FTIR Spectroscopic Studies of the Conformation and Amide Hydrogen Exchange of a Peptide Model of the Hydrophobic Transmembrane α-Helices of Membrane Proteins†

Yuan-Peng Zhang,‡ Ruthven N. A. H. Lewis,‡ Robert S. Hodges,‡,§ and Ronald N. McElhaney*,‡

Department of Biochemistry and MRC Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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ABSTRACT: The conformation and amide hydrogen exchangeability of the hydrophobic peptide Lys_2-Gly-Leu_{24}-Lys_2-Ala-amide were studied by Fourier transform infrared spectroscopy. In these studies information on the secondary structure of the peptide was obtained from an examination of the contours of both the amide I and amide II absorption bands. The conformationally sensitive amide I and amide II regions of the infrared spectra suggest that the peptide is predominantly α-helical and that it contains some non-α-helical structures which are probably in an extended conformation. Studies of the exchangeability of the amide protons of the peptide indicate that there are two populations of amide protons which differ markedly with respect to their exchangeability with the bulk solvent phase, whether the peptide is dissolved in methanol or dispersed in hydrated lipid bilayers. One population of amide protons is very readily exchangeable, and our data suggest that it arises primarily but not exclusively from the extended regions of the peptide. The other population exchanges very slowly with the bulk solvent and appears to originate entirely from the α-helical domain of the peptide. This latter population is virtually unexchangeable when the peptide is dispersed in hydrated phosphatidylcholine bilayers but can be largely exchanged when the peptide is solubilized with methanol. We suggest that this slowly exchanging population of amide protons arises from the central part of the hydrophobic polyleucine core which forms a very stable α-helix that would be deeply buried in the hydrophobic domain of hydrated lipid bilayers. Our results also suggest that the readily exchangeable population of amide protons probably arises from the amino acid residues near the ends of the peptide. These residues probably comprise the extended domains of the peptide and small contiguous portions of its α-helical polyleucine core. Our studies indicate that the α-helical structure of the peptide remains stable under all our experimental conditions. We also show that, by utilizing initial H/D exchange, the conformation of both the transbilayer domain and the hydrophilic end regions of this peptide, and presumably also those of transmembrane proteins, can be differentially probed by infrared spectroscopy.

The interactions of lipids and proteins in biological membranes and reconstituted model membranes have been studied by a variety of physical techniques [see Watts and DuPont (1985, 1986) and references cited therein]. Although the data generated by such studies have proven valuable in developing a qualitative picture of lipid–protein interactions in model and bilogical membranes, our understanding of these interactions at the molecular level is incomplete. This is due at least in part to our lack of detailed knowledge about the three-dimensional structure of many of the proteins studied and their exact arrangement within the lipid bilayer. One approach to this problem is to chemically synthesize model polypeptides which mimic the structure and conformation of various portions of natural membrane proteins and to reconstitute these with lipid bilayers formed from natural or synthetic lipids [for examples, see Davis et al. (1983), Jacobs and White (1986, 1987) and Mclean et al. (1991)]. A good example of this is the class of synthetic polypeptides Lys_2-Gly-Leu_n-Lys_2-Ala-amide (P_{16},[1] P_{20} and P_{24}, where n = 16, 20, and 24, respectively), which have been used as models of the hydrophobic transmembrane α-helices of natural membrane proteins (Davis et al., 1983). These molecules were designed such that the hydrophobic polyleucine core would form an α-helix and traverse the lipid bilayer with the charged terminal lysine residues anchoring the ends of the peptide to the bilayer surface and inhibiting lateral aggregation of the polypeptide (Davis et al., 1983). CD studies have shown that these peptides adopt a predominantly α-helical conformation when they are dissolved in either methanol or nondenaturing solvents and when dispersed in DPPC bilayers (Davis et al., 1983), and subsequent X-ray diffraction studies of oriented PC bilayers containing these peptides have shown that these peptides orient perpendicular to the plane of the bilayer surface (Huschilt et al., 1989). Thus, with respect to their secondary structures and their orientation within lipid bilayers, these peptides should be good models of the hydrophobic transmembrane α-helices which are presumed to form the hydrophobic domains of many integral transmembrane proteins.

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* Corresponding author.
‡ Department of Biochemistry.
§ MRC Group in Protein Structure and Function.

[1] Abbreviations: DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; TFE, trifluoroethanol; CD, circular dichroism; C=O, carbonyl; P_n, Lys_2-Gly-Leu_n-Lys_2-Ala-Amide (n = number of leucine residues); PC, phosphatidylcholine; PS, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine.

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Indeed, DSC studies of the interaction of this particular class of peptides with PC bilayers (Huschilt et al., 1985; Zhang et al., 1992) are consistent with the above expectations in that they appear to interact primarily with the hydrophobic domain of the lipid bilayer and can be considered class III integral membrane proteins or peptides as defined by Papahadjoupolos et al. (1975) and later refined by McElhaney (1986).

Interest in the interaction of peptides such as P_{24} with lipid bilayers stems not only from their being models of hydrophobic α-helical transmembrane segments of membrane proteins but also from considerations of the nature of the lipid–protein interactions which may occur when there is a mismatch between the hydrophobic length of a protein (or peptide) and the hydrophobic thickness of its host lipid bilayer [see Huschilt et al. (1985), Morrow et al. (1985), and Mouritsen and Bloom (1984)]. From the information currently available in the literature, it seems likely that the stress caused by a mismatching of the hydrophobic length of a peptide such as P_{24} and the hydrophobic thickness of its host lipid bilayer would result in conformational distortions of the membrane lipids rather than significant changes in the conformation of the peptide itself (Mouritsen & Bloom, 1984; Sperotto & Mouritsen, 1988). However, the possibility that conformational changes in this type of peptide may occur under the aforementioned conditions is not excluded by available data. Thus a knowledge of the structure of the peptide within a lipid bilayer, and the structural changes which can occur in response to changes in the physical properties of the lipid bilayer and to changes in other physical conditions, should be very useful. To this end, we have used FTIR spectroscopy to study the structure of P_{24} when it is dispersed in fully hydrated PC bilayers and to compare that structure with that of the solid phase and with that which occurs in methanolic solution. FTIR spectroscopy is well suited for such a study because reasonably good spectra could be obtained with relatively small samples and, unlike a technique such as CD, it is possible to examine the solid phase as well as aqueous dispersions of the lipid/peptide mixtures while avoiding problems of data distortion due to light scattering, etc. Our approach involves monitoring changes in the conformation of the peptide by an examination of the conformationally sensitive amide I band near 1650 cm^{-1} and a study of the exchangeability of the amide protons of P_{24} by an examination of changes in both the amide I and amide II bands when samples of proteated P_{24} are dissolved in deuterated methanol. The data obtained should assist the interpretation of previous studies of the interaction of P_{24} with DPPC bilayers (Huschilt et al., 1985; Morrow et al., 1985; Pauls et al., 1985; Roux et al., 1989) and the accompanying DSC and FT-IR spectroscopic study (Zhang et al., 1992).

MATERIALS AND METHODS

The peptide P_{24} was synthesized using standard solid-phase synthetic techniques as described previously (Davis et al., 1983). The PCs were synthesized and purified in this laboratory using procedures previously reported (Lewis et al., 1987). Before the samples were prepared for infrared spectroscopy, it was necessary to first remove the trifluoroacetate counterions which originated from the TFA used during the purification of the peptide. The trifluoroacetate moieties give rise to a strong absorption band near 1670 cm^{-1} (Surewicz & Mantsch, 1989) and this band overlaps the amide I absorption band of the peptide. The trifluoreacetate counterions were replaced with chloride ions by twice lyophilizing the peptide from 10 mM hydrochloric acid. The solid-phase infrared spectra of the peptide was obtained from thin films that were dried onto the surface of the barium fluoride windows of the sample holder from both methanol (CH_3OH) and deuterated methanol (CH_3OD). To obtain a dry, extensively deuterium-exchanged P_{24} sample, the sample was first dried from CH_3OD and then redissolved in the same solvent and heated in a sealed tube to temperatures near 100 °C for 2 h. After the sample was redried, the process (incubation near 100 °C and subsequent redrying) was repeated once with CH_3OD, once with perdeuterated TFE/CH_3OD (1:1), once with benzene/TFE (1:1), and twice with CH_3OD before being dried onto the surface of the barium fluoride window. The liquid samples (i.e., methanolic solutions and aqueous lipid dispersions) used for spectroscopic analysis were 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. The methanolic solutions used were of a peptide concentration of some 10–20 mg/mL. Aqueous dispersions of the lipid/peptide mixtures were prepared for infrared spectroscopy using (as appropriate) either CH_3OH- or CH_3OD-dried peptide samples as follows. The peptide and the lipid were codissolved in CH_3OH (or CH_3OD for CH_3OD-dried samples) in the required proportions, and the solvent was removed with a stream of nitrogen. After removal of residual traces of solvent in vacuo, the sample was hydrated by vigorous vortexing with D_2O at temperatures well above the gel/liquid-crystalline phase transition temperature of the lipid. The infrared spectra were recorded with a Digilab FTS-40 Fourier transform infrared spectrometer which was continually purged with dry, CO_2-depleted air. Spectra were acquired using the methodology which is now standard for these kinds of samples [see Mantsch et al. (1985)] and processed using software supplied by Digilab Inc. and other computer programs developed by the National Research Council of Canada. In cases where the spectra obtained consist of overlapping bands, data processing usually involved the use of Fourier deconvolution to obtain fairly accurate estimates of the number of component bands and their frequencies, followed by curve-fitting procedures to obtain estimates of bandwidth and band area. Typically band narrowing factors of 1.6–2.0 were used during deconvolution. The noise levels of our spectra were usually low enough such that band narrowing factors as high as 2.3 could be used without introducing significant distortions to the spectra. From considerations of instrument resolution, signal to noise, etc., we estimate that the accuracy of the band frequencies and band areas returned by the curve-fitting procedures are ±1–2 cm^{-1} and ±7–10%, respectively.

RESULTS

Infrared spectra of dried films of P_{24} were recorded between 3500 and 900 cm^{-1} (see Figure 1) and the frequencies and assignments of the main absorption bands are listed in Table I. As is typical of this region of the infrared spectra of proteins and peptides, the spectrum shown in Figure 1 contains four absorption bands (amide A, amide I, amide II, and amide III; see Table I) which are attributable to the characteristic vibrational modes of the amide moieties of the peptide. Of these, the amide I and amide II bands are structurally significant, since they convey information about the conformation and structural stability of the peptide, respectively. An examination of these bands and the structural implications thereof is the principal thrust of this paper. The infrared

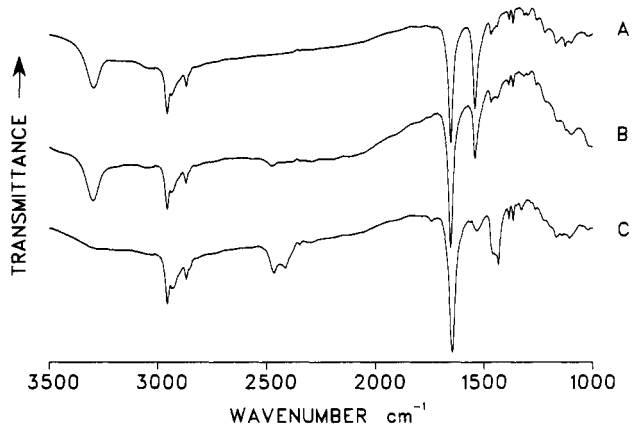

FIGURE 1: Infrared spectra of dry films of P_{24}. The spectra are shown in the transmittance mode for (A) P_{24} samples dried from CH_3OH, (B) P_{24} samples dried from CH_3OD, and (C) P_{24} samples after extensive deuterium exchange (see materials and methods).

Table I: Frequencies and Assignments of the Major Infrared Absorption Bands of Solid Films of the Peptide P_{24}

band	frequency (cm^{-1})		
	CH_3OH dried	CH_3OD dried	D exchanged
N–H stretch	3296	3296	≅3300 (broad)
N–D stretch		2473	2469
amide I	1656	1655	1648
amide II	1544	1543	1535
amide II′		1460	1460
CH3 sym-bend	1386, 1367	1386, 1367	1386, 1367
amide III	1295	1295	
amide III′			965

spectra shown in Figure 1 also contains two significant bands at 1386 and 1367 cm^{-1} which are characteristic of isopropyl groups (McMurray & Thornton, 1952; Sheppard & Simpson, 1953). These bands arise from the symmetric bending vibrations of the methyl groups present on isopropyl residues, and in the case of a peptide such as P_{24}, such residues are only present in its polyleucine core. Under our experimental conditions, we find that the properties of these two absorption bands are insensitive to the temperature changes, to the solvent changes, and to changes in the other environmental variables that were studied. This enabled us to compare the intensities of the absorption bands in different spectra of P_{24} by scaling the intensities of all peptide bands so as to normalize the integrated intensities of these methyl symmetric bending bands.

The amide I and amide II regions of infrared spectra of dried films of P_{24} are shown in Figure 2. With CH_3OH-dried films of this peptide, the amide I and amide II bands are observed near 1656 and 1543 cm^{-1}, respectively. The maxima of these bands remain essentially unchanged (±1 cm^{-1}) over the entire temperature range studied (10–50 °C). With the aid of Fourier deconvolution and curve-fitting procedures, we find that the amide I band of the CH_3OH-dried sample is resolvable into three component bands with maxima near 1678, 1656, and 1625 cm^{-1}, whereas the amide II band is resolvable into two components with maxima near 1543 and 1528 cm^{-1}. In the case of the amide I band, the component near 1656 cm^{-1} dominates the contours of the band (≅80% of the total integrated intensity) and can be assigned to the amide I absorption of the α-helical domains of the peptide (Miyazawa & Blout, 1961). The components near 1678 and 1625 cm^{-1} together contribute some 20% of the integrated intensity of the amide I band and are probably attributable to the amide I vibrations of the non-α-helical domains of the peptide. The absorption maxima of the "non-α-helical amide I absorptions"

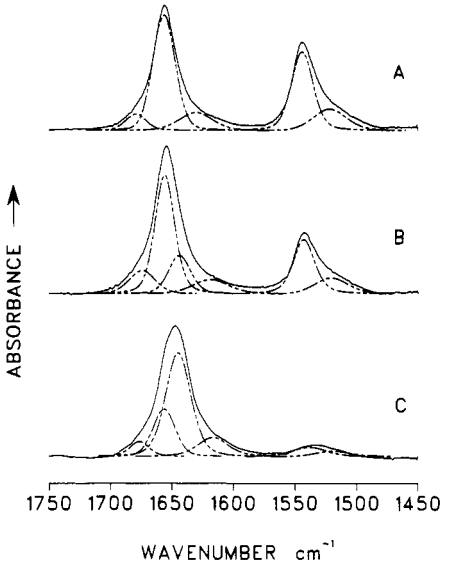

FIGURE 2: Amide I and amide II regions of the infrared spectra of dry films of P_{24}. The spectra shown were obtained from CH_3OH-dried samples (A), CH_3OD-dried samples (B), and extensively deuterium-exchanged smaples (C). To effect comparison of the various spectra, the intensities have been normalized to the total integrated intensity of the methyl symmetric bending bands of the peptide isopropyl groups. Spectra are shown in the absorbance mode with the solid lines representing the contours of the actual spectra obtained and the dashed lines representing the contours of the component bands resolved by a combination of Fourier deconvolution and curve-fitting techniques.

suggest that they arise from domains of the peptide which are in an extended conformation (Miyazawa & Blout, 1961). The fact the α-helical amide I absorption dominates this region of the infrared spectrum of P_{24} is consistent with previous CD studies of this peptide (Davis et al., 1983). In the case of the amide II band, Fourier deconvolution and curve-fitting analyses also indicate that the component near 1543 cm^{-1} contributes some 70% of the integrated intensity. The appearance of an amide II band in this frequency range has been observed in the infrared spectra of α-helical polypeptides (Miyazawa & Blout, 1961) and was also theoretically predicted by normal mode calculations [see Krimm (1983) and Dwivedi and Krimm (1984) and references cited therein]. Thus the observed contours of the amide II band are also consistent with the proposal that solid films of P_{24} are predominantly α-helical under our conditions.

We also examined the effects of deuterium exchange on the infrared spectrum of P_{24} so as to obtain some information on the stability of the conformation that it adopts. As is evident from Figures 1 and 2, a sizeable fraction of the amide protons of the peptide are not easily exchanged for deuterons by repeatedly drying the sample from CH_3OD. We find that upon drying the sample from CH_3OD there are small decreases in the relative integrated intensities of the amide A and amide II bands and that modest increases in infrared absorption are observed near 2450 and 1485 cm^{-1}. These new bands arise because of the N-deuteration of some of the peptide nitrogens and are attributable to the N–D stretching and amide II′ vibrational modes, respectively (Miyazawa et al., 1958). Our inability to completely exchange the amide protons of P_{24} by repeatedly drying from CH_3OD is structurally significant, since it implies that in methanolic solution the peptide adopts a very stable conformation (or possibly a highly aggregated state) which effectively shields a large fraction of the amide protons from exchange with the solvent phase. To investigate further the exchange of the amide protons, we also examined

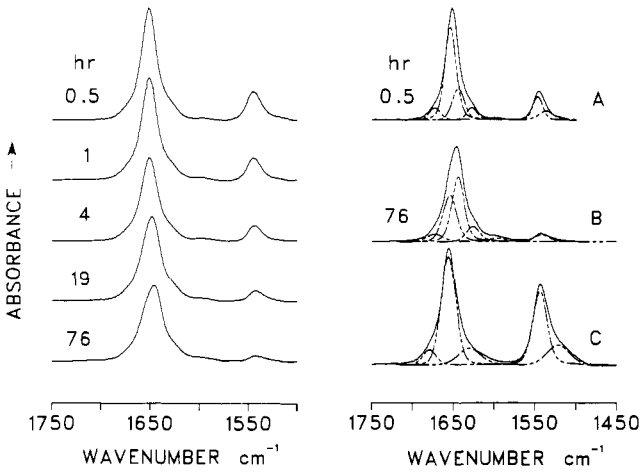

FIGURE 3: Time-dependent changes in the amide I and amide II bands of a CH_3OD solution of P_{24} at 40 °C. The spectra were acquired at the times indicated and are presented in the absorbance mode with their intensities scaled to normalize the integrated intensity of the methyl symmetric bending bands of the isopropyl groups of the peptide. The left panel shows typical spectra obtained during the proton/deuteron exchange experiment, and the right panel shows the band components of spectra obtained at the start (A) and completion (B) of the experiment. To enable some comparison with a completely unexchanged sample, the spectrum of the solid film dried from CH_3OH (spectrum C) is also shown. The solid lines represent the contours of the actual spectra obtained, and the dashed lines represent the contours of the component bands resolved by a combination of Fourier deconvolution and curve-fitting techniques.

spectra of P_{24} in CH_3OD solution.[2] The spectra were recorded as a function of time, and the changes in the contours of the amide I and amide II regions of the spectrum are shown in Figure 3. The progress of the exchange process an be conveniently monitored by the decrease in the integrated intensity of the amide II band near 1543 cm^{-1}. A comparison of the spectrum initially obtained with that expected of the fully proteated sample[3] indicates that the exchange of the some of the amide protons must be very rapid since the amide I and amide II regions of the spectrum obtained when the peptide is initially dissolved in CH_3OD are essentially similar to those of the solid films that are repeatedly dried from that solvent. At room temperature we find little evidence for further proton/deuteron exchange since the spectra remain essentially enchanged for several days. However, as shown in Figure 3, further H/D exchange is clearly accelerated at higher temperatures.[4] At 40 °C the process is still relatively slow (apparent half-life ≅2 days), and, as is evident from Figure 3, it is still incomplete after 4 days. It is thus clear that, on the basis of the exchangeability of the amide protons, methanololic solutions of P_{24} must contain two distinct populations of amide groups. Since these two populations obviously differ on the basis of their access to the solvent phase, their existence must be a reflection of either the stability of the peptide conformation or possibly the size and stability of any peptide aggregates which may exist in solution.

Exchange of the amide protons for deuterons was also reflected in subtle but significant changes in the contours of the amide I band. When initially dissolved in CH_3OD, the amide I band maximum is observed near 1652 cm^{-1}. Although this frequency is somewhat lower than that obtained for the fully proteated solid film (≅1656 cm^{-1}) or the CH_3OH solution (≅1654 cm^{-1}), it is still in the range expected of amide I absorptions of α-helical proteins or polypeptides (Miyazawa & Blout, 1961). However, with the progress of the H/D exchange, we find that there is a broadening of the contours of the amide I band accompanied by a progressive shift in the band maximum toward lower frequencies. Using a combination of Fourier deconvolution and curve-fitting analysis, we find that the observed changes are the result the appearance of a new band near 1644 cm^{-1} (see Figure 3). As shown in Figure 3, this band is absent from the spectrum of the fully proteated solid and is relatively small in the spectrum of the CH_3OD solutions initially prepared. However, with the progress of the amide proton exchange, it progressively grows in intensity at the obvious expense of the higher frequency band near 1654 cm^{-1}. The appearance of the band near 1644 cm^{-1} is attributable to the N-deuteration of the peptide, which in turn results in the growth of a population of deuterium-bonded amide carbonyl groups.[5] The shift to lower frequencies occurs because deuterium-bonded carbonyl groups tend to absorb infrared radiation at lower frequencies than do the hydrogen-bonded analogues [see Pinchas and Laulicht (1971) and Dwivedi and Krimm (1982a,b) and references cited therein]. The observed 8-cm^{-1} shift in the absorption maximum of the amide I band of P_{24} is comparable to that observed upon N-deuteration of the α-helical domains of other proteins and polypeptides (Chirgadze & Brazhnikov 1974; Rabolt et al., 1977) and has been theoretically predicted by normal mode calculations (Dwivedi & Krimm, 1984).

The fact that a significant increase in the rate and extent of amide proton exchange occurs in hot methanol made possible the design of a methodology to prepare an extensively deuterium-exchanged sample of P_{24} (see Materials and Methods). A spectrum of a dry film of the sample so prepared is shown in Figure 1, and the frequencies and assignments of the main absorption bands are listed in Table I. The fact that extensive H/D exchange has occurred is made evident by the virtual disappearance of the N–H stretching band (i.e., amide A) near 3300 cm^{-1} and its replacement with the N–D stretching counterpart near 2540 cm^{-1}, the marked changes in the contours of the amide I band near 1650 cm^{-1}, as well as the marked reduction in the intensity amide II band near 1543 cm^{-1} and the appearance of the amide II′ band[6] near 1450 cm^{-1}. However, a close inspection of the infrared spectrum also indicates that relatively small amide A and amide II absorption bands still persist. Moreover, from a Fourier

[2] In the spectrum of CH_3OD solutions of P_{24}, strong solvent absorptions mask the amide A, amide II′, and amide III regions of the spectrum. Spectra of the amide I and amide II regions could be obtained by subtraction of the contributions of the solvent and background water vapor present in the purge gas of the instrument.

[3] For a spectrum of the fully proteated sample, we used that of the CH_3OH-dried film. Spectra of P_{24} in CH_3OH solution were of very poor quality because of high background solvent absorptions. From such spectra, we determined that the maxima for the amide I and amide II bands occur near 1654 and 1543 cm^{-1}, respectively. The spectra were not of sufficiently good quality to enable any Fourier deconvolution or curve-fitting analysis of the band contours.

[4] The proton/deuteron exchange experiments were done at 40 °C. Although we believe that the process would probably have been faster and more extensive at higher temperatures, such studies were impractical with methanolic solutions because of rapid and extensive solvent losses by leakage and evaporation under our experimental conditions. Solvent losses at temperatures near 40 °C were sufficiently slow to enable use of the sample for 3–4 days.

[5] The amide I absorption band arises predominantly from the C=O stretching vibrations of the amide carbonyl group. In α-helical proteins or peptides this band is hydrogen bonded to an amide hydrogen.

[6] The vibrational motions responsible for the amide II band are very complex and involve the interaction of both N–H bending and C–N stretching vibrations (Miyazawa et al., 1958). N-deuteration of the amide bond uncouples this interaction, and this results in a disappearance of the amide II absorption band (≅1530–1550 cm^{-1}) and its replacement with the amide II′ absorption band near 1450 cm^{-1}.

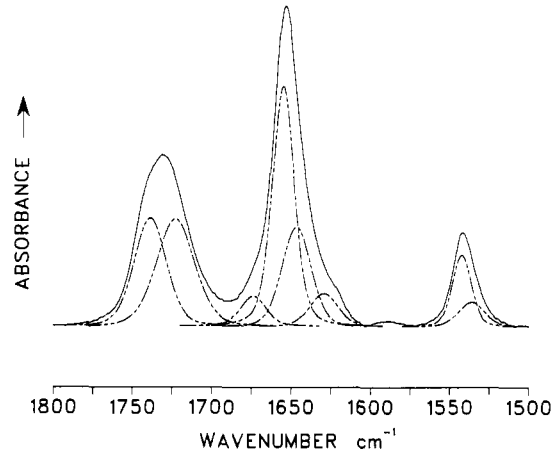

FIGURE 4: 1800–1500-cm^{-1} region of the infrared spectrum of a hydrated P_{24}/DPPC mixture. The spectrum is shown in the absorbance mode with the solid lines representing the data obtained and the dashed lines representing the component bands resolved by a combination of Fourier deconvolution and curve-fitting techniques.

deconvolution and curve-fitting analysis, we estimate that the amide I band of the sample contains a small absorption peak (≅1654 cm^{-1}) which accounts for some 20% of its total integrated intensity (see Figure 1). Since this band is probably attributable to the amide I absorption of hydrogen-bonded peptide carbonyl groups, it would appear that despite the relative severity of the exchange procedure, small populations of unexchanged amide protons evidently remain. The fact that complete exchange of the amide protons is so difficult is probably an indication of the extraordinary stability of the peptide conformation under the given experimental conditions.

Infrared spectra of hydrated P_{24}/DPPC mixtures were also examined. In this case it was necessary to disperse the lipid/peptide mixtures in D_2O,[7] and as a result useable data could only be obtained from the 1500–1800-cm^{-1} region of the spectrum because of strong solvent absorption in other areas of interest. The spectra obtained (see Figure 4) contained bands arising from the lipid C=O stretching vibration (≅1735 cm^{-1}) as well as the amide I and amide II vibrations of the peptide. Irrespective of the temperature and the phase state of the lipid, Fourier deconvolution and curve-fitting analyses of the C=O stretching band of the lipid indicate that it is a composite of two bands of comparable integrated intensities with maxima near 1743 and 1728 cm^{-1}. These two bands have been observed in the infrared spectra of virtually all hydrated diacyl-PCs studied so far, and their frequencies and relative intensities are fairly insensitive to the typical thermotropic gel/liquid-crystalline phase transition of these lipid bilayers [see Mendelsohn and Mantsch (1986) and references cited therein]. It is currently proposed that these two component bands arise from instantaneous populations of hydrogen-bonded and non-hydrogen-bonded ester carbonyl groups (Blume et al., 1988). We find that, irrespective of the temperature, peptide concentration, or the phase state of the lipid, the incorporation of P_{24} into these lipid bilayers has no identifiable effect on the contours of the lipid C=O stretching band or on the properties of its component bands. This observation suggests that P_{24} probably does not interact significantly with the polar/apolar interfacial regions of PC bilayers. We also find that once the PC/P_{24} mixture is dispersed in D_2O, the contours of the amide I and amide II bands are very similar to those of freshly prepared CH_3OD solutions of the peptide. Under those conditions, the amide I band can be resolved into components with maxima near 1675, 1656, 1644, and 1627 cm^{-1} with the bands arising from the non-α-helical domains (i.e., those near 1675 and 1627 cm^{-1}) accounting for some 20% of the total integrated intensity. Moreover, irrespective of whether the starting peptide sample was initially dried from CH_3OH or CD_3OD, the amide I absorption band arising from the α-helical domains of the peptide consists of components attributable to hydrogen-bonded (≅1654 cm^{-1}) and deuterium-bonded (≅1644 cm^{-1}) populations, of which the former is the dominant. Thus, like the CH_3OD-solubilized sample, the peptide dispersed in hydrated lipid bilayers evidently contains two populations of amide protons, one of which must be easily exchangeable and readily accessible to the bulk solvent phase. However, unlike the CH_3OD-dissolved sample, H/D exchange of the slowly exchangeable population of amide protons does not take place once the peptide is dispersed in PC bilayers. Thus, for example, we find that the relative intensity of the amide II band remains unchanged when a lipid-dispersed sample is held at temperatures near 90 °C for several hours, and during that time there were no further changes in the contours of the amide I band. The presence of this seemingly unexchangeable population of amide protons has important implications regarding the stability of the peptide conformation in the lipid bilayer and will be discussed below.

DISCUSSION

This work demonstrates the versatility of infrared spectroscopy for studying the secondary structure of peptides and proteins. The secondary structure of such molecules is usually obtained from an analysis of the contours of the conformationally sensitive amide I band which occurs between 1600 and 1700 cm^{-1} [see Surewicz and Mantsch (1988) and references cited therein]. In this study of P_{24}, such an analysis indicates that 80% of the integrated intensity of the amide I band can be assigned to the α-helical domain(s) of the peptide. Moreover, given that the intrinsic absorptivity of the amide I band of protein or peptide α-helices tends to be lower that of proteins or peptides in an extended conformation [see Chirgadze et al. (1973) and Chirgadze and Brazhnikov (1974)], one can conclude that at least 80% of the amino acid residues of this peptide must be arranged in an α-helical conformation. Thus, when viewed from the perspective of the number of amino acid residues involved, our data suggest that the α-helical domain of P_{24} incorporates a minimum of 24 of its 30 amino acid residues. Consequently, since poly(L-leucine) has a very strong α-helix-forming potential (Alter et al., 1972), this suggests that the 24 leucine residues which constitute the hydrophobic core of P_{24} must be included in its α-helical domain. The possibility that the α-helical domains of this class of peptides incorporates the entire polyleucine core was also deduced by an examination of the infrared spectra of the P_{16} and P_{20} homologues of the peptide P_{24} (unpublished experiments from this laboratory). Such conclusions are compatible with previous CD data, which suggest that P_{24} may well be up to 90% α-helical under our experimental conditions (Davis et al., 1983).

The conclusion that P_{24} should be predominantly α-helical under our experimental conditions is also supported by an examination of the amide II band of the peptide. The fact that one could obtain such conformational information from the amide II band of a protein or peptide has been theoretically

[7] The use H_2O was precluded by the fact that solvent absorption in the amide A, amide I, and amide II regions of the infrared spectrum is very strong. D_2O absorption in the amide I and amide II regions is also significant but considerably weaker than is the case with H_2O.

predicted by normal mode calculations (Krimm, 1983; Dwivedi & Krimm, 1982a,b, 1984) but is rarely exploited in practice. This is because transmission infrared spectroscopic studies of proteins and peptides under biologically relevant conditions almost always require that the spectra be acquired in D_2O, since water absorbs very strongly in this region of the infrared spectrum. Unfortunately, the H/D exchange which occurs in D_2O solution causes the disappearance of the amide II band and the conformational information which it contains. Although in principle one should be able to obtain comparable information from the amide II′ band of the deuterated peptide, this proves to be impractical because of greater solvent absorption in the amide II′ region of the infrared spectrum (1400–1500 cm^{-1}) and the overlap of the amide II′ band with a number of other bands arising from the side-chain vibrational modes. However, as illustrated in our studies of P_{24}, it is feasible to observe the amide II bands arising from domains of the protein (or peptide) which are not easily accessible to the bulk solvent phase. Under such conditions, one may be able to obtain conformational data specific to those domains of the protein (or peptide) which are shielded from the bulk solvent, and this may make it easier to analyze and interpret the data obtainable from a study of the conformationally sensitive amide I region of the spectrum. Such studies of P_{24} suggest that the amino acid residues which are buried in the hydrophobic domain of the bilayer are virtually inaccessible to the bulk solvent phase, and this may also be true of the transmembrane α-helices of many integral membrane proteins. The ability to obtain conformational information from the amide II bands may also prove to be useful in those instances where the contours of the amide I band of the protein are influenced by overlapping contributions arising from the polar headgroups of lipids such as PS or the amide I vibrations of the interfacial regions of lipids such as sphingomyelin.

The H/D exchange experiments provided useful insights into the conformational stability of the peptide. These experiments exploited the fact that we were able to observe changes in both the amide II and amide I bands of the peptide, thus obtaining semiquantitative information about the extent of H/D exchange and conformational information about the parts of the peptide molecule where such exchange occurred, respectively. This aspect of infrared spectroscopy has been previously been used in studies of rhodopsin (Englander et al., 1982; Downer et al., 1986) and the *Acholeplasma laidlawii* B membrane proteins (Mantsch et al., 1988). From such studies, we determined that there are two populations of amide protons which exchange with bulk solvent protons at markedly different rates. The readily exchangeable population is evidently the more accessible to the bulk solvent phase, and, whether dissolved in methanol or dispersed in hydrated lipid bilayers, it accounts for some 40% of the integrated intensity of the amide II band near 1543 cm^{-1}. Thus, considering that the intensities of amide II bands arising from peptides in extended conformations tend to be somewhat greater than that of peptides in a α-helical conformation (Chirgadze et al., 1973; Chirgadze & Brazhnikov, 1974), we suggest that the fast-exchanging population of amide protons arises from no more than 12 of the 30 amino acid residues of the peptide. Moreover, from the changes in the contours of the amide I band, we also suggest that these residues arise primarily but not exclusively from the extended, non-α-helical region of the peptide molecule.

The H/D exchange experiments on the peptide dispersed in a hydrated lipid bilayer indicate that the fast-exchanging population of amide protons must arise from amino acid residues that are located near to the bilayer surface. Thus, from an examination of the amino acid sequence of P_{24} and its probable orientation in the lipid bilayer [see Huschilt et al. (1989)], we conclude that the amino acid residues which give rise to the fast-exchanging population of amide protons should consist of the N- and C-terminal polar residues and a few of the adjacent leucine residues which form the polyleucine core. Given this, it also follows that the slowly exchanging population of amide protons must have arisen from regions of the α-helical polyleucine core that are deeply buried in the lipid bilayer. Such conclusions are compatible with the results of amide hydrogen exchange studies of the hydrophobic intramembrane segments of glycophorin (Sami & Dempsey, 1988) and the hydrophobic domains of some membrane proteins (Englander et al., 1982; Downer et al., 1986; Henry et al., 1987) and are consistent with the observation that with P_{24} the slowly exchanging population of amide protons appears to be virtually unexchangeable in lipid bilayer dispersions. Presumably the H/D exchange occurring in the α-helical domain of P_{24} is restricted to the end regions of α-helix because they are closer to the bilayer surface. However, the greater propensity for H/D exchange in the end regions of the P_{24} α-helix may also be a function of the reduced stability of the α-helical conformation in those regions. It is expected that the hydrogen bonds between amino acid residues located near the middle of an α-helix should be significantly stronger than those between residues located at or near to the ends of an α-helical segment (Nakanishi et al., 1972).

In the case of the methanolic solutions, however, the interpretation of the results of the H/D exchange experiments is somewhat more complicated. As is the case with the samples dispersed in lipid bilayers, there are two populations of amide protons which exchange with the bulk solvent protons at markedly different rates. Of these, one can probably attribute the fast-exchanging population to amino acyl residues near to the ends of the α-helical region, and the slowly exchanging population to the amide protons located in the middle of the peptide α-helix, because the conformational stability of the midregion of the α-helix should be greater than that found nearer to its ends (Nakanishi et al., 1972). Indeed, our inability to completely exchange all of the amide protons of the peptide under harsher conditions is probably attributable to the considerable conformation stability in the inner regions of the peptide α-helix. However, unlike the lipid-dispersed peptide samples, H/D exchange of the slowly exchanging population of amide protons of P_{24} is observable in methanolic solution at elevated temperatures. From the FTIR spectroscopic data (this work) and previous CD studies (Davis et al., 1983), it seems unlikely that this is the result of any major unfolding of the peptide when it is dissolved in methanol, because the α-helical conformation of this peptide is very stable and is maintained under all of our experimental conditions. However, the CD studies have also shown that thermally induced decreases in the fractional helicity of the peptide, though fairly small, are greater when it is dissolved in methanol than when it is dispersed in hydrated lipid bilayers or solubilized with aqueous detergents (Davis et al., 1983). Thus, although we do not envisage any major unfolding of the peptide in methanolic solution, it is possible that the frequency and/or amplitude of hydrogen-bond-making/breaking thermal fluctuations of the peptide α-helix may be significantly greater in that medium.

The other feature which clearly differentiates the hydrated bilayer dispersion from the methanolic solution is the dif-

ferential accessibility of the solvent phase to the H/D exchange sites. In principle, this result could also be due to differences in the aggregation state of the peptide in the two media. However, we believe this to be unlikely under our experimental conditions for the following reasons. From the results of DSC studies, it is estimated that when low concentrations of P_{24} are dispersed in PC bilayers, each peptide molecule perturbs the behavior of no more than 25 lipid molecules (Zhang et al., 1992), and since these estimates compare favorably with estimates of the minimum number of lipids required to form a "two-dimensional solvation" layer around a single molecule of the peptide [$\simeq$18 molecules; see Morrow et al. (1985)], it seems likely that P_{24} exists primarily as single-stranded helices in lipid bilayers. Also, high-performance size-exclusion chromatography of this peptide suggests that P_{24} exists primarily as monomeric species in methanolic solution (unpublished results from this laboratory). Thus, given that the P_{24} probably exists as single-stranded helices in hydrated lipid bilayers and in methanolic solution, one can thus reduce considerations of solvent accessibility to the H/D exchange sites to the fact that in methanolic solution the solvent is not as shielded from the H/D exchange sites as is the case with hydrated lipid bilayers. Although PC bilayer membranes are very permeable to water, the steady-state concentrations of water molecules in the hydrophobic core of the lipid bilayer are small (Griffith et al., 1974; Worchester & Franks, 1976). Thus one would expect that in methanolic solution the concentration of labile hydrogens in the vicinity of the exchange sites should greatly exceed that present in lipid bilayer membranes. Given this, mass action effects alone could account for the slower exchange rates which occur in hydrated lipid bilayers, even if the thermal fluctuations at the exchange sites are conducive to the exchange process. At this time there is no easy means of quantifying the effects of such factors on our experimental observations, and it may well be that the differences in the amide-hydrogen exchange rates observed in methanol and in hydrated lipid bilayers are the result of significant contributions from both differences in the hydrogen-bond-making/breaking thermal fluctuations accessible to the peptide and differences in the accessibility of the bulk solvent to the H/D exchange sites.

Finally, in these studies the capacity of infrared spectroscopy to study membrane proteins in situ has enabled us to determine that, when dispersed in hydrated lipid bilayers, the peptide P_{24} adopts a predominantly α-helical conformation which is stable to changes in temperature and the phase state of its host lipid bilayer. Moreover, through the use of infrared spectroscopy, we have been also able to infer that the α-helical domain of this peptide incoprorates its entire polyleucine core. This is a significant conclusion which utilized very small quantities of sample and could not have been easily obtained from studies using other physical techniques, except perhaps a solid-state NMR spectroscopic study of P_{24} in oriented lipid bilayers. Although this particular conclusion was not unexpected, its confirmation made possible better estimates of the hydrophobic length of the peptide and proved to be invaluable in the interpretation of our DSC studies [see Zhang et al. (1992)].

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