-Q UV Plus system (Millipore Co., Bedford, MA).

Lipids were dried under nitrogen; stock solutions (5 to 10 mg lipid/ml) in hexane/ethanol (9:1) or chloroform were stored under nitrogen at −20°C. Spreading solutions (0.9–1.1 mg lipid/ml) were prepared by placing an aliquot of a stock solution in preweighed flasks. Solvent was removed under nitrogen and lipid was dried under vacuum (300–700 mm Hg) for at least 3 hours. The dried lipid was weighed using an analytical balance (model B6, Mettler Instruments Corp., Hightstown, NJ) and dissolved in the spreading solvent, hexane/ethanol (9:1).

Surface measurements

π-A isotherms were measured with a Fromherz film balance equipped with a Teflon multicompartment trough and Wilhelmy plate device (Almog and Berns, 1981; Almog et al., 1988). The balance is interfaced with a real-time data acquisition and display system employing a 50-MHz IBM-compatible personal computer, a 16-bit high-accuracy analog input module (PC2036 M-1, Intelligent Instrumentation, Tucson, AZ), and acquisition software (Labtech Notebook Pro, Wilmington, MA). Data analysis and curve fitting were performed with TableCurve 2D and SigmaPlot software (Jandel Scientific, San Rafael, CA). The sensitivity of the surface pressure measurements was 0.1 mN/m and, the area could be measured to 0.07 cm^2

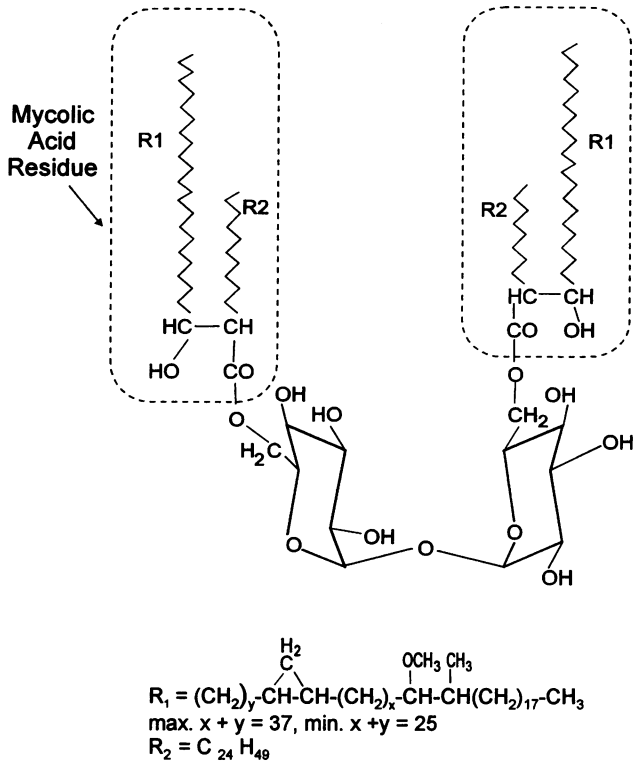

FIGURE 1 Chemical structure of cord factor from *M. tuberculosis*, Peurois strain (Goren, 1990; Adam et al., 1967).

(<0.3 $Å^2$/lipid molecule). The instrument was calibrated by recording the standard π-A isotherm of dipalmitoylphosphatidylcholine. Statistical analysis was performed using InStat 1.1 software (GraphPad, San Diego, CA). Significant differences were determined using nonparametric, unpaired, and one-tailed t-tests (as appropriate).

Monolayers were formed by adding 10- to 20-μl aliquots of spreading solution (containing ~10 to 20 μg of lipid) on a large subphase surface area (for CF, $A \approx 400$ $Å^2$/molecule) with an Agla microsyringe (Wellcome Reagents, Beckenham, UK) or with a Captrol III pipet filler (Becton Dickinson, Parsippany, NJ) and Accu-fill 90 microcapillary tubes (accuracy within ±0.25%). The surface of the subphase of water or buffer A (0.02 mM HEPES, 0.1 M NaCl, pH 7.4), kept at approximately rim level of the trough, was precleaned by repeatedly sweeping the barrier and removing surface-active materials by aspiration. After the initial addition, the spreading solvent was allowed to evaporate for 15 min. Monolayers were compressed continuously at barrier speeds of 2–6 $Å^2$/molecule/min, and the π-A isotherms were recorded at 22°C. The number of runs for each lipid preparation is given in Results. The π-A isotherms of pure CF were determined at air-water interfaces, and the isotherms of PI and of CF-PI mixtures were determined under nitrogen. In compression/expansion/recompression studies, monolayers were slowly compressed to a surface pressure near 35 mN/m, expanded to their original area, and (after 15 min) recompressed, all at constant barrier speed. (Increasing the waiting period before recompression up to 60 min did not significantly change the π-A isotherms.) It should be noted that, when a CF monolayer was compressed at relatively high compression speed (>30 $Å^2$/molecule/min) on a subphase the level of which was several millimeters above the rim of the trough, the monolayer slid over the rim of the trough. Therefore, these compression conditions were avoided.

The liftoff area (A_{lo}) was determined as the molecular area at which an increase in π was first detected during monolayer compression. The area occupied by one molecule in a close-packed orientation (A_{cp}) was obtained graphically by extrapolating to $\pi = 0$ the slope of the π-A isotherm between $\pi = 25$ mN/m and π of monolayer collapse (π_c; see Results)

(Gains, 1966). In measurements on mixed monolayers, mean molecular areas were determined as monolayer areas divided by the total number of lipid molecules. Values of the surface compressional modulus (SCM), which is the reciprocal of the two-dimensional compressibility $(-1/A)(dA/d\pi)_t$, were calculated from π-A isotherms using TableCurve 2D. The isotherms were smoothed and $d\pi/dA$ was calculated with the Savitsky-Golay differentiation procedure using a moving window containing 20% of the data points, and each point in the resulting curve was multiplied by the corresponding $-A$ value.

RESULTS

Surface properties of CF monolayers

π-A isotherms were measured from CF monolayers on pure water or buffer A at 22°C. The isotherms were obtained for compression cycles in which the monolayers were compressed, expanded, and recompressed at a constant barrier speed not exceeding 6 Å^2/molecule/min. The π-A isotherms of the initial compression and recompression of a typical CF monolayer are shown in Fig. 2.

The first compression of CF monolayers displayed a mean liftoff area (A_{lo}) of 300 ± 45 Å^2/molecule and a mean close-packed molecular area (A_{cp}) of 160 ± 9 Å^2/molecule (n = 18). A_{lo} corresponds (loosely) to the molecular area at which lipid molecules or aggregates in the monolayer begin to interact and exert force on one another as the space available to the molecules is reduced during compression. On the other hand, A_{cp} corresponds to the cross-sectional area at close packing of the acyl chains of the mycolic acid residues in one CF molecule. The relatively large difference between the values of A_{lo} and A_{cp} (~140 Å^2/molecule) indicates that initial packing of the acyl chains is not efficient.

Monolayer collapse occurred at 47–52 mN/m as indicated by a gradual decrease (without sharp fall-off) in the amplitude of the slope of the π-A curve (Fig. 2). Near collapse surface pressure (π_c) the monolayer was relatively stable, exhibiting a small decrease in π with time at fixed monolayer area (Fig. 3). The time-dependent change in π could be fit (r = 0.99) by a kinetic equation with two independent first-order decays, consistent with film collapse occurring by two independent processes (e.g., nucleation and growth of bulk-phase aggregates; Tomoaia-Cotisel et al., 1983). After a CF monolayer was compressed to collapse by going to high π (>40 mN/m), on recompression, the complete π-A isotherm was shifted to smaller molecular areas, indicating a loss of lipid probably due to formation of multilayers. Therefore, in experiments involving multiple compression/expansion cycles, pressures used did not usually exceed 35 mN/m. Values of π_c were the same for initial compressions or recompressions.

The π-A isotherms of CF monolayers were further analyzed in terms of SCM (see Materials and Methods). Values of maximal SCM (SCM_{max}), which generally occur at high π, are useful for characterizing lipid monolayers as liquid-expanded (L-E; 12.5–50 mN/m), liquid-condensed (L-C; 100–250 mN/m), or solid-condensed (S-C; 1000–2000 mN/m) (Davies and Ridel, 1961). The mean value of SCM_{max} for CF monolayers after initial compression was

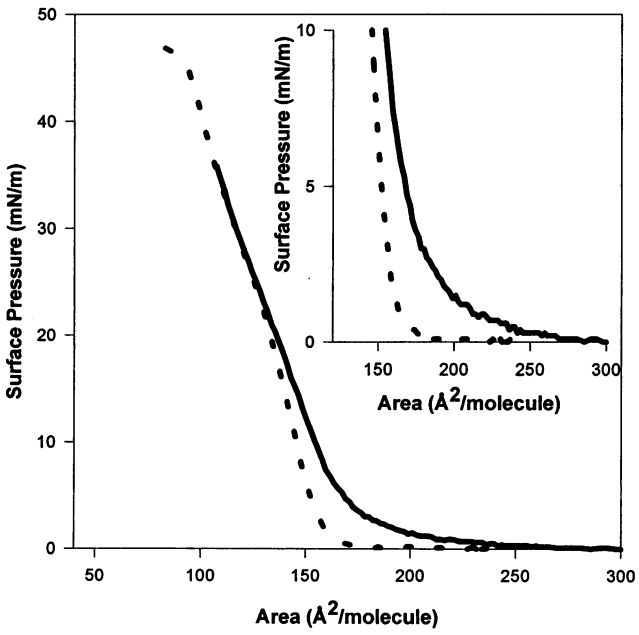

FIGURE 2 The π-A isotherm of a typical CF monolayer at 22°C, showing first compression (——) and recompression (- - -). The inset shows an expanded scale ($\pi \leq$ 10 mN/m) used for measuring A_{lo}.

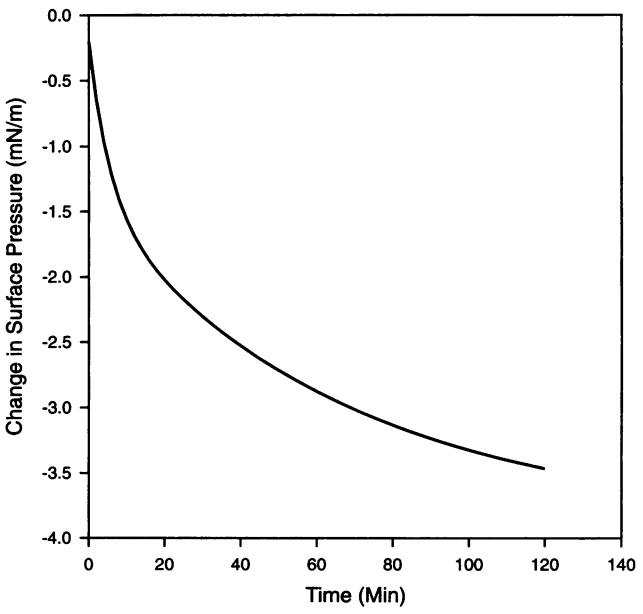

FIGURE 3 Plot of the change in surface pressure of a collapsed monolayer of CF with time. The monolayer is compressed at 3 Å^2/molecule/min to collapse (indicated by decrease in the amplitude of the slope of the π-A isotherm), and the change in surface pressure at constant area is recorded. The curve is fitted (r^2 = 0.99) by two-decay kinetics: $\Delta\pi = a + be^{[\text{minud}]ct} + de^{[\text{minud}]ft}$, where a = −3.8 mN/m, b = 1.2 mN/m, c = 0.18 min^{-1}, d = 2.4 mN/m, f = 0.015 min^{-1}, and t = min.

69 ± 17 mN/m (n = 18), which falls between that for L-E and L-C monolayers (Table 1). For comparison, the π-A isotherm of a DPPA monolayer on water was determined, and its surface parameters also are given in Table 1. The SCM_{max} value of this two-chain lipid was 375 mN/m, much larger than that of CF and falling between the L-C and S-C classes.

When the CF monolayer was recompressed as described in Materials and Methods, the A_{cp} value (161 ± 8 Å^2/molecule, n = 13) did not differ significantly from that obtained in the first compression (160 ± 9 Å^2/molecule). However, the π-A isotherm of recompressed CF monolayers yielded a significantly smaller A_{lo} (193 ± 30 Å^2/molecule, $p < 0.001$) and a significantly higher SCM_{max} (113 ± 27 mN/m, $p < 0.001$) relative to the first compression. The recompressed monolayers become more condensed at low π, whereas π-A curves of initially compressed and subsequently recompressed CF monolayers were essentially identical above 20 mN/m. This is observed as a bend in the π-A isotherm of the recompressed monolayer at approximately 15 mN/m (Fig. 2). Subsequent recompression cycles of CF monolayers did not result in further significant changes in the π-A isotherms.

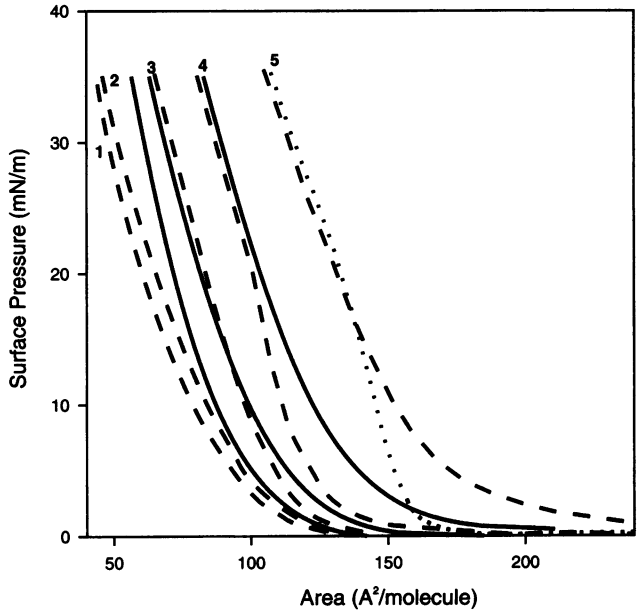

FIGURE 4 The π-A isotherms of (*1*) PI; (*2–4*) mixed CF-PI monolayers (- - -) and the corresponding ideal isotherms (——) for PI:CF ratios of 82:18, 67:33, and 33:67, respectively; (*5*) CF (- - -) first compression and (· · ·) recompression. Ideal isotherms were calculated using the additivity rule, as described in Results.

Surface properties of mixed monolayers of CF and PI

A typical π-A isotherm of a PI monolayer on buffer A is shown in Fig. 4. The SCM_{max} and A_{lo} values (n = 8) were 55 ± 10 mN/m and 125 ± 8 Å^2/molecule, respectively. These values are characteristic of a phospholipid in a fluid state and a L-E monolayer. The transition from monolayer to the collapsed state occurred smoothly at approximately 48–50 mN/m, in agreement with published reports (Hirasawa et al., 1981), and the isotherm did not change significantly on recompression (data not shown). To obtain information on the packing and possible interactions between CF and PI, isotherms of monolayers with different compositions of CF and PI were measured on buffer A (Fig. 4). The collapse surface pressures, π_c, of the mixed CF-PI monolayers over the complete compositional range were 49 ± 3 mN/m, indicating that the mixed monolayers were as stable as monolayers of the pure components.

The experimental π-A isotherms for various mixed monolayers (Fig. 4, dashed curves) were compared with isotherms for ideal mixing (Fig. 4, solid lines). The ideal isotherms were calculated using simple additivity: $A = N_1A_1 + N_2A_2$, where the areas per molecule, A_i, are obtained from the π-A isotherms of pure CF and PI, and N_i is the mole fraction of each lipid. Deviations between observed and calculated isotherms suggest miscibility and interactions between the two lipid components (Gains, 1966), with negative deviations in molecular area suggesting increased order and positive deviations suggesting increased disorder. Fig. 5 shows the mean molecular areas obtained by subtracting the measured isotherms of mixed CF-PI monolayers from the ideal π-A isotherms at various values of π. There are significant negative deviations from ideality near 0.6 mole fraction CF at 5–10 mN/m. At higher surface pressures (20–30 mN/m), there are smaller negative deviations near 0.2 mole fraction CF and small positive deviations near 0.5 and 0.7 mole fraction CF. Nonideal mixing of CF and PI was also observed as negative deviations in mean A_{lo} values from calculated values at mole fraction CF ranging from 0.3 to 0.7 (Fig. 6), suggesting monolayer condensation. The SCM_{max} values for the initial compression of mixed CF-PI monolayers (Fig. 7) fall on or slightly below the ideal mixing line below 0.3 mole fraction CF and are significantly above the ideal mixing line from 0.3 to 0.7 mole fraction CF ($p < 0.01$ at 0.5 mole fraction CF; Fig. 7). In fact, at 0.5 mole fraction CF, the SCM_{max} value is even well above that of a pure CF monolayer, gradually decreasing to that of a pure CF monolayer with increasing CF content. The same trends in SCM_{max} values were also observed with recompressed CF-PI monolayers

TABLE 1 Characteristics of various lipid monolayers

Lipid	SCM_{max} (mN/m)	A_{lo} (Å^2/molecule)	A_{cp} (Å^2/molecule)
CF (first compression)	69	300	160
CF (recompression)	113	193	161
CF + PI (1:1)	107	145	126
PI	55	125	–*
DPPA	375	58	43

*A_{cp} cannot be determined graphically from π-A curves of this L-C monolayer.

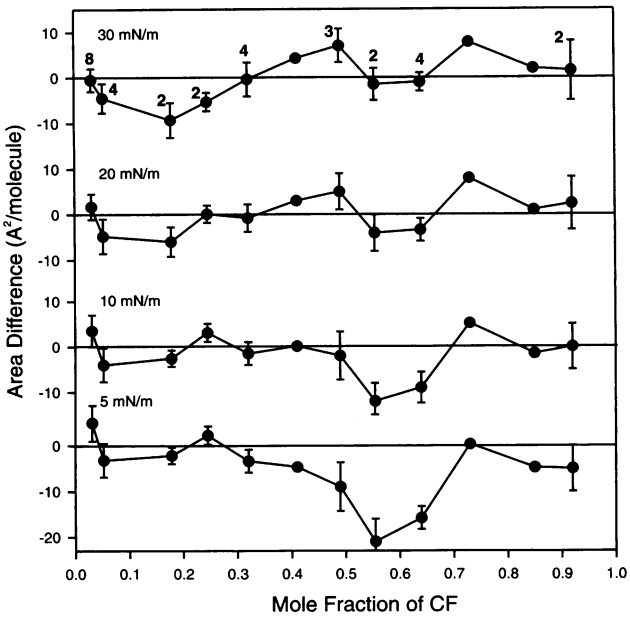

FIGURE 5 Plots of the differences in molecular area between observed π-A isotherms of mixed CF-PI monolayers and the corresponding ideal isotherms as a function of mole fraction CF at indicated monolayer surface pressures. Error bars for data points with more than one determination are indicated. At each composition, the number of determinations was the same at all surface pressures, as indicated on the 30 mN/m plot.

(not shown). The decreased compressibility of monolayers composed of roughly equimolar mixtures of CF and PI is consistent with specific interactions occurring between CF and PI in the monolayer.

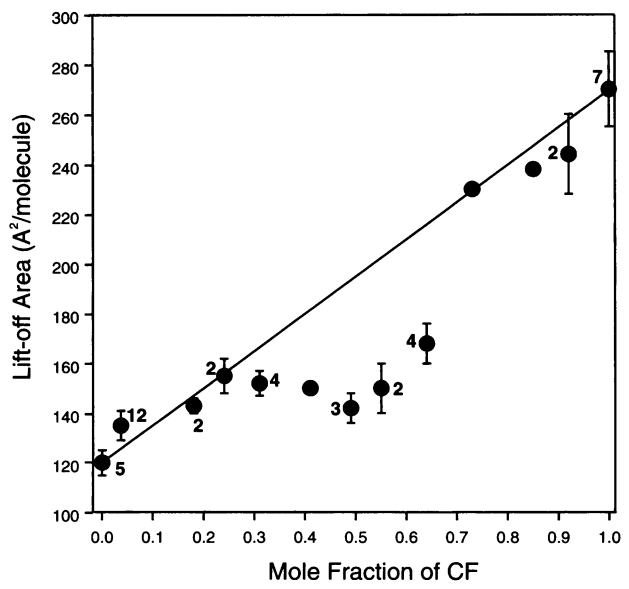

FIGURE 6 Plot of A_{lo} values of mixed CF-PI monolayers as a function of the mole fraction CF. The straight line connects the A_{lo} values of the pure lipid monolayers and represents ideal mixing. Error bars for data points with more than one determination and the number of determinations are given. Data points within 0.05 mole fraction CF were combined and plotted at the mean mole fraction CF.

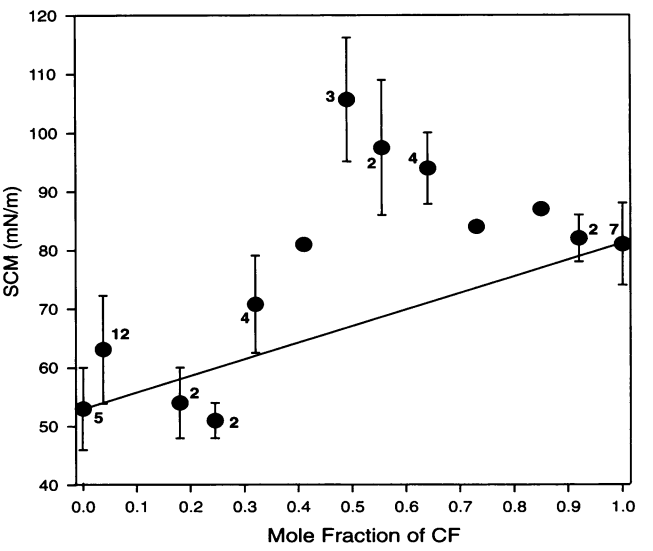

FIGURE 7 Plot of SCM_{max} values of mixed CF-PI monolayers as a function of CF composition. The straight line connects the SCM_{max} values of the pure lipid monolayers and represents ideal mixing. The number of runs and error bars for data points with more then one determination are indicated. Data points within 0.05 mole fraction CF were combined and plotted at the mean mole fraction CF.

The free energies of mixing (ΔG_m) of PI and CF in monolayers were calculated by integrating under the π-A isotherms of mixed and pure lipids (Goodrich, 1957):

$$\Delta G_m = \Delta G_{ex} + \Delta G_i = \Delta G_m$$

$$= \int_o^{\pi} (A_{12} - N_1A_1 - N_2A_2)d\prod + RT(N_1 \ln N_1 + N_2 \ln N_2)$$

where ΔG_{ex} is the excess Gibbs energy of mixing, and ΔG_i is the free energy of mixing for an ideal mixed monolayer. If the molecular areas of the mixed monolayers (A_{12}) are related by simple additivity of the molecular areas of pure monolayers at all surface pressures (i.e., $A_{12} = N_1A_1 + N_2A_2$), then ΔG_{ex} is zero and there are no specific interactions between components. Plots of ΔG_m and ΔG_i at 10 mN/m during the first compression of mixed monolayers spanning the complete range of CF compositions are shown in Fig. 8. The marked difference between ΔG_m and ΔG_i, at approximately 0.5–0.7 mole fraction CF, indicates strong attractive interactions between CF and PI consistent with the negative deviations in A_{lo} observed at the same π and monolayer composition (Fig. 5).

DISCUSSION

Surface properties of CF

As the cell wall of mycobacteria is a permeability barrier to many drugs, mycolic acid residues are expected to exhibit tight packing and high stability. A good indicator of the packing properties of mycolic acid residues of CF are the

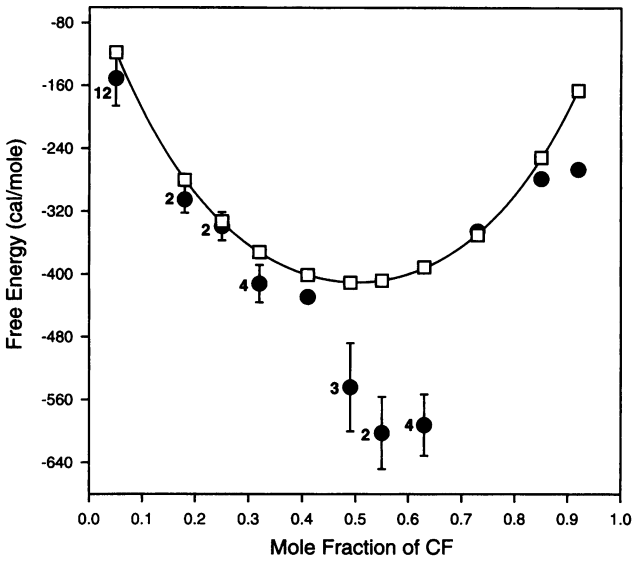

FIGURE 8 Plots of the measured free energy of mixing for CF-PI monolayers at 10 mN/m (●) and ideal free energy of mixing (□) as a function of mole fraction CF.

surface parameters A_{cp}, SCM_{max}, and A_{lo}. The A_{cp} of CF is approximately 160 Å^2/molecule. As the trehalose moiety has a cross-sectional area of approximately 80 Å^2 (Retzinger et al., 1981), molecular packing of CF must depend primarily on the acyl chains. The observed A_{cp} value corresponds to occupation by each of the four acyl chains in CF of an average area of 40 Å^2, almost twice that of saturated acyl chains in a phospholipid (Table 1). This suggests that the long chains of the mycolic acid residues in CF monolayers may be kinked (presumably near a methoxy group or a cyclopropane ring) and folded, resulting in six vertically packed acyl chains. This packing mode for CF in monolayers was first suggested by Retzinger et al. (1981), who determined the A_{cp} for CF from the Aoyama B strain of *M. tuberculosis* as 187 Å^2/molecule. The difference between this A_{cp} value and that reported here (160 Å^2/molecule) may be due to different sources of CF or different experimental conditions, such as compression speeds, which are not described by Retzinger et al. (1981). Assuming that the kinking model is correct and that six acyl chains are packed vertically in CF monolayers, the mean cross-sectional area for each acyl chain (27–31 Å^2/molecule) is larger than those of saturated fatty acids (20.3 Å^2; Ries and Kimball, 1957; Ries and Swift, 1982) and of saturated phospholipids (22–23 Å^2/molecule; Tomoaia-Cotisel et al., 1981, and Table 1). Thus, even assuming folding of the long acyl chains, the lateral chain packing in pure CF monolayers is not as tight as that of acyl chains in monolayers of fatty acids or phospholipids.

The SCM_{max} value of recompressed CF monolayers obtained in this study (113 ± 27 mN/m) and that of monolayers of CF from the Aoyama B strain of *M. tuberculosis* (152 mN/m; Retzinger et al., 1981) correspond to that of L-C monolayers. Although larger than the SCM_{max} values of initial compression, these values are considerably lower than those of fatty acids (Ries, 1957; Ries and Swift, 1982) and phospholipids (Table 1; Tomoaia-Cotisel et al., 1981) having shorter (16 to 36 carbons) saturated acyl chains, indicating that CF monolayers are significantly more compressible. Apparently, structural factors, such as the longer acyl chains in CF, the presence of methoxy or cyclopropane groups in the long acyl chain, and/or the folding of the long chain, interfere with the vertical packing of acyl chains, which is also suggested by the relatively large A_{cp} value per acyl chain noted above.

When CF is spread on an aqueous subphase, the polar headgroups are essentially immersed in the subphase and the acyl chains extend into the gas phase. The slow asymptotic rise in surface pressure observed during the first compression of CF monolayers, at very large molecular areas and low surface pressures (Fig. 2, inset), suggests the sequence of events shown schematically in Fig. 9 *a–c*. Initially, the acyl chains may extend nearly horizontally above the aqueous surface (Adamson, 1982; Hasmonay, 1994), with intermolecular contacts occurring between the ends of the long acyl chains. As the surface area is reduced during compression, the acyl chains move closer to each other, lift from the subphase, and orient more perpendicularly to the interface. This hypothesis is supported by the SCM value of approximately 8 mN/m at areas larger than 200 Å^2/molecule, which is considerably smaller than the minimal value (12.5 mN/m) assigned to L-E monolayers (Davies and Ridel, 1961). Apparently, the initial interactions between neighboring molecules in the monolayer occur with the long acyl chains of the mycolic acid residues widely separated and having relatively little contact with one another.

The changes in π-A isotherms at low π between the first and second compressions of CF monolayers are not likely to

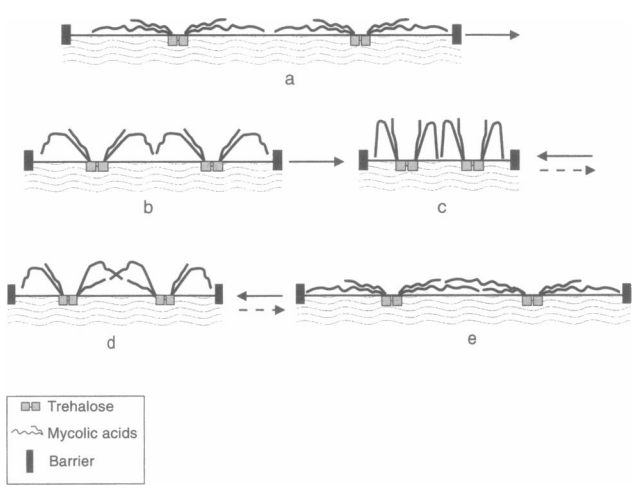

FIGURE 9 Possible monolayer compression models. Schematic representations of a CF monolayer on an aqueous subphase in initially expanded state (*a*), partly compressed (*b*) and close-packed (*c*) states without interdigitation, and partly compressed (*d*) and expanded (*e*) states with interdigitation. Initial compression, expansion, and recompression steps are indicated by solid arrows to the right, broken arrows, and solid arrows to the left, respectively.

be caused by partial solubilization of CF molecules into the subphase, e.g., the π-A isotherms of the two compressions are similar above 20 mN/m (Fig. 2). A more plausible mechanism is that the CF molecules realign during the first compression/expansion/recompression cycle. In particular, some acyl chains may adopt an arrangement, at large molecular areas, whereby the long acyl chain of one molecule contacts end-to-end the short, rather than the long, acyl chain of an adjacent CF molecule (Fig. 9, *d* and *e*). Fig. 9 *e* depicts the interactions between acyl chains of two CF molecules positioned parallel to the air/aqueous interface at low coverage of the interface by the lipid (Hasmonay, 1994), organized in interdigitated-like structures. Interdigitated molecules would be mixed with those that are not involved in interdigitation (Fig. 9 *a*) and form a fluid monolayer when pressure is not applied. However, interdigitation would enhance the cohesive interactions between the acyl chains, consistent with the smaller A_{lo} and higher SCM_{max} values (lower compressibility) of recompressed monolayers. The model in Fig. 9 presumes that the same final packing (noninterdigitated) is obtained for initial compression and for recompression, as the π-A isotherms of both are identical at high π. Simple calculations show that full interdigitation would lead to approximately a 50% smaller area (assuming random lateral packing), compared to the 36% reduction in A_{lo} observed on recompressing CF monolayers. Interdigitation may also play an important role during collapse of CF monolayers. As shown in Figs. 2 and 3, there is no sharp drop in monolayer stability at molecular areas below that required for collapse, suggesting the absence of precipitous fracture as observed with cardiolipin monolayers (Ries and Swift, 1982). The gradual, poorly defined transition of CF monolayers to the collapsed state and the fact that the change in collapse surface pressure follows two independent first-order decays (Fig. 3) suggest that CF may form multilayered structures at high surface pressure (Tomoaia-Cotisel et al., 1983; Ries and Swift, 1982), perhaps stabilized by interdigitation. (Note that layering of the monolayer does not account for the changes between first and subsequent compressions of CF monolayers, as these experiments were done below the collapse surface pressure.)

Interdigitation models have been invoked to explain the organization of asymmetric acyl chains of phospholipids in a variety of bilayer systems and biological membranes (e.g., Boggs and Tummler, 1993; Huang and Mason, 1986). Such models are particularly favored when one acyl chain is approximately one-half as long as the other (Hasmonay et al., 1994), as in the CF molecule. However, it should be noted that the surface properties of recompressed CF monolayers also may be influenced by other factors, e.g., alteration in the hydrogen bonding of the β-hydroxy group (Durand et al., 1979b), although it seems unlikely that these other factors alone could account for the magnitude of the changes observed.

Surface properties of mixed monolayers of CF and PI

The packing of CF and PI molecules in mixed monolayers is sensitive to surface pressure and lipid composition (Figs. 4 and 5). The large negative deviations in molecular area from those of ideal π-A isotherms (Fig. 5) and the large excess free energy of mixing (Fig. 8) at 0.5–0.7 mole fraction CF and low surface pressure ($\pi \leq 10$ mN/m) suggests more efficient packing in mixed than in pure monolayers (Cadenhead and Phillips, 1968). The improved packing of the lipid molecules is supported by the progressive decrease in compressibility of mixed CF-PI monolayers as the CF mole fraction increases from 0.2 to 0.5 (Fig. 7). Near equimolar mixtures of CF and PI, the monolayers have L-C characteristics with significantly smaller A_{lo} values (Figs. 4 and 6) and significantly higher SCM_{max} values (Fig. 7) than expected from simple additivity, and closer to those of recompressed CF monolayers. Evidently, at low π in the presence of roughly equimolar amount of PI, CF adopts a configuration during initial compression that is similar to that of recompressed CF monolayers. Thus, PI may promote the interdigitation of the acyl chains of CF molecules or interdigitation may take place between the acyl chains of PI and those of CF.

The observation that minimal compressibility and maximal packing density of mixed CF-PI monolayers occurs at 0.5–0.7 mole fraction CF is consistent with the current bilayer paradigm of lipid organization in the mycobacterial cell wall (Nikaido, 1993; Brennan and Nikaido, 1995). The tight lipid structure, inferred from x-ray diffraction measurements and from the low permeability of the cell wall (Nikaido, 1993), may result, in part, from interdigitation of the mycolic acid residues covalently bound to arabinogalactan with those of the loosely bound CF, located in the outer leaflet. However, CF is present in relatively small amounts in the outer leaflet of the mycobacterial cell wall (Minnikin, 1982; Goren, 1990). Instead, the long acyl chains of the bound mycolic acid residues may interdigitate more frequently with the shorter chains of the outer-leaflet phospholipids and glycolipids, which are present at concentrations roughly equal to those of the bound mycolic acid residues (Nikaido, 1993; David et al., 1988).

Significant negative deviations from ideal molecular areas are also observed in mixed CF-PI monolayers at surface pressures similar to those in phospholipid bilayers ($\pi \geq 20$ mN/m) at a CF mole fraction of approximately 0.2 (Fig. 5). This suggests that attractive (presumably van der Waals) forces between PI and CF molecules result in increased ordering of the phospholipid acyl chains. Loss of host membrane fluidity may be an important mechanism of CF toxicity, consistent with the previous observation that loss of mitochondrial function by CF was associated with membrane rigidification (Sut et al., 1990).

It should be noted that earlier studies of mixed monolayers of CF and phospholipids having a choline headgroup (Durand et al., 1979b; Crowe et al., 1994) reported mono

layer expansion at small mole fractions of CF, as opposed to the condensation observed for CF-PI monolayers in Fig. 5 at and above 20 mN/m. This suggests that the nature of the phospholipid headgroup may play a role in the behavior of mixed CF-phospholipid monolayers. However, comparisons with the results reported by Durand et al. (1979b) are complicated by the fact that the molecular areas determined from the π-A isotherms of CF (extracted from a mixture of human strains of *M. tuberculosis*) in that work are substantially smaller than those determined in this study (Fig. 2) and in Retzinger et al. (1981). This may be due to differences in experimental conditions, in particular, compression rates, which are incompletely described in the earlier studies. (Note precaution in Materials and Methods regarding compression rates.) Crowe et al. (1994) studied CF having unsaturated groups and substantially shorter acyl chains (C_{52}) than the CF from *M. tuberculosis* (C_{80}) (Goren, 1990) used in this study.

In conclusion, monolayers of CF are initially loosely packed and compressible and become significantly more condensed by compression or by interactions with PI, suggesting realignment of the mycolic acid residues. One of the major implications of this study is that the surface properties of mycolic acid residues may be governed by their chain-length asymmetry. This characteristic may allow interdigitation of the mycolic acid residues with other lipids in the mycobacterial cell wall to compensate for chain-length differences (Nikaido, 1993; Brennan and Nikaido, 1995) and so produce higher packing density and reduced permeability. This raises the possibility that mycobacteria might regulate the compressibility and thus the permeability of their cell walls by varying the specific composition and structure of the loosely bound lipids. Thus, improved drug penetration may be achieved by altering the organization of the outer cell wall lipids, which might in turn disrupt the organization of the covalently bound mycolic acid residues.

The exact mechanism of the pathogenicity of CF is not clear. It may involve an increase in acyl chain ordering in host cell membranes as observed for mixed CF-PI monolayers at $\pi \geq 20$ mN/m. This effect may be compounded in bilayers by interdigitation of CF with phospholipids, in effect cross-linking the two leaflets of the bilayer. This could influence the ability of one monolayer to communicate signals across the bilayer, limit the ability of the phospholipid to form domains, adversely affect the activity of integral membrane proteins, and alter the permeability of the membrane (Huang and Mason, 1986).

We thank Donald Mousseau (Instrumentation and Automation, Wadsworth Center) for his technical assistance with computer interfacing of the monolayer device and Dr. Mayer Goren (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) for stimulating discussions and for kindly furnishing the two cord factors, one of which he synthesized and purified with Dr. Avraham Liav.

This work was supported in part by a grant from Health Research, Inc.

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