Study of the Effect of Melittin on the Thermotropism of Dipalmitoylphosphatidylcholine by Raman Spectroscopy†

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ABSTRACT: The effect of amphiphilic toxin melittin (Mel) on the thermotropic behavior of dipalmitoylphosphatidylcholine (DPPC) has been studied by Raman spectroscopy. The spectra show that for complexes that were incubated above 40 °C, melittin does not penetrate DPPC bilayers in the gel state as an intrinsic protein since the conformation of the lipid acyl chains is just slightly perturbed by the toxin. Instead, at the DPPC/Mel molar ratios investigated (R_i = 5 and 15), Raman results suggest the formation of discoidal particles as complexes of apolipoproteins with phosphatidylcholines. These lipid/protein assemblies are characterized by a high conformational order, low intermolecular chain–chain interactions due to the size of the particles, and a low cooperativity of the gel to liquid-crystalline transition. The latter is biphasic for samples studied. It is believed that aggregation of these particles into larger ones occurs when the bilayers become less stable at higher temperature and that melittin is partially embedded into the hydrophobic core of the larger lipid/protein units. The freezing of the dispersion at approximately 0 °C also causes a reversible aggregation of the particles that leads to the formation of domains in which the interchain interactions are very similar to that of the pure lipid. The small particles of DPPC/Mel are also metastable, and with time, they form larger aggregates from which melittin is expulsed.

It is now well established that melittin (Mel)[1] has a marked affinity for phospholipids. This toxin causes the lysis of natural as well as synthetic membranes (Haberman, 1972), and it has recently been shown by NMR spectroscopy (Dufourc et al., 1986a), quasi-elastic light scattering, and freeze-fracture electron microscopy (Prendergast et al., 1982; Dufourcq et al., 1986) that it can change drastically the lipidic arrangement by inducing under some conditions the formation of small lipid/melittin particles. This phenomenon may be associated to the mechanism of cell membrane lysis by melittin.

Melittin displays an amphiphilic character and, therefore, is an interesting model peptide to study lipid/protein interactions. Due to the strong electrostatic interactions between the basic residues of the toxin and the polar head-group charges of phospholipids, melittin has a greater affinity for anionic lipids (Dufourcq & Faucon, 1977). Since the hydrophobic part of the peptide tends to penetrate the apolar zone of lipid bilayers, the effect of the toxin is also modulated by the cohesion of the hydrophobic core of this membrane. For example, the binding of melittin to DPPC dispersions is much stronger for lipid bilayers in the fluid phase than in the gel phase (Dufourcq et al., 1986). Also, the importance of hydrophobic interactions is nicely shown by the fact that the chain length of charged lipids has a critical effect on the thermotropic behavior of lipid/melittin complexes. For example, melittin causes the complete disappearance of the gel to liquid-crystalline phase transition of phosphatidylglycerol with short chains (C_{14} or shorter) while there is only a downward shift of the transition that remains cooperative when the cohesion of the lipidic structure is assured by longer chains (Bernard et al., 1982).

In a previous study (Dasseux et al., 1984), we have shown by Raman spectroscopy that in DMPC/Mel complexes, the toxin increases the intra- and intermolecular disorders of the bilayer in the gel phase. In the liquid-crystalline phase, melittin affects the molecular packing at a lipid to peptide incubation molar ratio (R_i) of 10, without appreciable change of the number of gauche bonds. At this molar ratio, the gel to liquid-crystalline transition is shifted to a lower temperature and is less cooperative. When a larger quantity of toxin is added to DMPC (R_i = 4, for example), the transition is abolished, behavior that has been associated with the formation of micellar particles (Prendergast et al., 1982; Dasseux et al., 1984).

In order to determine the influence of the dispersive force between the aliphatic hydrogens of the lipidic chains, we have extended our study of the effect of melittin on DMPC to DPPC. We report here the Raman results on the lipidic moiety of DPPC/Mel complexes between −20 and 70 °C, as well as on the time-dependent stability of these complexes.

Materials and Methods

Dipalmitoylphosphatidylcholine was purchased from Avanti Polar Lipids Inc. and used without further purification. Highly

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[1] Abbreviations: Mel, melittin; QLS, quasi-elastic light scattering; NMR, nuclear magnetic resonance; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; T_m, gel to liquid-crystalline phase transition temperature; R_i, phospholipid to protein incubation molar ratio; DSC, differential scanning calorimetry; PC, phosphatidylcholine.

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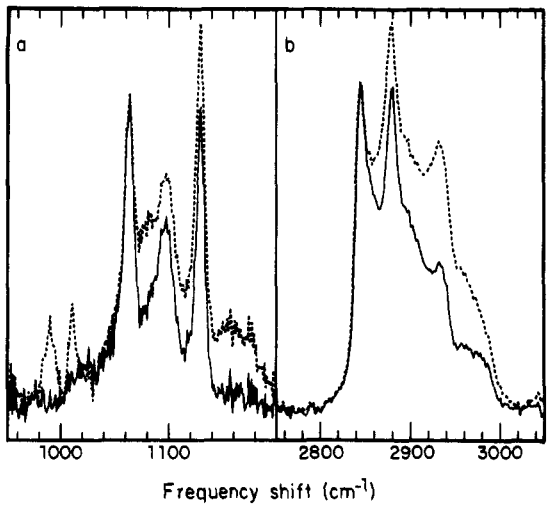

FIGURE 1: (a) C–C (950–1200 cm^{-1}) and (b) C–H (2750–3050 cm^{-1}) stretching mode regions of the Raman spectra of a DPPC/Mel complex with R_i = 5 and 10 °C before (dotted line) and after (solid line) correction for the spectral contribution of melittin.

purified melittin was isolated from bee venom (Sigma Chemical Co.) as described previously by Dasseux et al. (1984). However, the following modifications to the purification procedure were followed. To increase the efficiency of the reduction, 10 mM EDTA and 10 mM EGTA were added to the sulfite solution. For the last salt removal, melittin was reinjected on the C_{18} column preconditioned with pure water, and the column was successively eluted with 30 mL of H_2O, 20 mL of 100 mM NaCl solution, and 30 mL of H_2O; this sequence of elution also exchanges the various residual salts. After a methanol chase, the melittin-containing fractions were pooled, and the methanol was freeze–evaporated under vacuum. Melittin was then solubilized in water and lyophilized. Melittin from Bachem Feinchemikalien AG was also used after the sulfite treatment followed by only one step of purification by HPLC.

DPPC/Mel complexes were prepared by hydrating solid DPPC with a given volume of a melittin solution of appropriate concentration to yield the desired incubation molar ratio as well as a final lipid concentration of 10% by weight. Under these conditions, melittin is mainly in the tetrameric form. DPPC/Mel mixtures were then subjected to several heating and cooling cycles through the transition temperature until they were completely transparent at room temperature. When this point was reached, additional incubation did not affect the observed Raman spectra. Melittin solutions were prepared in 100 mM phosphate buffer, pH 7.5, containing 10 mM EDTA in order to avoid any lipid degradation by phospholipase A_2, potentially present in melittin.

Raman spectra were recorded with a computerized Spex Model 1400 double monochromator (Savoie et al., 1979) with a spectral resolution of 5 cm^{-1}. They were excited with the 514.5-nm line of a Spectra Physics Model 165 argon ion laser at a power of approximately 70 mW at the sample. DPPC/Mel complex spectra were corrected for the spectral contribution of melittin by subtracting the spectrum of the hydrating melittin solution using the 1012 cm^{-1} tryptophan line as an internal intensity standard. The effect of such a correction on the C–C and C–H stretching mode regions is shown in Figure 1. This correction also eliminated the phosphate buffer band at 980 cm^{-1}. Depending on their signal to noise ratio, spectra used to construct the temperature profiles given below were averages of two to four scans at 2 s/cm^{-1} for the C–C stretching mode region and one to two scans at 2 s/2 cm^{-1} for the C–H stretching mode region.

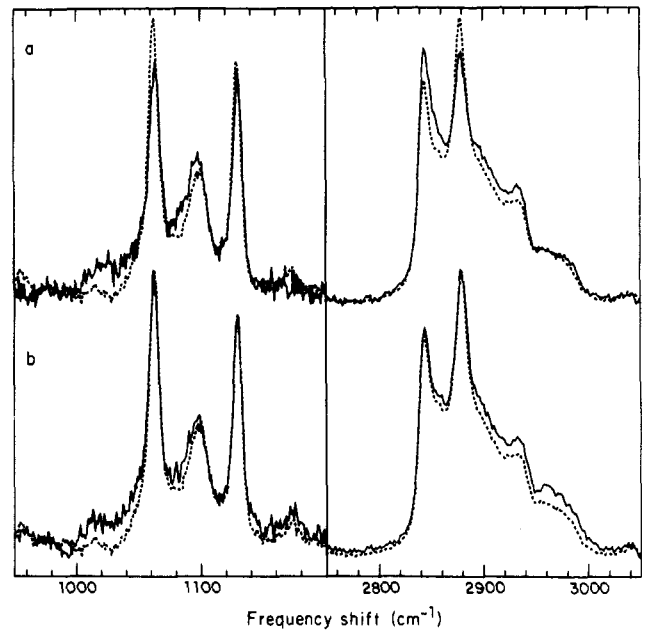

FIGURE 2: Comparison between the C–C (950–1200 cm^{-1}) and C–H (2750–3050 cm^{-1}) stretching mode regions in the Raman spectra taken at 10 °C of a pure DPPC dispersion (dotted line) and of a DPPC/Mel complex with R_i = 5 (solid line) in (a) the transparent phase and (b) the opaque phase.

RESULTS

Raman spectra in the C–H and C–C stretching mode regions of a DPPC/Mel complex at R_i = 5, taken at 10 °C immediately after heating the sample above the transition temperature (spectrum a) and after 4 days of incubation at 20 °C (spectrum b), are shown in Figure 2. We have chosen these two regions since they are particularly sensitive to both intra- and intermolecular orders.

Transparent Samples. The spectra of the complex with R_i = 5 taken immediately after heating the sample above the gel to liquid-crystalline transition temperature (T_m) were first recorded (Figure 2a). In the C–C stretching region, the addition of melittin causes only a slight increase of the intensity of the 1080 cm^{-1} band associated with the presence of gauche conformers relative to the intensity of the bands at 1060 and 1130 cm^{-1} due to the trans conformers (Gaber & Peticolas, 1977; Yellin & Levin, 1977). Therefore, for the gel phase, the toxin slightly decreases the intramolecular order of the lipidic acyl chains. The C–H stretching region is much more perturbed by the addition of melittin. First, there is a marked decrease of the intensity of the 2880 cm^{-1} band relative to that of the 2850 cm^{-1} band, these two bands being respectively assigned to the acyl chain methylene antisymmetric and symmetric stretching vibrations (Spiker & Levin, 1975). This decrease of the h_{2880}/h_{2850} ratio is partly due to the diminution of the intermolecular vibrational coupling in less tightly packed acyl chains and to their higher rotational mobility (Gaber & Peticolas, 1977; Snyder et al., 1980). At the same time, there is an increase of the intensity of the 2935 cm^{-1} feature. This change results in part from underlying infrared-active methylene asymmetric stretching modes that become Raman active when the chain symmetry is lowered (Bunow & Levin, 1977), and also from a change in the Fermi resonance between the symmetric C–H stretching mode of the terminal methyl groups and the first overtone of the symmetric CH_2 bending mode (Hill & Levin, 1979). This band is particularly sensitive to the intramolecular order, and its intensity increase confirms

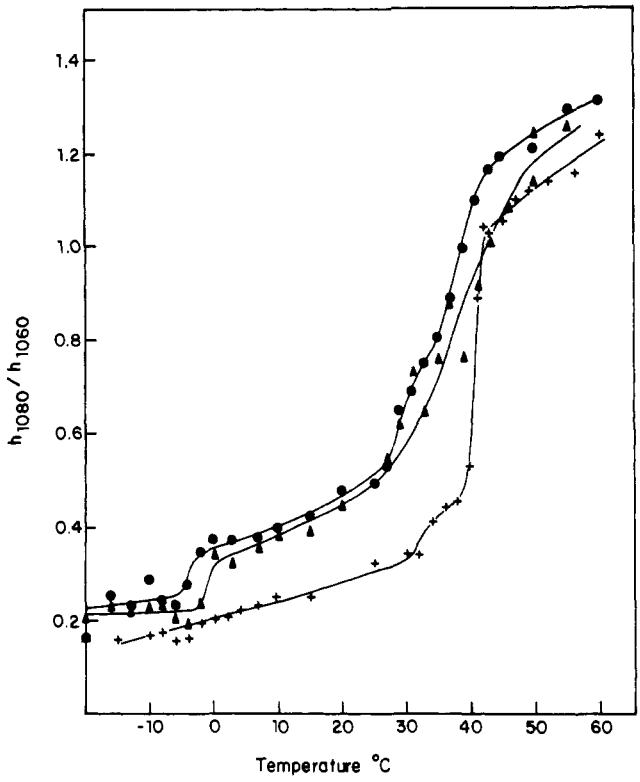

FIGURE 3: Temperature profiles of pure DPPC (+) and of DPPC/Mel complexes with R_i = 15 (▲) and R_i = 5 (●) derived from the h_{1080}/h_{1060} peak height intensity ratio.

the result obtained from the C–C region that melittin increases the intramolecular disorder.

Figure 3 presents the temperature dependence of the h_{1080}/h_{1060} intensity ratio for a dispersion of DPPC as well as for DPPC/Mel complexes at R_i = 15 and 5. Since this ratio is derived from bands that are sensitive to the conformation of the acyl chains, it is a very useful probe to follow the evolution of the intramolecular order (Gaber & Peticolas, 1977; Yellin & Levin, 1977). Below 0 °C, the acyl chain conformation is slightly affected by melittin since the h_{1080}/h_{1060} ratio increases with concentration of the toxin. The melting of ice at 0 °C does not seem to affect the almost all-trans conformation of the pure lipid acyl chains. However, for DPPC/Mel complexes, the h_{1080}/h_{1060} ratio increases at 0 °C, thus showing that more gauche bonds are then present along the chains. Between 0 and 30 °C, the conformational order is clearly perturbed by the toxin. For example, at 26 °C, the h_{1080}/h_{1060} ratio has a value of 0.32 for the pure lipid compared to 0.49 for the complex at R_i = 5. This conformational perturbation by melittin on DPPC is, however, smaller than that detected previously for DMPC (Dasseux et al., 1984). For this latter case, the value of the h_{1080}/h_{1060} ratio was 0.65 for a complex at R_i = 10 at the same temperature interval below the transition (14 °C).

Our Raman results on the C–C region show that the gel to liquid-crystalline transition of DPPC becomes less cooperative and is shifted toward lower temperatures in the presence of melittin. This effect increases with the concentration of the peptide, and as opposed to DMPC/Mel complexes for which no phase transition was detected at R_i = 4 by Raman spectroscopy and at R_i = 2 by differential scanning calorimetry (Dasseux et al., 1984), the transition is still present on the Raman temperature profiles at R_i = 2 (unpublished observations).

Above the transition temperature, the conformational order of DPPC also seems slightly perturbed by melittin (Figure 3).

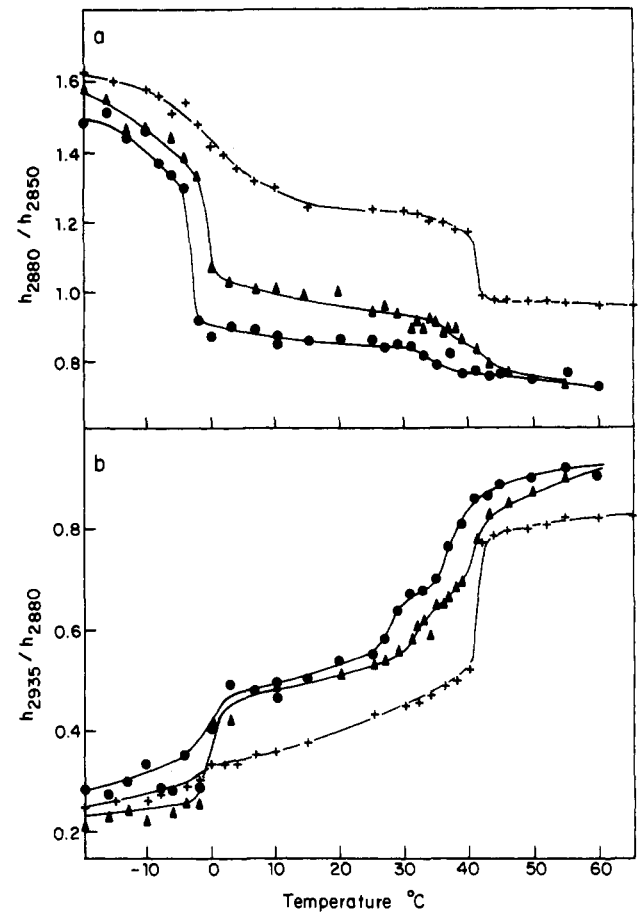

FIGURE 4: Temperature profiles of pure DPPC (+) and of DPPC/Mel complexes with R_i = 15 (▲) and R_i = 5 (●) derived from (a) the h_{2880}/h_{2850} peak height intensity ratio and (b) the h_{2935}/h_{2880} ratio.

The augmentation of the conformational disorder in the liquid-crystalline phase of phospholipids is difficult to follow by Raman spectroscopy since the number of gauche bonds is already quite high above T_m, which makes the measurement of the intensity of the bands due to the trans conformation less accurate. Nevertheless, our results are in good agreement with those obtained by NMR spectroscopy by Dufourc et al. (1986a), showing that there is an apparent general chain disordering above T_m.

Figure 4 shows the temperature profiles derived from the C–H stretching region of the Raman spectra of pure DPPC and of DPPC/Mel complexes with R_i = 15 and 5. It follows from the band assignment given above for this spectral region that the h_{2880}/h_{2850} intensity ratio is a highly sensitive probe of the intermolecular vibrational coupling and consequently of the lateral packing of the acyl chains; it is also sensitive to the chain dynamics (Gaber & Peticolas, 1977; Snyder et al., 1980). On the other hand, the h_{2935}/h_{2880} ratio monitors essentially the overall disorder of the lipid acyl chain matrix and in particular the conformational order (Bunow & Levin, 1977; Huang et al., 1983). Below 0 °C, the h_{2935}/h_{2880} ratio in the Raman spectrum of DPPC is almost not affected by melittin while the h_{2880}/h_{2850} ratio decreases with increasing concentration of the toxin. Therefore, melittin perturbs the lipidic packing even when the system is frozen. Simultaneously with the melting of ice, the lipid undergoes a major cooperative transition as revealed by the drastic decrease of the h_{2880}/h_{2850} ratio. For example, for the complex with R_i = 5, this ratio drops from 1.30 to 0.90 at 0 °C. This transition, that is also detectable from the h_{2935}/h_{2880} ratio, shifts to lower temperature when the melittin concentration increases and reaches

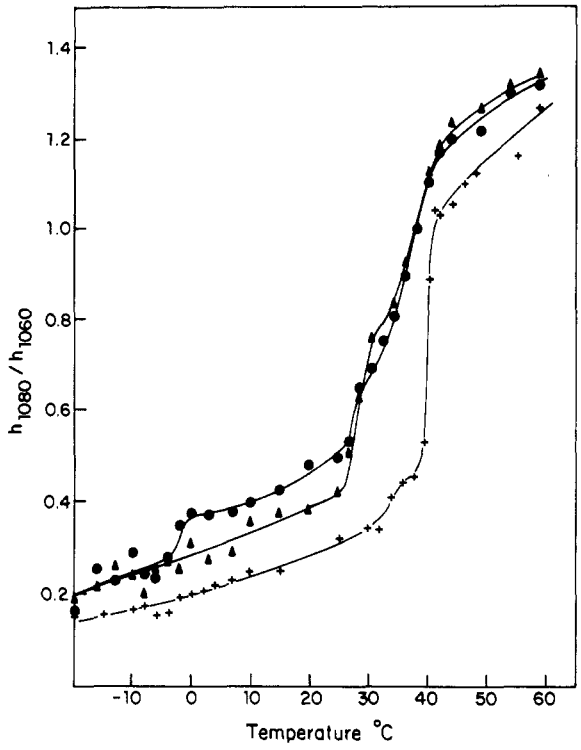

FIGURE 5: Temperature profiles of pure DPPC (+) and of a DPPC/Mel complex with R_i = 5 in the transparent phase (●) and in the opaque phase (▲) derived from the h_{1080}/h_{1060} peak height intensity ratio.

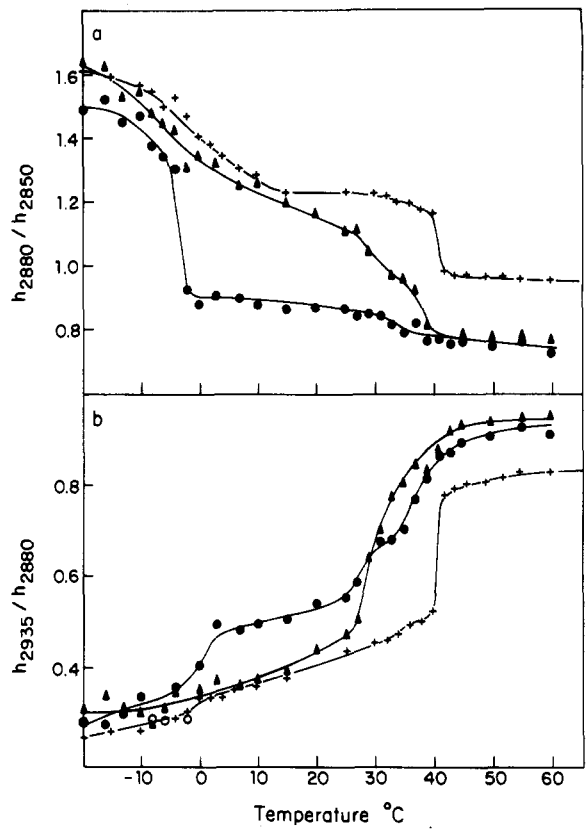

FIGURE 6: Temperature profiles of pure DPPC (+) and of a DPPC/Mel complex with R_i = 5 in the transparent phase (●) and in the opaque phase (▲) derived from (a) the h_{2880}/h_{2850} peak height intensity ratio and (b) the h_{2935}/h_{2880} ratio.

−4 °C at R_i = 2 (unshown results). No similar transition is detected on the pure lipid temperature profiles. For the phase between 0 and 30 °C, the intensity ratios in the C–H stretching region show that the intermolecular vibrational coupling is much weaker than that of the pure lipid and that the overall disorder is significantly higher. Again, these effects are a function of the melittin concentration. These observations, coupled with the fact that the samples are transparent between 0 and 30 °C, are compatible with the existence of small particles also detected by others physical techniques (Prendergast et al., 1982; Dufourcq et al., 1986; Dufourc et al., 1986a). The smaller size of the lipid units would be responsible for the lower vibrational coupling and consequently for the low h_{2880}/h_{2850} intensity ratio.

As for the results derived from the C–C stretching mode region, the C–H temperature profiles shown in Figure 4 demonstrate that the gel to liquid-crystalline phase transition of DPPC is considerably shifted toward lower temperature by melittin and that its cooperativity is also decreased. The amplitude of the transition on the h_{2880}/h_{2850} temperature profile is rather small since, as discussed above, the value of the ratio is already quite low in the gel phase. In contrast, the profiles observed from the h_{2935}/h_{2880} ratio show a more pronounced and biphasic transition.

Above T_m, both the h_{2880}/h_{2850} and h_{2935}/h_{2880} ratios reveal that the disorder of the lipidic structure is very high in DPPC/Mel complexes and that the intermolecular vibrational coupling is very low.

Opaque Samples. When DPPC/Mel samples are kept at room temperature for a few days, they become opaque as pure lipid dispersions in the gel state. Therefore, the transparent phase that occurs between 0 and 30 °C after the complexes are heated above T_m is a metastable one. Figure 2b displays the Raman spectra of an opaque sample of DPPC/Mel at R_i = 5 taken at 10 °C 4 days after its preparation. As can be seen, the effect of the toxin has essentially disappeared, and the spectrum of the complex is very similar to that of a pure DPPC dispersion.

The comparison between the temperature profiles derived from the C–C and C–H regions of opaque and transparent samples of DPPC/Mel complexes with R_i = 5 as well as of a pure DPPC dispersion is given in Figures 5 and 6. Below 0 °C, the curves obtained from the C–H region for the opaque samples are very close to those of the pure lipid while the curve derived from the C–C region reveals that the conformation of the acyl chains of DPPC in both opaque and transparent complexes is the same and is slightly more disordered than that of the pure lipid. None of the curves obtained for opaque samples shows any evidence of the 0 °C transition. Consequently, between 0 and 30 °C, the three intensity ratios used to construct the temperature profiles are quite different for the two types of complexes. In opaque samples, there is less conformational disorder, as revealed by the h_{1080}/h_{1060} intensity ratio, and much more intermolecular vibrational coupling than in transparent samples (higher h_{2880}/h_{2850} ratio). For example, at 10 °C, the h_{2880}/h_{2850} and h_{2935}/h_{2880} ratios are 0.87 and 0.49, respectively, for the transparent complex (Figure 6), compared to 1.25 and 0.37 for both the opaque sample and the dispersion of pure DPPC. Therefore, the metastable phase of DPPC/Mel complexes undergoes a rearrangement toward a thermodynamically more stable one in which the packing of the acyl chains of the lipid is very similar to that of pure DPPC.

The gel to liquid-crystalline transition of opaque samples starts at 28 °C which is 12 °C lower than for pure DPPC. In addition, the transition is broad and biphasic. In the liquid-crystalline phase, the intensity ratios are within experimental errors identical for the opaque and transparent samples. Therefore, the perturbation induced by melittin on DPPC

reappears when some defects are present in the bilayers by going through the phase transition. When the samples are cooled down, they are transparent and require a few days to become opaque again.

Discussion

The study of the thermotropism of complexes of melittin with DPPC by Raman spectroscopy provides valuable information regarding their structure and stability especially when they form small particles that have already been investigated by NMR spectroscopy (Dufourc et al., 1986a), QLS, and freeze–fracture electron microscopy (Dufourcq et al., 1986). The presence of the hydrophobic part of proteins and polypeptides within the apolar core of phospholipid membranes generally gives rise to the appearance of more gauche conformers along the lipid acyl chains in the gel phase (Dunker et al., 1979; Curatolo et al., 1978; Susi et al., 1979; Taraschi & Mendelshon, 1979). For DPPC/Mel complexes, the conformational order is slightly perturbed as revealed by the h_{1080}/h_{1130} intensity ratio. Consequently, it seems that in the gel phase, melittin is not embedded deeply inside the bilayer as an intrinsic protein. In addition, a phase transition is detectable for these complexes even at $R_i = 2$. These results lead to the conclusion that the lipids in the small particles of DPPC/Mel are organized in bilayers. This hypothesis is also reinforced by the fact that the order parameters obtained by NMR spectroscopy are also very close to those of bilayers (Dufourc et al., 1986a). In spite of these similarities with pure lipid bilayers, complexes of DPPC/Mel are characterized by a very low intermolecular vibrational coupling as indicated by the C–H stretching region. One can then ask how can the intermolecular coupling be reduced without a significant increase of the number of gauche bonds. As discussed above, the h_{2880}/h_{2850} ratio is a sensitive probe of the vibrational coupling of the C–H stretching vibrations between adjacent chains (Gaber & Peticolas, 1977; Kouaouci et al., 1985) since it decreases markedly when the coupling is lowered by isotopic dilution with deuteriated chains, for example. Our results suggest that this ratio is also sensitive to the size, or to the number of molecules, of the lipid units in which the intermolecular coupling occurs.

The Raman results presented here all favor a structure for DPPC/Mel complexes that is very similar to that of disks of phospholipids with apolipoproteins. These "bicycle tire" like complexes consist of phospholipid bilayer disks whose hydrophobic edges are shielded from the aqueous phase by a layer of proteins (Segrest, 1977; Tall et al., 1977; Wlodawer et al., 1979). Raman results for phosphatidylcholine/apolipoprotein complexes have several common features with our results on DPPC/Mel. Lavialle and Levin (1980) and Gilman et al. (1981) have observed that before the phase transition, the number of gauche bonds in PC/apolipoprotein complexes is similar to that found in a dispersion of pure lipid while the spectra in the C–H region are considerably different. In addition, the cooperativity of the transition of discoidal particles formed by this system is much lower. Therefore, these lipid/protein assemblies are characterized by a high conformational order, a low intermolecular chain–chain interaction, and a low cooperativity of the gel to liquid-crystalline phase transition. Such a "bicycle tire" like structure seems to be associated with the high potential of some proteins like melittin (Dasseux et al., 1984) to form amphipathic α-helices in the presence of phospholipids (Segrest, 1977). This interpretation of our Raman results is also supported by the electron microscopy results (Dufourcq et al., 1986) indicating that DPPC/Mel complexes at $R_i = 5$–50 resemble flat disks which are approximately one bilayer thick.

When small particles of DPPC/Mel are cooled down below 0 °C, there is in their Raman spectra a sudden augmentation of the h_{2880}/h_{2850} ratio associated with the freezing of water. This result can be explained by the aggregation of the discoidal particles with a partial expulsion of the melittin molecules from the hydrophobic core of the bilayers. The driving force of this reorganization seems to be associated with the change of structure occurring when water crystallizes. It is well-known that, during the crystallization of liquids, solute particles are excluded from the growing lattice resulting in an increased concentration of solutes in the liquid. During the freezing of water, it seems that the exclusion of the DPPC/Mel complexes from the ice lattice induces more collisions and aggregation of the particles. As a result of the phenomenom, there is a sharp increase of the interchain vibrational coupling, and the h_{2880}/h_{2850} ratio reaches a value that is very close to that of the pure lipid. When opaque samples of DPPC/Mel are cooled down below 0 °C, no such transition is detected.

The Raman spectra of transparent samples of DPPC/Mel complexes change with time at room temperature, and after a few days, the h_{2880}/h_{2850} ratio becomes very close to that of the pure lipid. The only explanation for the increase of vibrational coupling is again the aggregation of the small particles into larger ones accompanied by the partial or total expulsion of melittin. The fact that the samples become opaque also supports this conclusion. Therefore, the small particles that are formed spontaneously at $T < T_m$ after incubation of DPPC and melittin at high temperature are metastable, and with time, melittin is expulsed with the reassociation of the lipids. Such a dissociation of DPPC/Mel complexes was also detected by intrinsic fluorescence polarization (Faucon et al., 1979).

The type of structure obtained below 0 °C for transparent and opaque samples is not the same. On the one hand, Raman spectra in the C–H stretching region are slightly different. For example, the intermolecular coupling is lower for transparent samples. On the other hand, when the temperature is increased above 0 °C, samples that were opaque at the beginning will remain opaque while samples that were transparent will also come back transparent above 0 °C. This "memory" of the samples for their initial state is inconsistent with the same structure below 0 °C, even though both types of structure are due to the aggregation of particles with expulsion of melittin. We believe that the freezing of water induces a less important structural reorganization of the lipidic assembly than the reach of the thermodynamical equilibrium at room temperature, since the formation of small particles for originally transparent samples is instantaneous after the melting of ice. This requires that melittin is still attached to the bilayers even at low temperature. On the opposite hand, the rebinding of melittin in the case of opaque samples to form small particles requires heating the samples above 30 °C. As has already been shown, free melittin binds to DPPC only at high temperature when defects are present on the membrane surface (Dasseux et al., 1984).

The interaction of melittin with DPPC bilayers affects considerably the phase transition of this lipid. For transparent samples, the onset of the transition is shifted to 28 °C, and the temperature profiles for all samples investigated show a biphasic transition. Anomalous transitions have also been observed by other physical techniques. By differential scanning calorimetry, an exothermic peak followed by an endothermic one has been detected around 30 °C for DPPC/Mel complexes with $R_i = 5$ (Dasseux, 1983). A discontinuity of the first

moment of NMR bands has also been measured just prior to the transition at $R_i = 20$ (Dufourc et al., 1986a).

Several concomitant phenomena can be at the origin of the biphasic phase transition of DPPC/Mel complexes. For instance, lipid phase separation can give rise to multiple phase transitions. However, we have observed a similar behavior for complexes with $R_i = 5$ and 15, and the results of electron microscopy show that transparent samples of DPPC/Mel with $R_i < 15$ are rather homogeneous and composed only of small particles (Dufourcq et al., 1986). Since the concentrations used in electron microscopy and Raman spectroscopy are very close, it is very unlikely that the major factor giving rise to the biphasic transition is the presence of two populations of lipids. However, as the temperature is increased, the bilayers become less stable due to the presence of some defects, and this can lead to a reorganization of the lipid assembly such as the aggregation of the discoidal particles into larger ones. We have observed that the phase transition of DPPC/Mel complexes was accompanied by an increase of the turbidity of the samples and a decrease of the signal to noise ratio in the spectra which was reestablished after the transition. This phenomenom can be associated with the fact that the size of the particles was then closer to the wavelength of the exciting laser beam. In addition, for some complexes we have observed a significant and reproducible increase of the h_{2880}/h_{2850} ratio prior to the main transition at 30 °C. This aggregation process would be at the origin of the exothermic peak observed by DSC. Since both the intramolecular and overall disorders increase with the aggregation of the discoidal particles, the melittin molecules have to be at least partially embedded into the hydrophobic core of the larger lipid/protein units.

For opaque samples, the transition can result both from the binding of the toxin and the formation of small particles and also from the melting of the lipid acyl chains. At $T > 30$ °C, the temperature profiles of opaque and transparent complexes become much closer (Figures 5 and 6).

For the liquid-crystalline phase, Raman results are essentially the same for transparent and opaque samples. In all cases, the overall disorder is very high, and the number of gauche bonds of the lipid acyl chains is slightly higher than that of the pure lipid. However, the results of freeze–fracture electron microscopy (Dufourc et al., 1986b) show that the size of the complexes is strongly dependent on the lipid to protein molar ratio. For example, at $R_i = 15$, the complexes are approximately 300–600 nm in diameter while at $R_i = 5$ they are only 10–25 nm. Since we have shown that the h_{2880}/h_{2850} ratio is sensitive to the size of the units involved in intermolecular coupling, it is surprising to have identical values of this ratio for complexes with $R_i = 5$ and 15. In order to explain this finding, melittin, at least at $R_i = 15$, has to be uniformly incorporated into the lipidic assembly as we have postulated during the aggregation of the small particles. In such a case, the intermolecular coupling is partially blocked by the toxin within the bilayer. This interpretation is further reinforced by the electron microscopy results (Dufourcq et al., 1986) that show protein particles on the surface of the $R_i = 15$ complexes in the fluid phase due to the presence of intrinsic melittin. Since the conformational disorder is already quite high in the liquid-crystalline phase, incorporation of melittin inside the bilayer increases only slightly the number of gauche bonds.

The behavior of complexes of melittin with DPPC and DMPC provides some indication that the affinity of the toxin, at least in the gel phase, is different for the two lipids. Even though QLS results (J. Dufourcq, private communication) show the formation of small particles in both cases, our results indicate that in the presence of the same quantity of melittin the conformation of the acyl chains of DPPC is much less perturbed by the toxin than in the case of DMPC. The smaller cohesion of the apolar region of DMPC bilayers that favors hydrophobic interactions between the toxin and the lipid is probably at the origin of the difference of affinity of the two lipids. The cooperativity of the phase transition is also much more affected in DMPC/Mel than in DPPC/Mel complexes at high concentrations of melittin. We believe from these results that the existence of lamellar DMPC/Mel complexes is possible only in the presence of a small quantity of toxin and that the formation of small particles which look like mixed micelles is readily induced in the case of DMPC/Mel complexes where hydrophobic interactions are more favored. The higher stability of DPPC bilayers leads to the existence of the "bicycle tire" like disks for a greater range of lipid to protein molar ratios.

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Characterization of the Cation Binding Sites of the Purple Membrane. Electron Spin Resonance and Flash Photolysis Studies†

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ABSTRACT: The binding of Mn^{2+} and La^{3+} to the blue membrane prepared by deionization of the *Halobacterium halobium* purple membrane has been studied by electron spin resonance (ESR) spectroscopy, visible absorption spectroscopy, and flash photolysis. ESR studies indicated that 10 Mn^{2+} binding sites are present per bacteriorhodopsin monomer. Five high- and medium-affinity sites, normally occupied by Ca^{2+} and Mg^{2+} in the purple membrane, as well as five low-affinity sites were found. Proteolysis and chemical modification experiments indicated that the low-affinity sites are located on the bacteriorhodopsin C-terminal segment, while the high- and medium-affinity sites involve other carboxyl groups of the protein. Competition experiments indicated that La^{3+} binds much more strongly than Mn^{2+} to these sites. Visible absorption spectroscopy and flash photolysis experiments indicated that binding of Mn^{2+} or La^{3+} regenerates both the purple color and formation of the M_{412} intermediate. The effect occurs progressively as cations bind to the high- and medium-affinity sites, bound La^{3+} being more effective than bound Mn^{2+}. In addition, La^{3+} was also shown to inhibit the M_{412} decay but at concentrations higher than those required for binding to divalent cation sites. It is suggested that divalent cations support both the purple color and proton-pumping activity by rendering less negative the surface potential of the purple membrane. This process may promote deprotonation of the counterion of the retinal Schiff base and possibly of other functional groups. On the other hand, it is proposed that the inhibitory effect of La^{3+} is mainly due to binding to a site distinct from those of divalent cations. This latter site may be involved in Schiff base reprotonation.

Bacteriorhodopsin (BR),[1] the retino protein of the *Halobacterium halobium* purple membrane, acts as a light-driven proton pump. Much is known about this protein, with regard to both its structure and its function [for reviews, see Stoeckenius and Bogomolni (1982), Dencher (1983), and Stoeckenius (1985)]. From the functional point of view, absorption of a photon initiates a photocycle during which trans–cis isomerization of the retinal chromophore occurs as well as sequential deprotonation and reprotonation of the retinal–protein Schiff base. Changes in the protonation of several amino acid residues appear to be also involved in the photocycle (Scherrer et al., 1981; Siebert et al., 1982; Hanamoto et al., 1984; Engelhard et al., 1985; Rothschild et al., 1986). How these features relate to the mechanism of active proton transport is the subject of current interest.

Recent studies have indicated that the purple membrane binds about 5 mol of Ca^{2+} and Mg^{2+}/mol of BR and that these divalent cations are essential for the proton transport function (Chang et al., 1985). Indeed, extraction of the cations by various procedures shifts the absorption maximum of light-adapted BR from 568 to 605 nm, yielding the so-called blue membrane (BR_{605}). The pigment thus formed has a severely perturbed photocycle with no deprotonated Schiff base intermediate and does not pump protons (Mowery et al., 1979; Kobayashi et al., 1983; Dupuis et al., 1985; Chang et al., 1985). The purple membrane (BR_{568}) can be regenerated by the addition of mono- or divalent cations with parallel recovery of the proton-pumping activity (Kimura et al., 1984; Chang et al., 1985, 1986; Dupuis et al., 1985). The trivalent cation La^{3+} also restores the purple color but, on the other hand, inhibits proton pumping by perturbing later stages of the photocycle (Drachev et al., 1984; Chang et al., 1986).

Proposals have been made to explain the requirement of cations for the purple color and for the formation of the Schiff base deprotonated M_{412} intermediate (Kimura et al., 1984; Dupuis et al., 1985; Chang et al., 1986). Presently, little is known concerning the nature and properties of the various cation binding sites of the purple membrane as well as their relative involvement in the proton-pumping activity. In this

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[1] Abbreviations: BR, bacteriorhodopsin; BR_{568}, purple form of bacteriorhodopsin; BR_{605}, blue form of bacteriorhodopsin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ESR, electron spin resonance.

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