Interaction of a Peptide Model of a Hydrophobic Transmembrane α-Helical Segment of a Membrane Protein with Phosphatidylcholine Bilayers: Differential Scanning Calorimetric and FTIR Spectroscopic Studies†

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ABSTRACT: High-sensitivity differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy were used to study the interaction of a synthetic model hydrophobic peptide, Lys_2-Gly-Leu_{24}-Lys_2-Ala-amide, and members of the homologous series of *n*-saturated diacylphosphatidylcholines. In the low range of peptide mole fractions, the DSC thermograms exhibited by the lipid/peptide mixtures are resolvable into two components. One of these components is fairly narrow, highly cooperative, and exhibits properties which are similar to but not identical with those of the pure lipid. In addition, the fractional contribution of this component to the total enthalpy change, the peak transition temperature, and cooperativity decrease with an increase in peptide concentration, more or less independently of acyl chain length. The other component is very broad and predominates in the high range of peptide concentration. These two components have been assigned to the chain-melting phase transitions of populations of bulk lipid and peptide-associated lipid, respectively. Moreover, when the mean hydrophobic thickness of the PC bilayer is less than the peptide hydrophobic length, the peptide-associated lipid melts at higher temperatures than does the bulk lipid and vice versa. In addition, the chain-melting enthalpy of the broad endotherm does not decrease to zero even at high peptide concentrations, suggesting that this peptide reduces but do not abolish the cooperative gel/liquid-crystalline phase transition of the lipids with which it is in contact. Our DSC results indicate that the width of the phase transition observed at high peptide concentration is inversely but discontinuously related to hydrocarbon chain length and that gel phase immiscibility occurs when the hydrophobic thickness of the bilayer greatly exceeds the hydrophobic length of the peptide. The FTIR spectroscopic data indicate that the peptide forms a very stable α-helix under all of our experimental conditions but that small distortions of its α-helical conformation are induced in response to any mismatch between peptide hydrophobic length and bilayer hydrophobic thickness. These results also indicate that the peptide alters the conformational disposition of the acyl chains in contact with it and that the resultant conformational changes in the lipid hydrocarbon chains tend to minimize the extent of mismatch of peptide hydrophobic length and bilayer hydrophobic thickness.

The interactions between integral membrane proteins and the lipids of biological membranes are of fundamental importance to membrane biology, and this fact continues to provide the impetus for many investigations of lipid–protein interactions in both model and biological membranes. Although there have been fairly thorough characterizations of the relationship between membrane lipid structure and physical properties and the function of several membrane proteins [see Watts and De Pont (1985, 1986) and references cited therein], our understanding of the physicochemical principles underlying this relationship remains rather rudimentary. In part this is because most of the studies attempted so far has been done with natural proteins, many of which are very large, multisubunit aggregates of unknown three-dimensional structure which interact with even single membrane lipids in complex, multifaceted ways [for examples, see McElhaney (1986) and George et al. (1989, 1990)]. To circumvent these problems, a few workers have synthesized model peptides which are designed to interact specifically with either the polar, the interfacial, or the hydrophobic domains of the lipid bilayer and have begun to study the interaction between membrane lipids and these peptides using a variety of physical techniques [for examples, see Jacobs and White (1986, 1987) and Mclean et al. (1991)]. This approach has been successfully used by Davis, Bloom, and co-workers in their studies of a class of synthetic hydrophobic peptides, Lys_2-Gly-Leu_n-Lys_2-Ala-amide[1] (Davis et al., 1983; Huschilt, et al., 1985, 1989; Morrow et al., 1985; Pauls et al., 1985; Roux et al., 1989). These peptides each contain a long sequence (16–24 residues) of hydrophobic leucine residues capped at both the N- and the C-termini with two polar lysine residues and were designed to spontaneously form hydrophobic α-helices and to partition into the hydrophobic domain of a lipid bilayer with their

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[1] Abbreviations: P_n, Lys_2-Gly-Leu_n-Lys_2-Ala-Amide (*n* = number of leucine residues); PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine, DSPC, distearoylphosphatidylcholine; DSC, differential scanning calorimetry; DTA, differential thermal analysis; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; R_p, peptide/lipid molar ratio; T_m, gel/liquid-crystalline phase transition temperature; d_m, mean hydrophobic thickness of lipid bilayer; TFA, trifluoroacetate; C=O, carbonyl; $\Delta T_{1/2}$, transition width measured at peak half-height; ΔH, transition enthalpy.

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charged lysine terminal residues located at the polar headgroup/interfacial regions. To date, techniques such as circular dichroism (Davis et al., 1983), X-ray diffraction (Huschilt et al., 1989), and DSC and ^{2}H NMR spectroscopy (Roux et al., 1989; Huschilt et al., 1985; Davis et al., 1983) have been used to study the interaction of these peptides with hydrated DPPC bilayers, and such studies have shown that these peptides do form stable α-helices which insert into the lipid bilayer and which orient perpendicular to the bilayer surface. These studies have also shown that the incorporation of these peptides into DPPC bilayers results in a progressive decrease in the ΔH and cooperativity of the gel/liquid-crystalline phase transition of this phospholipid but changes its T_m only slightly (Morrow et al., 1985). Thus these peptides appear to interact primarily with the hydrocarbon chains of DPPC molecules and can be considered typical class III (i.e., integral, transmembrane) peptides or proteins, as first defined by Papahadjoupoulos et al. (1975) and later refined by McElhaney (1986).

In their studies of the interactions of these peptides with DPPC bilayers, Huschilt et al. (1985) also investigated the nature of the lipid–peptide interactions which occur when there is a mismatch between the hydrophobic thickness of the lipid bilayer and the hydrophobic lengths of the α-helices of the peptide. Their approach involved a ^{2}H NMR spectroscopic study of acyl chain dynamics in DPPC/peptide complexes formed by the incorporation of peptides of different hydrophobic lengths, and from their results there did not appear to be any major differences between the interaction of DPPC with either the P_{24} or P_{16} peptide. However, ^{2}H NMR spectroscopy is probably not sufficiently sensitive for such studies, especially under conditions where relatively low levels of the peptide are present. Here, we present the results of an alternative approach in which the thermotropic phase behavior of hydrated lipid/peptide complexes formed with the peptide P_{24} and *n*-saturated diacyl-PCs of various chain lengths (13–21 carbon atoms) are studied by high-sensitivity DSC and FTIR spectroscopy. The advantages of this approach are that these lipids incrementally cover a wide range of bilayer thicknesses ranging from somewhat shorter to much longer than the hydrophobic length of the peptide and that bilayer thickness can also be changed by inducing a gel to liquid-crystalline phase transition in the lipid phase. In addition, unlike the previous study, the combination of physical techniques used enables us to study the effects of variations of the lipid/peptide ratio, to examine the system when the lipids are in either the gel or the liquid-crystalline state, and to monitor any peptide conformational changes which may occur as a result of changes in bilayer thickness or bilayer phase state. We expect that studies such as these will provide some of the basic thermodynamic data necessary for the development, testing, and refinement of the theoretical models of lipid–protein interactions in general, and of the effects of the mismatching of the bilayer hydrophobic thickness and the hydrophobic length of the protein or peptide in particular [for examples, see Owicki and McConnell (1979), Mouritsen and Bloom (1984), Riegler and Möhwald (1986), Peschke et al. (1987), and Sperotto and Mouritsen (1988, 1991)].

MATERIALS AND METHODS

The *n*-saturated diacyl-PCs were from a stock of highly purified materials that were synthesized in this laboratory [see Lewis et al. (1987)]. The hydrophobic peptide P_{24} was synthesized using solid-phase methods and purified by high-performance liquid chromatography as described Davis et al. (1983). The pure sample was then twice lyophylized from 10 mM hydrochloric acid to remove the TFA counterions to which the peptide was bound at the end of the synthesis. This step was essential in the case of the FTIR spectroscopic samples because the strong C=O stretching band of the TFA group ($\cong$1670 m^{-1}) overlaps with the both ester C=O stretching band of the lipid and the amide I band of the peptide. We found that the removal of the TFA counterions was not necessary in the case of the DSC samples because the thermotropic properties of the peptide/PC mixtures were insensitive to its presence.

The PC/peptide vesicle suspensions used for DSC were prepared as follows. The lipid and the peptide were codissolved in methanol in a clean glass test tube to obtain the required peptide/lipid ratio, and the solvent was evaporated with a stream of nitrogen. After removal of the last traces of the organic solvent in vacuo overnight, the mixture was hydrated by vigorous vortexing with water at temperatures well above the T_m of the respective lipid to obtain a lipid concentration of 1–2 mg cm^{-3}. The DSC thermograms were recorded with a computer-controlled Microcal-MC2 high-sensitivity differential scanning calorimeter operating at heating scan rates between 11 and 30 °C h^{-1}. The data acquired were analyzed with DA2 software (Microcal Inc) and other computer programs available in the laboratory. In cases where the observed DSC thermograms were the summation of overlapping peaks, curve-fitting methods were used to obtain estimates of the transition temperatures and enthalpies of the component peaks. The procedure used for the deconvolution of the DSC thermograms was based on the assumption that the observed thermogram can be described in terms of a linear combination of multiple independent transitions, each of which approximate a two-state transition. The lipid content of the samples used for DSC was quantified by the gas chromatographic method routinely used in this laboratory (Lewis & McElhaney, 1985).

Lipid/peptide samples for FTIR spectroscopy were prepared either freshly or from the samples used for DSC experiments. The first involved of the preparation of a dry lipid/peptide sample as described above for the DSC sample except that deuterated methanol (CH_3OD) was used as the solvent. Alternatively, the sample used for DSC experiments was lyophilized and twice dissolved in deuterated methanol before being dried in vacuo overnight. The dry sample (containing 3–4 mg of lipid) was then hydrated with 50 μL of D_2O at temperatures well above the T_m of the respective lipid and then squeezed between the BaF_2 windows of a heatable liquid cell to form a 25-μm film and mounted in a cell holder attached to a computer-controlled circulating water bath that was used to regulate the temperature. Infrared spectra were recorded with a Digilab FTS-40 Fourier transform infrared spectrometer using the standard methodology for these types of samples (Mantsch et al., 1985). The data acquired were processed using DDS software (Digilab, Inc.) and other computer programs developed by the National Research Council of Canada. In cases where the spectra obtained consisted of broad overlapping bands, data processing usually involved the use of Fourier deconvolution to obtain fairly accurate estimates of the frequencies of the component bands, followed by curve-fitting procedures to obtain estimates of bandwidth and intensity. Typically band narrowing factors of 1.8–2.0 were used during deconvolution. Under our conditions, band narrowing factors of 2.5 could be used without introducing significant distortions to the spectra.

RESULTS

In considering the DSC and FTIR spectroscopic data presented below, it is important to compare the hydrophobic

Table I: Hydrophobic Thicknesses of the Bilayers Formed by Various Phosphatidylcholines

PC	hydrophobic thickness (Å)[a]		
	gel phase	liquid-crystalline phase	mean[b]
13:0	31.5	21.0	26.3
14:0	34.2	22.8	28.5
15:0	36.8	24.5	30.7
16:0	39.4	26.3	32.9
18:0	44.7	29.8	37.3
21.0	52.5	35.0	43.8

[a] Hydrophobic thicknesses were calculated using the equations provided in Sperotto and Mouritsen (1988). [b] The mean of the hydrophobic thicknesses of the gel and liquid-crystalline phases.

length of the P_{24} peptide with the hydrophobic thicknesses of the various PC bilayers used in this study. On the basis of measurements of a molecular model of the P_{24} peptide and the assumption that its entire polyleucine sequence adopts an "ideal" α-helical conformation, we estimate that the mean hydrophobic length[2] of P_{24} should be some 31–32 Å. Given this value, and the hydrophobic thicknesses expected of the various PC bilayers used in this study (see Table I), the following points should be noted. First, in the gel phase, matching of peptide hydrophobic length and lipid hydrophobic thickness should only occur with 13:0 PC (the shortest chain lipid used in this study). Thus, with all of the other lipids used, there should be a progressively greater mismatch between peptide hydrophobic length and bilayer gel phase hydrophobic thickness with increases in acyl chain length. Second, with the exception of 21:0 PC, the hydrophobic thickness of each of the lipid bilayers used in this study is shorter than the hydrophobic length of P_{24} when the lipids are in the liquid-crystalline phase. Third, the expected mean hydrophobic thicknesses of 15:0 PC is very close to the calculated hydrophobic length of P_{24}. Thus a mismatch between peptide hydrophobic length and bilayer mean hydrophobic thickness should occur with all of the other lipids used. Finally, 21:0 PC is the only lipid sample studied for which bilayer hydrophobic thickness exceeds peptide hydrophobic length in both the gel and liquid-crystalline phases. The above observations are important and in part define the perspective from which most of the data presented below will be interpreted.

Differential Scanning Calorimetry

Thermotropic Phase Behavior of the Pure Phosphatidylcholines. As illustrated in Figure 1, in the absence of peptide all of the PCs utilized in this study exhibit on heating a lower temperature, lower enthalpy, less cooperative pretransition and a higher temperature, higher enthalpy, and more cooperative main transition. The pretransition is known to arise from a conversion of the lamellar gel ($L_{\beta'}$) phase to the rippled gel ($P_{\beta'}$) phase and the main phase transition from a conversion of the $P_{\beta'}$ phase to the lamellar liquid-crystalline (L_α) phase. Both the pretransition and main phase transition increase in temperature with increases in the length of the hydrocarbon chains. However, the pretransition exhibits a steeper dependence on fatty acyl group length so that the temperature interval between these two transitions decreases as the hydrocarbon chain length increases. Thus for the 21:0 PC, the pretransition and main phase transition overlap. The 13:0 PC (and 12:0 PC) are unique in this series of PCs in exhibiting a high-temperature shoulder on the main phase transition endotherm. Although the physical basis of this behavior is not fully understood, both thermal events are known to involve chain melting (Morrow & Davis, 1987; Lewis and McElhaney, unpublished observations) and will thus be considered together in the following analysis. For a more thorough discussion of the thermotropic phase behavior of PCs containing linear saturated fatty acyl chains, the reader is referred to Lewis et al. (1987) and references cited therein.

The Effect of Peptide Incorporation on the Pretransition. The 13:0 PC exhibits its pretransition near −1 °C, which is below the temperature range accessible to our high-sensitivity DSC instrument, while the pretransition of the 21:0 PC overlaps with the main phase transition. Therefore, the effects of P_{24} incorporation on the pretransition of these two PCs could not be investigated. However, the incorporation of P_{24} does alter the pretransition of the PCs of intermediate hydrocarbon chain lengths but in somewhat different ways. The pretransition of 14:0 PC is very sensitive to the incorporation of peptide and has already become undetectable at the lowest R_p investigated (0.017). The pretransition of 15:0 PC is less sensitive to peptide incorporation and persists to R_p values of at least 0.033, while for 16:0 PC and 18:0 PC the pretransitions are less sensitive still and can be detected at R_p values of 0.067. However, the incorporation of increasing quantities of peptide tend to progressively reduce the pretransition temperature, enthalpy, and cooperativity in all of these longer-chain PCs. These results indicate that this peptide must be incorporated into at least one of the lamellar gel phases formed by these phospholipids and that the effect of P_{24} on the gel-state thermotropic phase behavior is greatest for PCs in which the hydrophobic thickness of the gel-state bilayer approaches the hydrophobic length of the peptide.

The Effect of Peptide Incorporation on the Main Transition. For all of the PCs studied, the incorporation of increasing amounts of P_{24} initially produces a two-component DSC endotherm (or, in the case of the 13:0 PC, a three-component endotherm) as well as progressively decreasing the enthalpy and reducing the cooperativity of the overall gel to liquid-crystalline phase transition of the phospholipid. At intermediate peptide levels, the DSC thermograms can be well fit by two components, which are clearly resolved by the curve-fitting procedures described under Materials and Methods (see Figure 2). The relative contribution of the sharper component, which possesses phase transition thermodynamic parameters similar to those of the pure phospholipid, decreases as the proportion of peptide increases, and this component disappears entirely when the R_p value approaches 0.1 (see Figures 1 and 4). In contrast, the relative contribution of the broader component to the total endotherm increases as the peptide concentration increases, and it is the only component which persists at the highest levels of peptide incorporation. From these observations, it is clear that the behavior of the lipid peptide mixtures does not approximate that of an ideal "two-dimensional solution" even at low R_p. However, at low to moderate R_p the behavior of the system seems closer to that expected of a macroscopic mixture of peptide-rich and peptide-poor domains. We thus assign the sharp component of the DSC endotherm to the melting of the peptide-poor or "bulk" PC population (i.e., to those PC molecules not directly interacting with the incorporation peptide molecules) and the broad component to the peptide-rich or "boundary" PC population (i.e., to those PC molecules which do interact directly with the incorporated protein). We stress here that the term "boundary" lipid is used empirically as a convenient means of describing the population of lipid molecules whose phase behavior is directly perturbed by interaction with the peptide and is not meant to imply the formation of a "lipid/

[2] Note that this is different from "end-to-end" distance of this α-helical peptide [35–36 Å; see Davis et al. (1983)].

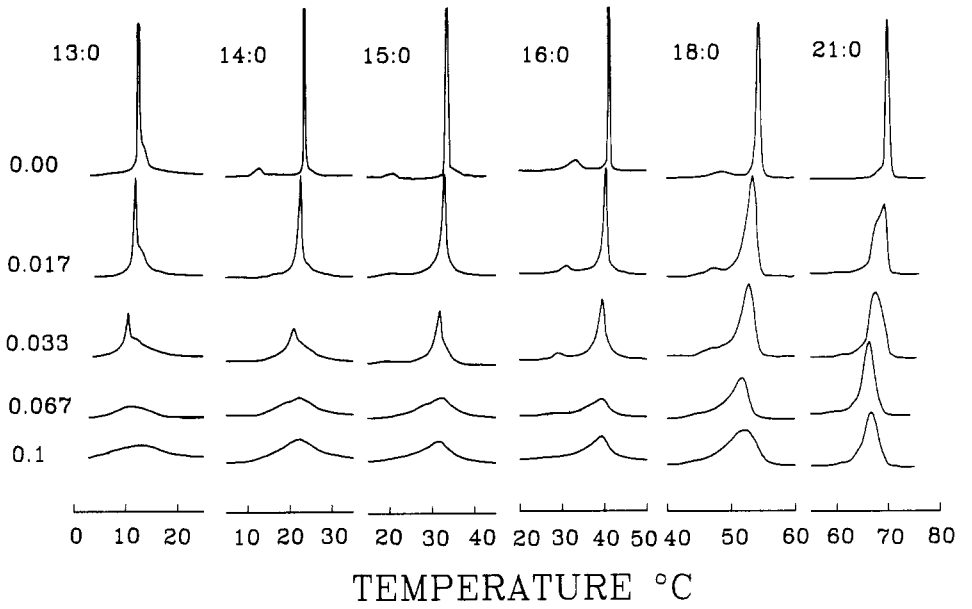

FIGURE 1: Effect of P_{24} on the DSC heating thermograms of a series of *n*-saturated diacyl-PCs. Thermograms are shown as a function of the acyl chain length (*N*:0) of the lipids, and the approximate P_{24}/lipid ratios are indicated in the column of numbers printed on the left side of the figure.

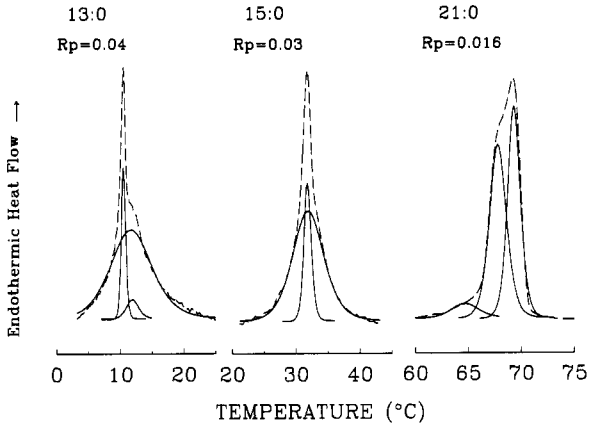

FIGURE 2: Illustration of the curve-fitting procedure used to resolve the components of the DSC heating thermograms exhibited by mixtures of P_{24} with PC bilayers. The examples shown are P_{24}/13:0, R_p = 0.04 (left panel); P_{24}/15:0, R_p = 0.03 (middle panel); P_{24}/21:0, R_p = 0.016 (right panel).

peptide complex" per se. As mentioned above, the thermodynamic parameters of the bulk lipid gel to liquid-crystalline phase transition are similar to but not identical with those of the corresponding pure lipid. In particular, the phase transition temperature is slightly but progressively lowered by the incorporation of increasing quantities of peptide (see Figure 3), and the cooperativity of the chain-melting phase transition is reduced modestly (see Figure 1). These effects are observed with all the PCs utilized in this study, and their magnitudes do not depend on the chain length of these phospholipids. We attribute this small reduction in phase transition temperature and cooperativity in the bulk lipid population to the indirect effects of the presence of the peptide in the lipid phase, these peptide molecules essentially acting as impurities in the PC gel-phase bilayer [see Jain (1988) for a further discussion of this phenomena].

In contrast to the comparable effects of peptide incorporation on the thermotropic phase transition of the bulk domains, the effect of the incorporation of peptide on the boundary lipids depends on the hydrocarbon chain length of the host PC bilayer. Thus the phase transition temperature of the broad component of the DSC endotherms is shifted to higher temperatures relative to the bulk lipid phase transition with the shorter chain PCs but to lower temperatures in the case of the longer chain PCs (see Figure 3). In fact, as shown in Figure 5, the magnitude and direction of this relative shift in the phase transition temperature is clearly if nonlinearly related to the difference between the mean hydrophobic thickness of the PC bilayer and the hydrophobic length of the incorporated peptide. Only in the case of 15:0 PC, where these hydrophobic lengths nearly match, is there no relative shift in the bulk and boundary lipid phase transition temperatures. Very similar results have been previously been reported by Riegler and Mohwald (1986) when bacterial photosynthetic reaction center proteins were reconstituted into PCs of various chain lengths.

The overall lipid phase transition enthalpy for all the PCs examined decreases as a linear function of increasing peptide incorporation, as shown in Figure 4. However, the enthalpy contribution of the bulk lipid component initially decreases and that of the boundary lipid component initially increases, both in a clearly nonlinear manner. The enthalpy of the bulk lipid component eventually decreases to zero as R_p values near 0.07 while the enthalpy of the boundary lipid component reaches a maximum at a similar or slightly lower R_p value and then decreases. However, an appreciable enthalpy due to the boundary lipid population remains even at relatively high R_p values where the bulk lipid phase transition has completely disappeared. At R_p values of 0.10 and above, the phase transition enthalpy of all the PCs studied has been reduced by approximately 2–3 kcal/mol. Expressed on the basis of a percentage reduction in enthalpy as compared to the corresponding pure lipid, the relative decrease in enthalpy ranges from a high of about 60% for the 13:0 PC to a low of about 25% for the 18:0 and 21:0 PCs.

The effect of peptide incorporation of the cooperativity of the lipid phase transition is also affected by the hydrocarbon chain length of the host PC bilayer (see Figure 3). Although the width of the boundary lipid phase transition increases in all cases with increasing peptide incorporation, this decrease in cooperativity is greatest for the shorter chain (13:0–15:0) PCs but becomes progressively less as the hydrocarbon chain length of the PC increases further. Thus if the mean hydrophobic thickness of the PC bilayer is less than or equal to that of the hydrophobic length of the peptide, the

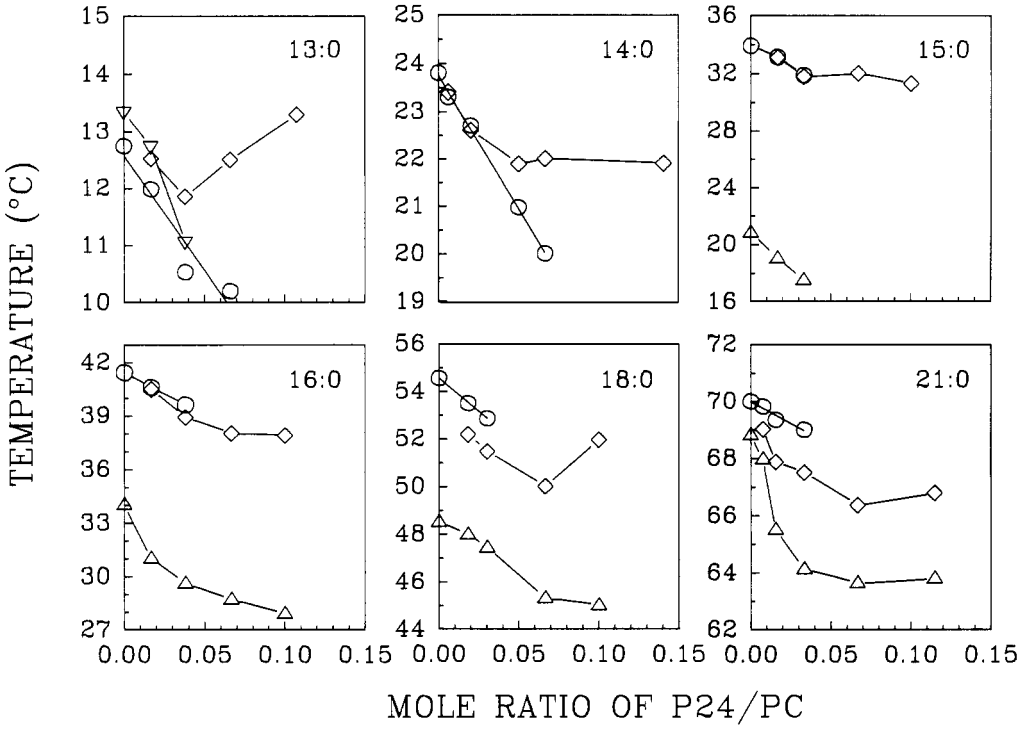

FIGURE 3: Effect of P_{24} on the peak transition temperature of the components of the DSC thermograms exhibited by mixtures of P_{24} and the *n*-saturated diacyl-PCs. (○) Bulk lipids. (◇) Peptide-associated lipids. (△) Pretransitions. (▽) Unassigned broad peak of 13:0 PC.

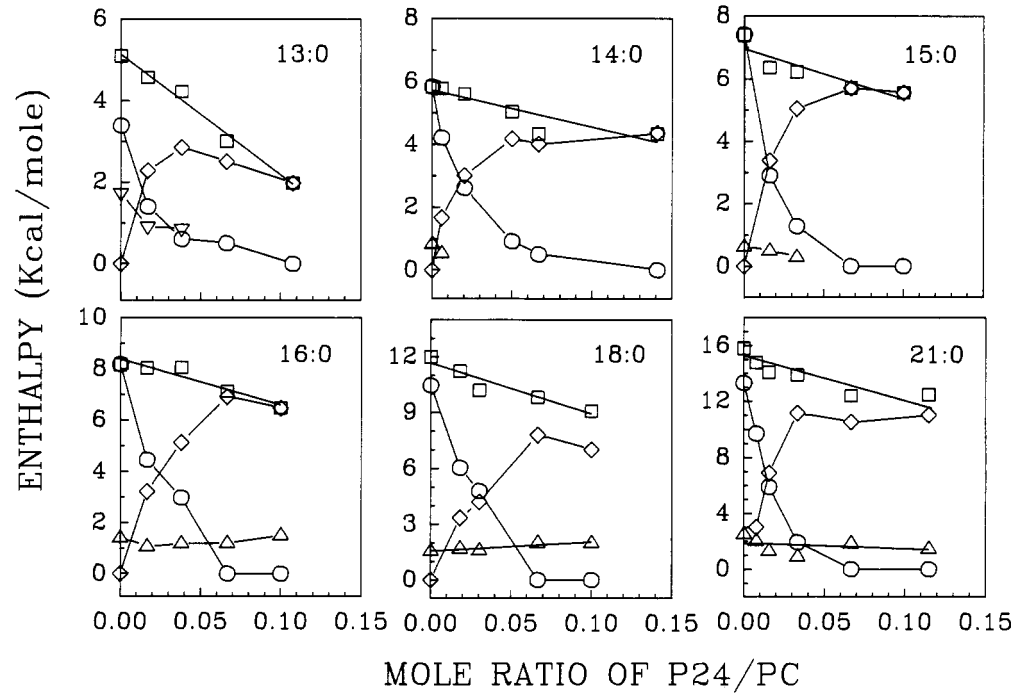

FIGURE 4: Effect of P_{24} on the transition enthalpies of the components of the DSC thermograms exhibited by mixtures of P_{24} and the *n*-saturated diacyl-PCs. (□) Total enthalpy. (○) Bulk lipids. (◇) Peptide-associated lipids. (△) Pretransitions. (▽) Unassigned broad peak of 13:0 PC.

temperature range over which hydrocarbon chain melting occurs is increased. The possible molecular basis for this effect will be discussed later.

The mixtures of P_{24} and 21:0 PC differ fundamentally from those of the other peptide/PC systems as regards the stability of the complexes formed, particularly those containing high mole fractions of the peptide. Unlike the other peptide/lipid mixtures, the shape of the DSC heating themograms exhibited by the P_{24}/21:0 PC mixtures changes upon repeated scanning through the temperature range of their chain-melting transitions. We found that repeated scanning of the sample results in the progressive growth of a high-temperature shoulder in the DSC thermogram and a slight shift of the major peak to lower temperatures. As illustrated in Figure 6, this effect seems to be primarily a function of the length of time that the sample is incubated at low temperatures, since the contribution of the high-temperature shoulder to the overall ΔH clearly increases with incubation time at room temperature. Interestingly, the growth of this high-temperature shoulder occurs in the temperature range in which the so-called bulk lipid melts, but these changes in peak distribution do not result in any significant change in the total ΔH measured. This result suggests that the gel phases of the multilamellar vesicles initially formed by the dispersal of these particular peptide/

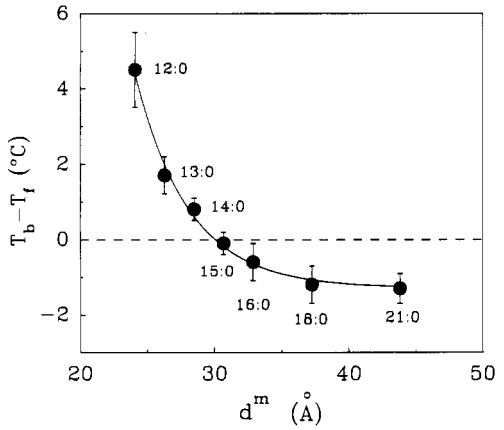

FIGURE 5: Plot of the difference of the transition temperatures of the peptide associated (T_b) and bulk (T_f) lipids versus the mean hydrophobic thickness (d^m) of the lipid bilayer. With the exception of the data point for 12:0 PC, the data were obtained from the DSC thermograms of mixtures of P_{24} (R_p = 0.033) with the *n*-saturated diacyl-PCs studied. The data point for 12:0 PC was estimated from our FTIR spectroscopic studies of comparable mixtures of DLPC and P_{24}.

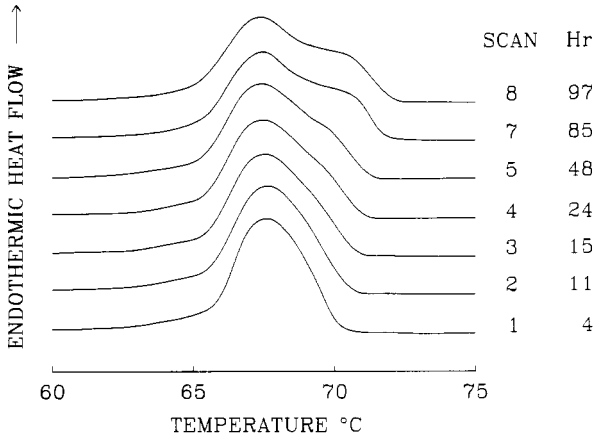

FIGURE 6: DSC thermograms of P_{24}/21:0 (R_p = 0.033) recorded in eight consecutive scans over a period of 4 days. The sample was stored at room temperature in between scans.

lipid mixtures are not stable and that with time there is phase separation to produce relatively peptide-rich and peptide-poor domains. This observation is particularly interesting, especially when one considers that the hydrophobic thickness of 21:0 PC bilayers greatly exceeds the hydrophobic length of P_{24} in both the gel and liquid-crystalline states. The suggestion that under such conditions there could be phase separation of the peptide from the lipid has been inferred from previous studies of bacteriorhodopsin/PC (Lewis & Engelman, 1983) and of bacterial photosynthetic reaction center protein/PC (Riegler & Mohwald, 1986) systems.

Fourier Transform Infrared Spectroscopy

In these studies, infrared spectra of mixtures of the peptide with short-chain (13:0 PC), intermediate-chain (16:0 PC), and long-chain (21:0 PC) lipids were recorded as a function of temperature and as a function of the mole fraction of the peptide. The use of FTIR spectroscopy permits a noninvasive monitoring of both the structural organization of the lipid bilayer and the conformation of the peptide. Thus the gel/liquid-crystalline phase transitions of the lipid bilayer can be conveniently monitored by changes in the frequency of CH_2 symmetric stretching band near 2850 cm^{-1}, changes in solid-state hydrocarbon chain packing by changes in the CH_2 scissoring band near 1468 cm^{-1}, changes in the hydration and/or polarity of the polar/apolar interfacial region of the lipid

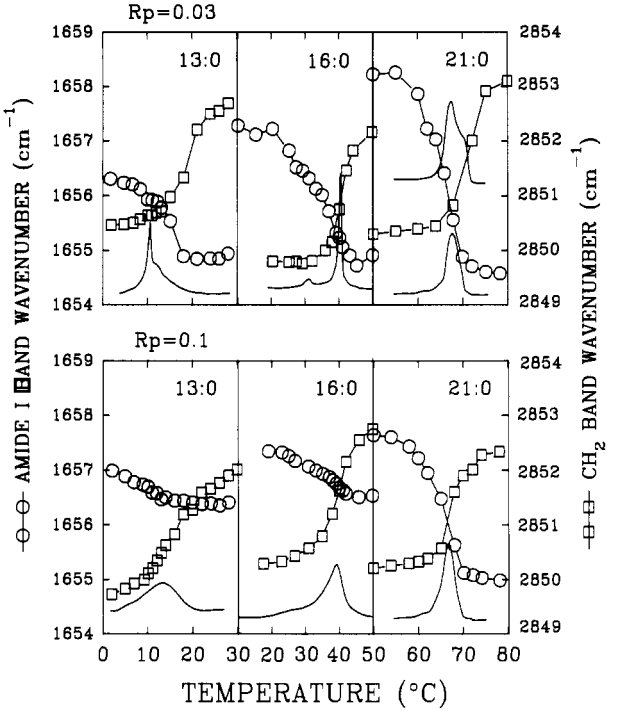

FIGURE 7: Combined plots of CH_2 symmetric stretch (□), peptide amide I band (○), and calorimetric thermograms as a function of temperature for systems of P_{24}/13:0, 16:0, and 21:0. The peptide/lipid molar ratios are 0.03 (top panel) and 0.1 (bottom panel).

bilayer by changes in the contours of the ester carbonyl stretching bands nears 1735 cm^{-1}, and changes in peptide structure can be monitored by changes in the conformationally sensitive amide I band near 1650 cm^{-1} [see Mendelsohn and Mantsch (1986) and Mantsch and McElhaney (1991)]. We find that the incorporation of the peptide into the lipid bilayer does not result in discernible changes in the hydration or the polarity of the polar/apolar interfacial regions of the lipid bilayer but does severely inhibit the formation of lipid subgel phases, with the result that, at low temperature, the gel-phase frequencies of the CH_2 scissoring band near 1468 cm^{-1} are always typical of rotationally disordered hydrocarbon chains. Thus the only spectroscopic parameters we examined in detail were the amide I band of the peptide and the CH_2 symmetric stretching bands of the phospholipid hydrocarbon chains.

Illustrated in Figure 7 are the frequency changes exhibited by the lipid CH_2 symmetric stretching and the peptide amide I bands coincident with the thermotropic phase transitions of peptide-rich and peptide-poor mixtures of P_{24} with the three PCs studied. With all of the samples, it is clear that the heating endothermic transitions reported by DSC are accompanied by an increase in the frequency of the CH_2 symmetric stretching band near 2850 cm^{-1}, indicating that melting of the lipid hydrocarbon chains is an integral part of the process(es) under observation. In addition, all of the peptide/lipid mixtures prepared exhibit a relatively sharp amide I vibrational band near 1655 cm^{-1}, which is consistent with a predominantly α-helical conformation for the peptide [see Zhang et al. (1992) and references cited therein.] Figure 7 also shows that a small decrease in the frequency of the amide I band consistently occurs at the chain-melting transitions of all of the lipid/peptide mixtures studied. This frequency change is both reproducible and reversible, suggesting that the conformation of the peptide is slightly altered at the gel/liquid-crystalline phase transition of the lipid bilayer. However, as was observed in our FTIR spectroscopic studies (Zhang et al., 1992), the frequency of the amide I band is not dramatically affected by changes in the absolute temperature,

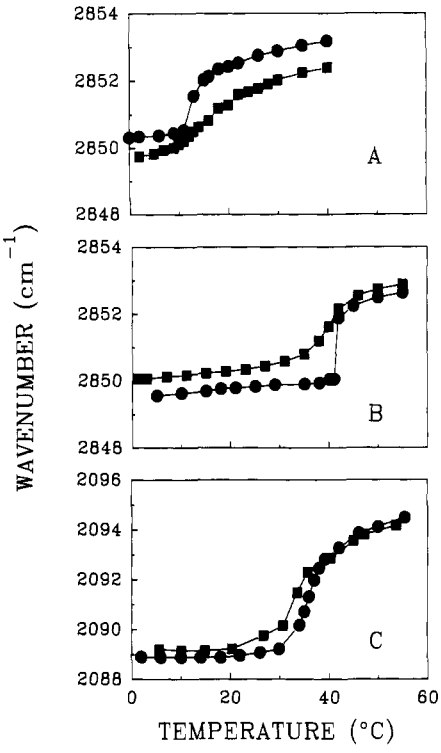

FIGURE 8: Effect of P_{24} on the temperature dependence of the frequency of (A) the symmetric CH_2 stretching band of 13:0 PC, (B) the symmetric CH_2 stretching band of 16:0 PC, and (C) the symmetric CD_2 stretching band of chain perdeuterated 16:0 PC. (●) Pure lipid; (■) P_{24}/lipid mixtures (R_p = 0.01).

changes in the lipid phase state, changes in the bilayer thickness, or changes in the peptide concentration. Thus, as expected, the peptide retains a predominantly α-helical conformation irrespective of changes in the above parameters. Therefore, the conformational changes which occur as a result of changes in any of those parameters probably represent minor distortions of the peptide α-helix (Chirgadze et al., 1976). Interestingly, such structural/conformational changes always seem to occur whenever there is a decrease in the thickness of the lipid bilayer, however caused. Thus, small decreases in the amide I frequency are observed when the lipid chains melt and when the peptide is intercalated into thinner gel-phase lipid bilayers (see Figure 7).

The effect of peptide concentration on the properties of the chain-melting transition of the lipids was also monitored by a comparison of the changes in the frequency of the symmetric CH_2 stretching bands of the pure lipids with those of the lipid/peptide mixtures containing relatively high concentrations of peptide (see Figure 8). For this particular purpose, the data from mixtures containing "nonsaturating" peptide concentrations were not useable since we could not accurately and/or reliably separate the CH_2 stretching band components of the bulk and the peptide-rich lipid domains. In addition, the phase separation which occurs with the long-chain lipids results in complex and irreproducible spectroscopic data which are very difficult to interpret, and as a result only data obtained with 13:0 and 16:0 PC were examined. Figure 8 shows that the pure lipid samples exhibit a relatively sharp increase in frequency ($\cong$2–2.5 cm^{-1}) which is typical of the chain-melting transitions of these types of phospholipids [see Mendelsohn and Mantsch (1986) and Mantsch and McElhaney (1991) and references cited therein]. With the short-chain lipid (i.e., 13:0 PC), the perturbing effect of the peptide is manifest by an increase in the width of the transition and by overall decreases in the frequency of the CH_2 symmetric stretching band in both the gel and the liquid-crystalline states (see Figure 8A). Moreover, at high peptide mole fractions the increase in frequency which occurs at the gel/liquid-crystalline transition is smaller than that which occurs in the pure lipid. Given that these parameters have been correlated with the extent of conformational disorder in hydrocarbon chains (Snyder, 1967; Maroncelli et al., 1982, 1985), one can conclude from these data that the presence of the peptide in the short-chain lipid bilayer results in an overall reduction of the conformational disorder in the hydrocarbon chains both above and below the phase transition temperature. In the case of 16:0 PC, however, we find that the incorporation of relatively large amounts of the peptide results in an overall elevation of the frequencies of the CH_2 symmetric stretching band in the gel and the liquid-crystalline states (see Figure 8B). Thus, with this medium-chain lipid, the data suggest that incorporation of the peptide into these bilayers results in an increase in the conformational disorder of the acyl chains in both the gel and liquid-crystalline states. The suggestion that further disordering of the liquid-crystalline state of this lipid may occur as a result of the incorporation of the peptide seems surprising, since the it is currently believed that the incorporation of such transmembrane moieties should cause a reduction in the conformational disorder of liquid-crystalline lipid bilayers [see Marsh (1985) and references cited therein]. To determine whether our observations are "real" or an artifact of the interference from the methylene groups present on the peptide, we examined the effect of the peptide on the CD_2 stretching bands of chain-perdeuterated DPPC (see Figure 8C). It is clear that with the exception of the effects directly attributable to the perdeuteration of the acyl chains (i.e., the lowering of the gel/liquid-crystalline phase transition temperature and the frequency of the methylene symmetric stretching bands), the data presented in Figure 8B,C are essentially similar. The CD_2 symmetric stretching band of pure chain-perdeuterated DPPC also shows a sharp increase in frequency at temperatures near 37 °C, and the effect of incorporating relatively large amounts of the peptide is generally similar to what occurs with the fully proteated sample of DPPC. As was the case with the fully proteated sample, the gel-phase frequency of the CD_2 stretching band is greater than that of the pure lipid, but in the liquid-crystalline phase the frequency of the CD_2 stretching band is not significantly changed by the incorporation of large amounts of the peptide. Thus, it would appear that, with DPPC bilayers, the incorporation of the peptide does cause an increase in the conformational disorder of the gel phase, but in the liquid crystalline phase the incorporation of the peptide does not result in any significant change in the conformational disposition of the fatty acyl chains. The latter conclusion, though similar to that derived from ^{2}H NMR studies of P_{24}/DPPC mixtures (Morrow et al., 1985), seems contrary to the expectations of previous studies of lipid–protein interactions [see Marsh (1985)], and the reasons why this may be the case will be explored further below.

DISCUSSION

A common feature of all of the calorimetric data presented here is that at low mole fractions of the peptide the thermotropic phase behavior of the various mixtures can be approximated by that expected of a macroscopic mixture of peptide-rich and peptide-poor domains. Thus, even at low R_p, the mixture does not behave as an "ideal two-dimensional solution", a conclusion which is similar to that reached in previous DSC and ^{2}H NMR studies of the interaction of this peptide with DPPC bilayers (Morrow et al., 1985). Our data also indicate that the bulk lipid component exhibits properties which are similar to but not identical with those of the pure lipid. This observation is generally compatible with the colligative effects

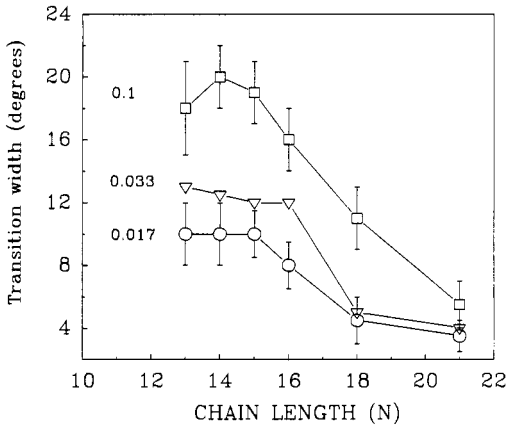

FIGURE 9: Effect of acyl chain length on the width of the chain-melting transition of the peptide-associated lipids. Data are presented for the peptide/lipid molar ratios indicated, and the transition width is determined by the difference between the initiation and completion temperatures of the fitted peaks.

which occur when "pure" lipids are contaminated by low levels of noninteracting lipophilic impurities [see Jain (1988) and references cited therein].

Although the P_{24} peptide was designed to meet the structural requirements for the class III type proteins (or peptides) defined by Papahadjoupolos et al. (1975), our results are at variance with the expected effects of such proteins on the thermotropic phase behavior of its host lipid bilayer. As outlined by those authors, class III integral, transmembrane proteins are expected to interact primarily with the hydrophobic core of lipid bilayers. Thus their effects on the T_m and $\Delta T_{1/2}$ of the lipid phase transition should be minimal, but there should be a progressive decrease in ΔH to zero because the lipid molecules which interact with the protein or peptide are not expected to participate in the cooperative chain-melting process. Our data indicate that with the incorporation of P_{24} into these PC bilayers there are in fact modest changes in T_m but considerable increases in $\Delta T_{1/2}$. Moreover, it is also evident that the P_{24} peptide does not prevent the lipids from participating in a gel/liquid-crystalline phase transition, since chain-melting phase transitions (albeit of reduced cooperativity and enthalpy) are calorimetrically detectable even at very high peptide mole fractions. We find that lipids in contact with the peptide participate in a chain-melting transition which is fairly broad and that the width of the transition increases with increases in R_p and tends to decrease with increases in acyl chain length. In addition, as shown in Figure 9, there is a sharp and discontinuous decrease in the width of this melting peak for mixtures of P_{24} with the two longer chain lipids studied. Currently, the physical basis of this particular observation is unclear, but we suspect that it may be related to an increased predisposition towards the kind of phase separation that occurs with P_{24}/21:0 PC mixtures. In previous DSC studies of the interaction of bacteriorhodopsin with DMPC bilayers, Heyn et al. (1981) suggested that the lipid molecules in contact with the protein are not prevented from participating in the chain-melting phase transition of the lipids but undergo a "partial" chain-melting transition of significantly lower cooperativity and enthalpy. Our results are compatible with such an interpretation. Thus, given the above and the fact that the calorimetric data upon which the classification scheme of Papahadjoupolos et al. (1975) was proposed predate the widespread availability of high-sensitivity differential scanning calorimeters, we suggest that the main reason for the difference between our data and that proposed for such class III type proteins is the result of the poor resolution of the instruments available prior ot the late 1970's. Indeed, with low-resolution instruments operating at moderate scan rates, the only major observable effect of incorporating low mole fractions of a peptide such as P_{24} would be a decrease in ΔH, since the broad melting transition of the peptide-rich domain would not be accurately resolved nor would the relatively small changes in T_m which we have detected (see Figures 1 and 3). Thus we propose that, with respect to their effects on the thermotropic phase behavior of their host lipid bilayers, class III proteins should be calorimetrically defined as those which cause modest changes in T_m, significant increases in $\Delta T_{1/2}$, and some reduction in ΔH. We also suggest that lipid molecules in contact with this class of protein can undergo some form of chain-melting transition at all protein concentrations at which a continuous lipid bilayer can be formed. Calorimetrically, this would be reflected by a reduced but nonvanishing ΔH at high protein concentrations.

The fact that the peptide-associated lipid population evidently contributes to the observed enthalpy change complicates the task of estimating the number of peptide molecules which are perturbed by the presence of the peptide. In many of the studies performed to date, estimates of the number of lipids associated with the peptide have been obtained by linear extrapolation a plot of the total ΔH versus mole fraction to zero enthalpy. However, such a treatment is based on the premise that the protein or peptide completely removes a given and constant number of lipids from the chain-melting phase transition, and from our data it is clear that such a premise is not applicable here. Nevertheless, a possible approach to the solution of this problem arises naturally from the observation that the ΔH of the bulk lipid population decreases with increasing R_p. Thus it may be possible to estimate the maximum number of lipids perturbed by the peptide by extrapolating the initial slope of a plot of bulk lipid enthalpy versus R_p to zero enthalpy. As shown in Figure 4, this plot is clearly curved, but the curvature decreases considerably as R_p approaches zero. However, for all of the lipids used in this study, a linear extrapolation of the initial slopes of their respective curves intercepts the x axis at mole ratios near 0.04. Therefore, from this value we can calculate that for each of the PCs we studied, the chain-melting transitions of some 25 lipid molecules are perturbed by the intercalation of the P_{24} peptide into lipid bilayer. Although this particular treatment of the data is compatible with the approximation of the system as a macroscopic mixture of peptide-rich and peptide-poor domains, we do caution, however, that the value we have calculated ($\simeq$25 lipid molecules) should be regarded as a crude estimate of the maximum number of lipid molecules which are perturbed (presumably by direct interaction with the peptide) at very dilute peptide concentrations. Nevertheless, this value is in reasonably good agreement with the results of Morrow and co-workers, who estimated that some 18 molecules of phospholipid would be required to form a single "solvation layer" around each molecule of P_{24} [see Morrow et al. (1985)].

This work is one of the few systematic physical characterizations of the effect of a mismatch between bilayer hydrophobic thickness and protein (or peptide) hydrophobic length on the nature of the lipid–protein interactions which occur. In the particular case of the hydrophobic peptide used here (P_{24}), our spectroscopic data show conclusively that major structural/conformational changes in the peptide do not occur in response to any such mismatch. Given this, one must conclude that it is primarily the membrane lipids which make the required "adjustments" in response to any mismatch between the hydrophobic lengths of the lipid bilayer and the peptide. This conclusion is similar to that arrived at in studies

of the interaction of photosynthetic reaction center proteins with PC bilayers (Riegler & Möhwald, 1986). With a peptide such as P_{24}, this conclusion is probably not surprising, since P_{24} adopts a very stable α-helical conformation which is little affected by both temperature and denaturing solvents (Davis et al., 1983; Zhang et al., 1992). Interestingly, however, our data suggest that a small distortion of the α-helical conformation of the peptide structure does occur at the gel/liquid-crystalline phase transition or possibly whenever there is a change in the bilayer thickness. Although it is clear that any change in the conformation of P_{24} is minor, the fact that some change does occur suggests that under appropriate conditions it may be possible to induce substantial changes in the conformation of hydrophobic peptides in response to changes in the physical state or in the thickness of the lipid bilayer. Hence, the fact that this peptide exhibits very little sensitivity to the changes in the physical state or thickness of the lipid bilayers studied may simply be a reflection of the extraordinary stability of its α-helical structure. Thus it would be interesting and informative to perform a similar set of studies with other hydrophobic peptides which adopt less stable α-helical conformations.

This study has also provided data which may be useful in the development of an understanding of the probable "adjustments" made by the lipid in response to any mismatch of peptide hydrophobic length and bilayer hydrophobic thickness. With the short-chain lipids, the peak transition temperature of the broad component is higher than that of the bulk lipid component whereas the opposite is the case with the long-chain lipids (see Figure 5). In principle, this may be the result of a net "ordering" (stretching) and "disordering" (shortening) of the membrane lipid hydrocarbon chains when the hydrophobic thickness of the bilayer is, respectively, thinner and thicker than the hydrophobic length of the peptide. Such an interpretation is compatible with theoretical models of lipid–protein interaction based on the elastic properties of the lipid bilayer [see Owicki and McConnell (1979), Mouritsen and Bloom (1984), Riegler and Möhwald (1986), and Sperotto and Mouritsen (1988, 1991) and references cited therein], though we find that the magnitude of the changes predicted by such models (particularly the changes in T_m) tend to be considerably greater than what is observed experimentally. Nevertheless, it is clear that our experimental observations are at least qualitatively compatible with the trends predicted by those models, since all conformational adjustments of the lipid hydrocarbon chains appear to be those which minimize the mismatch between peptide hydrophobic length and bilayer hydrophobic thicknesses. In fact, with the short chain lipids, our observation that the presence of the peptide results in a net lowering of the frequencies of the CH_2 symmetric stretching band is also consistent with that suggestion. However, with medium chain lipids such as DPPC, where mismatch between bilayer mean hydrophobic thickness and peptide hydrophobic length is small, our FTIR spectroscopic data suggest that the peptide nevertheless produces a net increase in acyl chain conformational disorder of the gel phase while not affecting the conformational disposition of the hydrocarbon chains in the liquid-crystalline phase. That the presence of the peptide results in a net "disordering" of the gel phase of these lipids is not unexpected and can be rationalized within the framework of the theoretical models referenced above. This is because an increase in the conformational disorder of the gel phase should enable a closer matching of hydrophobic thickness of the peptide and these lipid bilayers, aside from the general disordering of the gel phase expected when a lipophilic impurity (in this case the P_{24} peptide) is incorporated into the lipid bilayer. However, by the same reasoning, one would have also expected to observe a net decrease in the conformational disorder of the liquid-crystalline phase. Indeed, one would have expected that the presence of the relatively rigid P_{24} molecule next to any given fatty acyl chain would restrict its conformational freedom more than would another fatty acyl chain. One possible explanation for this seemingly anomalous observation arises naturally from considerations of the packing of the flexible hydrocarbon chains of the lipid molecules around the fairly rigid body of the peptide. From an examination of molecular models it is clear that if a lipid all-trans hydrocarbon chain were aligned next to the peptide, van der Waals contacts between the peptide and the methylene groups of the hydrocarbon chain will not be ideal and there will be significant amounts of "free volume" around the peptide. We think that this nonideality of the lipid/peptide hydrophobic packing contacts, the relative lack of flexibility of the peptide (a natural result of the extraordinary stability of its α-helical conformation), and the inherent flexibility of the fatty acyl chain together provide a powerful driving force favoring a general increase in the conformational disorder in the hydrocarbon chains around the peptide. However, in the particular case of the peptide mixtures with lipids such as DPPC, any tendency toward an increase in conformational disorder will have to be tempered, since an increase in the conformational disorder of the acyl chain will inevitably result in a decrease in bilayer thickness which will, in turn, increase the mismatch between peptide hydrophobic length and bilayer hydrophobic thickness. Thus, the extent and nature of the conformational disorder in the fatty acyl chains probably represent a compromise between a number of opposing tendencies, which in the particular case of the liquid-crystalline phase of mixtures of P_{24} with DPPC (and most probably DSPC as well) seems to result in acyl chain disorder which is comparable to that of the liquid-crystalline phase of the pure lipid.

The effect of the P_{24} on the thermotropic properties of the lipid pretransition seems directly related to the extent of the mismatch between peptide hydrophobic length and bilayer hydrophobic thickness in the gel phase. With the lipids used in this study, there is an increase in the mismatch between peptide hydrophobic length and bilayer gel-state hydrophobic thickness, and a concomitant decrease in the effect of the peptide on the lipid pretransition, as the length of the PC hydrocarbon chain increases. Thus the pretransition of 14:0 PC was abolished by the incorporation of peptide mole fractions near 0.02, whereas with DPPC and DSPC pretransitions were clearly resolvable at peptide mole fractions in excess of 0.06 (see Figure 1). These results suggest that any significant mismatch between peptide hydrophobic length and bilayer gel phase hydrophobic thickness markedly reduces the miscibility of the peptide (or possibly the "lipid/peptide complex") with the gel phase of the host lipid. This aspect of our data is qualitatively compatible with the general predictions of most of the theoretical models presented so far [see Owicki and McConnell (1979), Mouritsen and Bloom (1984), Peschke et al. (1987), Riegler and Möhwald (1986), and Sperotto and Mouritsen (1988, 1991) and references cited therein], though there appears to be some disagreement as regards the details. Thus, for example, whereas most models predict that a mismatch of peptide hydrophobic length and bilayer hydrophobic thickness should result in phase separation of the peptide from the lipids, phase separation was only observed in mixtures with the longest chain lipid studied (21:0 PC). However, with peptides such as P_{24}, their phase separation from the lipids will also result in the concentration of positively charged lysine groups at the surfaces of the peptide-rich domains.

Consequently, although a mismatch of peptide hydrophobic length and bilayer hydrophobic thickness should provide a strong driving force toward phase separation, charged-group repulsion between the terminal lysine residues of the peptide may effectively inhibit that process except in those peptide/lipid mixtures where the forces promoting phase separation are very strong (i.e., where there is a considerable mismatch of peptide hydrophobic length and bilayer hydrophobic thickness). Moreover, with the peptide/21:0 PC mixtures, we also find that there is a significant kinetic component to the processes which lead to the phase separation. Thus, one cannot even be certain whether any disagreement between our data and theoretical predictions is "real" or an artifact of considerably slower kinetics. The possibility that a considerable mismatch between protein (or peptide) hydrophobic length and bilayer hydrophobic thickness can be tolerated before phase separation actually occurs was noted in previous studies of bacteriorhodopsin reconstituted into PC bilayers (Lewis & Engleman, 1983).

Finally, it is clear that the behavior of bilayer lipid molecules is radically altered by their interaction with even simple model hydrophobic peptides such as P_{24} in ways which are not fully understood at present. Here, most of our data can be qualitatively accommodated within the framework of several theoretical models that have been proposed [see Owicki and McConnell (1979), Mouritsen and Blood (1984), Peschke et al. (1986), Riegler and Möhwald (1986), and Sperotto and Mouritsen (1988, 1991) and references cited therein], but we generally find that the magnitude of the effects we have observed tend to be a lot smaller than what is theoretically predicted. Thus, despite studies of "conceptually simple model systems" such as the one described here, and many studies of other more complex systems [for a review, see McElhaney (1986)], a comprehensive and satisfactory framework within which one can rationalize the interaction between membrane lipids and integral membrane proteins has yet to be formulated. In part, this is the result of the fact that the critical database of reasonably accurate calorimetric data (the basic seed data for the development of theoretical models) is still very small, because a lot of the data in the literature are of studies performed with low-sensitivity instruments with which an accurate characterization of the energetics of lipid–protein interactions is not feasible. Moreover, most of the available data (including that presented here) have been obtained from studies of proteins or peptides reconstituted with PC model bilayers, and there is no reason to suppose that the results would be the same if similar studies were performed with other lipids or mixtures of lipids. It thus seems unlikely that any satisfactory framework for understanding the basics of lipid–protein interactions will be devised until a larger database of accurate calorimetric and other measurements of mixtures of various model peptides with a wide range of different lipid mixtures has been accumulated. Clearly, additional work in which lipid hydrocarbon chain length and chain structure and lipid polar headgroup size and charge are systematically varied will be required for a complete understanding of the molecular basis for the interaction of peptides such as P_{24} with lipid bilayers. Such work is currently in progress in this laboratory.

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