# A Polyalanine-Based Peptide Cannot Form a Stable Transmembrane α-Helix in Fully Hydrated Phospholipid Bilayers†

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ABSTRACT: The conformation and amide proton exchangeability of the peptide acetyl-$K_2$-$A_{24}$-$K_2$-amide ($A_{24}$) and its interaction with phosphatidylcholine bilayers were examined by a variety of physical techniques. When dissolved in or cast from methanol as a dried film, $A_{24}$ is predominantly α-helical. In aqueous media, however, $A_{24}$ exists primarily as a mixture of helical (though not necessarily α-helical) and random coiled structures, both of which allow rapid H−D exchange of all amide protons. When incorporated into phospholipids in the absence of water, $A_{24}$ also exists primarily as a transmembrane α-helix. However, upon hydration of that system, rapid exchange of all amide protons also occurs along with a marked change in the amide I absorption band of the peptide. Also, when dispersed with phosphatidylcholine in aqueous media, the conformation and thermal stability of $A_{24}$ are not significantly altered by the presence of the phospholipid or by its gel/liquid-crystalline phase transition. Differential scanning calorimetric and electron spin resonance spectroscopic studies indicate that $A_{24}$ has relatively minor effects on the thermodynamic properties of the lipid hydrocarbon chain-melting phase transition, that it does not abolish the lipid pretransition, and that its presence has no significant effect on the orientational order or rates of motion of the phospholipid hydrocarbon chains. We therefore conclude that $A_{24}$ has sufficient α-helical propensity, but insufficient hydrophobicity, to maintain a stable transmembrane association with phospholipid bilayers in the presence of water. Instead, it exists primarily as a dynamic mixture of helices and other conformers and resides mostly in the aqueous phase where it interacts weakly with the bilayer surface or with the polar/apolar interfacial region of phosphatidylcholine bilayers. Thus, polyalanine-based peptides are not good models for the transmembrane α-helical segments of natural membrane proteins.

Lipid−protein interactions are of fundamental importance for both the structural integrity and the various functions of all biological membranes (see refs *1* and *2*). In particular, the chemical composition and physical properties of the host lipid bilayer can markedly influence the activity, thermal stability, and the location and disposition of a large number of integral membrane proteins in both model and biological membrane systems (see refs *1*−*5*). For these reasons many studies of the interactions of membrane proteins with their host lipid bilayers have been carried out, in both biological and reconstituted model membrane systems, employing a wide range of different physical techniques (see refs *6*−*10*). However, our understanding of the physical principles underlying lipid−protein interactions remains incomplete, and the molecular mechanisms whereby associated lipids actually alter the activity, and presumably also the structure and dynamics, of integral membrane proteins are largely unknown. One reason for this situation is the fact that most transmembrane proteins are relatively large, multidomain macromolecules of complex and often unknown three-dimensional structure and topology that can interact with lipid bilayers in complex, multifaceted ways (see refs *1*−*10*). To overcome this problem, a number of workers have designed and synthesized peptide models of specific regions of natural membrane proteins and have studied their interactions with model lipid membranes of defined composition (see refs *11* and *12*). Physical studies of such relatively tractable model membrane systems have already significantly advanced our understanding of the molecular basis of lipid−protein interactions.

The synthetic peptide acetyl-$K_2$-G-$L_{24}$-$K_2$-A-amide ($P_{24}$)[1] and its analogues have been successfully utilized as a model of the hydrophobic transmembrane α-helical segments of integral membrane proteins (see refs *12* and *13*). These peptides contain a long sequence of hydrophobic and strongly α-helical promoting leucine residues capped at both the N-

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and C-termini with two positively charged, relatively polar lysine residues. Moreover, the normally positively charged N-terminus and the negatively charged C-terminus have both been blocked in order to provide a symmetrical tetracationic peptide which will more faithfully mimic the transbilayer region of natural membrane proteins. The central polyleucine region of these peptides was designed to form a maximally stable, very hydrophobic α-helix which will partition strongly into the hydrophobic environment of the lipid bilayer core, while the dilysine caps were designed to anchor the ends of these peptides to the polar surface of the lipid bilayer and to inhibit the lateral aggregation of these peptides. In fact, CD (*13*) and FTIR (*14*−*16*) spectroscopic studies of $P_{24}$ have shown that it adopts a very stable α-helical conformation both in solution and in lipid bilayers, and X-ray diffraction (*17*), fluorescence quenching (*18*), and FTIR spectroscopic (*14*−*16*) studies have confirmed that $P_{24}$ and its analogues assume a transbilayer orientation with the N- and C-termini exposed to the aqueous environment and the hydrophobic polyleucine core embedded in the hydrocarbon core of the lipid bilayer when reconstituted with various PCs. DSC (*13*, *15*, *19*−*21*) and $^2$H NMR spectroscopy (*13*, *19*−*21*) studies have shown that $P_{24}$ and the closely related peptide $L_{24}$ (*22*) broaden the gel/liquid-crystalline phase transition and reduce its enthalpy. The phase transition temperature is shifted either upward or downward, depending on the degree of mismatch between the hydrophobic length of the peptide and the hydrophobic thickness of PC lipid bilayers (*15*), but this is not observed in PE bilayers, where $P_{24}$ substantially decreases the phase transition temperature in a hydrocarbon chain length-independent manner (*21*). Moreover, $^2$H NMR (*13*, *19*, *20*, *22*) and ESR (*23*) spectroscopic studies have shown that both $P_{24}$ and $L_{24}$ order the phospholipid hydrocarbon chains in liquid-crystalline PC bilayers. As well, $L_{24}$ and its analogues lower the lamellar/nonlamellar phase transition temperatures in PE bilayers in a manner not primarily dependent on hydrophobic mismatch (*24*). Moreover, small distortions of the α-helical conformation of $P_{24}$ are also observed in response to peptide−lipid hydrophobic mismatch (*15*). $^2$H NMR (*25*) and ESR (*23*) spectroscopic studies have shown that the rotational diffusion of $P_{24}$ about its long axis perpendicular to the membrane plane is rapid in the liquid-crystalline state of the bilayer and that the closely related peptide $L_{24}$ exists at least primarily as a monomer in liquid-crystalline POPC bilayers, even at relatively high peptide concentrations.

A related peptide, $(LA)_{12}$, in which the polyleucine core of $L_{24}$ is replaced by alternating leucine and alanine residues, has also been investigated to examine whether the replacement of one-half of the leucine residues by smaller and less hydrophobic alanine residues would influence the stability of the helical form of the peptide and if the rougher surface topology of $(LA)_{12}$ would alter its effects on lipid bilayers. The application of a variety of physical techniques has revealed that the behavior of $(LA)_{12}$ in solution and in lipid micelles or PC or PE bilayers is generally similar to that of $P_{24}$ (22, *26*−*28*). However, $(LA)_{12}$ perturbs the gel/liquid-crystalline phase transition of PC and PE bilayers to a greater extent than does $P_{24}$ at comparable concentrations, as inferred from the greater decrease of the temperature and enthalpy of the gel/liquid-crystalline phase transition, possibly due in part to its rougher surface topology. However, the influence of the hydrophobic mismatch between the peptide and the host PC bilayer on the shift in the phase transition temperature is less pronounced for $(LA)_{12}$ than for $L_{24}$, perhaps in part because of the greater conformational plasticity of $(LA)_{12}$ in response to alterations of the bilayer thickness (*27*, *28*). As well, $(LA)_{12}$ does not appear to order the hydrocarbon chains of saturated PC bilayers as much as does $L_{24}$, perhaps also due to its rougher surface topology (*22*).

We report here the results of an extension of our previous studies of peptide models of the α-helical transmembrane segments of membrane proteins to the peptide acetyl-$K_2$-$A_{24}$-$K_2$-amide ($A_{24}$). In these studies we wished to address two major questions: first, whether the polyalanine core of this peptide possesses a sufficiently strong α-helical propensity to form a stable transbilayer helix in the hydrophobic core of a phospholipid bilayer, and, second, whether the hydrophobicity of the polyalanine core of this peptide is sufficiently large to form a stable association with phospholipid bilayers in the presence of excess water. In brief, we find that $A_{24}$ probably does exist as a transmembrane α-helix when reconstituted with PCs in the absence of water. However, when water is added to the peptide/phospholipid complex, $A_{24}$ partitions strongly into the aqueous phase, where it exists primarily in a non-α-helical conformation which interacts only weakly with phospholipid bilayers. These results suggest that polyalanine-based peptides are poor models of the α-helical transmembrane segments of membrane proteins and may also be unsuitable for studies of the insertion of such peptides into preformed phospholipid bilayers.

## MATERIALS AND METHODS

The phospholipids used in this study were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and were used without further purification. Commercially supplied solvents of at least analytical grade quality were redistilled prior to use. The peptide $A_{24}$ was synthesized and purified as the TFA salt using previously published solid-phase synthesis and reversed-phase high-performance liquid chromatographic procedures (*26*). Circular dichroism measurements were performed on both aqueous and methanolic $A_{24}$ solutions using procedures and equipment similar to those described by Zhang et al. (*26*). Interpretation of the CD spectra in terms of secondary structure content was achieved by the methodology developed by Baldwin and co-workers (*29* and references cited therein). Electron spin resonance measurements were performed using materials, sample preparation methodologies, and data acquisition and analysis protocols similar to those described by Subczynski et al. (*23*, *30*).

Samples were prepared for DSC as follows. Lipids and peptides were codissolved in methanol to attain the desired lipid-to-peptide ratio, and the solvent was removed with a

[1] Abbreviations: $P_{24}$, acetyl-$K_2$-G-$L_{24}$-$K_2$-A-amide; $(LA)_{12}$, acetyl-$K_2$-$(LA)_{12}$-$K_2$-amide; $L_{24}$, acetyl-$K_2$-$L_{24}$-$K_2$-amide; $A_{24}$, acetyl-$K_2$-$A_{24}$-$K_2$-amide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPDPC, dipentadecanoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; TFA, trifluoroacetate; TFE, 2,2,2-trifluoroethanol; CD, circular dichroism spectroscopy; PBS, phosphate-buffered saline; DSC, differential scanning calorimetry; ESR, electron spin resonance spectroscopy; FTIR, Fourier transform infrared; ATR-FTIR, attenuated total reflection Fourier transform infrared; LMV, large multilamellar vesicle.

stream of nitrogen, leaving a thin film on the sides of a clean glass test tube. This film was subsequently dried in vacuo for several hours to ensure removal of the last traces of solvent. Samples containing ~1 mg of lipid were then hydrated by placing some wet cotton wool into the tube (without contacting the lipid/peptide film) and allowing the sample to absorb water from the water vapor-saturated air by warming the sample to temperatures slightly above the lipid gel/liquid-crystalline phase transition temperature. Subsequently, the cotton wool was removed, and the sample was dispersed in 2 mL of either water or phosphate-buffered saline (50 mM phosphate, 100 mM NaCl, pH 7.4) by vigorous vortexing at room temperature. DSC thermograms were recorded with a high sensitivity CSC Nano-DSC instrument (Calorimetry Sciences Corp., Provo, UT), operating at heating and cooling rates of 10 °C/h. The data were analyzed and plotted with the Origin software package (OriginLab Corp., Northampton, MA).

Transmission FTIR spectroscopic measurements were performed on dried films, on aqueous solutions of the peptide, and on aqueous dispersions of lipid/peptide mixtures. Peptide and lipid/peptide films were prepared by spreading methanolic solutions of the same on the face of the $CaF_2$ windows of the sample cell. The film was dried in vacuo for several hours prior to data acquisition. Samples containing 2−3 mg of lipid were hydrated as described above for the DSC experiments and subsequently dispersed by vigorous mixing with 75 $\mu$L of $D_2O$ or $D_2O$-based PBS. The paste obtained was then squeezed between the $CaF_2$ windows of a heatable, demountable liquid cell (NSG Precision Cells, Farmingdale, NY) equipped with a 25 $\mu$m Teflon spacer. Once the cell was mounted in the sample holder of the spectrometer, the sample temperature could be varied between −20 and 90 °C by an external, computer-controlled water bath. Infrared spectra were acquired as a function of temperature with a Digilab FTS-40 Fourier transform spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) using data acquisition parameters similar to those described by Mantsch et al. (*31*). The experiment involved a sequential series of 2 °C temperature ramps with a 20 min interramp delay for thermal equilibriation and was equivalent to a scanning rate of 4 °C/h. Polarized ATR-FTIR spectra measurements were performed on lipid/peptide films prepared by drying methanolic solutions of the lipid/peptide (30:1) mixture onto the surface of a zinc selenide ATR crystal (angle of incidence = 45°). The latter was mounted on a trough plate of a horizontal ATR accessory (Spectra-Tech, Shelton, CT). Spectra were acquired at room temperature with a Mattson RS1 spectrometer equipped with a broadband MCT detector. A wire grid polarizer (Optometrics, Ayer, MA) mounted in a rotation stage was positioned in the IR beam path before the detector such that rotation of the polarizer could be accomplished without breaking the continuous dry air purge. Background and sample spectra were acquired with both s- and p-polarized radiation. Data acquisition involved the coaddition of 1024 scans at a resolution of 4 $cm^{-1}$ and with one level of zero-filling. The transmission and ATR spectra obtained were analyzed using computer programs obtained from the instrument manufacturer and from the National Research Council of Canada and were plotted with the Origin software package.

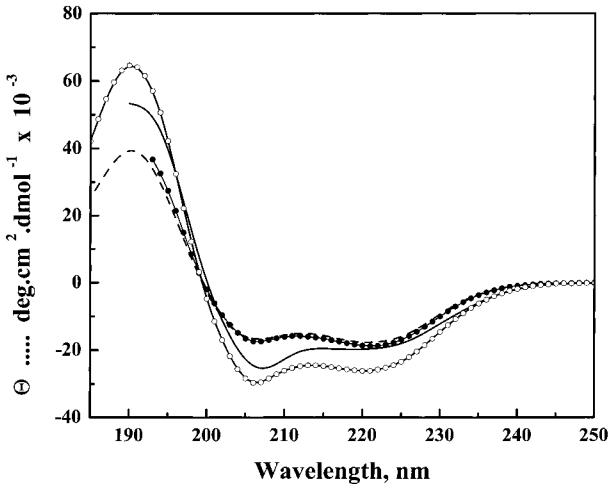


FIGURE 1: Circular dichroism spectra of peptide $A_{24}$ dissolved in methanol (—), unbuffered water (- - -), phosphate-buffered saline (●), and 50% TFE (○).

## RESULTS

*Circular Dichroism Studies.* CD spectra of $A_{24}$ dissolved in aqueous media, methanol, and in 50% TFE/water at 25 °C are presented in Figure 1. In aqueous media $A_{24}$ exhibits molar ellipticity minima of almost comparable intensity (~−17 000 and −19 000 deg·$cm^2$·dmol) at wavelengths near 206 and 222 nm, respectively. In both methanol and 50% TFE/$H_2O$, this peptide exhibits more skewed ellipticity profiles in which the minimum near 206 nm is somewhat more pronounced. The molar elliplicity values measured in 50% TFE/$H_2O$ (~−30 000 and ~−26 000 at 206 and 222 nm, respectively) are significantly higher than those measured in aqueous solvents (see above) or in methanol (~−25 000 and ~−20 000 at 206 and 222 nm, respectively). The observed increase in molar elliplicity of $A_{24}$ in 50% TFE/$H_2O$ is consistent with the well-known tendency of TFE to stabilize helical structures (29 and references cited therein). Analysis of the CD spectra observed in aqueous and methanolic solution suggests that $A_{24}$ is predominantly (~70−75%) helical in those media. Upon heating, the double minima CD profile shown in Figure 1 is progressively replaced by a spectrum containing a single minimum centered near 202 nm (data not presented), indicating that a marked decline in peptide secondary structure occurs at higher temperatures. The thermal instability of this peptide contrasts sharply with the marked thermal stability of the peptides $L_{24}$ and $(LA)_{12}$, which remain almost totally helical under all conditions examined (*14*, *26*).

*FTIR Spectroscopic Studies.* Illustrated in Figure 2 are the C═O stretching and amide I regions of FTIR spectra exhibited by $A_{24}$ and by a mixture of this peptide with POPC. Methanol-dried films of $A_{24}$ alone exhibit sharp amide I and amide II absorption bands centered at frequencies near 1659 and 1545 $cm^{-1}$, respectively, as well as a higher frequency component centered between 1672 and 1685 $cm^{-1}$. The latter component arises from the TFA counterions bound to the peptide (*32*) and will not be discussed further. The spectra exhibited by dried films cast from a methanolic solution of a mixture of $A_{24}$ and POPC also exhibit sharp amide I and amide II absorption bands centered near 1659 and 1545 $cm^{-1}$, respectively, in addition to a broad absorption band centered near 1738 $cm^{-1}$, which arises from the stretching vibrations

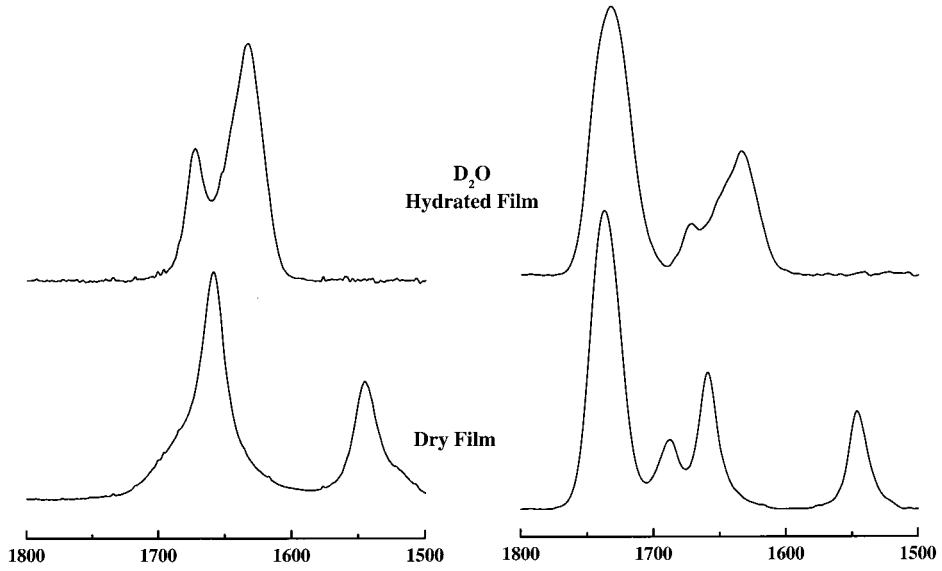


FIGURE 2: Transmission FTIR spectra exhibited by peptide $A_{24}$ (left panel) and by a 1:30 (mol/mol) mixture of $A_{24}$ with POPC (right panel). The data presented show the C═O stretching, amide I, and amide II regions of the FTIR spectrum of methanol-dried films and spectra of the same films after hydration with $D_2O$. Spectra of the hydrated films were acquired at 25 °C within 5 min of the addition of $D_2O$ to the dry films.

of the ester C═O groups of the lipid. The frequencies of the amide I and amide II absorption exhibited by the lipid-free and lipid-containing $A_{24}$ films are typical of predominantly α-helical proteins (*33*). Comparable results were also obtained when $A_{24}$ and POPC/$A_{24}$ mixtures were cast from TFE solution (data not shown).

Figure 2 also shows that, upon hydration with $D_2O$, the amide II absorption band of both lipid-free and lipid-containing $A_{24}$ films is rapidly abolished (≤5 min), indicating that the peptide N−H protons are quickly and completely exchanged with the solvent, even when the peptide is mixed with lipid. Concomitantly, the peptide exhibits a considerably broader amide I absorption band envelope which contains the main amide I peak centered near 1633 $cm^{-1}$ and a smaller TFA peak near 1673 $cm^{-1}$. Interestingly, the main amide I absorption band maximum (∼1633 $cm^{-1}$) is well outside of the range expected of either fully proteated or fully deuterated α-helices, for which values near 1657−1659 and 1647−1649 $cm^{-1}$, respectively, are more typical (*34*). Thus, whether mixed with lipid or not, α-helical structures may not be the preferred conformational motif of $A_{24}$ in the presence of water. Moreover, given that complete H/D exchange of the amide protons of the peptide occurs when the lipid/peptide mixtures are exposed to deuterated aqueous media, we can also conclude that, even in the lipid/peptide mixture, the amide protons of $A_{24}$ are all available for exchange with the bulk solvent. In turn, this suggests that $A_{24}$ may not be fully inserted into the lipid bilayer. This possibility was examined by a comparing the properties of $A_{24}$ in aqueous solution with those exhibited by aqueous dispersions of $A_{24}$/lipid mixtures.

Illustrated in Figure 3 is a comparison of the temperature-induced changes in the amide I band contours of peptide $A_{24}$ in the presence and absence of lipid. The lipid membranes of this preparation (DPDPC) are in the gel state at low temperatures and undergo a transition to the liquid-crystalline state at temperatures near 35 °C. In the absence of lipid, PBS solutions of $A_{24}$ exhibit amide I band maxima ranging from 1633 to 1635 $cm^{-1}$ at temperatures below 40 °C (Figure 3), and at higher temperatures, an irreversible decline in the amide I band maximum to frequencies near 1622 $cm^{-1}$ occurs. This change is attributed to the appearance of a sharp band centered near 1622 $cm^{-1}$ at the obvious expense of the 1633 $cm^{-1}$ band described earlier (spectra not shown). These temperature-induced changes in the amide I band envelope are essentially identical to those exhibited by the PBS-dispersed $A_{24}$/DPDPC mixture. It should be noted, however, that the spectroscopic changes described above are independent of the gel/liquid-crystalline phase transition of the phospholipid bilayers present. This point is vividly demonstrated by a comparison of the temperature dependences of the peptide amide I band maximum and the

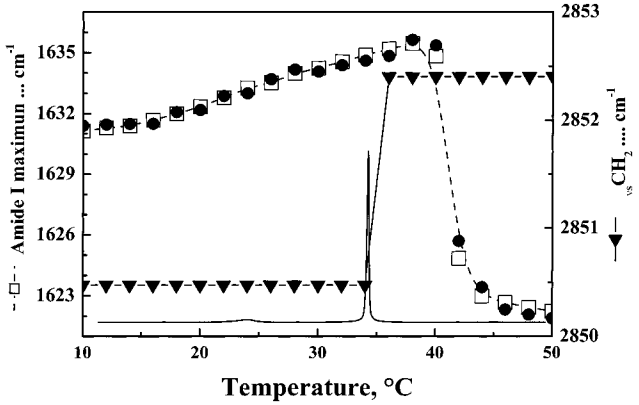


FIGURE 3: Comparison of the temperature-induced changes in the amide I band maxima exhibited by peptide $A_{24}$ in PBS (●) and those of the amide I (□) and $CH_2$ symmetric stretching (▼) band maxima exhibited by a 1:30 (mol/mol) mixture of $A_{24}$ with DPDPC. A DSC thermogram (—) of the same lipid/peptide mixture is superimposed to illustrate the correspondence between the spectroscopic changes and the gel/liquid-crystalline phase transition of the phospholipid membranes.

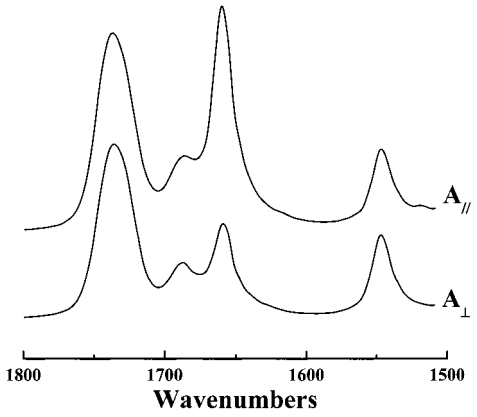


FIGURE 4: The C═O stretching, amide I, and amide II regions of attenuated total reflectance FTIR spectra exhibited by dried films of a POPC/$A_{24}$ mixture (30:1 mol/mol). The absorbance spectrum shown in the top panel ($A_{\parallel}$) was acquired with p-polarized infrared radiation, and the spectrum in the bottom panel ($A_{\perp}$) was acquired with s-polarized infrared radiation.

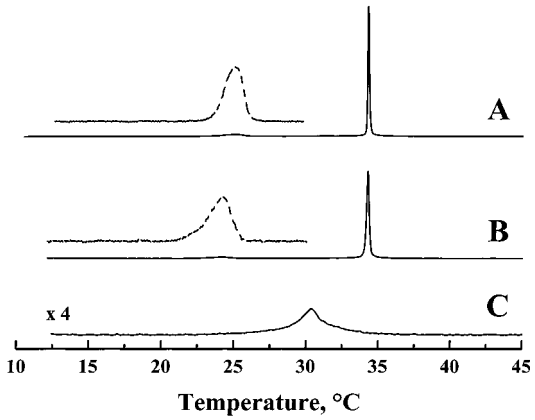


FIGURE 5: DSC heating thermograms exhibited by dispersions of DPDPC in PDS (A) and PBS dispersed mixtures of DPDPC and peptide $A_{24}$ (B) with the dashed lines representing the lipid pretransition endotherms on an expanded scale. The DSC thermogram (C) was obtained from an aqueous dispersion of the peptide $L_{24}$ in DPDPC and is presented to facilitate comparison between the effects of peptide $A_{24}$ on PC bilayers and those typical of hydrophobic transmembrane peptides such as $L_{24}$ and $(LA)_{12}$. All thermograms shown have been normalized with respect to scan rate and to the mass of the lipid in the sample. Lipid:peptide ratios are all 30:1 (mol/mol).

lipid symmetric $CH_2$ stretching band maximum (Figure 3). The data clearly show a discontinuity in the lipid hydrocarbon chain $CH_2$ symmetric stretching band maxima at temperatures between 34 and 36 °C, temperatures which coincide with the calorimetrically detected gel/liquid-crystalline phase transition of this lipid membrane. This result contrasts sharply with the temperature-dependent changes in the amide I band maxima, which exhibit a slow upward drift in frequency at temperatures up to 40 °C and a sharp decrease to frequencies near 1622 $cm^{-1}$ at temperatures above 42 °C. The absence of any correlation between changes in the amide I band of $A_{24}$ with the gel/liquid-crystalline phase transition of the admixed lipid was also observed in comparable studies of $A_{24}$ mixed with POPC (data not shown) and raised the issue of whether the peptide was actually inserted into the lipid bilayer. The latter issue was addressed by the polarized ATR-FTIR spectroscopic experiment presented below and by an examination of the thermotropic phase behavior of lipid/peptide mixtures and the effect of these peptides on the order and dynamics of the lipid hydrocarbon chains (see DSC and ESR results that follow).

Illustrated in Figure 4 are polarized attenuated total reflectance FTIR spectra of a dry film composed of POPC and the peptide $A_{24}$ (30:1 mol/mol). As observed with the transmission FTIR data shown in Figure 2, these spectra also exhibit sharp amide I and amide II absorption bands centered near 1658 and 1547 $cm^{-1}$, consistent with the peptide adopting a predominantly α-helical conformation under these conditions. However, Figure 4 also shows that the intensity of the amide I band in spectra acquired with p-polarized radiation is considerably greater (4−5 times the integrated intensity) than that of acquired with s-polarized radiation. This observation provides clear evidence that the $A_{24}$ helices are preferentially oriented in the dry film such that the amide I transition moment is aligned essentially along the normal to the surface of the ATR crystal, indicating that the long axes of the peptide helices are preferentially oriented perpendicular to the surface of the crystal. A comparable examination of the absorption bands arising from the symmetric C−H stretching vibrations of the terminal methyl groups on the lipid hydrocarbon chains indicates that the fatty acyl chains of the lipid are also preferentially aligned perpendicular to surface of the ATR crystal (data not shown). Together, these observations provide strong evidence that the $A_{24}$ is actually incorporated into the dried lipid/peptide films and that it exists in those films as a transbilayer α-helix.

*Differential Scanning Calorimetric Studies.* The effects of the presence of $A_{24}$ on the thermotropic phase behavior of LMVs composed of DPDPC were studied by high-sensitivity DSC, and some typical results are presented in Figure 5. In the absence of peptide, DSC thermograms of DPDPC alone exhibit two phase transitions, a lower temperature, less enthalpic pretransition centered near 25 °C and a higher temperature, more energetic main transition centered near 35 °C. These transitions correspond to the lamellar gel/rippled gel and rippled gel/liquid-crystalline phase transitions of DPDPC, respectively (*35*). Both of these transitions are reversible on cooling, with the pretransition exhibiting a small degree of hysteresis (cooling scans not shown). Figure 5 also shows that the DSC heating thermograms exhibited by aqueous dispersions of the $A_{24}$/DPDPC mixtures are generally similar to those exhibited by DPDPC in the absence of peptide. However, the temperature, cooperativity, and the enthalpy of the pretransition exhibited by the DPDPC/$A_{24}$ mixture are all slightly reduced relative to those observed in the absence of peptide. These calorimetric results contrast sharply with those obtained when comparable amounts of more hydrophobic peptides such as $L_{24}$ and $(LA)_{12}$ are incorporated into DPDPC bilayers (compare Figure 5, traces B and C). In such cases the lipid pretransition is not observed, and the temperature, enthalpy, and cooperativity of the main phase transition are all markedly reduced (for examples, see refs *15*, *22*, and *27*). Since it is well established that peptides such as $L_{24}$ and $(LA)_{12}$ exist exclusively as transbilayer α-helices when reconstituted with phospholipids (see the introduction), the absence of significant effects of $A_{24}$ on the thermotropic phase behavior of DPDPC strongly suggests that this peptide is not incorporated into these phospholipid bilayers in a transmembrane orientation. Indeed, the very small effect of $A_{24}$ on the lipid pretransition (a process which

is very sensitive to the incorporation of, or association with, even small amounts of peptide or other materials; *36*, *37*) suggests instead that $A_{24}$ is present primarily in the aqueous phase where it may be interacting only weakly with the bilayer surface. However, these results do not completely rule out the possibility that the peptide $A_{24}$ is inserted into these lipid bilayers, because similar calorimetric results might be obtained if the peptide is laterally segregated into peptide-rich domains within the lipid bilayer. This latter possibility was addressed in the ESR spectroscopic studies presented below.

*Electron Spin Resonance Spectroscopic Studies.* ESR spectra of LMV's composed of POPC containing 1 mol % of the spin label 1-palmitoyl-2-(*n*-doxyl)stearoyl-PC were acquired as a function of temperature and peptide content. Our results reveal three very important points. First, as noticed in our previous studies of $L_{24}$-containing POPC membranes (*23*), the spectra did not exhibit any features consistent with the presence of two components which may be assigned to the so-called bulk and boundary lipids (spectra not shown). Thus the lateral diffusion of the probe molecules, and the exchange rates of probe molecules between any lipid domains that may be present, must both be fast on the ESR time scale (i.e., $>10^7\ s^{-1}$). The absence of "two-compartment spectra" is especially significant because such spectra are commonly observed when membrane proteins with large intramembrane domains are incorporated into lipid bilayers (see ref *38*). With such systems, the appearance of the two-component spectra is generally attributed to longer residence time (>0.1 $\mu$s) of lipids in the boundary and/or to self-clustered peptide-rich regions (*23*). The absence of comparable two-component spectra here therefore suggests that it is very unlikely that laterally segregated clusters of $A_{24}$ were present in these membranes. A similar conclusion was inferred from the results of comparable studies of $L_{24}$-containing POPC membranes (*23*).

Second, over the whole range of peptide concentrations examined (0−10 mol %), $A_{24}$ did not induce any significant changes in the contours of the observed ESR spectra, nor did it affect the maximal splittings of the doxyl labels located at any depth within the POPC membrane (spectra not shown). This observation differs sharply from what has been observed with hydrophobic transmembrane peptides such as $L_{24}$, for which subtle changes in spectral contours generally occur along with peptide concentration-dependent increases in the maximal splittings (~10% at 10 mol % of peptide). Third, the presence of $A_{24}$ also had no significant effect on the order and dynamics of the probe molecules, as can be inferred from the calculated rotational correlation times (Figure 6, panels A and B) and the molecular order parameters (Figure 6, panel C). This result also differs markedly from that observed with transmembrane peptides such as $L_{24}$ and $(LA)_{12}$, which cause significant increases in both the rotational correlation times and the molecular order parameters determined at all depths in the lipid bilayer (see Figure 6). This observation, and the evidence that laterally segregated clusters of $A_{24}$ do not exist in these membranes, provides strong evidence that $A_{24}$ is not located within the hydrophobic core of hydrated lipid bilayers.

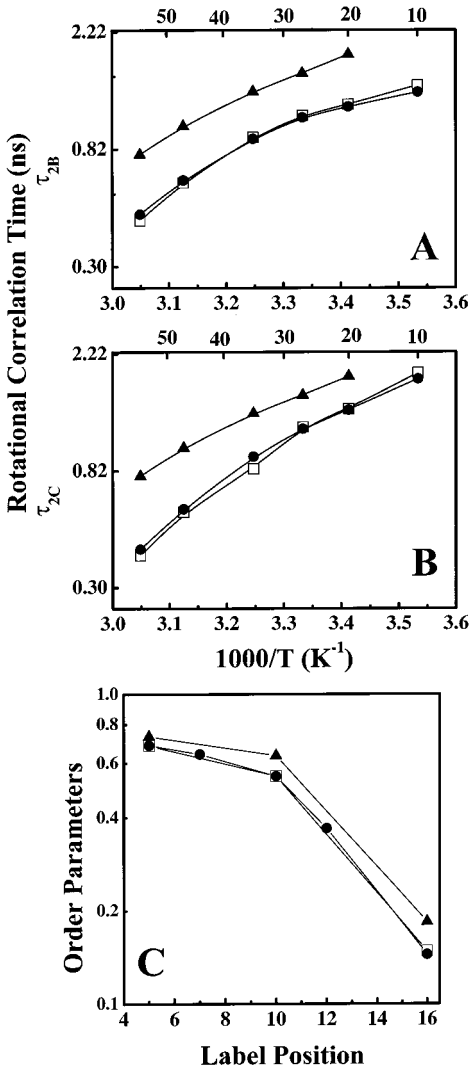


FIGURE 6: Effect of peptide $A_{24}$ on the effective rotational correlation times of the doxyl spin label of 1-palmitoyl-2-(16-doxyl)stearoyl-PC in POPC membranes (A and B) and on the order parameter profiles (C) of 1-palmitoyl-2-(*n*-doxyl)stearoyl-PC in POPC membranes. The data shown were obtained in the absence (●) and presence (□) of peptide $A_{24}$. Also, comparable data obtained with peptide $L_{24}$ (▲) are presented to facilitate comparison between the effects of peptide $A_{24}$ and those typical of hydrophobic transmembrane peptides. Order parameters were all determined at 20 °C, and the peptide-containing mixtures all contained 10 mol % peptide.

## DISCUSSION

This work provides strong evidence that helical conformations are thermodynamically favored when the peptide $A_{24}$ is dissolved in aqueous media at low temperatures, a result consistent with the thesis that alanine is a strongly α-helical-forming amino acid residue (*39*−*41*). It is should be noted, however, that helical structures are not always thermodynamically favored when alanine-rich peptides are dissolved in aqueous media (*42*−*46*). It is also interesting to note that although our CD results suggest that α-helical structures predominate (~70−75%) when $A_{24}$ is dissolved in aqueous media, our FTIR spectra are incompatible with the presence of large amounts of α-helical structure when $A_{24}$ is dissolved in such media. Similar discrepancies between the results of CD and FTIR spectroscopic studies have been reported in studies of other alanine-rich peptides, for which amide I

frequencies comparable to those reported here were also observed in aqueous solution (*43*, *47*). Interestingly, however, such studies have also suggested that the $3_{10}$-helix may be the preferred conformational motif when relatively short alanine-rich peptides are dissolved in aqueous media, with α-helices predominating at substantially longer peptide lengths (*42*). Given these observations, and the observation that CD does not adequately differentiate between $3_{10}$- and α-helical motifs (see ref *48* and references therein), our FTIR spectroscopic data may actually be indicating that $3_{10}$-helices may be preferred to the α-helical conformational motif when $A_{24}$ is dissolved in aqueous media.

Recent studies of the intrinsic helix-forming propensities of various hydrophobic amino acid residues in water and of the overall stability of peptide helices formed by such amino acids indicate that whereas the intrinsic helix-forming propensity decreases in the order Ala > Leu >Ile > Val, helical stability actually decreases in the order Leu > Ile > Ala > Val (*49*). Given that the hydrophobicities of these peptides decrease in the order Ile ∼ Leu > Val > Ala (*49* and references cited therein), we suggest that the stability of peptide helices in aqueous media is actually a reflection of both the intrinsic helix-forming propensity of their amino acid residues and the hydrophobicities of the amino acid side chains. It should be noted, however, that other studies suggest that the α-helical propensities of the same hydrophobic amino acids decrease in the order Ile > Leu > Val > Ala (*50*−*52*). These latter studies could be reconciled with the results of those alluded to above if one assumes that parameters measured in the latter studies were actually a reflection of helical stability and not intrinsic helix-forming propensity per se. Nevertheless, our structural studies of the peptides $L_{24}$, $(LA)_{12}$, and $A_{24}$ are generally in accord with the results of both sets of studies. It should also be noted that both sets of studies also imply that the intrinsic α-helical-forming propensities of the various amino acids are higher in nonpolar solvents such as butanol (dielectric constant ∼17.8 units at 25 °C) than in water. Given this, one would also surmise that, in the less polar environment of the hydrophobic core of phospholipid bilayers, the α-helical-forming propensities of these aliphatic amino acid residues would be even greater than observed in water or butanol and that the differences between the α-helical-forming propensities of the individual acid residues would be even greater. Our observations of the relative α-helical propensities of the peptides $L_{24}$, $(LA)_{12}$, and $A_{24}$ reported in this and previous work (*14*, *26*) are compatible with these general principles, as are our observations of the relative α-helical-forming propensities of $A_{24}$ in lipid, methanol, and aqueous media. It is also interesting to note that the ranking order Ile > Leu > Val > Ala is also the order of the decreasing abundance of these aliphatic amino acid residues in the α-helical transmembrane segments of monotopic membrane proteins (*53*, *54*). This suggests that these amino acids may have been selected for enrichment in the transmembrane segments of integral membrane proteins at least in part on the basis of their α-helical-forming propensities in the nonpolar environment of the membrane interior (*52*).

The major conclusion that may be drawn from our experimental results is that $A_{24}$ does not form a stable transmembrane association with fully hydrated lipid bilayers. It is therefore interesting to compare our experimental results for $A_{24}$ with those predicted by molecular dynamics simulations of polyalanine peptides in phospholipid/water and octane/water systems (*55*, *56*). One of these studies (*55*) predicts that a 20-residue polyalanine peptide with amino- and carboxy-blocked termini should preferentially localize at the lipid/water interface rather than partition into either the aqueous phase or the nonpolar hydrocarbon phase. Such predictions are compatible with our experimental findings for $A_{24}$, which appears to be localized, at least in part, at the phosphatidylcholine bilayer/water interface. Moreover, the same molecular dynamics simulations predict that although the polyalanine peptide should form a fairly stable α-helix when inserted into the octane phase or when localized at the octane/water surface, this peptide should partially unfold when present in aqueous media and that different helical forms may be formed locally during this reversible loss of structure. These latter predictions are also compatible with our experimental findings for $A_{24}$, which appears to exist in an α-helical conformation when inserted into phospholipid bilayers in the absence of water but in a partially helical (but non-α-helical) conformation when dissolved in aqueous media or associated with hydrated phospholipid bilayers. Interestingly, however, exclusion of polyalanine helices from the hydrocarbon core of the lipid bilayer was not predicted in other molecular dynamics simulations (*56*), which also suggest that the polyalanine helices should unfold when partitioned into water. The basis of the discrepancies in the predictions made by the two molecular dynamics simulation studies is currently unclear but could be related to the lengths of the simulations performed (∼1.6 and 20−200 ns in refs *56* and *55*, respectively).

Unlike peptides such as $L_{24}$ and $(LA)_{12}$, $A_{24}$ does not partition into the hydrophobic domains of phospholipid bilayers. This finding can be rationalized on the basis of the relative overall hydrophobicities of the three peptides and, in particular, by the experimentally determined hydrophobicity scales derived from the partitioning of the amino acid residues of short unstructured peptides into the polar/apolar interfaces and into the hydrocarbon core of phospholipid bilayers (*57*−*59*). On the basis of these hydrophobicity scales, Leu residues are expected to strongly prefer the lipid bilayer interface and especially the lipid bilayer hydrophobic core over water, whereas Ala residues should exhibit a slight preference for water over the phospholipid bilayer polar/apolar interface and a fairly strong preference for water over the hydrophobic core of the lipid bilayer. Indeed, using these hydrophobicity scales, free energies for the transfer of the peptides $L_{24}$, $(LA)_{12}$, and $A_{24}$ (as α-helices) from water to the hydrophobic cores of phospholipid bilayers were estimated as −24.7, −3.7, and +17.3 kcal/mol, respectively, and values of −22.5, −13.7, and −5 kcal/mol were estimated for their transfer from water to the bilayer polar/apolar interfaces, respectively. It is also interesting to note that if the dilysine caps of these peptides are omitted from the calculations, free energy estimates for transferring the central regions of $L_{24}$, $(LA)_{12}$, and $A_{24}$ (as α-helices) from water to the hydrophobic core of a phospholipid bilayer were −35.9, −14.9, and +6.1 kcal/mol, respectively, and −24.8, −16.1, and −7.3, respectively, for their transfer from water to the bilayer polar/apolar interfacial region. Given these free energy estimates, $A_{24}$, unlike $L_{24}$ and $(LA)_{12}$, would not be expected to partition into the hydrophobic domains of lipid

bilayers, a conclusion consistent with our experimental observations and with the predictions of recent molecular dynamics simulations (*54*).

Recent studies of alanine-rich peptides containing varying numbers of leucine residues by Lafleur and co-workers have shown that there are both minimal length and minimal hydrophobicity requirements for the spontaneous insertion of such peptides into preformed phospholipid vesicles (*60*). Interestingly, however, these workers also showed that, for peptides which meet these criteria, the presence of lysine caps at both ends of the peptide prevents spontaneous insertion into preformed phospholipid vesicles, despite the fact that such peptides remain associated with lipid bilayers if first cosolubilized with phospholipid prior to vesicle formation. It was therefore concluded that the translocation of the charged lysine residues through the hydrophobic core of the preformed lipid vesicles was a significant energy barrier against their spontaneous insertion into the preformed lipid bilayers. In our studies, lipid/$A_{24}$ mixtures were all prepared by cosolubilization in organic solvent prior to the formation of the lipid vesicles, and the peptide is shown to be α-helical and in a transbilayer orientation prior to addition of water. However, given our experimental observations, it is apparent that translocation of the dilysine termini of $A_{24}$ through the hydrophobic regions of the hydrophobic core of the lipid bilayer occurs quite readily upon hydration. Apparently the free energy of partitioning of $A_{24}$ into water is large enough to overcome this energy barrier.

In concluding, we note that $A_{24}$ clearly does not form a stable transmembrane association with fully hydrated phospholipid bilayers. Although it is feasible to insert this peptide into dry phospholipid films as a transmembrane α-helix, this association is not maintained upon hydration of these films, presumably because the peptide preferentially partitions into the aqueous phase. Preferential partitioning of $A_{24}$ into the aqueous phase is undoubtedly the combined result of its appreciable solubility in aqueous media and the fact that the volume of the aqueous compartment greatly exceeds that of the lipid phase. Our findings can therefore be attributed to the fact that the helical surface of $A_{24}$ is not sufficiently hydrophobic for the peptide to form a stable association with the hydrophobic core of the lipid bilayer either as dispersed monotopic helices or as a peptide aggregate. This property makes alanine-rich peptides such as $A_{24}$ very poor models for the helical transmembrane segments of membrane proteins and, most probably, poor candidates for spontaneous insertion into preformed lipid bilayers. These latter conclusions are compatible with the results of a number of other experimental observations (*59*−*62*) and with the finding that alanine is the least abundant of the aliphatic amino acids present in the transmembrane segments of monotopic membrane proteins (*53*). We therefore suggest that both α-helical propensity and hydrophobicity are important parameters in determining the suitability of aliphatic amino acid residues to form stable transmembrane α-helices in a lipid bilayer environment.

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