Infrared Spectroscopic Study of the Secondary Structure of Melittin in Water, 2-Chloroethanol, and Phospholipid Bilayer Dispersions†

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ABSTRACT: The conformations of melittin, an amphipathic polypeptide consisting of 26 amino acid residues, and its hydrophobic (residues 1–19) and hydrophilic (residues 20–26) fragments were examined in various solvent systems, including H_2O, ^2H_2O, 2-chloroethanol, and 1,2-dimyristoylphosphatidylcholine (DMPC) multilayers, by infrared spectroscopy. Water and 2-chloroethanol were used as reference solvents for characterizing the amide I and II vibrational frequencies of the polypeptide in systems reflecting unordered, β-structure, or α-helical forms. In DMPC bilayer assemblies both melittin and its hydrophobic fragment F_1 exhibit α-helical conformations. In contrast, infrared spectra for the hydrophilic F_2 fragment are suggestive of a β conformation with perhaps spectral contributions from random-coil configurations. The α-helical conformation of intact melittin in DMPC multilayer dispersions remains unchanged as the bilayer passes from the gel to liquid-crystalline state. For melittin–water solutions the infrared spectra monitor changes in population of specific conformations as the temperature is varied. Thus, for melittin concentrations in which tetramers are dominant, high temperatures (31 °C) favor the α-helical form, while low temperatures (8 °C) lead to populations of both β and α-helical structures. At lower melittin concentrations for which monomers persist, high temperatures favor an unordered polypeptide form, while low temperatures induce an α-helical conformation. Although peak-height intensity ratios A_{II}/A_I for the amide I and II regions are difficult to interpret rigorously, values of this parameter for aqueous solutions of melittin suggest a sensitivity to structural changes involving the aggregation properties of the polypeptide.

Although the perturbing effects of lipid–protein associations on the physical and chemical behavior of bilayer systems are well appreciated [see, for example, Seelig & Seelig (1980), Chapman et al. (1979), Rice et al. (1979), Pink & Chapman (1979), Griffith & Jost (1978), and Papahadjopoulous et al. (1975)], relatively little spectroscopic information exists concerning the influence of the lipid environment upon protein conformation. For examination of this problem, a number of the spectroscopic techniques generally used to determine protein conformations in aqueous media have also proven advantageous for clarifying the structural arrangements assumed by proteins either embedded within lipid bilayers or associated with micellar assemblies (Massey et al., 1981; Bösch et al., 1980; Wallace & Blout, 1979; Keniry & Smith, 1979, 1981). For example, lysozyme (Lippert et al., 1980) and the B protein of F_d phage (Dunker et al., 1979) were reported from Raman studies to be in β chain pleated sheet structures, while glucagon (Epand et al., 1977; Bösch et al., 1980), bovine rhodopsin (Osborn & Nabedryk-Viala, 1978), myelin basic protein (Keniry & Smith, 1981, 1979), various apolipoproteins (Jonas & Krajnovich, 1977; Assman & Brewer, 1974), and lipophilin (Cockle et al., 1978) were shown by circular dichroism studies to adopt α-helical conformations.

Infrared spectroscopy, in particular, represents an established spectroscopic technique that has been widely used for qualitative determinations of the secondary structure of proteins and polypeptides (Thomas & Kyogoku, 1977; Fawcett & Long, 1973; Susi, 1969), although, again, relatively few studies have been explicity directed toward assessing the conformational possibilities available to a protein component within a lipid environment (Rothschild et al., 1980; Rothschild & Clark, 1979). In deducing secondary structural features of proteins and polypeptides from vibrational data, the characteristic vibrational frequencies for the trans CONH amide unit are specifically monitored. Because of either instrumental considerations or of spectral interference of critical bands from the solvent and lipid media, not all the amide vibrational modes are necessarily experimentally accessible. Consequently, in this report we will be concerned with only the amide I and II modes occurring in the 1700–1500-cm^{-1} spectral interval. The amide I feature, located approximately in the 1680–1597-cm^{-1} region, results primarily from the C=O stretching vibration coupled to the in-plane NH bending and CN stretching modes (Thomas & Kyogoku, 1977; Susi, 1969). Falling generally within the 1480–1575-cm^{-1} interval, the amide II band is mainly associated with the coupled CN stretching and in-plane NH deformation modes of the peptide group (Thomas & Kyogoku, 1977; Susi, 1969). Although neither of these two amide bands are overlapped by lipid vibrations in spectra of membrane bilayers, the bending mode of water at approximately 1640 cm^{-1}, which absorbs strongly in the infrared spectra of liposomal preparations, must be appropriately subtracted from the 1650-cm^{-1} amide I region prior to making structural inferences concerning protein conformations. In attempting to circumvent the problem of water interference in this spectral area, investigators have used ^2H_2O, whose deformation mode is shifted to lower frequencies, as a solvent medium (Schnarr & Maurizot, 1980; Rothschild et al., 1980; Osborn & Nabedryk-Viala, 1978). Unfortunately, even under these conditions, solvent interference remains a problem, but more importantly, the amide I frequencies are sensitive in varying degrees to hydrogen exchange from the deuterated solvent. In addition, the ratio of α-helix structures to random-coil forms for a given system tend to be affected by deuteration (Calvin et al., 1959). Since developments in the coupling of microcomputers to dispersive infrared spectrometers allow reliable spectral subtractions of water and other constituents to be performed on multicomponent systems, we choose to use H_2O as the preferred solvent for the systems to be examined in this report.

In this paper we discuss the infrared spectra of bilayer

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systems containing melittin, an amphipathic polypeptide composed of 26 amino acid residues, in an effort to obtain a clearer understanding of the conformational changes induced in proteins by lipid interactions. Specifically, we examine the temperature dependence of spectra of melittin and its hydrophobic F_1 (residues 1–19) and hydrophilic F_2 (residues 20–26) fragments both as recombinants with dimyristoylphosphatidylcholine (DMPC) liposomes and as components in solvent media. Since various conformational forms of melittin are known for a number of solvent systems (Talbot et al., 1979; Brown et al., 1980), spectral observations of melittin in specific solvent mixtures provide tractable reference systems both for assessing the sensitivity of infrared techniques and for use in distinguishing conformational forms of the polypeptide when the molecule is associated specifically with a lipid bilayer. For interpreting reference spectra, we note that melittin as a monomer exists predominantly in an extended, flexible conformation, while self-association leads to the formation of α helices (Lauterwein et al., 1979; Talbot et al., 1979). Also, in the presence of either micelles prepared from detergents or bilayers of dilaurylphosphatidylcholine, melittin exhibits characteristic nuclear magnetic resonance and circular dichroism spectra for a polypeptide in an α-helical form (Drake & Hider, 1979; Lauterwein et al., 1979).

Experimental Procedures

High-purity samples of 1,2-dimyristoyl-L-α-phosphatidylcholine (DMPC) were commercially obtained from Sigma Chemical Co. 2-Chloroethanol and deuterium oxide (99.8 mol %) were purchased from Fisher Scientific Co. and Bio-Rad Laboratories, respectively. All samples were used without prior purification. Melittin and its fragments were prepared from whole bee venom as previously described by Mollay et al. (1976) and Mollay (1976).

Lyophilized melittin, F_1, and F_2 were added to aqueous dispersions of DMPC (50% by weight) to yield final lipid:polypeptide molar ratios of 14:1, 25:1, and 25:1, respectively. These systems were chosen for comparison purposes with Raman data (Levin et al., 1982). The mixtures were first mechanically shaken for about 10 min and then kept at 40 °C for 1 h to allow the peptide or its fragments to interact completely with the DMPC liposomes. For preparations involving H_2O, ^2H_2O, and 2-chloroethanol, the appropriate solvent was added to either lyophilized melittin or a melittin fragment to yield final concentrations of 6×10^{-2} or 6×10^{-3} M.

Infrared spectra were obtained with a Perkin-Elmer 580B spectrophotometer coupled to a Perkin-Elmer Model 3500 data station, consisting of a microprocessor module, keyboard, and video display component. Since broad infrared bands are observed, spectra were recorded under conditions of moderate resolution with spectral slit widths of the order of 2.5 cm^{-1}. A jacketed, variable path length cell with CaF_2 windows was generally set for approximately a 4-μm path length, as determined from interference fringes. In preparing the cell for a spectral scan, the sample is placed directly onto the center portion of the lower salt plate. The upper assembly of the cell is then attached and the path length is adjusted. By carefully varying the path length of the assembled unit, bubbles, in particularly the gel-state samples, are easily eliminated. During spectral scans, the instrument was constantly purged with dry nitrogen gas. Water vapor and polystyrene were used for calibration; vibrational frequencies are reported to ± 2 cm^{-1}. The jacketed cell was connected to a thermostatically controlled temperature bath containing an ethylene glycol–water mixture. Temperatures, measured by a copper–constantan thermocouple inserted between the salt plates at the top of the window assembly, were maintained to within ± 0.1 °C.

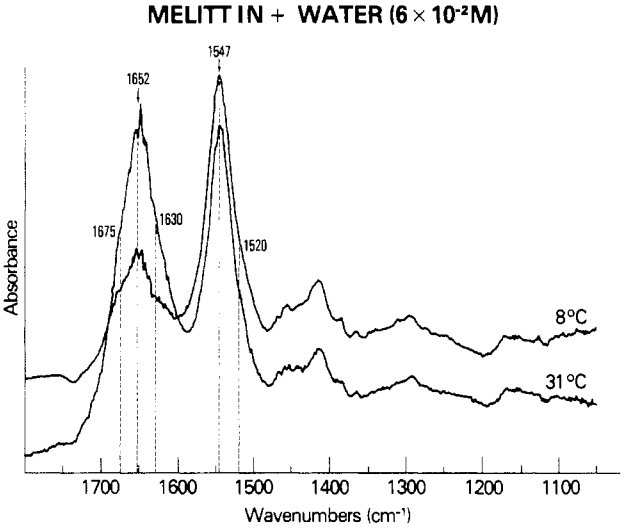

FIGURE 1: Infrared spectra at 8 and 31 °C of aqueous solutions of melittin (6×10^{-2} M) in the amide I and II regions. Spectral contributions from water have been subtracted.

Computer manipulations of spectra were performed in the absorbance mode. Both temperature and path-length conditions were matched for the reference and solute-containing spectra prior to the subtraction step. This procedure entails correcting the spectrum for slight differences in water concentrations. Although we originally thought that the computer subtraction of water from the liposomal preparations could be based upon the 2150-cm^{-1} water association band, small contributions to this region from melittin and its fragments precluded this approach. (The greatest contribution to this spectral area occurred for F_2.) Once temperature, path-length, and concentration differences were considered in the reference spectrum, a match point for the reference and sample spectra was taken in the 1950–1850-cm^{-1} window regions before performing the computer subtraction of the solvent. At most, a 1% change in the base line of the reference water spectrum was required to complete the adjustments before its subtraction. Band frequency measurements were determined from expanded, unsmoothed spectra.

Results and Discussion

In the following sections we examine the infrared spectra of melittin in a variety of environments in an attempt to identify the conformational changes in the polypeptide system as a function of solvent and temperature. In particular, our strategy is to obtain sets of reference spectra that can be applied toward delineating the structure of the polypeptide within a lipid bilayer matrix. An examination of the conformational behavior of the melittin fragments allows us to assess separately the effects of the bilayer milieu on the hydrophobic and hydrophilic segments as the polypeptide integrates itself within the membrane. In assigning the amide I and II vibrational frequencies to specific conformational species, we appeal to the wealth of information available from both empirical studies and correlations from normal coordinate treatments. [See, for example, Krimm & Bandekar (1980), Thomas & Kyogoku (1977), Fawcett & Long (1973), Koenig (1972), Susi (1969), and reference cited therein.]

Aqueous Solutions. Figure 1 presents the infrared spectra of melittin–water solutions at a moderately high concentration, 6×10^{-2} M (185 mg/mL), at 8 and 31 °C. Spectral contributions from the water solvent have been subtracted by following the above procedures. For these concentrations aqueous solutions of melittin have been reported to be tet-

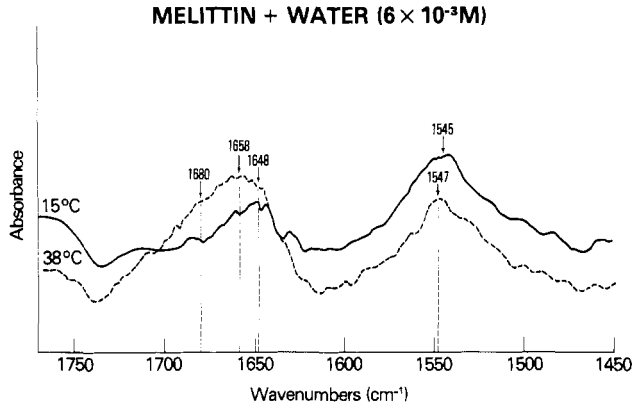

FIGURE 2: Infrared spectra at 15 and 38 °C of aqueous solutions of melittin (6×10^{-3} M) in the amide I and II regions. Spectral contributions from water have been subtracted.

rameric (Talbot et al., 1979; Brown et al., 1980). At 8 °C the amide I maximum at 1652 cm^{-1} reflects a predominantly α-helical conformation. Shoulders at 1675 and 1630 cm^{-1} are consistent, however, with the presence of a small population of β antiparallel chain pleated sheet structures. Recently, normal coordinate calculations on β-turn conformations have predicted vibrational modes that overlap the α-helix and β-sheet modes in the amide I region (Krimm & Bandekar, 1980; Bandekar & Krimm, 1979). (The β turn represents a peptide conformation that reverses its direction by about 180°.) Thus, one must exert care in interpreting spectra for the amide I region in terms of the traditional vibrational assignments. Since normal coordinate analyses indicate that one of the characteristic modes for the most common type I β turn occurs at significantly higher frequencies within the amide II region than those usually observed for the β sheet (Krimm & Bandekar, 1980), the absence of a frequency predicted to occur at ~1575 cm^{-1} (Krimm & Bandekar, 1980) precludes, at least, the existence of this structure for melittin in water. It is difficult, however, to unambiguously eliminate the possibility of the presence of the less common β-turn forms, since their amide II vibrational modes span the 1562–1536-cm^{-1} interval. The intense amide II mode at 1547 cm^{-1}, seen in Figure 1, is consistent with an α-helical conformation.

The spectrum in Figure 1 for melittin in water at 31 °C indicates that the primary peak in the amide I region remains centered at 1652 cm^{-1}; however, the shoulders at 1675 and 1630 cm^{-1}, associated with a β structure, are significantly diminished. Apparently, higher temperatures promote an α-helical structure, in a manner analogous to that observed for poly(γ-benzylglutamate) system (Scheraga, 1960). The amide II region supports this conclusion as the peak frequency shifts slightly from 1547 to 1545 cm^{-1} at 31 °C. For the change in temperature from 8 to 31 °C, the peak-height intensity ratio A_{II}/A_I (amide II mode/amide I mode) undergoes a significant decrease from 1.8 to 1.1. Thus, for the moderately concentrated water solutions of melittin the virtual disappearance of the 1675- and 1630-cm^{-1} shoulders and the decrease in the A_{II}/A_I ratio on increasing temperature indicate a conformational rearrangement favoring an α-helical state.

For low melittin concentrations, 6×10^{-3} M, infrared spectra in Figure 2 obtained at 15 °C exhibit amide I and II peaks at 1648 and 1545 cm^{-1}, which also suggest an α-helical arrangement for the peptide chain. (The spectrum is identical with spectra obtained at 8 °C.) As before, the spectra reflect systems for which water has been subtracted. [From the data quoted by Susi (1969), a β parallel chain polar sheet cannot be definitely ruled out.] Previously, for data determined at

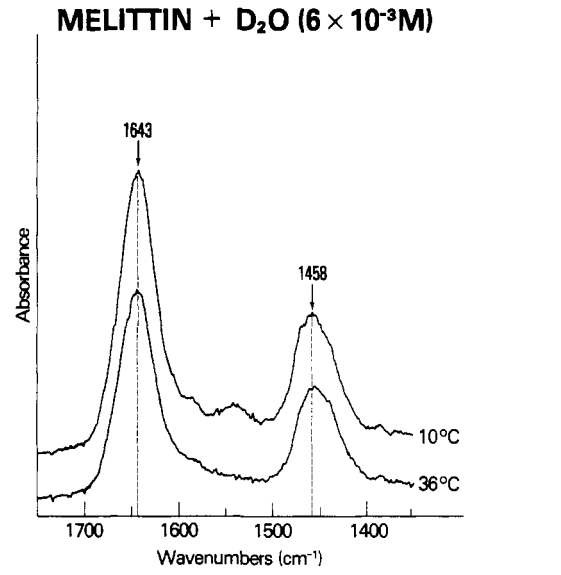

FIGURE 3: Infrared spectra at 10 and 36 °C of solutions of melittin (6×10^{-3} M) in ^2H_2O in the amide I and II regions.

ambient temperatures and above, melittin solutions at low concentrations and at low pH in the absence of salt were reported to be monomeric and in a random-coil conformation (Talbot et al., 1979; Brown et al., 1980). As noted in Figure 2, the spectrum at 38 °C shows an increase in intensity in the amide I mode at 1658 cm^{-1} and a filling in of intensity around 1520 cm^{-1}, frequencies consistent with the presence of unordered structures. The shoulders around 1680 and 1530 cm^{-1} in the 38 °C spectrum (Figure 2) also suggest the presence of some β-sheet structures. In contrast to the results obtained for the more concentrated 6×10^{-2} M solutions, the predominant α-helical structure is observed at low temperatures, with an increase in temperature promoting the unfolded conformation.

The A_{II}/A_I peak-height ratio in Figure 2 changes from 2.0 to 0.75 as the temperature increases from 15 to 38 °C, a somewhat larger change in comparison to that observed for the more concentrated system. It is interesting that the A_{II}/A_I ratios are similar for both the concentrated and diluted melittin samples at low temperatures, although the more concentrated system consists of both α-helical and β structures. This suggests that both frequency and intensity parameters are important for fully characterizing the state of the polypeptide in solution.

Melittin in ^2H_2O. For melittin dissolved in ^2H_2O, Figure 3, a symmetrical contour centered at 1643 cm^{-1} appears for the amide I mode in the spectrum recorded at 10 °C. This value represents about a 5-cm^{-1} decrease in comparison to the same concentration of melittin (6×10^{-3} M) dissolved in H_2O. The observed frequency for melittin in ^2H_2O reflects an unordered structure and is consistent with the unordered form found for β-lactoglobulin and α-casein (Susi, 1969). In contrast, an α-helical structure for β-lactoglobulin would result in a peak at 1649 cm^{-1} (Susi, 1969). The amide II peak at 1545 cm^{-1} in H_2O is shifted to 1458 cm^{-1} because of deuterium exchange in the peptide groups. (The weak 1545-cm^{-1} feature in Figure 3 for the spectrum recorded at 10 °C arises from incomplete hydrogen exchange. This transition disappears in the 36 °C spectrum.) Comparable frequency shifts for the amide II mode are also observed, for example, in the case of membrane- micellar-bound rhodopsin (Osborn & Nabedryk-Viala, 1978). In contrast to solutions of melittin (6×10^{-2} and 6×10^{-3} M) in H_2O, the spectra in Figure 3 for melittin in ^2H_2O indicate no change in conformation on increase in

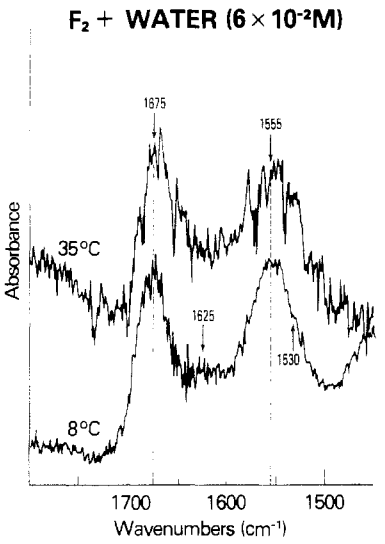

FIGURE 4: Infrared spectra at 8 and 36 °C of aqueous solutions of the hydrophilic fragment F_2 of melittin (6×10^{-2} M) in the amide I and II regions. Spectral contributions from water have been subtracted.

temperature. The effects of ^2H_2O on melittin conformation may originate in alterations in the strength of the hydrogen bonds on deuteration (Scheraga, 1960). In any event, these data further emphasize the chemical difficulties involving the use of ^2H_2O as a solvent system for obtaining infrared spectra. That is, the advantage gained from shifting the solvent absorption away from the amide I region could be lost as a consequence of the effect of deuterium exchange on conformational stability.

F_2 Fragment (6×10^{-2} M) in H_2O. Figure 4 displays the water-subtracted spectrum for the hydrophilic F_2 fragment of melittin at both 8 and 35 °C for the solute at a concentration of 6×10^{-2} M. Since these signals are intrinsically weak, the signal to noise levels are increased compared to the previous spectra. Both the 1555-cm^{-1} transition and very weak ~1625-cm^{-1} feature, present at only 8 °C, correspond to calculated frequencies for poly(L-alanine) in an antiparallel chain pleated structure (Moore & Krimm, 1976a,b). The 1675-cm^{-1} feature corresponds to the feature often associated with the Raman active, infrared inactive mode usually observed at ~1674 cm^{-1} (Fawcett & Long, 1973). Inasmuch as this mode for F_2 is weak and is observed in solution, its origin may arise from a relaxation of the $\nu(0,0)$ selection rule. Alternatively, the 1675-cm^{-1} feature could reflect the presence of a population of molecules with a β-turn structure (Krimm & Bandekar, 1980; Bandekar & Krimm, 1979).

Although some ambiguity may arise in the precise conformational form for F_2, the system is stable between 8 and 35 °C, as evidenced both by the absence of frequency shifts in the amide I and II regions and by the constant A_{II}/A_I peak-height ratio ($A_{II}/A_I \simeq 0.7$).

Anhydrous Melittin. The infrared spectrum of melittin cast as a film onto a KBr plate from a chloroform solution is displayed in Figure 5. Prominent amide I features appear at 1655 and 1628 cm^{-1} with shoulders at 1695 and 1680 cm^{-1}. For the amide II region the central maximum is located at 1540 cm^{-1}. The contour displays a shoulder at 1570 cm^{-1} and an asymmetry toward lower wave numbers. The strong 1655-cm^{-1} feature is consistent with a random-coil structure (Koenig, 1972; Susi, 1969), while the 1695-, 1680-, and 1628-cm^{-1} features are consistent with a β structure (Moore & Krimm, 1976a,b). Although the amide II generally appears ~1535 cm^{-1} for the random coil, we associate the 1540-cm^{-1}

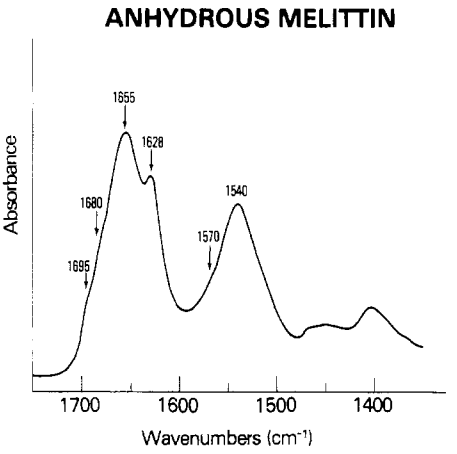

FIGURE 5: Infrared spectrum at room temperature of anhydrous melittin.

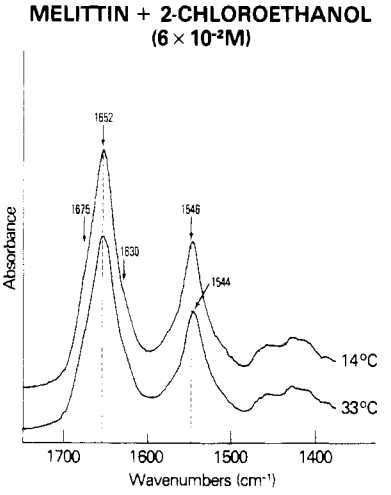

FIGURE 6: Infrared spectra at 14 and 33 °C of 2-chloroethanol solutions of melittin (6×10^{-2} M) in the amide I and II regions. Contributions from solvent spectra have been subtracted.

feature with the unordered structure. As noted above, the amide II mode for an α-helix conformation appears at ~1546 cm^{-1} in solution. The combination of the 1695- and 1570-cm^{-1} features may indicate the presence of a population of type I β turns, since only this conformation is predicted to give a distinctively high amide II frequency, as noted above (Krimm & Bandekar, 1980). Although these assignments must be judged as tentative, the spectrum suggests a predominance of the random-coil structure. Although we do not show the spectra for the 1200–1300-cm^{-1} amide III region, a weak, broad feature is observed at ~1246 cm^{-1}, a frequency consistent with unordered forms (Frushour & Koenig, 1975). In this preparation of a solid film the A_{II}/A_I peak-height intensity ratio is 0.70.

Solutions of Melittin in 2-Chloroethanol. For concentrations of melittin of 6×10^{-2} M in 2-chloroethanol, the spectrum at 14 °C (with the solvent subtracted) in Figure 6 displays a nearly symmetrical contour centered at 1652 cm^{-1} with minor inflections at 1675 and 1630 cm^{-1}. This amide I feature closely approximates that expected for a predominantly α-helical conformation, which is in agreement with the properties of protein solutions in 2-chloroethanol (Tanford, 1961). The weak shoulders in the amide I region are perhaps suggestive of a small amount of β conformation. For an increase in temperature from 14 to 33 °C, no frequency shifts occur and the A_{II}/A_I intensity ratio remains ~0.6. Spectra were also recorded for melittin in 2-chloroethanol at concentrations of

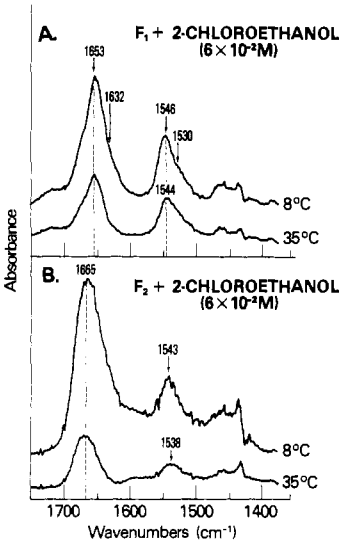

FIGURE 7: Infrared spectra at 8 and 35 °C of 2-chloroethanol solutions of melittin fragments (6×10^{-2} M). Contributions from solvent spectra have been subtracted. (A) Spectra of the hydrophobic fragment F_1 and (B) spectra of the hydrophilic fragment F_2.

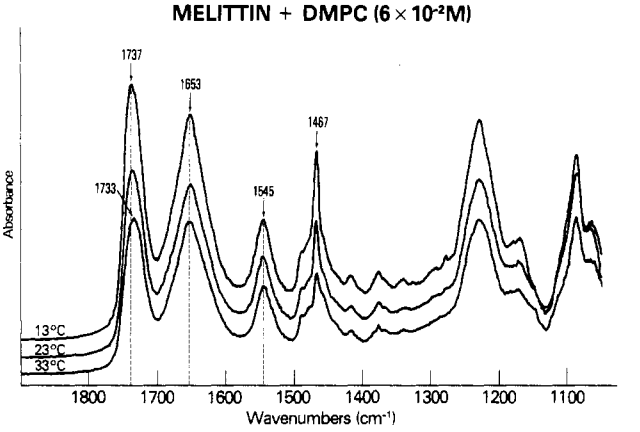

FIGURE 8: Infrared spectra at 13, 23, and 33 °C of multilayer dispersions of DMPC + melittin (6×10^{-2} M). Spectral contributions from water have been subtracted.

the order of 6×10^{-3} M (spectra not shown). Except for an increase in the noise level for the signal, the characteristics of the amide I and II regions were essentially the same as those for more concentrated (6×10^{-2} M) melittin solutions.

Figure 7A shows the solvent-subtracted spectra of the F_1 segment solvated in 2-chloroethanol at 8 and 35 °C. The 1653- and 1546-cm^{-1} features for the amide I and II regions, respectively, clearly indicate an α-helical conformation. The inflection at 1630 cm^{-1} and asymmetry of the amide II contour at ~1530 cm^{-1} may reflect the presence of some β structure.

For the F_2 fragment in 2-chloroethanol in Figure 7B the amide I band appears as a broad band centered at 1665 cm^{-1}, indicating the presence of β-like conformations (Koenig, 1972; Lord & Yu, 1970). The broadness of the feature for the 1538-cm^{-1} amide II band at 35 °C is also associated with disordered structures (Koenig, 1972). Again, ambiguities persist in these assignments as the amide I and II frequencies are also consistent with calculated frequencies for a type I β turn (Krimm & Bandekar, 1980). Comparison of the ratios of the amide I and II features show that for F_1 and F_2 A_{II}/A_I is 0.5 and 0.3, respectively, at 8 and 35 °C.

Melittin–DMPC Multilayer Complexes. Figure 8 displays spectra from 1800 to 1050 cm^{-1} at 13, 23, and 33 °C for reconstituted DMPC multilayers containing melittin at a concentration level of 6×10^{-2} M. For these spectra the background water spectrum has been appropriately subtracted, yielding a flat base line in the amide I region for pure DMPC liposomes (spectrum not shown). Temperature profiles derived from Raman spectroscopic data indicate that for this polypeptide concentration (lipid:polypeptide mole ratio = 14:1) two thermal order–disorder transitions are observed (Lavialle et al., 1980). The lower transition at 17 °C represents the depression of the main DMPC gel to liquid-crystalline phase transition, while the higher transition at approximately 29 °C is associated with the melting behavior of the boundary layer of lipids surrounding the intruding, hydrophobic portion of melittin. By recording the infrared spectra at 13, 23, and 33 °C, we examine the recombinant system at points, respectively, (a) in the gel phase, (b) after the gel to fluid phase transition but before the melting of the boundary layer, and (c) after the melting of the immobilized boundary lipid molecules. Through this 13–33 °C temperature range the amide I and II frequencies at 1653 and 1545 cm^{-1} remain constant. [The change in intensity ratios for the methylene CH_2 deformation modes at 1467 and 1488 cm^{-1} (Figure 8) monitors the fluidity changes in the bilayer on increasing temperature.] Further, the A_{II}/A_I peak-height intensity ratio also remains unchanged with a value of 0.75. Since the observed amide I and II frequencies reflect an α-helical conformation (Koenig, 1972; Thomas & Kyogoku, 1977) for melittin in each temperature region, we conclude that the conformation of the polypeptide remains unchanged and that probably no polypeptide aggregation occurs as the bilayer lipid passes from the gel phase to a completely liquid-crystalline state. This clear determination of an α-helix structure in the lipid bilayer is consistent with studies of melittin in sodium dodecyl sulfate micelles (Dawson et al., 1978) and in egg yolk phosphatidylcholine vesicles (Drake & Hider, 1979), for which α-helical conformations of the peptide were deduced by circular dichroism methods.

Previous studies suggested that an interaction with phospholipids leads to a disruption of melittin aggregates (Strom et al., 1978; Lauterwein et al., 1979). Although it cannot be definitely established that monomers result from the dissociation of the melittin tetramer (Strom et al., 1978), the close correspondence between the infrared spectra of melittin–DMPC recombinants with that of a dilute solution of melittin (6×10^{-3} M) in 2-chloroethanol implies the likelihood of either a monomeric or perhaps dimeric form in the liposome. Geometrical arguments involving the melting of the boundary layer of lipid around melittin in DMPC liposomes (Lavialle et al., 1980; Levin et al., 1982) strongly suggest the existence of monomeric melittin monomers within the bilayer.

Figure 8 also shows a shift for the lipid carbonyl stretching modes from 1737 to 1733 cm^{-1} for increasing temperatures. For pure DMPC liposomes undergoing the gel to liquid-crystalline phase transition, this analogous frequency shift is accompanied by a 10% decrease in peak-height intensity for the carbonyl stretching mode. In the gel to liquid-crystalline temperature range for the melittin-containing system shown in Figure 8, the peak-height intensity of the carbonyl mode decreases by 40%. The Raman spectra for melittin–DMPC complexes indicate a significant perturbation of the lipid acyl chains in this temperature range (Levin et al., 1982; Lavialle et al., 1980). The present infrared data for the lipid carbonyl stretching mode intensities demonstrate the existence of a highly perturbed interface region in comparison to the pure liposome. This does not necessarily imply a direct interaction between the polypeptide and lipid carbonyl groups but may simply reflect for the melittin-containing system an increase

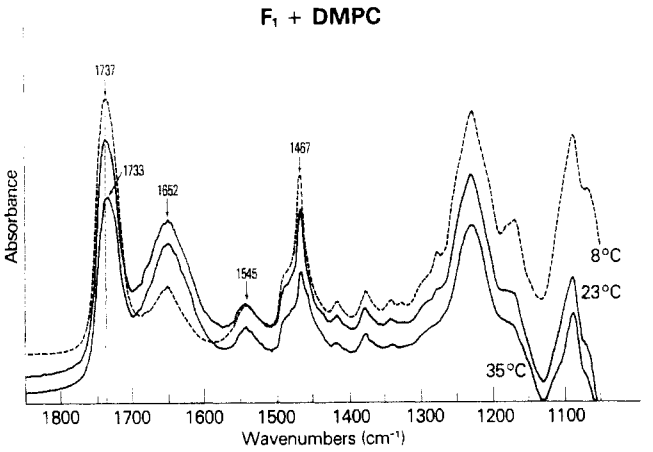

FIGURE 9: Infrared spectra at 8, 23, and 35 °C of multilayer dispersions of DMPC + the hydrophobic fragment F_1 of melittin (6×10^{-2} M). Spectral contributions from water have been subtracted.

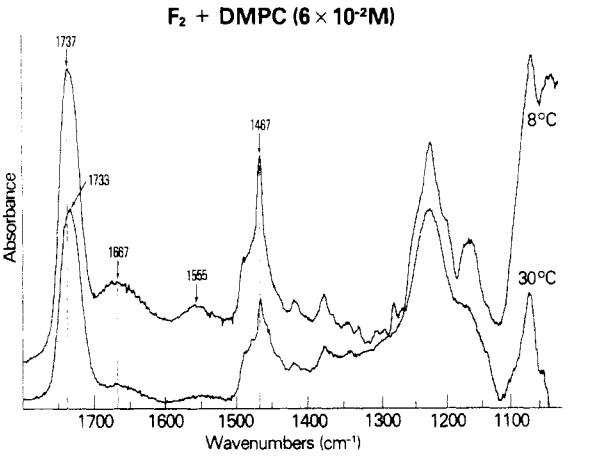

FIGURE 10: Infrared spectra at 8 and 30 °C of multilayer dispersions of DMPC + the hydrophilic fragment F_2 of melittin (6×10^{-2} M). Spectral contributions from water have been subtracted.

in the *inter*molecular dimensions for the liquid-crystalline state in comparison to the average intermolecular distances for the pure multilayer system.

For the hydrophobic fragment F_1–DMPC complex the infrared spectrum in Figure 9 also shows a dominant α-helical conformation for the 1–19 melittin segment. A temperature increase from 8 to 35 °C decreases the peak-height intensity for the carbonyl stretching modes by ~32%, as compared to the 40% decrease for intact melittin. Also, the A_{II}/A_I intensity ratios for the F_1–DMPC complex exhibit a quite different behavior in comparison to the effects observed for the polypeptide recombinants with intact melittin. As noted above for the intact polypeptide, no change was observed in the A_{II}/A_I ratios in the 13–33 °C temperature range. For the liposomal assembly containing F_1, the A_{II}/A_I intensity ratio decreases by about a factor of 2 on increasing the temperature of the system from 8 to 35 °C. In contrast to a moderate change observed in the intact system, for F_1 the amide I/carbonyl intensity ratio increases by a factor of about 2.7. Although the carbonyl intensity exhibits a temperature dependence in the various melittin-containing systems, the changes observed for the amide I and amide II intensities of F_1 reflect significant alterations in the field effects associated with the medium surrounding the oscillators. It is reasonable to conclude that an increase in temperature results in an aggregation of the hydrophobic segments within the lipid bilayer. Since no frequency shifts occur, one can probably rule out the concomitant introduction of conformational changes within the polypeptide units forming the aggregate. Since intact melittin fails to undergo these dramatic intensity changes, it is tempting to associate a stabilizing influence to the hydrophilic segment present in the intact polypeptide.

Figure 10 shows the spectrum for the hydrophilic F_2 fragment associated with DMPC. Although the 1667- and 1555-cm^{-1} features in the amide I and II regions suggest a β structure, the broad contour may also be indicative of contributions from the unordered form (Fawcett & Long, 1973; Koenig, 1972; Susi, 1969). Obviously, further clarifications concerning vibrational contributions from β turns would require additional detailed Raman studies on these systems. Although no change in the amide I and II frequencies occurs on changes in temperature, the carbonyl stretching mode intensity decreases by 35% on increasing the temperature from 8 to 30 °C, which is compared to decreases in intensity of about 10% for pure DMPC, 32% for F_1, and 40% for intact melittin complexes undergoing the analogous thermal changes. A previous comparison of Raman spectroscopic temperature profiles for F_2 associated with DMPC liposomes to the profiles for pure DMPC multilayers showed only subtle lipid acyl chain perturbations for the F_2 system (Levin et al., 1982). That is, an ~1 °C increase occurs in the gel to liquid-crystalline phase transition. However, in the Raman experiment the existence of a population of immobilized lipids is observed in the liquid-crystalline state (Levin et al., 1982). The changes in the infrared intensities for the carbonyl modes indicate, in addition, that important interactions occur between the polypeptide fragment and the bilayer lipid interface region.

Conclusion

Although infrared and Raman techniques have been extensively applied in conformational determinations of polypeptide and protein systems, relatively few studies involve structural studies of membrane components specifically within the lipid bilayer [see, for example, Wallach et al. (1979), and Lord & Mendelsohn (1981)]. For multicomponent systems involving membrane constituents, the use of group frequency arguments in deducing structural behavior often becomes complicated as a consequence of overlapping band contours. In the three component lipid, water, melittin systems examined here, the vexing effects of water interference in the amide I region can be greatly minimized through the reliable elimination of the temperature-dependent water spectrum by means of systematic computer subtraction methods. Thus, the infrared spectra for the conformationally sensitive amide I and II regions may be clearly delineated for analysis and comparison purposes. (The amide III region is unfortunately buried in the infrared spectrum under the strongly absorbing antisymmetric PO_2^- stretching mode at ~1240 cm^{-1}.) Although interference from overlapping water bands can generally be overcome in spectra of liposomal dispersions, conformational assignments deduced from the amide I and II spectral features may still prove somewhat ambiguous from effects due to band broadening and to spectral contributions from more than one conformational species. Despite the uncertainties that arise in assigning vibrational modes to specific secondary structures, infrared techniques may still be used to advantage by carefully basing one's spectral interpretations on both empirical and quantitative correlations derived from model compounds. Although the vibrational assignments for protein systems may prove elusive, our results indicate that it is clearly possible to gain insight into the structural properties exhibited by polypeptides associated with lipid bilayers.

In this report water and 2-chloroethanol were used as reference solvent systems for characterizing the frequencies of

Table I: Observed Infrared Amide I and II Frequencies and Intensity Ratios for Melittin and Its Fragments in Various Media

polypeptide	concn (M)	solvent	temp (°C)	amide I frequency (cm^{-1})	amide II frequency (cm^{-1})	A_{II}/A_I [a]
intact melittin	6×10^{-2}	H_2O	8	1675 (sh) [b]	1547	0.8
				1652	1520 (vw)	
				1630 (sh)		
			35	1675 (vw)	1547	1.1
				1652	1520 (vw)	
				1630 (vw)		
intact melittin	6×10^{-3}	H_2O	15	1648	1545	2
			38	1680 (sh)	1547	0.75
				1658		
				1648 (sh)		
intact melittin	6×10^{-2}	^2H_2O	10	1643	1458	0.5
			36	1643	1458	0.5
intact melittin	anhydrous		25	1695 (sh)	1570 (sh)	0.7
				1680 (sh)	1540	
				1655		
				1628		
intact melittin	6×10^{-2}	2-chloroethanol	14	1675 (vw)	1546	0.6
				1652		
				1632 (sh)		
			33	1652	1544	0.6
intact melittin	6×10^{-2}	DMPC multilayers	13	1653	1545	0.75
			23	1653	1545	0.75
			33	1653	1545	0.75
F_1	6×10^{-2}	2-chloroethanol	8	1675 (vw)	1546	0.5
				1653	1530 (vw)	
				1632 (vw)		
			35	1675 (vw)	1544	0.5
				1653	1530 (w)	
				1632 (vw)		
F_1	6×10^{-2}	DMPC multilayers	8	1652	1545	0.5
			35	1652	1545	0.25
F_2	6×10^{-2}	H_2O	8	1675	1555	0.7
				1625 (w)	1530 (?)	
			35	1675	1555	0.7
					1530 (?)	
F_2	6×10^{-2}	2-chloroethanol	8	1665	1543	0.3
			35	1665	1548	0.3
F_2	6×10^{-2}	DMPC multilayers	8	1667	1555	
			30	1667	1555	

[a] A_{II}/A_I represents the peak-height intensity ratio between the amide I and amide II vibrational transitions. [b] sh and vw represent shoulder and very weak, respectively.

melittin in the unordered, extended, and α-helical forms. On the basis of vibrational frequency comparisons in the amide I and II regions, we conclude that melittin exists predominantly in an α-helical conformation when introduced into DMPC bilayers. Further, no change in the secondary structure of melittin is observed when the bilayer passes through the gel to liquid-crystalline phase. The infrared spectra also indicate that a large proportion of melittin exist in the α-helical form for concentrations in water of 6×10^{-2} M, an observation in agreement with Lauterwein et al. (1979) and Talbot et al. (1979). By examining the temperature dependence of the spectra, we observed that an increase in temperature from 8 to 35 °C favors the formation of α helices at the expense of the small amount of β structure present at low temperatures. For low concentrations of melittin dissolved in water, Talbot et al. (1979) and Lauterwein et al. (1979) proposed a largely unordered structure. Their experiments, which were performed above 25 °C, are in agreement with our infrared results obtained at 35 °C. At lower temperatures (~15 °C), however, the infrared spectra of melittin solutions (6×10^{-3} M) reflect an α-helical structure.

For the hydrophobic F_1 fragments of melittin in lipid systems and 2-chloroethanol solutions, the infrared spectra again reflect the existence of an α-helical conformation. In contrast, the smaller hydrophilic F_2 fragment of melittin assumes extended β structures in water and in 2-chloroethanol solutions. For F_2 in DMPC bilayers the spectra are suggestive of populations of both the β and unordered forms. Table I summarizes the amide I and amide II infrared frequencies observed for melittin and its fragments in the various solvent systems.

Table I also summarizes the peak-height intensity ratios A_{II}/A_I obtained from the amide I and II modes of the polypeptide in differing environments. A strict interpretation of these intensity values would be extraordinarily difficult, primarily because the more relevant integrated band areas are hard to obtain accurately and because more than one conformational structure may be contributing intensity to the band interval. Further, the interpretation of the experimental values in terms of molecular parameters becomes clouded since one must consider reaction field and dielectric effects, changes in the normal coordinates specifying the vibrational frequencies, and existing intermolecular charge-transfer effects, phenomena all requiring formidable treatments for these complex systems [see, for example, Person & Steele (1974)]. Despite the obstacles that generally pervade intensity discussions, the peak-height ratios demonstrate a variability as a function of aggregation. Thus, a high A_{II}/A_I (1.8–1.1) ratio is observed for melittin existing as a tetramer in water, while a low value (0.75) is obtained for experimental conditions reflecting the monomeric form in water. For these water solutions of melittin (6×10^{-2} and 6×10^{-3} M) the intensity ratios change significantly as the relative populations of contributing structures

are altered for either the tetrameric or monomeric unit (vide supra).

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