three symmetric hemins, 2,4-dimethyldeuterohemin (**9**), protohemin III (**10**), and protohemin XIII (**11**) (Figure 2 and Table II), the one that exhibits the largest differences in heme methyl contact shift patterns between bovine and rat cytochrome b_5 is protohemin III, the only one that places a vinyl at position c. A more datailed interpretation of the shifts must await the development of a quantitative basis for the interpretation of the hyperfine shifts.

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Topography of the Membrane-Binding Domain of Cytochrome b_5 in Lipids by Fourier-Transform Infrared Spectroscopy†

Peter W. Holloway* and Catherine Buchheit
Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908
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ABSTRACT: Fourier-transform infrared spectroscopy was used to examine the secondary structure of the membrane-binding domain (nonpolar peptide) of rabbit liver cytochrome b_5 in D_2O and in the presence of phospholipids and deoxycholate. In all situations, the predominant structure was α helix, but an examination of the components of the amide I band in the spectrum of the nonpolar peptide showed that the major peak was shifted from 1655 cm^{-1} in the lipids to 1650 cm^{-1} in deoxycholate. This shift to lower frequency, together with a decrease in intensity of the amide II band, is indicative of N–H to N–D exchange of the peptide backbone. A semiquantitative analysis indicated that the α helix of the peptide is over 95% exchanged in the presence of deoxycholate but is only 10% exchanged in the presence of lipid. These data suggest that the membrane-inserted portion of the peptide is α helical and is largely protected from N–H to N–D exchange by the bilayer. We suggest that this technique appears to provide a general method for determining the type of secondary structure involved in membrane interaction and the percentage of this structure which is involved in the interaction.

Cytochrome b_5, an amphipathic integral membrane protein found in the endoplasmic reticulum, has been a popular subject for model membrane studies. The protein has two distinct domains: a hydrophilic heme-containing domain and a hydrophobic membrane-binding domain (nonpolar peptide, NPP).[1] The structure of the former, in isolation, has been

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determined by X-ray crystallography (Mathews et al. 1971), but information on the structure of the latter has had to depend on low-resolution techniques: circular dichroism (Visser et al., 1975; Dailey & Strittmatter, 1978) and, more recently, FT-IR (Holloway & Mantsch, 1989a). The predominant, unanswered, question is the topography of the membrane-binding domain in the membrane. Fluorescent quenching by depth-dependent probes has attempted to locate the Trp in this domain in the depth of the membrane, but this tells little about the penetration of the domain as a whole in the bilayer (Fleming et al., 1979; Kleinfeld & Lukacovic, 1985; Markello et al., 1985). This problem is not unique to cytochrome b_5, there is a need for a technique which would determine the type of secondary structure in a membrane-inserted domain of a protein and also, if possible, the amount of insertion of the domain in the bilayer. During our prior FT-IR studies of cytochrome b_5 (Holloway & Mantsch, 1989b), considerable effort was taken to validate the utility of obtaining spectra in both D_2O and H_2O as an aide to assigning particular spectral bands to specific secondary structure. It was found with the polar domain that a rather uniform shift to lower frequency of 5 cm^{-1} occurred for each band, characteristic of a particular type of secondary structure, in changing from H_2O to D_2O. In the present study, aimed at determining the secondary structure of the hydrophobic domain of cytochrome b_5 in lipids, use has been made of this shift to estimate the amount of a particular secondary structure which cannot undergo exchange in changing from H_2O to D_2O. This "unexchangeable" peptide backbone is suggested to be protected from exchange by being buried in the membrane, and this technique appears to provide a method for determining the type of secondary structure involved in membrane interaction and the percentage of this structure which is involved in the interaction.

Materials and Methods

Cytochrome b_5 and the NPP were isolated as described previously (Holloway & Mantsch, 1989a). Lipids were obtained from Avanti Polar Lipids Inc., Birmingham, AL, and sodium deoxycholate was from Sigma, St. Louis, MO. Infrared spectra were recorded at 25 °C with a Nicolet 740 instrument with an MCTA detector. For each spectrum, 256 interferograms were collected, co-added, apodized with a Happ Genzel function, and Fourier-transformed to give a resolution of 2 cm^{-1}. Lipid vesicles, at approximately 100 mM, were prepared in 50 mM NaHEPES in D_2O (pH* 7.8) by sonication for 5–6 h in glass tubes sealed under argon in a bath-type sonicator. After phosphate analysis, they were adjusted to 50 mM lipid phosphorus with HEPES/D_2O buffer. Samples containing NPP (0.5 mM) were prepared by adding the buffer, lipid vesicles (50 mM), or DOC (50 mM) to a sample of lyophilized NPP, and the mixtures were then assembled between CaF_2 windows with a 50-μm Teflon spacer. Spectra were collected after 18 h at room temperature. A previous report has shown that the binding of NPP to lipid vesicles is complete in 30 min (Fleming & Strittmatter, 1978). The spectrum of NPP in H_2O was obtained by using a 1.5 mM solution of NPP and a 6-μm spacer.

Subtraction of the corresponding lipid spectrum from the lipid plus NPP was performed interactively using Spectra Calc (Galactic Industries Corp., Salem, NH), aiming for a flat base line between 1900 and 1800 cm^{-1}, and the resultant "difference spectra" were subjected to Fourier self-deconvolution with Spectra Calc. The Spectra Calc algorithm is based on that of Griffiths and Pariente (1986) and requires the input of two constants, γ and X, which define the shape of the exponential filter used to multiply the transformed spectrum in the Fourier domain. The form of the filter is $\exp(2\pi\gamma X)$, and X is the normalized spectral array values ranging from 0 to 1. Optimal deconvolution was obtained with a γ of 5.5 and an X of 0.2. These Spectra Calc parameters gave deconvolved spectra which were visually similar to spectra obtained by using the deconvolution procedure of Mantsch et al. (1986) using a Lorentzian of 22 cm^{-1} half-bandwidth and a resolution enhancement factor (k value) of 2.3. The deconvolved spectra were subjected to a curve-fitting routine with Spectra Calc, which uses an algorithm based on the Levenberg–Marquardt method (Press et al., 1989), with the constraints that peaks at 1655 and 1650 cm^{-1} had to be included in the fit. In addition, the peak positions obtained from the deconvolved spectra were used as fixed input values for curve fitting the original, undeconvolved, spectra.

Circular dichroism spectra were obtained on a Jasco J-500A at 25 °C in a 0.5-mm cell. The sample contained NPP (0.8 mg/mL) and DOC (50 mM) in D_2O-containing buffer, and from the spectrum was subtracted a spectrum of DOC in buffer. Fluorescence measurements were obtained on a Perkin-Elmer MPF44A using an excitation wavelength of 280 nm, and the emission at 338 nm was followed with time. One sample was an aliquot from an FT-IR study and contained NPP (0.5 mM) and POPC (50 mM) which had been kept at 25 °C for 24 h. The concentrated mixture (1.5 μL) was added to 10 mM HEPES buffer (1.5 mL), and after 1 min, a 2-fold excess of bromolipid (100 μM) [1-palmitoyl-2(6,7-dibromostearoyl)phosphatidylcholine] was added. A second sample of NPP, in the absence of POPC, was added to 10 mM HEPES buffer, and after 1 min, the same volume of bromolipid as before was added.

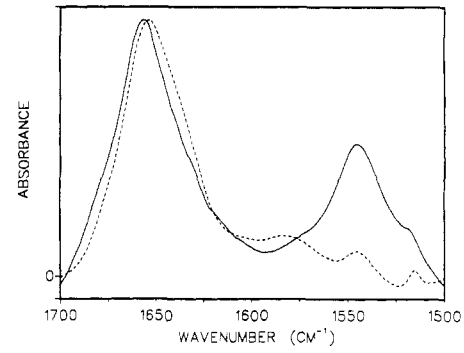


FIGURE 1: FT-IR spectra of the nonpolar peptide of cytochrome b_5 in H_2O and D_2O in the region of the amide I and amide II bands. Spectra of the nonpolar domain were obtained as described under Materials and Methods. The spectrum in H_2O-containing buffer was obtained with 1.5 mM nonpolar peptide using a 6-μm path-length cell (—), and the spectrum in D_2O-containing buffer was obtained with 0.5 mM peptide using a 50-μm path-length cell (- - -). The absorbance maxima of the bands at 1650 cm^{-1} are 0.05 for the sample in H_2O and 0.13 for the sample D_2O.

Results

The FT-IR spectra of the NPP in H_2O buffer and D_2O buffer are shown in Figure 1, and the former spectrum shows the amide I band between 1700 and 1600 cm^{-1}, due predominantly to the C=O stretching vibrations of the amide groups coupled to the in-plane NH bending and CN stretching modes,

[1] Abbreviations: NPP, nonpolar peptide, the hydrophobic membrane-binding domain of cytochrome b_5; FT-IR, Fourier-transform infrared spectroscopy; DOC, sodium deoxycholate; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; pH*, *pH meter reading in D_2O-containing buffers; the pD was not corrected* for the glass electrode reading error.

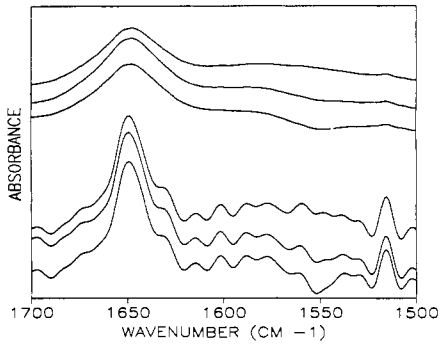


FIGURE 2: FT-IR spectra of the nonpolar peptide of cytochrome b_5 in the presence of deoxycholate: effect of over- and undersubtraction of solvent. The spectrum of 50 mM deoxycholate in D_2O-containing buffer was subtracted from a spectrum of nonpolar peptide (0.5 mM) in the same solvent, as described under Materials and Methods. The upper three curves are the resultant difference spectra: with 5% undersubtraction (top curve), optimal subtraction (middle curve), and 5% oversubtraction (lower curve). The lower three curves are the corresponding spectra after Fourier self-deconvolution.

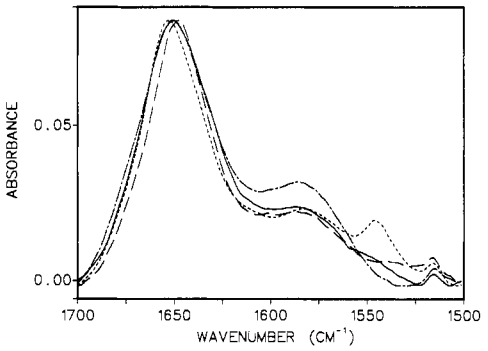


FIGURE 3: FT-IR spectra of the nonpolar peptide of cytochrome b_5 in the presence and absence of lipids and deoxycholate. The spectra of the nonpolar peptide (0.5 mM) were obtained in D_2O-containing buffer with or without 50 mM DMPC or deoxycholate. (—) In buffer alone; (- - -) with DMPC; (– –) with deoxycholate; (– - –) in buffer alone after exposure to CD_3OD.

and the amide II band between 1600 and 1500 cm^{-1}, due to N–H in-plane bending and CN stretching. As can be seen, in changing the solvent from H_2O to D_2O, there is a shift of 4 cm^{-1} to lower frequency of the amide I band and a large decrease in the relative intensity of the amide II band. These changes are due to the N–H to N–D exchange of the hydrogens of the peptide bond.

FT-IR spectra are obtained in a single-beam mode, and spectra such as those shown in Figure 1 are the result of subtracting the spectra of solvent from solvent plus NPP in an "interactive" fashion. The aim of such a subtraction is to obtain a flat base line in the 1900–1800 cm^{-1} region. It has previously been shown (Holloway & Mantsch, 1989b) that deliberate under- or oversubtraction even of the H_2O band at 1640 cm^{-1} does not overly perturb the shape of the amide I or amide II bands but in the present series of experiments there is the additional complication of the amphiphile absorbance. Although no bands in the spectrum of DMPC are in the amide I or amide II region, DOC alone shows a large broad band at 1510 cm^{-1} due to the antisymmetric stretching mode of the COO^- of the DOC. To ensure that over- or undersubtraction of the carboxylate band of the DOC does not influence the shape or position of the amide I band, the subtraction of the spectrum of DOC in buffer from that of the NPP in DOC and buffer was first performed to give the optimal base line in the 1900–1800 cm^{-1} region, and the subtraction was then repeated to give a deliberate over- and undersubtraction of the DOC-

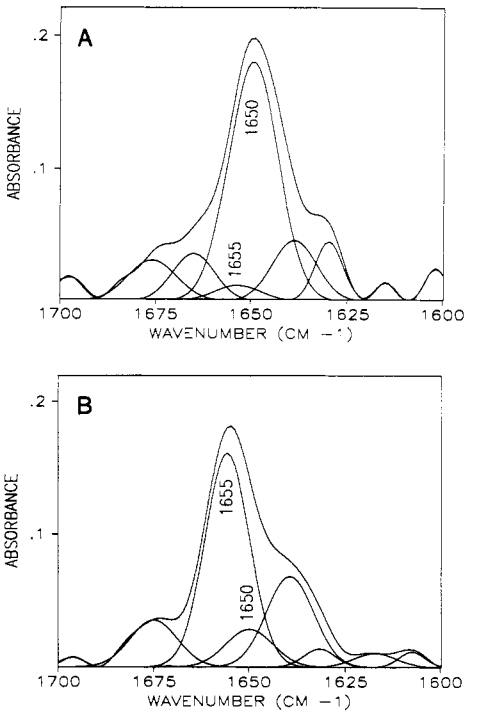


FIGURE 4: Curve fitting to the deconvolved spectra of the nonpolar peptide of cytochrome b_5 in the presence of deoxycholate or DMPC. The spectra of the nonpolar peptide (0.5 mM) were obtained in the presence of deoxycholate or DMPC and were subjected to Fourier self-deconvolution as described under Materials and Methods. The resultant spectra were then subjected to a standard curve-fitting routine with the constraints of including bands at 1655 and 1650 cm^{-1}, which are marked in the two spectra. (A) Sample in the presence of deoxycholate. (B) Sample in the presence of DMPC.

containing buffer by 5%. In Figure 2 are shown these spectra of the NPP in the presence of DOC, after subtraction of the DOC and buffer absorbances, together with the corresponding deconvolved spectra. It can be seen that in all the original spectra the amide I peak is centered at the same frequency, approximately 1650 cm^{-1}, and that the major band in the deconvolved spectra is also not displaced by the various degrees of subtraction. Figure 3 shows a comparison of the spectra of the NPP in D_2O, DOC, and DMPC. The amide I band of the NPP in D_2O is quite broad with a width at half-height of 47 cm^{-1} while in the amphiphiles it is much sharper with a width of only 40 cm^{-1}. It can also be seen that the amide I band is at 1653 cm^{-1} in DMPC and at 1648 cm^{-1} in DOC, compared to its position of 1651 cm^{-1} in D_2O. These differences in frequency of the amide I band are consistent with the peptide bond hydrogen atoms being less exchanged with solvent when the NPP is bound to DMPC than when it is bound to DOC. This conclusion is supported by the intensity of the amide II band which decreases in the order DMPC > D_2O > DOC. That these changes in the amide I and amide II bands are due to N–H to N–D exchange came from examination of the spectrum of NPP which had been dissolved in CD_3OD, heated to 50 °C, and evaporated to dryness prior to being dissolved in D_2O. This latter spectrum, also shown in Figure 3, has a very broad amide I band with a width of 53 cm^{-1}, but shows no evidence of an amide II band.

Previous infrared studies of the polar domain of cytochrome b_5 (Holloway & Mantsch, 1989a) had shown that the seven discrete bands which make up the deconvolved amide I profile are each shifted 5 cm^{-1} to lower frequencies as the solvent is changed from H_2O to D_2O; the band ascribed to α helix moves from 1655 to 1650 cm^{-1}. The same study showed that the NPP was predominently α helix when in H_2O or D_2O. In

Table I: Areas of α-Helical Amide I Bands and Amide II Bands of Nonpolar Peptide[a]

conditions	% at 1655 cm^{-1}		% at 1650 cm^{-1}		% total helix[b]		% as N–H[c]		amide II area[d]
	D[e]	U	D	U	D	U	D	U	
in D_2O	37	41	7	2	44	43	84	95	0.020
in DMPC	52	54	9	4	61	58	85	93	0.053
in POPC	46	45	7	7	53	52	87	87	0.040
in DOC	2	1	53	51	55	52	4	2	0.009
after CD_3OD in D_2O	8	5	41	37	49	42	16	12	0

[a] The FT-IR spectra of the NPP were processed by subtraction of the solvent, Fourier self-deconvolution, and curve fitting, as described under Materials and Methods, to give the relative areas of the bands making up the amide I envelope in the deconvolved (D) and undeconvolved (U) spectra. The percentages of this area found in the bands at 1655 and 1650 cm^{-1} are shown. [b] The total area in the 1655 and 1650 cm^{-1} peaks is considered to be the total α helix in the sample. [c] The ratio of the percentage found in the 1655 cm^{-1} band to the total in the 1655 and 1650 cm^{-1} bands is taken as unexchanged (buried in the bilayer) α-helical peptide N–H. [d] The areas of the amide II bands were normalized by setting the lowest area, that for the NPP which had been exposed to CD_3OD, to zero and then dividing these areas by the area of the corresponding amide I band. The area of the amide I band was determined between 1690 and 1610 cm^{-1}, and the amide II between 1555 and 1530 cm^{-1}. [e] The percentage peak areas shown are from fitting the deconvolved (D) or undeconvolved (U) spectra as described under Materials and Methods.

order to quantitate the changes seen in the amide I bands in the spectra of the NPP in different situations, the deconvolved spectra were subjected to a curve-fitting routine with the constraint that peaks at 1655 and 1650 cm^{-1} had to be included in the fit. The band at 1655 cm^{-1} would be indicative of unexchanged (shielded) α helix, the band at 1650 cm^{-1} of exchanged (exposed) α helix, and the sum a measure of the percentage of the secondary structure of the NPP which is in α helix. In this quantitation, it is assumed that the absorptivities of the amide I bands due to different types of secondary structure are equal and these absorptivities do not change upon N–H to N–D exchange. Examples of these fits for NPP in DOC and in DMPC are shown in Figure 4. In addition, an alternative procedure was used to quantitate the changes seen in secondary structure. In this procedure, the peak positions, as determined by deconvolution, were used as input parameters for curve fitting of the original, undeconvolved, spectra. The disadvantage of the latter procedure is that the relatively featureless amide I envelope may be fit adequately by many different combinations of peaks if complete freedom is allowed for optimization of height and width. In this study, an initial trial with each spectrum indicated that, when moderately good fits were obtained, the majority of the fitted peaks were between 25 and 30 cm^{-1} half-bandwidth. As a preliminary procedure to the subsequent optimal fitting procedure, the program was constrained to allow optimization of the fit by only varying the height of each peak: the width, the fractional contribution of Lorentzian to each peak, and the position were held constant. Values for width and fractional Lorentzian contribution were originally set at 28 cm^{-1} and 0.5, respectively. After this preliminary procedure (300 rounds of iteration), the values obtained were used as input for a final round of fitting with only the centers of the bands being fixed, at those positions obtained from examination of the deconvolved spectra. In Table I are compared these values (columns headed "U") with those values obtained from fitting the deconvolved spectra (columns headed "D"). It can be seen that there is quite good agreement between the two sets of data.

It was of concern that curve fitting the relatively featureless undeconvolved spectra, while mathematically more correct, could be subject to bias by input of expected peak heights of the bands at 1655 and 1650 cm^{-1}. Accordingly, the spectra of NPP in DOC and NPP in DMPC were subjected to the above-described procedure except that the initial values entered into the preliminary procedure for peak heights of the 1655 and 1650 cm^{-1} bands were equal, rather than entering a large peak at 1655 cm^{-1} for the spectrum with DMPC and a large peak at 1650 cm^{-1} for the spectrum in DOC. Although the final areas obtained were not identical with those shown in

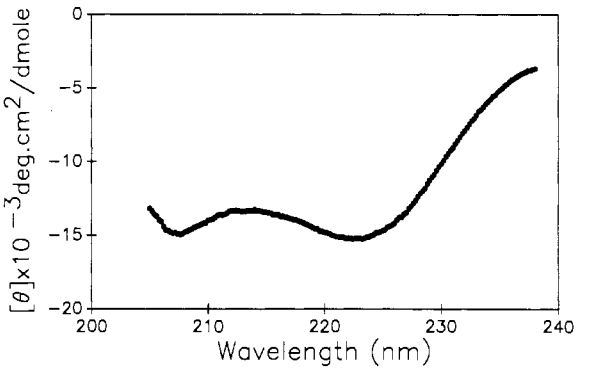


FIGURE 5: Circular dichroism spectrum of the nonpolar peptide of cytochrome b_5 in the presence of deoxycholate. The sample contained nonpolar peptide (0.8 mg/mL) and deoxycholate (50 mM) in D_2O-containing buffer, and spectra were obtained as described under Materials and Methods.

Table I, the program was able to approach the values obtained before. For the spectrum with DMPC, the percentage areas of the bands at 1655 and 1650 cm^{-1} were 46% and 15% (compared to 54% and 4% in Table I), and for the DOC-containing sample, the two areas were 9% and 42% (compared to 1% and 51%).

In Table I, taking the average values for the percentages found by using the deconvolved versus undeconvolved fits, it can be seen that the total α helix increases from 44% for the sample in D_2O to 60% in DMPC; the slightly lower value of 53% seen in POPC is probably not significantly different from the value found for NPP in DMPC. Of these percentages, the majority (87–89%) is in the band at 1655 cm^{-1} and so is "shielded" from solvent exchange by some mechanism. In DOC, although there is still approximately the same (54%) amount of α helix, only 3% of it is at 1655 cm^{-1} and is "shielded" from the solvent.

Confirmation of the assignment of the 1655 and 1650 cm^{-1} bands to α helix came from a circular dichroism analysis of NPP in DOC. Figure 5 shows a circular dichroism curve typical of a highly helical protein; by the standard algorithm of Chang et al. (1978), it is greater than 90% α helix. This value for α helix is higher than seen with the FT-IR and may be due to the much lower concentration of NPP which was used for the circular dichroism, or the latter may be influenced by the high content of aromatic residues in the NPP.

These comparisons of the NPP in the DOC micelle and the phospholipid bilayer suggest that the lack of exchange of approximately 90% of the α-helical amide N–H in the phospholipid-bound NPP is due to their being buried in the bilayer. A key assumption in this analysis is that the peaks at 1655

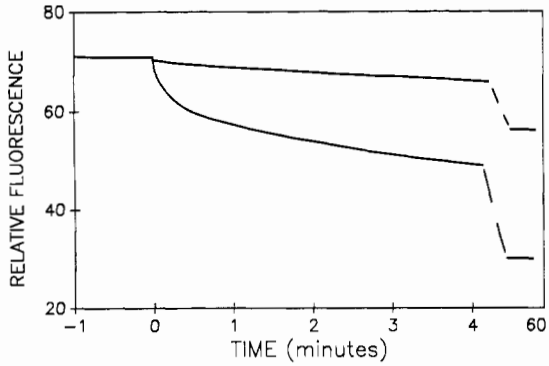


FIGURE 6: Time course of fluorescence change upon adding the nonpolar peptide of cytochrome b_5 to bromolipid vesicles. The fluorescence measurements were obtained as described under Materials and Methods. The bromolipid was added at time zero, and the upper curve is the sample of nonpolar peptide in the presence of POPC and the lower curve is the nonpolar peptide in buffer. The fluorescence was followed continuously for 4 min and then intermittently up to 60 min.

and 1650 cm^{-1} are due to unexchanged and exchanged α helix, respectively. To validate this assumption, the NPP was deliberately exposed to conditions which would favor exchange. After dissolution and heating in CD_3OD, the NPP, when dissolved in D_2O and examined by FT-IR, still contained 46% α helix, but with only 14% of the total α helix being in the unexchanged form.

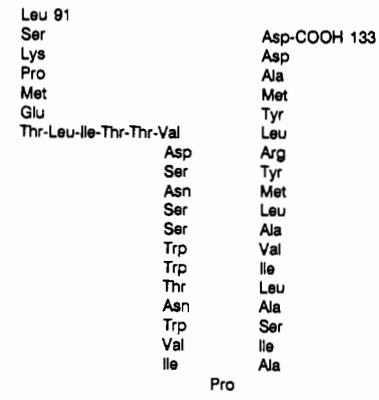
All these conclusions are made from a study of the amide I band, but the same general trends are seen with the amide II band, although no real quantitation can be made. As shown in Table I, the area under the curve between 1555 and 1530 cm^{-1}, taken as the position of the amide II band, decreases in the order DMPC > POPC > D_2O > DOC > CD_3OD. The amide II band is a measure of all the peptide N–H to N–D exchange, but if most of the other types of secondary structure are exchanged under all conditions, then this residual amide II does show a ranking identical with the 1655/(1655 + 1650) ratio. Because of the existence of the DOC carboxylate at 1551 cm^{-1}, the value of the area of the amide II peak in DOC is somewhat arbitrary, but as can be seen in Figure 2, deliberate undersubtraction does not give a spurious band which we are interpreting as amide II.

One problem with FT-IR studies is the relatively high concentrations of protein and lipid which are required to obtain the spectra, compared to, for example, the amounts used for fluorescence, and it was of concern that the NPP had not completely bound to the vesicles under the conditions of the experiment. The state of the NPP in the experiment with POPC was examined by fluorescence quenching by bromolipid (Leto et al., 1980). As shown in Figure 6, the fluorescence of the sample of NPP mixed with POPC which was used in the FT-IR studies did not decrease appreciably with time after addition of excess bromolipid (upper tracing). In contrast, the fluorescence of a sample of NPP in buffer alone decreased rapidly after bromolipid was added (lower tracing). This rapid quenching is the result of binding of the NPP to the bromolipid vesicles, and the lack of a rapid phase of quenching with the NPP–POPC mixture indicates that there is no appreciable amount of unbound NPP in this latter mixture. The rapid quenching seen when bromolipid is added to NPP also gives an indication of the rapidity of binding of the NPP to lipid vesicles. The half-time for the quenching process is approximately 4 min.

Discussion

Although numerous studies have been made of cytochrome b_5, there is still little consensus as to the conformation of the membrane-binding domain (Fleming et al., 1979; Takagaki et al., 1983; Gogol & Engelman, 1984; Markello et al., 1985; Kleinfeld & Lukacovic, 1985; Everett et al., 1986; Rzepecki et al., 1986; Arinc et al., 1987; Ozols, 1989). The situation is, in fact, made even more confusing by the existence of a "loose" and "tight" form of interaction with membranes (Enoch et al., 1979: Leto et al., 1980). As the membrane-binding domain contains Trp residues, several attempts have been made to locate the position of these in the depth of the membrane by fluorescence quenching techniques (Fleming et al., 1979; Markello et al., 1985; Kleinfeld & Lukacovic, 1985). Again, the data will be clouded by the existence of three closely spaced Trp residues in this domain with some debate as to whether all or only one of these are/is fluorescent. Scheme I shows a possible structure for the membrane-binding domain of rabbit cytochrome b_5, based on a very similar structure recently proposed by Ozols (1989) for the rat liver form. This "hairpin" structure is one of two structures originally proposed by Visser et al. (1975) and is supported by the recent experiments of Arinc et al. (1987) and Ozols (1989). The location of the Trp as shown is supported by the bromolipid quenching studies from this laboratory for the cytochrome in both "loose" and "tight" forms (Tennyson & Holloway, 1986).

Scheme I



The secondary structure of the membrane-binding domain has been examined by several techniques in both the intact protein and the isolated NPP. Two circular dichroism studies indicate the NPP is highly helical in buffer, detergent, and lipid (Visser et al., 1975; Dailey & Strittmatter, 1978), which is in agreement with the Chou-Fasman calculations. Our recent FT-IR studies are in agreement with the circular dichroism data, although the NPP was only examined by FT-IR in the aqueous (aggregated) form (Holloway & Mantsch, 1989a). The present study was aimed at using FT-IR to determine the secondary structure of the NPP in phospholipid bilayers.

The mid-infrared spectra of proteins is characterized by the amide I and amide II bands. The position of both of these bands is sensitive to the extent of deuteration of the peptide N–H, and the ratio of the areas of the amide I and amide II bands has been used extensively to follow N–H to N–D exchange (Hvidt & Nielsen, 1966). Numerous studies of globular proteins have shown that amide hydrogens involved in stable internal H-bonding exchange very slowly with half-times of up to many days (Wagner & Wuthrich, 1982). The process of exchange is subject to catalysis by both H_3O^+ and OH^- with the intermediate reacting with water to complete the hydrogen transfer (Englander et al., 1972). The exchange of bonds within the interior of a protein will depend on either the diffusion of these reactants through pores in the molecule or the local unfolding of the protein (Woodward et al., 1982; Englander & Kallenbach, 1984). The barriers to this exchange which are evident in globular proteins would also exist in

membrane-inserted peptides. Although water can diffuse freely through membranes its equilibrium concentration in the membrane will be low; likewise, the ionic species will be excluded, and the low dielectric of the membrane will tend to stabilize any internal peptide hydrogen bonds. These conclusions prompted us not only to attempt to elucidate the secondary structure of the NPP in lipids but also to use the possible lack of N–H exchangeability as a measure of peptide insertion in the membrane.

Our previous studies with the intact cytochrome b_5 (Holloway & Mantsch, 1989a), and the two domains which can be separated by trypsin cleavage, indicated that the NPP was predominantly α helical (43%) but in D_2O did not undergo complete N–H to N–D exchange even when kept in D_2O for 24 h. This lack of exchange of such a small peptide is probably due to the high aggregation state in aqueous media. In contrast, the polar heme-containing domain was shown to undergo complete N–H to N–D exchange in 1 h. When the amide I band of the polar domain in H_2O was subjected to Fourier self-deconvolution, it was found to be made up of seven distinct bands between 1700 and 1600 cm^{-1}; remarkably, the bands were exactly duplicated in the spectrum in D_2O, except that each band was shifted 5 cm^{-1} to lower frequencies, indicative of the complete N–H to N–D exchange (Holloway & Mantsch, 1989b). The band due to α helix was at 1655 cm^{-1} in H_2O and at 1650 cm^{-1} in D_2O, and we have used these assignments in the present report to assess the "exchangeability" of the α-helical N–H in the membrane-bound NPP.

Secondary structure assignments have been made by curve fitting either the amide I band or the deconvolved amide I band. In this study, we performed the curve-fitting procedure with both deconvolved and undeconvolved spectra with the constraint that bands at 1655 cm^{-1} (unexchanged α helix) and 1650 cm^{-1} (exchanged α helix) were forced to be included in the curve fitting. The results with the two procedures were very similar, and the average values from the two types of fitting will be used in the following discussion. From an evaluation of these data, a semiquantitative evaluation of the exposure, or lack of, of the NPP to solvent was obtained. This evaluation is subject to several assumptions: that peak areas of the fitted bands are proportional to the relative contribution of the different types of secondary structure; that is, the absorptivities of the bands are the same, that these absorptivities do not change as the peptide N–H is converted to N–D and that there is no structural change of the system in replacing H_2O by D_2O. The studies of Susi and Byler (1986), where the secondary structures of several proteins as determined from X-ray structures are compared with those from curve fitting of deconvolved FT-IR spectra of the samples in D_2O, suggest that the above assumptions are reasonable.

In D_2O alone, the (aggregated) NPP was 44% helix with approximately 90% of the helix unexchanged; in DMPC, it was 60% helix with 90% of the helix unexchanged; and in POPC, it was 53% helix with 87% of the helix unexchanged. The lack of exchange is not solely due to the presence of α helix as the NPP in DOC was also 54% α helix, but only 3% was unexchanged. It was also confirmed by circular dichroism that in DOC the NPP is largely α helical, as reported previously (Visser et al., 1975; Dailey & Strittmatter, 1978). That the changes in the deconvolved spectra, which we admittedly are forcing to include bands at 1655 and 1650 cm^{-1}, are the result of exchange was confirmed by exposing the NPP to deuterated methanol under conditions where extensive exchange should occur. The NPP, after exposure to deuterated methanol, evaporation, and dissolution in D_2O, still contained 46% α helix, but now only 14% of the helix was unexchanged. Finally, the intensity of the amide II band in the different situations decreased in approximately the same order as the amount of unexchanged α-helical N–H decreased, as assessed by the amide I changes.

The reason for the lack of N–H to N–D exchange in DMPC and POPC is most likely due to the reasons mentioned above: the bilayer would be a barrier to entry of solvent and catalytic ionic species. It is unlikely that the mere presence of α helix is sufficient to prevent exchange, as in DOC, where the NPP is still highly helical, almost complete exchange occurs. Instead, we consider that the fundamental differences between the bilayer and the micelle are sufficient to explain the exchange in the latter. DOC forms a micelle containing 4–13 monomers in aqueous solution, and Robinson and Tanford (1975) had previously shown that the NPP binds a total of 20–31 DOC molecules at equilibrium. The structure of the DOC micelle is not known, but it is certainly a much less "tight" structure than the phospholipid bilayer and is subject to a rapid desorption–absorption of the monomeric units on a rather rapid time scale because of the high critical micelle concentration of DOC (5 mM). Both of these properties of the DOC micelle would be expected to allow quite easy solvent entry to the interior of the NPP–DOC complex. In contrast to this rapid exchange, a recent NMR study of amide–hydrogen exchange of detergent-solubilized M13 coat protein found that residues approaching the hydrophobic region exchanged quite slowly, although the ones measured still had half-times of approximately 1 h (Henry et al., 1987). In our preliminary attempts to monitor the rate of exchange of NPP in DOC, we saw apparently complete exchange after 30 min. The slower exchange seen with the M13 coat protein may be a result of using the much more structured detergent sodium dodecyl sulfate; the M13 coat protein complex is reported to consist of a dimeric protein with approximately 85 sodium dodecyl sulfate molecules, which may be enough to provide a more "membrane-like" environment.

It was of concern that the lack of N–H to N–D exchange of the NPP when mixed with lipids may be due to a lack of binding of the highly aggregated NPP, although it had previously been reported that NPP is completely bound to lipid vesicles after 30 min (Fleming & Strittmatter, 1978). Proof that the NPP was bound to the vesicles came from fluorescence quenching experiments with bromolipid. The supposed POPC-bound NPP did not suffer a rapid decrease in fluorescence when mixed with bromolipid whereas NPP suspended in buffer underwent a rapid quenching when bromolipid was added. Our previous studies with cytochrome b_5 have shown that binding of cytochrome b_5 to vesicles is rapid, even when the initial slow step is dissociation of the octameric form of the cytochrome to the monomer, whereas the exchange of the protein between vesicles is approximately 100 times slower (Leto & Holloway, 1979; Leto et al., 1980). The relatively rapid rate of fluorescence quenching seen when NPP aggregate was added to bromolipid vesicles ($t_{1/2}$ = 4 min.) suggests that the dissociation of the NPP aggregate to individual molecules would be followed by a rapid binding to the lipid vesicles on too fast a time scale to allow exchange of the α-helical amide bonds. This has allowed us to trap the α helix in the unexchanged state.

The membrane-binding domain of cytochrome b_5 is thought to be in a "hairpin" structure in the membrane (Scheme I) (Ozols, 1989). Previous quenching studies of tryptophan fluorescence have variously placed the quenchable Trp at the

center of the bilayer (Fleming et al., 1979; Kleinfeld & Lukacovic, 1985) or 0.4 nm below the surface (Markello et al., 1985); the latter data are consistent with the structure shown in Scheme I, although there is debate as to which Trp is responsible for the fluorescence (Fleming et al., 1979; Markello et al., 1985). By our analysis, the total α helix, when the NPP is in a lipid environment, is 53–60%, and of this, 87–90% is buried in the membrane. This total buried NPP α helix, obtained directly from the value of the peak at 1655 cm^{-1}, is therefore 46–53%. The structure shown in Scheme I is in agreement with this analysis. If Asp-103 and Arg-127 are in the interface region of the bilayer, then residues 104–126 would be buried in the membrane; this latter segment accounts for 54% of the total peptide and by Chou–Fasman calculations is predicted to be α helix (Dailey & Strittmatter, 1978). The Pro residue will of course disrupt the α helix and so decrease the actual value to somewhat less than 54%, very close to our 46–53%. It is conceivable that further analysis of the spectra could indicate the percentage of other structures, turns for example, which are not subject to exchange, and these analyses are underway.

It is suggested in this report that FT-IR is a versatile technique for the study of membrane proteins. Not only can the amide I band give information on the secondary structure of the total protein but also the position of the resolved peaks of the amde I, and their shift upon addition of D_2O, together with the intensity of the amide II band, can give information on the amount of the protein buried in the membrane and the types of secondary structure involved in this interaction.

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