Fourier Transform Infrared Spectroscopy and Site-Directed Isotope Labeling as a Probe of Local Secondary Structure in the Transmembrane Domain of Phospholamban

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ABSTRACT Phospholamban is a 52-amino acid residue membrane protein that regulates Ca^{2+}-ATPase activity in the sarcoplasmic reticulum of cardiac muscle cells. The hydrophobic C-terminal 28 amino acid fragment of phospholamban (hPLB) anchors the protein in the membrane and may form part of a Ca^{2+}-selective ion channel. We have used polarized attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy along with site-directed isotope labeling to probe the local structure of hPLB. The frequency and dichroism of the amide I and II bands appearing at 1658 cm^{-1} and 1544 cm^{-1}, respectively, show that dehydrated and hydrated hPLB reconstituted into dimyristoylphosphatidylcholine bilayer membranes is predominantly α-helical and has a net transmembrane orientation. Specific local secondary structure of hPLB was probed by incorporating ^{13}C at two positions in the protein backbone. A small band seen near 1614 cm^{-1} is assigned to the amide I mode of the ^{13}C-labeled amide carbonyl group(s). The frequency and dichroism of this band indicate that residues 39 and 46 are α-helical, with an axial orientation that is approximately 30° relative to the membrane normal. Upon exposure to ^2H_2O (D_2O), 30% of the peptide amide groups in hPLB undergo a slow deuterium/hydrogen exchange. The remainder of the protein, including the peptide groups of Leu-39 and Leu-42, appear inaccessible to exchange, indicating that most of the hPLB fragment is embedded in the lipid bilayer. By extending spectroscopic characterization of PLB to include hydrated, deuterated as well as site-directed isotope-labeled hPLB films, our results strongly support models of PLB that predict the existence of an α-helical hydrophobic region spanning the membrane domain.

INTRODUCTION

Phospholamban (PLB) is a small 52-residue membrane protein (see sequence below) associated with regulation of Ca^{2+}-ATPase activity in the sarcoplasmic reticulum of cardiac muscle cells (Tada and Kadoma, 1989). In response to β-adrenergic stimulation, PLB is phosphorylated at two sites, Ser 16 and Thr 17, which both lie in the cytosolic domain (Wegener et al., 1989; Toyofuku et al., 1994). Evidence indicates that PLB functions either directly by regulating Ca^{2+}-ATPase activity through protein-protein interactions (Tada, 1992) and/or indirectly as a Ca^{2+}-selective channel (Kovacs et al., 1988). Recently, a mechanism has been proposed that focuses on changes in the interaction of the cytosolic domain of PLB with Ca^{2+}-ATPase (Arkin et al., 1995).

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          5    10    15    20    25
MDKVQYLTRSAIRRASTIEMPQQAR

       30    35   *40 *  45    50
QNLQNLFINFCLILICLLLICIIVMLL
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The functional form of PLB consists of five identical subunits that form a pentameric cluster. A model of PLB has been proposed, with each subunit consisting of two connected α-helical domains (Adams et al., 1995; Arkin et al., 1995). An hydrophilic N-terminal domain (residues 1–24) is predicted to extend into the cytoplasm, whereas the predominantly hydrophobic C-terminal sequence of phospholamban (hPLB) (residues 25–52) is believed to span the membrane domain and anchor the protein in the membrane (Simmerman et al., 1989; Arkin et al., 1994). An overall pentameric clustering of the hydrophobic region is postulated to form the membrane-embedded Ca^{2+} channel (Arkin et al., 1994) and has been modeled as a left-handed coiled-coil consisting of five helices (Arkin et al., 1995).

To further test this model, we have focused in this work on the hydrophobic C-terminal fragment of phospholamban (hPLB). For this purpose we have combined site-directed isotope labeling (SDIL) with polarized attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy and hydrogen/deuterium (H/D) exchange. As shown recently for several model polypeptides (Tadesse et al., 1991; Martinez et al., 1994), placement of a ^{13}C label in the peptide group of specific amino acid residues allows FTIR spectroscopy to probe the localized protein backbone structure. In the case of hPLB, we have placed ^{13}C labels near the middle of the amino acid sequence at Leu residues 39 and 42 (indicated by asterisks in sequence) by chemical synthesis. Our results indicate that the overall hPLB structure as well as the local region around positions 39 to 42 consists of a transmembrane-oriented α-helix, which is protected from H/D exchange. These findings support current models of PLB that predict an helical hydro-

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phobic region spanning the membrane domain. More generally, this work serves as a model for FTIR analysis of larger membrane proteins, such as bacteriorhodopsin, whose peptide backbone can be isotopically labeled at specific sites using genetic methods (Sonar et al., 1994, 1995; Ludlam et al., 1995).

MATERIALS AND METHODS

Preparation of hPLB membranes

Transmembrane domain peptide corresponding to human phospholamban residues 25–52 was synthesized using standard t-BOC solid-phase chemistry. Lyophilized peptides were purified on a perfusion (C4 equivalent) reverse-phase column (Perceptive Biosystems, Cambridge, MA), offering significant recovery yields as opposed to standard reverse-phase chromatography. The lyophilized peptides were dissolved in TFA (~4 mg/ml) and injected on the above column equilibrated in 95% H_2O, 2% acetonitrile, and 3% 2-propanol. Peptide elution was achieved by reducing the polarity of the eluent to a final concentration of the 38% acetonitrile and 57% 2-propanol. A constant 0.1% TFA was present in all of the solvents. Fractions containing peptides as detected by absorption were subsequently lyophilized. Peptide purity was assessed by amino acid analysis, mass spectrometry, and SDS-PAGE, indicating pentamerization of the peptides. Reconstitution of the peptides into liposomes was achieved by dissolving the lyophilized peptides in a solution of 20% octyl-β-glucoside (Sigma, St. Louis, MO). To that solution was added a solution containing 20 mg/ml dimyristoylphosphatidylcholine (DMPC) or perdeuterated DMPC (DMPC-d_{54}) (Avanti Polar Lipids, Alabaster, AL) and 10% octyl-β-glucoside. Vesicles were formed by exhaustive dialysis into a buffer containing 1 mM Tris:HCl, pH 7.4 (Sigma, St. Louis, MO). Lipid-to-protein ratios were on the order of 1:100 (molar).

ATR-FTIR measurements

Sample films for ATR-FTIR measurements were prepared according to methods previously reported (Marrero and Rothschild, 1987a,b; Braiman and Rothschild, 1988; Ludlam et al., 1995). Fifty microliters (~0.5 mg) of hPLB in a buffered solution (pH 7.0, 1 mM NaP_i, 1 mM KCl, 0.4 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 50 mM NaCl) was uniformly dried under a continuous stream of argon gas onto the surface of a germanium crystal internal reflection element (l = 50 mm; w = 20 mm; d = 2 mm). This procedure resulted in an hPLB film that remained tightly bound to the Ge crystal, even upon exposure to a flowing or injected solution (Marrero and Rothschild, 1987a,b). The hPLB sample was placed in an ATR sample cell (Harrick Scientific, Ossining, NY) at room temperature. Hydration or deuteration of hPLB was performed by injecting 1 ml of H_2O or D_2O, respectively, into the sample compartment of the ATR cell, which was sufficient to completely immerse the sample film.

Polarized ATR-FTIR spectra were recorded on a Nicolet 510P spectrometer (Nicolet Analytical, Madison, WI) equipped with a Brewster angle polarizer (Harrick Scientific). The polarizer was placed in the IR beam, and rotating the polarizer 90° allowed for spectra to be collected at orientations of the electric field perpendicular and parallel to the plane of incidence. All spectra were recorded by accumulating and co-adding a minimum of 1000 interferogram scans at a spectral resolution of 2 cm^{-1}.

Transmission FTIR measurements

Multilamellar samples for polarized transmission FTIR measurements were prepared by depositing 15 μl (~0.15 mg) of hPLB membranes onto an AgCl window either by the isopotential spin-dry method (Clark et al., 1980) or by slowly air-drying concentrated drops of membrane suspension. The former method results in multilamellar films of purple membrane that exhibit low mosaic spread (Clark et al., 1980). Polarized FTIR measurements were made using a KRS-5 wire-grid polarizer using methods previously described (Rothschild and Clark, 1979; Clark et al., 1980). Spectra were obtained at α_o = 0°, 38°, 45°, and 52° (where α_o refers to the tilt angle between the normal to the AgCl window and the direction of the incident light) of dehydrated films at room temperature. All spectra were recorded on a 60SX spectrometer (Nicolet Analytical) at a spectral resolution of 2 cm^{-1} and consisted of a minimum of 4000 scans.

Calculation of α-helix orientation from transmission FTIR and ATR-FTIR dichroism

The net α-helix orientation, defined by the tilt angle θ of the helix with respect to the membrane normal, was calculated from the measured dichroism using methods previously reported for polarized FTIR transmission measurements (Rothschild and Clark, 1979; Earnest et al., 1990) and polarized ATR-FTIR measurements (Frey and Tamm, 1991; Arkin et al., 1995). The dichroic ratio was defined as

$$R = \frac{A_\parallel}{A_\perp},$$

where $A_\parallel$ and $A_\perp$ are the absorption by the sample of infrared light polarized parallel and perpendicular, respectively, to the plane of incidence. In the ATR-FTIR case, the electric field amplitudes of the evanescent wave were determined by the following relations as given by (Harrick, 1967):

$$E_x = \frac{2\cos\gamma\sqrt{sin^2\gamma - n_{21}^2}}{\sqrt{(1-n_{21}^2)[(1+n_{21}^2)sin^2\gamma - n_{21}^2]}},$$

$$E_y = \frac{2\cos\gamma}{\sqrt{1-n_{21}^2}};$$

$$E_z = \frac{2\sin\gamma\cos\gamma}{\sqrt{(1-n_{21}^2)[(1+n_{21}^2)\sin^2\gamma - n_{21}^2]}},$$

where γ is the angle of incidence between the infrared beam and the internal reflection element (45°) and n_{21} is the ratio between the refractive indices of the sample (n_2 = 1.43) and the Ge internal reflection element (n_1 = 4.0). Here, the index of refraction of the DMPC-containing hPLB membranes was assumed to be 1.43, the same as that of pure DMPC (Wolfe and Zissis, 1978; Fringeli et al., 1989; Tamm and Tatulian, 1993). The order parameter S was subsequently determined by the following equation:

$$S = \frac{2(E_x^2 - R^{ATR}E_y^2 + E_z)}{(3\cos^2\alpha - 1)(E_x^2 - R^{ATR}E_y^2 - 2E_z^2)},$$

where the angle α between the transition dipole moment of the amide I mode and the α-helix axis was assumed to be 35°. The angle between the helix director and the normal of the Ge internal reflection element, θ, was then determined by the following relation (also from Harrick, 1967):

$$S = (3\langle\cos^2\theta\rangle - 1)/2.$$

This equation assumes that the membrane disorder relative to the substrate is minimal (i.e., the order parameter for sample disorder is 1). In addition, it should be noted that the calculated θ value can reflect a complex α-helix distribution, such as two different orientations within the same protein. In the case of ATR-FTIR, the semi-infinite bulk case approximation was used, because the average thickness of the deposited film (~20 μm) was larger than the penetration depth (~1.6 μm) (Harrick, 1967).

Several additional points should be noted. First, α values in the literature range from 29° to 40° (Miyazawa and Blout, 1961; Bradbury et al., 1962; Tsuboi, 1962). A higher value of α would have resulted in a lower tilt angle, θ. Second, the mosaic spread order parameter, which is a measure of the degree of orientation of the membrane relative to the sample plane, was

assumed to be 1 (i.e., no membrane disorder). This resulted in an overestimate of θ. Finally, for purposes of calculating the difference dichroism spectra as well as for presentation of ATR-FTIR spectra, all ATR-FTIR polarized spectra were interactively scaled by global baseline-correction and integration of the region between 1800 and 1000 cm^{-1}. This method of interactive scaling was verified by examining bands that exhibit low dichroism as revealed by polarized transmission FTIR (e.g., the 1450 cm−1 region of hPLB in perdeuterated DMPC). Importantly, the absolute values of $A_{\parallel}$ and $A_{\perp}$ (unscaled) were used in the calculation of R and, subsequently, θ.

RESULTS

The secondary structure of hPLB is predominantly α-helical

Fig. 1 shows the ATR-FTIR absorption spectra recorded using parallel polarization of hPLB and hPLB ^{13}C labeled at both the Leu 39 and Leu 42 amide carbonyl groups ([1-^{13}C]-L39,L42-hPLB). In addition to the intense ester carbonyl band at 1738 cm^{-1} arising from the DMPC ester carbonyl stretch mode and the CH_2 scissoring mode absorbing near 1468 cm^{-1} (Casal and Mantsch, 1984; Lewis et al., 1994), amide I and amide II modes with frequencies typical of α-helical structure appear at 1658 cm^{-1} and 1544 cm^{-1}, respectively (Parker, 1983; Byler and Susi, 1986; Haris et al., 1989; Cladera et al., 1992). A weak shoulder is also detected at 1677 cm^{-1}, which might reflect the presence of small amounts of β-turn structure (Bandekar and Krimm, 1979; Cladera et al., 1992). In contrast, the absence of subcomponent bands near 1680–1690 cm^{-1} and 1630–1640 cm^{-1} indicates that little β-structure is present in hPLB (Byler and Susi, 1986). Similar conclusions were reached in recent ATR-FTIR studies of hPLB that utilized curve fitting (Tatulian et al., 1995) and spectral deconvolution (Arkin et al., 1995).

Of special significance is the small band that appears near 1612 cm^{-1} in the spectra of [1-^{13}C]-L39-hPLB (single ^{13}C label) and 1614 cm^{-1} in [1-^{13}C]-L39,L42-hPLB (double ^{13}C label) (Fig. 1, *inset*). Because a band in this region is not detected in the spectrum of unlabeled hPLB, it is assigned to the isotope-induced downshifted amide I C=O stretching mode. This assignment is also supported by the fact that the intensity of this band increases by approximately 2 times between the single and double 1-^{13}C label. Normal mode calculations and model compound studies (Tadesse et al., 1991; Martinez et al., 1994) show that an approximate 40-cm^{-1} downshift of the amide I mode is expected due to 1-^{13}C labeling. Thus, this band appears to downshift from the main amide I band at 1658 cm^{-1} of hPLB assigned to α-helical structure. In agreement, a well-resolved shift in frequency (2 cm^{-1} for double ^{13}C label and 1 cm^{-1} for single ^{13}C label) and a drop in intensity of this band are observed, most likely because of the uncoupling of the Leu 39 and Leu 42 amide carbonyl mode from the main amide I mode of the α-helix. A small 1-cm^{-1} shift is also observed in the amide II bands of [1-^{13}C]-L39-hPLB and [1-^{13}C]-L39,L42-hPLB because of 1-^{13}C labeling. As discussed below, this result indicates that the entire region from Leu 39 to Cys 46 in hPLB has an α-helical structure.

Orientation of the α-helical structure in hPLB

In agreement with two earlier studies (Arkin et al., 1995; Tatulian et al., 1995), our measurements show that hPLB

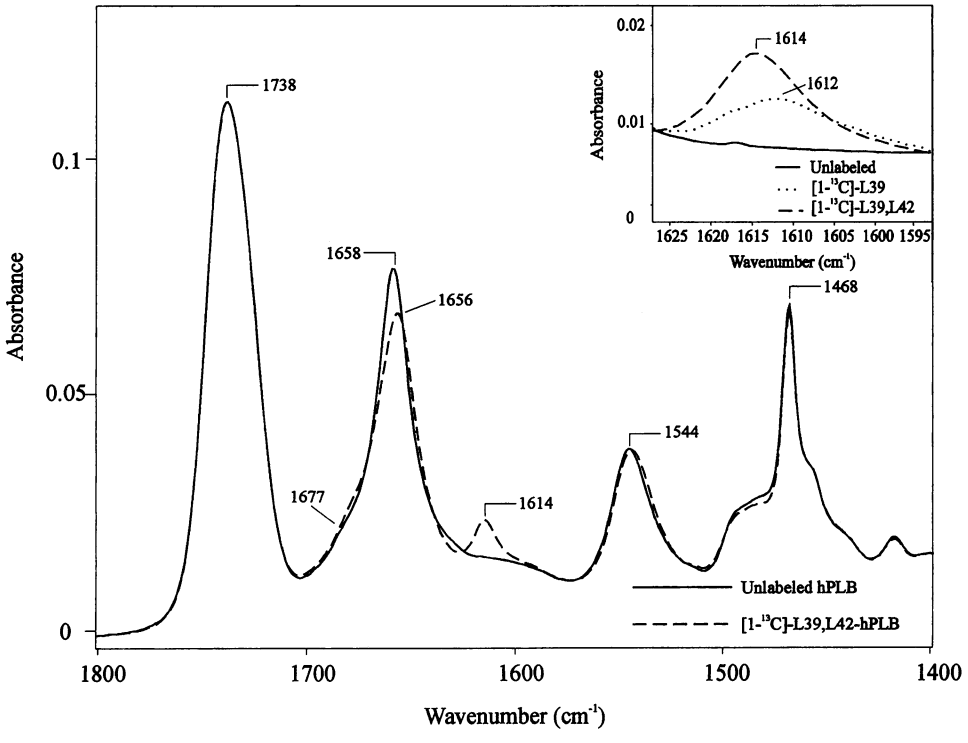

FIGURE 1 Comparison of ATR-FTIR spectra of unlabeled hPLB and [1-^{13}C]-L39,L42-hPLB recorded using parallel polarization (see text for details). Measurements were performed on dehydrated films. The absorbance scale shown is for unlabeled hPLB. (*Inset*) Comparison of expanded region near 1614 cm^{-1} of unlabeled hPLB, [1-^{13}C]-L39-hPLB, and [1-^{13}C]-L39,42-hPLB. Absorbance scale is shown for unlabeled hPLB.

has a net α-helix orientation that is predominantly perpendicular to the membrane plane. Fig. 2 shows the polarized absorption spectra ($A_{\parallel}$, $A_{\perp}$) and difference dichroism spectra ($A_{\parallel}$-$A_{\perp}$) of [1-^{13}C]-L39-hPLB reconstituted in perdeuterated DMPC and recorded using transmission FTIR spectroscopy (*left*) and ATR-FTIR spectroscopy (*right*). The sign of the dichroism of the amide I band (+) and amide II band (−) for both cases is consistent with a net transmembrane orientation of the α-helical structure (Earnest et al., 1990). Furthermore, the negative dichroism of the DMPC ester carbonyl band at 1738 cm^{-1} confirms that the overall orientation of the bilayer membrane is parallel to the germanium IRE surface or the AgCl window, as is expected when vesicles are dried onto a flat surface (Büldt et al., 1978).

To obtain a more precise estimate of the orientation of the α-helical structure, we analyzed a series of polarized transmission FTIR measurements of a [1-^{13}C]-L39-hPLB sample deposited on an AgCl window and tilted at different angles (α_o) relative to the incident beam (see Materials and Methods). As described previously (Rothschild and Clark, 1979), the dichroic ratio of the amide I band plotted as a function of the $\sin^2\alpha_o$ (Fig. 2, *left inset*) provides an accurate method for calculating the overall order parameter for the α-helical structure. Assuming no membrane disorder, the data indicate that hPLB has an average α-helix tilt angle of 48 ± 1° (see Materials and Methods). Introducing orientation disorder of the hPLB membrane lowers the calculated tilt angle. As a check on the accuracy of the original calculation, the orientation of the ester carbonyl bond of DMPC was found to be 61 ± 1°, compared to literature values of 64–65° for well-ordered multilamellar films of DMPC (Nabedryk et al., 1982; Ter-Minassian-Saraga et al., 1988).

Compared to transmission dichroism measurements, polarized ATR led to a lower estimate of the α-helix tilt angle of 37 ± 3° (i.e., α-helices are more perpendicular to the membrane plane), with a corresponding order parameter S of 0.44 ± 0.16. This lower value for θ compared to the transmission result is most likely due to the fact that the infrared light, which penetrates a short distance into the sample (calculated to be 1.6 μm; Harrick, 1967), effectively probes the most highly ordered membrane near the substrate-sample interface (Clark et al., 1980). In comparison, transmission measurements probe the entire membrane

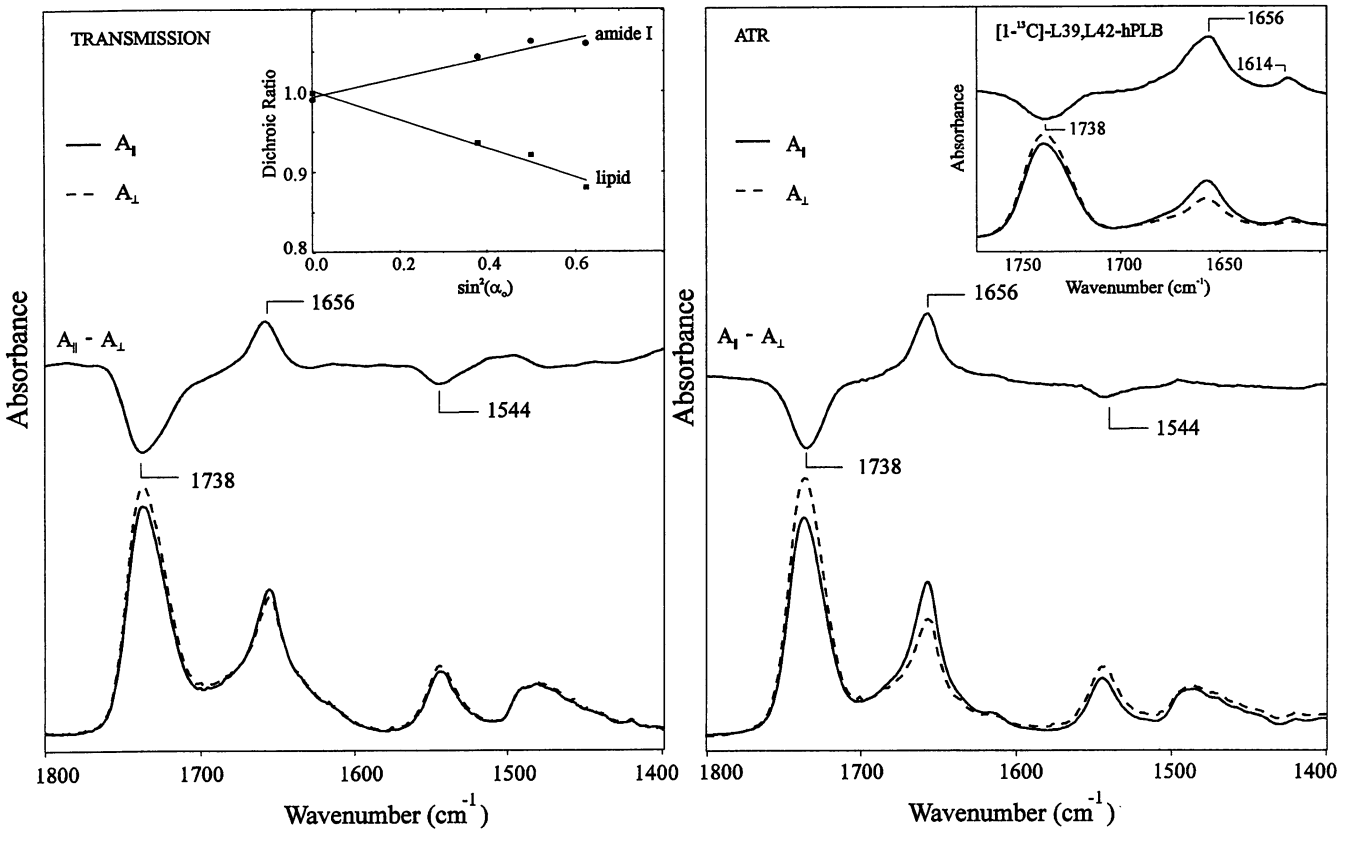

FIGURE 2 Absolute absorption and difference dichroism from 1400–1800 cm^{-1} of dehydrated [1-^{13}C]-L39-hPLB. (*Left*) Transmission FTIR spectra recorded for ∥ and ⊥ polarized light (*bottom traces*) and difference ($A_{\parallel} - A_{\perp}$) dichroism (*top trace*). The sample was tilted at 45° with respect to the window plane and incident beam. (*Right*) ATR-FTIR spectra for ∥ and ⊥ polarized light (*bottom traces*) and difference ($A_{\parallel} - A_{\perp}$) dichroism (*top trace*). All ATR-FTIR spectra shown were scaled (see Materials and Methods). This resulted in low dichroism of the 1450 cm^{-1} band, similar to results obtained for unscaled polarized transmission spectra. (*Inset left*) Plot of dichroic ratio vs. $\sin^2\alpha_o$ for polarized transmission FTIR spectra of hPLB. (*Inset right*) ATR-FTIR spectra of [1-^{13}C]-L39, L42-hPLB in expanded region between 1600 and 1750 cm^{-1} for ∥ and ⊥ polarized light (*bottom traces*) and difference ($A_{\parallel} - A_{\perp}$) dichroism (*top trace*).

thickness, which in the case of the hPLB films were calculated to be approximately 4 μm. Thus, the ATR measurements may give the more accurate estimate of the α-helix tilt angle, because the effective membrane disorder is lower. Lower values of θ were also obtained from earlier polarized ATR studies on hPLB based on data analysis using resolution enhancement (Arkin et al., 1995) and curve fitting techniques (Tatulian et al., 1995). It should also be noted that in the present work the average of the measured dichroic ratio was used (e.g., $R = 3.0$, corresponding to $\theta = 37 \pm 3°$), whereas in an earlier work (Arkin et al., 1995), the maximum measured dichroic ratio was used ($R = 3.43$, corresponding to $\theta = 27.5 \pm 4.5°$).

To probe the backbone orientation of hPLB in the region near Leu 39 and Leu 42, we calculated the dichroism of the 1614 cm^{-1} band assigned to the amide I mode of these residues. As seen in the difference dichroism spectrum of [1-^{13}C]-L39,L42-hPLB (Fig. 2, *right inset*), this band exhibits a positive dichroism consistent with the participation of the Leu 39 and Leu 42 carbonyl groups in an α-helix with a predominantly transmembrane orientation. The α-helix tilt angle θ for this local region (i.e., the region associated with the 1614 cm^{-1} band) was found to be approximately 30° with respect to the membrane normal, corresponding to an order parameter S of approximately 0.6. As discussed above, an even lower value of θ would be obtained if membrane disorder were taken into account or a higher value of α were used in the calculation.

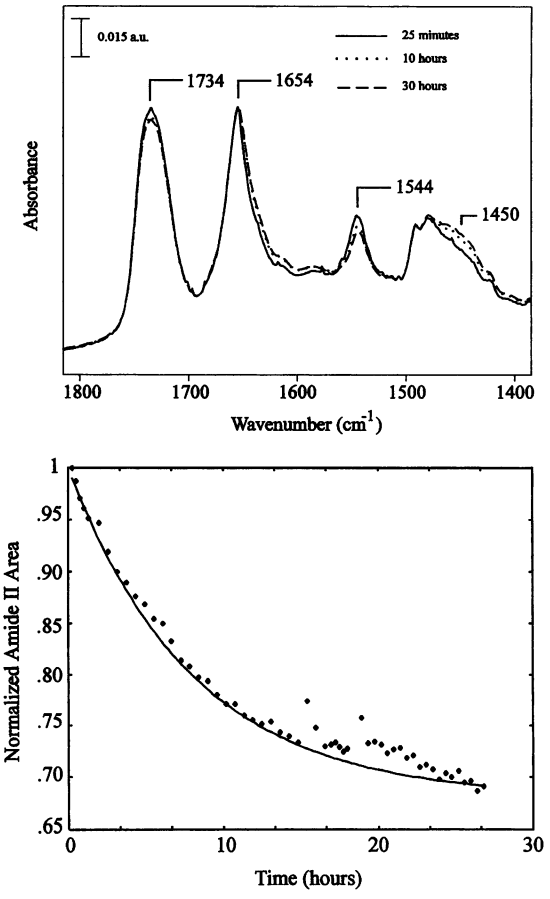

FIGURE 3 H/D exchange of [1-^{13}C]-L39-hPLB in perdeuterated DMPC. (*Top*) Kinetic traces of the 1544 and 1450 cm^{-1} bands in the region between 1400 and 1800 cm^{-1}. (*Bottom*) Plot of normalized amide II area vs. time. The single exponential decay fit resulted in a time constant of 8.1 h. The amide II areas over time were calculated by baseline correction and integration of the region between 1567 and 1530 cm^{-1}.

The hPLB fragment is resistant to H/D exchange

H/D exchange rates can be used to probe the accessibility of membrane protein structure to the external medium. Although the amide I band of α-helical structure undergoes only a small downshift in frequency due to H/D exchange, the amide II band typically shifts approximately 100 cm^{-1} from 1545 to 1455 cm^{-1} (amide II′) (Blout et al., 1961). To avoid interference with measurements in the amide II′ region, we utilized for these measurements [1-^{13}C]-L39-hPLB reconstituted in perdeuterated DMPC, which lacks the CH_2 scissoring mode absorbing near 1468 cm^{-1}. As seen in Fig. 3, exposure of an hPLB film ([1-^{13}C]-L39-hPLB) to D_2O causes a gradual shift of the amide II band from 1544 cm^{-1} to near 1450 cm^{-1}. A single exponential fit of the 1544 cm^{-1} band area in the 1530–1567 cm^{-1} range as a function of time (Fig. 3, *bottom*) shows that the H/D exchange is exponential, with a time constant of 8.1 h. After 30 h of exchange, at which point the exchange rate is very slow, the amide II band intensity has decreased by approximately 30%. Note that hydration of the dry multilayer film is expected to cause an effective drop in the infrared absorption due to swelling and subsequent removal of material outside the penetration depth of the evanescent light. However, the integrated intensities of the amide I band near 1655 cm^{-1} and of a second band near 970 cm^{-1} plotted as a function of time are constant after 15 min subsequent to the addition of D_2O. This indicates that swelling occurs on a much shorter time scale than the relatively slow H/D exchange kinetics observed.

As shown in Fig. 3 (*top*), [1-^{13}C]-L39-hPLB after 30 h of H/D exchange exhibits amide I and II band frequencies (1654 and 1544 cm^{-1}, respectively) similar to unexchanged dehydrated hPLB (Fig. 2, *right panel*). Furthermore, no significant shoulders on the amide I band were observed. In contrast, random coil exhibits an H/D exchange-induced shift of more than 20 cm^{-1} (Downer et al., 1986). This indicates that, as in the case of dehydrated membrane, the secondary structure of hPLB in a hydrated environment is predominantly α-helical with a transmembrane orientation. Similar results were also obtained for hPLB immersed in H_2O (data not shown), although the strong water band at 1640 cm^{-1} interferes with absolute absorption measurements of the amide I region. The small downshift of the amide I band to 1654 cm^{-1} in D_2O, which is correlated with the amide II downshift, is characteristic of the amide I H/D exchange-induced shift of α-helical structure (Susi et al.,

1967; Rath et al., 1991). (Part of the shift, however, might also be due to the effects of hydration, because only 30% of the amide groups have undergone H/D exchange.)

As seen in the dichroism difference spectra of [1-^{13}C]-L39,L42-hPLB (Fig. 4), the 1614 cm^{-1} band assigned to the amide I mode of Leu 39 and 42 is unaffected by exposure of the sample to D_2O for 30 h. This is a further indication that the local structure around Leu 39 and Leu 42 is α-helical, because less structured hydrogen bonded carbonyl groups (e.g., random coil) would be expected to undergo a significant downshift in frequency (Earnest et al., 1990). The absence of even a smaller 1–2-cm^{-1} shift typical of α-helices that undergo H/D exchange (Susi et al., 1967) also suggests that the Leu 39 and Leu 42 peptide groups are resistant to H/D exchange (see Discussion).

DISCUSSION

Current knowledge of how integral membrane proteins function is limited by the difficulty of obtaining high-resolution structural information. Although infrared spectroscopy is able to probe the overall secondary structure of membrane proteins (Braiman and Rothschild, 1988; Surewicz et al., 1993; Goormaghtigh et al., 1994a,b,c) as well as peptide models (Zhang et al., 1995), few studies have been able to probe local structure. A fundamental problem is that although specific types of secondary structure produce different band frequencies in the amide I region, vibrational modes arising from different parts of the protein with similar structure are normally indistinguishable by infrared spectroscopy.

In this work we have combined site-directed isotope labeling with polarized ATR-FTIR spectroscopy and H/D exchange to probe the local as well as overall secondary structure of hPLB reconstituted in DMPC bilayer membranes. hPLB was chosen because it is sufficiently small to synthesize chemically, thereby affording a convenient method for incorporating site-directed isotope labels, and is of considerable interest because of its role in Ca^{2+}-ATPase regulation and ion channel activity. hPLB also serves as an ideal model for interpreting the results of FTIR analysis of larger membrane proteins such as bacteriorhodopsin, for which SDIL analogs can now be produced through genetic methods and in vitro expression (Sonar et al., 1994, 1995). As described below, our results show that SDIL combined with FTIR provides an effective means of probing the local secondary structure in a membrane protein and adds to our knowledge of the basic structure of hPLB.

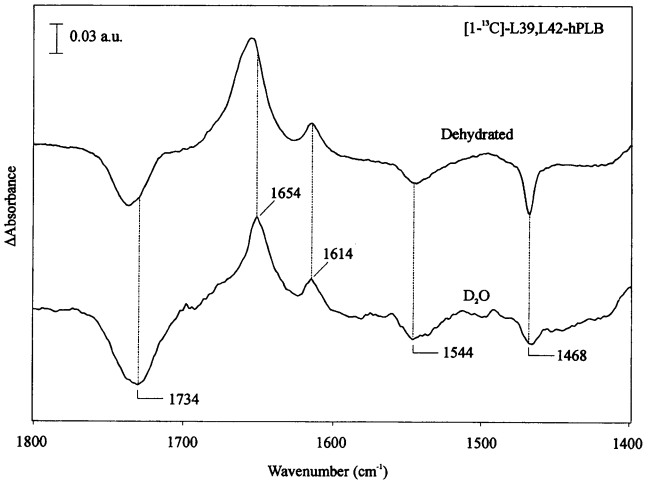

FIGURE 4 Comparison of difference ($A_{\parallel} - A_{\perp}$) dichroism spectra of dehydrated and deuterated (30 h) [1-^{13}C]-L39, L42-hPLB in the region between 1400 and 1800 cm^{-1}.

The local structure of hPLB between residues 39 and 46 is α-helical

This work demonstrates the ability of FTIR spectroscopy to probe the local backbone conformation in a membrane protein by exploiting SDIL-induced shifts in the amide I band. Previously it had been shown that the isotope shift induced by [1-^{13}C] labeling at specific positions in a 25-residue soluble polypeptide was approximately 30–40 cm^{-1} (Tadesse et al., 1991; Martinez et al., 1994), in agreement with normal mode calculations (Dwivedi and Krimm, 1984). In the case of hPLB, the magnitude of the isotope shift (44 cm^{-1}) and the drop in intensity of the intense band at 1658 cm^{-1} strongly support the conclusion that the peptide groups of both Leu 39 and Leu 42 participate in an α-helical structure. The spectral changes in the amide I band can be attributed to vibrational uncoupling of the ^{13}C labeled carbonyl group from the rest of the α-helix amide I mode, which involve the coupled (C═O stretch) vibrations of multiple carbonyl groups (Parker, 1983, and references therein; Miyazawa et al., 1958). Furthermore, because the Leu 39 and Leu 42 carbonyls participate in α-helical secondary structure (i.e., the observed amide I frequency reflects standard C═O···H-N hydrogen bonding between neighboring turns), this implies that residues 43 (39 + 4) and 46 (42 + 4) also participate in this structure. Extending this idea, a single label can report on the local secondary structure of four residues. Hence, six isotopic labels would be the minimum number needed to define a transmembrane helix (~40 Å).

Interestingly, the results of this work also support the conclusion of a recent study on the conformational changes occurring during the photocycle of bacteriorhodopsin (Ludlam et al., 1995). Using genetic methods based on amber mutagenesis, suppressor tRNA, and cell-free expression, a single carbonyl group at Tyr 185 was [1-^{13}C]-labeled and the bR→N ATR-FTIR difference spectrum was recorded. Similar to hPLB, a drop in intensity and small downshift in frequency of the amide I band (1670 cm^{-1}) assigned to buried α-helical structure (Rothschild et al., 1993) along with the appearance of a new negative band at 1637 cm^{-1} was observed.

The net secondary structure of hPLB is α-helical

The frequencies of the amide I and II bands, along with the absence of an appreciable shift of the amide I band upon H/D exchange, is a clear indication that hPLB has an α-helical structure when reconstituted into a lipid bilayer. Unlike most membrane proteins, which are predominantly α-helical, the amide I band of phospholamban is relatively symmetric and does not exhibit significant shoulders, even when resolution-enhanced (Arkin et al., 1995). For example, the α-helical protein bacteriorhodopsin exhibits a main amide I band near 1661 cm^{-1} with shoulders near 1680 and 1642 cm^{-1}, including additional subcomponent bands that are revealed with resolution-enhancement techniques. (The 1642 cm^{-1} band of bacteriorhodopsin arises partially from contributions of the protonated Schiff base C=N stretching mode (Ebrey et al., 1987; Earnest et al., 1990).) Rhodopsin exhibits even more intense sidebands near 1680 and 1640 cm^{-1}. This difference most likely reflects the almost complete embedding of hPLB into the bilayer, in contrast to larger membrane proteins, which contain significant extramembrane components, including loop regions that give rise to subcomponent amide I bands. A similar conclusion has previously been reached on the basis of resolution enhancement (Arkin et al., 1995) and curve fitting of the hPLB FTIR absorption spectrum (Tatulian et al., 1995).

The local structure of hPLB near Leu 39 and Leu 42 has a transmembrane orientation

Infrared dichroism has been used previously to probe the orientation of a number of α-helical membrane proteins, including bacteriorhodopsin (bR) (Rothschild and Clark, 1979; Nabedryk et al., 1985), the photoreceptor rhodopsin (Rothschild et al., 1980), and the C-terminal channel-forming peptide of colicin E1 (Rath et al., 1991; see, for a recent review, Goormaghtigh et al., 1994a,b,c). In each of these cases, a net out-of-plane orientation was detected for the α-helical structure. Our studies of hPLB using similar techniques show that hPLB reconstituted in DMPC has a predominantly transmembrane orientation, in agreement with earlier studies based on polarized ATR-FTIR and spectral deconvolution (Arkin et al., 1995; Tatulian et al., 1995). In this work we were able to go beyond this result by measuring the dichroism of a small band assigned to the isotope-shifted amide I mode of Leu 39 and Leu 42. This indicates that the orientation of the local α-helical structure in this region of hPLB is approximately 30° relative to the membrane normal. Importantly, this result is consistent with the proposed model of the pentameric phospholamban assembly forming a helical bundle with a central selective ion channel in Ca^{2+} transport control (Arkin et al., 1993).

hPLB is predominantly located in the bilayer domain

A variety of membrane proteins, including the acetylcholine receptor (Methot et al., 1994), rhodopsin (Rothschild et al., 1980), and colicin E1 (Rath et al., 1991), as well as peptide models (Zhang et al., 1992), exhibit significant H/D exchange, indicating that a major portion of their structure is accessible to water. In the case of bR, transmembrane α-helices were found to be resistant to H/D exchange, confirming that the α-helical structure of bR is embedded in the membrane interior and protected from the external medium. However, even in this case, non-α-helical structure undergoes rapid H/D exchange in the first 2 h of D_2O exposure (Earnest et al., 1990). Relative to these proteins, hPLB exhibits a relatively small H/D exchange and does not show a rapid phase of H/D exchange, as is the case with bR. This is consistent with a protein structure that is almost completely buried in the bilayer; i.e., there are no significant peripheral regions available for rapid exchange.

The ability to assign the amide I modes of the [1-^{13}C]-labeled peptide groups of Leu 39 and Leu 42 allowed us also to observe the effects of H/D exchange on these peptide groups. Because no band shift was detected, even at the level of 1–2 cm^{-1}, which is typical for α-helical structure that undergoes H/D exchange, we surmise that these specific amide groups are essentially unavailable for H/D exchange. This is consistent with the positioning of these groups within the bilayer, as expected on the basis of their position midway in the amino acid sequence of hPLB. It is possible that the absence of H/D exchange also reflects the positioning of these residues outside of the water-filled core of an hydrophilic channel predicted to be formed by the pentameric arrangements of the hPLB subunits. This would be possible if the α-helices in this bundle were rotated so that both Leu 39 and Leu 42 faced toward the lipid interior of the bilayer. ATR-FTIR-H/D exchange studies of additional SDIL analogs of hPLB will be necessary before this possibility can be confirmed.

CONCLUSIONS

In contrast to earlier infrared studies of the PLB structure (Arkin et al., 1995; Tatulian et al., 1995), we have in this work combined for the first time site-directed isotope labeling, infrared dichroism, and H/D exchange to probe the structure of a local region of the hydrophobic portion of PLB (hPLB) from Leu 39 to Cys 46. We also find that hPLB reconstituted into DMPC bilayer membranes is largely inaccessible to H/D exchange. These results are consistent with current models of PLB structure, which picture a core membrane embedded region forming a pentameric helical bundle with a central selective ion channel (Kovacs et al., 1988; Arkin et al., 1993; Adams et al., 1995). Future studies combining SDIL, polarized ATR-FTIR, and H/D exchange should be useful for analyzing the structure of other membrane proteins.

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