Deuterium NMR Reveals Helix Packing Interactions in Phospholamban

Weiwen Ying,[†] Scott E. Irvine,[‡] Richard A. Beekman,[‡] David J. Siminovitch,[‡] and Steven O. Smith*,[†]

Contribution from the Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, New York 11794-5215, and Department of Physics, University of Lethbridge, Lethbridge, AB T1K 3M4 Canada

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Abstract: Phospholamban is an integral membrane protein having a single membrane-spanning helix which forms a pentameric complex in cardiac and smooth muscle cell membranes. Deuterium NMR measurements of leucine residues in the transmembrane domain of the protein provide a novel approach for establishing the rotational orientation of the phospholamban monomer within the complex. At 5 °C, the spectra of Leu43 and Leu44 are similar and exhibit a quadrupole splitting of 33 kHz. This splitting is slightly narrower than the ∼40 kHz splitting which results solely from rapid methyl group rotation. The deuterium line shape of Leu42 has lost the distinctive 33-kHz quadrupole splitting due to increased librational motion of the side chain and/or rotation about the $C_\alpha-C_\beta$ and $C_\beta-C_\gamma$ bonds. The observed line shapes of the three consecutive leucine residues in phospholamban are consistent with Leu42 being oriented toward the lipids, where it exhibits fewer steric contacts, and Leu43 and Leu44 being oriented toward helix interfaces which restrict their motion. Possible packing arrangements of the three transmembrane leucine residues in the phospholamban pentamer are examined using computational methods to assess the packing restrictions of the leucine side chains. The results are discussed in terms of models of the phospholamban pentamer previously proposed on the basis of mutational data.

Introduction

Phospholamban is a 52-residue membrane protein involved in the regulation of calcium levels across sarcoplasmic reticulum (SR) membranes in cardiac and smooth muscle cells.[1] The C-terminal 22 amino acids are largely hydrophobic, adopt α-helical secondary structure, and anchor the protein in the membrane. Specific noncovalent interactions between the transmembrane helices stabilize a pentameric complex.[2] Phospholamban inhibits the calcium ATPase resident in SR membranes.[3] Phosphorylation of Ser16 and Thr17 in the cytoplasmic domain of the phospholamban reverses inhibition of the ATPase.[4] In addition, a central ion pore formed by the pentamer allows Ca^{2+}-selective ion conductance.[5] In this report, deuterium NMR measurements of three consecutive transmembrane leucine residues are used to constrain the rotational orientation of the phospholamban monomer within the pentameric complex. Deuterium NMR line shapes provide direct information on side-chain dynamics.[6] Leucine residues which are packed within helix interfaces of the phospholamban pentamer exhibit restricted motion relative to those leucines oriented toward the surrounding lipid bilayer. The line shapes observed for Leu43 and Leu44 exhibit distinctive quadrupole splittings characteristic of side-chain motion, which is restricted only to fast rotation about the $C_\gamma-C_\delta$(D3) bond. In contrast, the Leu42 line shape is characteristic of fast methyl group rotation and additional rotation or librations about the $C_\alpha-C_\beta$ and $C_\beta-C_\gamma$ bonds, consistent with an orientation toward the surrounding lipids, These results are used to assess structural models of phospholamban which have been proposed previously[4,7,8] on the basis of mutational studies of the transmembrane domain of the pentamer.

Experimental Section

Materials. 5-D_3-Leucine was obtained from Cambridge Isotope Laboratories (Andover, MA). Other amino acids and octyl β-glucoside (β-OG) were obtained from Sigma Chemical (St. Louis, MO). Dimyristoylphosphocholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL).

Peptide Synthesis and Reconstitution into DMPC Bilayers. Full-length deuterated phospholamban peptides were synthesized and purified at the Keck Peptide Synthesis Facility at Yale University. In a typical procedure, phospholamban peptide (62 mg, 0.010 mmol), DMPC (271 mg, 0.40 mmol), and β-OG (750 mg) were dissolved in trifluoroethanol and then lyophilized. The resulting mixture was rehydrated with phosphate buffer (10 mM phosphate, 50 mM NaCl, pH 7) and then dialyzed for 24 h with frequent changing of the dialysis solution. After dialysis, the sample was pelleted by ultracentrifugation

* Corresponding author. Phone: 631-632-1210. Fax: 631-632-8575. E-mail: steven.o.smith@sunysb.edu.
† SUNY Stony Brook.
‡ University of Lethbridge.

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and lyophilized. The dried peptide was suspended in deuterium-depleted water and incubated at 30 °C for more than 24 h. Excess water was removed by spinning the sample in a magic angle spinning rotor. The remaining water amounts in all samples were 49 ± 5 wt %.

NMR Spectroscopy. All NMR experiments were performed on a Bruker Avance spectrometer. ^{2}H NMR data were collected using a Chemagnetics ^{2}H probe at a ^{2}H frequency of 55.2 MHz. A standard quadrupole echo pulse sequence was employed with a 2.8-μs 90° pulse, a 30-μs pulse separation, and a 0.4-s recycle delay. A total of 40 000 scans were averaged for each spectrum and processed using a 1 kHz exponential line broadening function.

Polarized IR Spectroscopy. After the NMR experiments, the phospholamban sample was removed from the NMR rotor and resuspended in phosphate buffer. The sample was gently sonicated in a bath sonicator and incubated at 30 °C for 2 h. The sample was then layered on a germanium window by slow removal of water by blowing a stream of nitrogen gas over the sample at an oblique angle. Attenuated total reflection (ATR) IR spectra were recorded on a Bruker IFS 66V/S spectrometer and processed as described previously by Arkin et al.[7] The frequency (1654 cm^{-1}) and dichroic ratio (2.7) of the amide I vibrational mode observed in the polarized IR spectrum were similar to those obtained previously [7] and argue that the pentamer remains helical with an orientation roughly perpendicular to the membrane plane. These data suggest that the protein retains its native structure.

Occluded Surface (OS) Calculations. The PDB coordinates of transmembrane phospholamban models of Herzyk and Hubbard (1PLN)[21] and by Adams et al. (1PLS)[20] were obtained from the Protein Data Bank. Packing of the three consecutive leucine residues was measured using the method of occluded surfaces.[9,10] The OS method provides a direct measure of molecular packing and allows the fractioning of the atomic or molecular surface; i.e., the packing interactions can be analyzed at either the atomic, amino acid, or molecular level, and the packing of both buried and surface-exposed residues may be quantified directly, in contrast to the more commonly used Voronoi procedure.[11] The packing calculation yields a packing value for each leucine residue. The packing value is composed of two parameters, the occluded surface area and the distribution of distances to occluding atoms. A molecular dot surface is calculated for each residue with a 1.4-Å probe. A normal is extended radially from each dot until it either intersects the van der Waals surface of a neighboring atom or reaches a length of 2.8 Å (the diameter of a water molecule). The occluded surface, OS_{atom}, is defined as that molecular surface area on the originating atom associated with normals that intersect with another atom surface as opposed to reaching the 2.8 Å limit. All other molecular surface area is considered nonoccluded or exposed. The average occluded surface packing value (OSP) for each residue is defined as[12]

$$\mathrm{OSP} = \sum_{\mathrm{atom}}^{\mathrm{res}} [\mathrm{OS}_{\mathrm{atom}} \langle 1 - \mathrm{RL} \rangle_{\mathrm{atom}}]/\mathrm{MS}_{\mathrm{res}}$$

where MS_{res} is the total surface of the residue (sum of occluded and nonoccluded areas) and RL is the length of the extended normal from one surface to the other, divided by 2.8 (actual length in angstroms divided by 2.8 Å).

Results and Discussion

Deuterium NMR. The predominant motion for narrowing the static 127-kHz quadrupole splitting of the Leu CD_3 deuterons is methyl group rotation. Fast (>10^7 s^{-1}) rotation of the side-chain methyl groups occurs at temperatures well below −25 °C and results in reduction of the quadrupole splitting to ∼40 kHz. The spectra described below all exhibit fast methyl rotation.

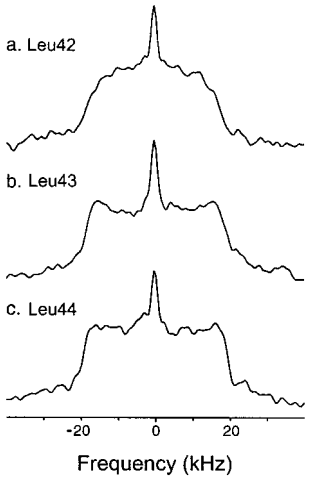

Figure 1. Deuterium NMR spectra of δ-D_3-labeled Leu42, Leu43, and Leu44 phospholamban at 5 °C. Phospholamban peptides were reconstituted into DMPC at a 1:40 peptide/lipid molar ratio and hydrated with approximately 50 wt % deuterium-depleted water. The spectra were obtained using a quadrupolar echo pulse sequence at a deuterium frequency of 55.2 MHz. The 90° ^{2}H pulse length was 2.8 μs, and the τ delay was 30 μs. Each spectrum represents the average of 40 000 transients. An exponential line broadening of 1 kHz was applied.

The most revealing motions in terms of side-chain dynamics are associated with rotation or jumps about the $C_\alpha-C_\beta$ and $C_\beta-C_\gamma$ bonds. Rotation, jumps, or librational motions about these bonds result in distinctive deuterium line shapes. Importantly, steric clashes due to helix−helix packing can restrict these larger amplitude motions and leave deuterium line shapes narrowed by predominately methyl group rotation. We targeted three consecutive leucine residues (Leu42, -43, and -44) in the transmembrane domain of phospholamban to establish the relative motion of the side chains and the rotational orientation of the phospholamban helices in the pentameric complex. Leucine residues packed within helix interfaces of the phospholamban pentamer should exhibit restricted motion relative to those leucines oriented toward the surrounding lipid bilayer. Since the three leucines are consecutive in the sequence, at least one leucine should lie within a helix interface, and one leucine should be oriented toward the lipid acyl chains.

Deuterium NMR spectra were obtained using a standard quadrupole echo pulse sequence at 36 °C, above the phase transition temperature of DMPC, and at 5 and −25 °C, below the DMPC phase transition temperature. Figure 1 presents the deuterium NMR spectra obtained at 5 °C of phospholamban labeled with deuterated Leu42, Leu43, or Leu44. The spectra of Leu43 and Leu44 are similar and exhibit a quadrupole splitting of 33 kHz. This splitting is slightly narrower than the ∼40 kHz splitting which results solely from rapid methyl group rotation. A distinct difference in line shapes is observed between Leu42 and the other two samples. The deuterium line shape of Leu42 has lost the distinctive 33-kHz quadrupole splitting due to increased librational motion of the side chain and/or rotation about the $C_\alpha-C_\beta$ and $C_\beta-C_\gamma$ bonds.

Figure 2a presents a simulation of the deuterium line shape of the δ-D_3-labeled Leu methyl deuterons resulting solely from fast rotation about the $C_\gamma-C_\delta$ bond using a three-site hop model and a jump rate of 10^8 s^{-1}. As the jump rate is reduced, the quadrupolar splitting increases, resulting in a distinctive series of line shapes.[13] Assuming only methyl rotation and changing only the jump rate, it is not possible to generate an $\eta = 1$ line

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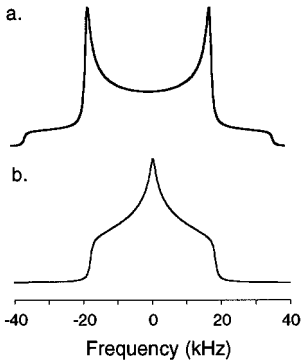

Figure 2. Simulations of δ-D_3-labeled Leu NMR line shapes. (a) Deuterium line shape of methyl deuterons resulting solely from fast rotation about the $C_\gamma-C_\delta$ bond. A three-site hop model with a jump rate of 1×10^8 s^{-1} was used in the simulation. (b) Deuterium line shape of methyl deuterons resulting from a two-site hop superimposed on a fast methyl group rotation. The jump rate was 5×10^9 s^{-1}, and the hop angle was set at 109.4°. Simulations were run with the program MXET1.[28] The starting asymmetry parameter η was 0.05, the 90° pulse length was 3.5 μs, and the echo delay was 50 μs.

shape such as that seen in Figure 1a. A major difference between the simulation in Figure 2a and the experimental spectrum in Figure 1b is the presence of residual deuterated water in the sample, which fills in the region between the horns of the deuterium powder pattern. Figure 2b presents a simple simulation of the deuterium line shape of methyl deuterons resulting from a two-site hop superimposed on a fast methyl group rotation. As described below, a two-site hop is a reasonable approximation for leucine side-chain motion involving transitions between the two predominant rotamers of leucine observed in proteins and rotations about the $C_\alpha-C_\beta$ and $C_\beta-C_\gamma$ bonds.

Deuterium NMR spectra obtained at −25 °C were nearly identical for the three samples and exhibited the deuterium line shapes observed in Figure 1b,c with a quadrupole splitting of 34 kHz. Similarly, the deuterium NMR spectra obtained at 36 °C were nearly identical for the three samples and exhibited the bell-shaped pattern seen in Figure 1a. Polarized ATR-FTIR spectra were obtained following the NMR measurements to establish that the phospholamban helix remained in the folded conformation and transmembrane orientation (see Experimental Section).

The 33-kHz splittings observed in the deuterium NMR spectra of Leu43 and Leu44 in phospholamban are similar to those observed for δ-D_3-labeled leucine in the membrane protein bacteriorhodopsin by Oldfield and co-workers.[14] The 35 δ-D_3-labeled leucines in bacteriorhodopsin exhibit distinct quadrupole splittings (31−36 kHz) up to 37 °C, indicating that their average motion is dominated by fast methyl group rotation.

The bell-shaped deuterium NMR spectrum observed for Leu42 is similar to that observed for D_{10}-labeled leucine in collagen.[15] In this case, methyl group rotation is accompanied by larger amplitude side-chain motions. Batchelder and co-workers[16] were able to simulate the temperature-dependent line shapes of leucine methyl deuterons in collagen and quantitate the motional rates between the predominant side-chain conformers of leucine. Their analysis was based on a motional model involving fast methyl group rotation and transitions between

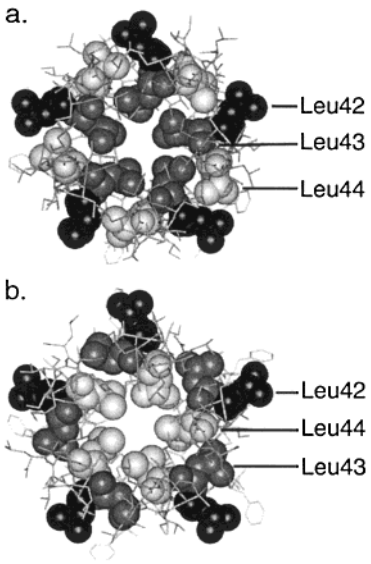

Figure 3. Helix packing in the transmembrane domain of phospholamban from Leu42 to Leu44. (a) Cross section of the model of Adams et al. (1PSL).[20] Leu42 is oriented outward toward the surrounding lipid bilayers, while Leu43 is oriented toward the central ion pore but tightly packed against Leu43 and Leu44 of the adjacent monomers. (b) Cross section of the model of Herzyk and Hubbard (1PLN).[21] Rotation of the transmembrane helix by ∼50° relative to the model in (a) leads to a more exposed orientation for Leu42. Leu44 faces the central ion pore packed against Leu43 and Leu44 of the adjacent monomers.

the two predominant rotamers of leucine observed in proteins.[17,18]

The analysis of leucine dynamics in phospholamban parallels studies of valine dynamics in gramicidin A, where differences in side-chain packing also result in drastically different deuterium line shapes.[19] In gramicidin A at 5 °C, Val6 and Val8 exhibit characteristic Pake powder patterns with quadrupole splittings of ∼40 kHz, whereas Val1 and Val7 exhibit bell-shaped powder patterns similar to those of Leu42 in phospholamban. Cross and co-workers[19] were able to simulate the Val1 and Val7 spectra using a three-state motional model involving hops about the $C_\alpha-C_\beta$ bond. They interpreted the differences in line shapes in terms of steric hindrance of the Val6 and Val8 side chains.

The observed line shapes of the three consecutive leucine residues in phospholamban are consistent with Leu42 being oriented toward the lipids, where it exhibits fewer steric contacts and Leu43 and Leu44 being oriented toward helix interfaces which restrict their motion. This orientation of the three leucine residues is in agreement with two models of the phospholamban pentamer[7,8] based on mutational data. Both mutational studies show that the residues most sensitive to mutation are Leu37, Ile40, Leu44, and Ile47, and both models place Leu42 in a lipid-exposed position.

Occluded Surface Analysis. Figure 3 presents cross sections at the level of Leu42−44 of molecular models of the phospholamban pentamer. The model developed by Adams et al.[20] was based on saturation mutagenesis of the transmembrane residues and a computational search algorithm that produced several clusters of structures. The model in Figure 3a was selected as the cluster that best explained the mutational results. The model

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developed by Herzyk and Hubbard[21] (Figure 3b) took advantage of mutational results from both Arkin et al.[22] and Simmerman et al.[2] Those researchers converted the mutational results into loose structural constraints which guided their molecular dynamics-simulated annealing calculations. They noted that, while the mutational results were very similar between the two studies, there are several distinct differences. The most striking differences are for Leu39, Cys46, Val49, and Met50, and the best fit occurs between the mutational results and their structural model when Leu39, Cys46, and Val49 have an outside orientation and Met50 has an inside orientation. Their final model is consistent with the proposal of Simmerman et al.[4] that the transmembrane residues (37−52) form a pentameric coiled-coil structure having a leucine zipper motif.

The cross sections shown in Figure 3 suggest that Leu43 and -44 are tightly packed even though they are oriented toward the ion pore and the helix interface, while Leu42 is more loosely packed. To quantitatively assess the packing of the three transmembrane leucine residues, we calculated the residue packing values for the leucine residues using the method of occluded surfaces.[9,10] The average residue packing value for the transmembrane region of helical membrane proteins is 0.431.[23] Surface residues generally have packing values in the range of 0.2−0.3, while the most tightly packed buried residues have packing values in the range of 0.5−0.6. In the model used by Herzk et al.,[21] the occluded surface analysis yields a low packing value for Leu42 (0.297), consistent with its larger amplitude motions and largely surface orientation. Leu43 (0.539) and Leu44 (0.548) have similarly high packing values, consistent with these residues both exhibiting similar rigid line shapes characteristic of restricted side-chain motion. The highest packing value was calculated for Leu44, which is scored as more sensitive than Leu43 in both mutational studies.[2,22] This residue is packed against both adjacent monomers and oriented toward the central ion pore in Figure 3b. In contrast, the packing values for these three transmembrane residues (Leu42, 0.371; Leu43, 0.521; Leu44 0.420) in the model by Adams et al.[20] are distributed over a narrower range, and there is not a clear correlation between the packing value and deuterium line shape.

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Conclusions

The deuterium NMR studies described here provide the basis of a new approach for establishing the rotational orientation of helices in membrane proteins. Leucine is the most common residue in transmembrane helices. Langosch and co-workers have recently shown that an artificial heptad motif of leucine residues is sufficient for association of transmembrane helices and have proposed that leucine zippers form a general motif for interactions in membrane proteins.[24] The deuterium line shapes indicate that, at −25 °C, the only significant motion is methyl group rotation, while increasing the temperature results in increased rotation or libration about the $C_\alpha-C_\beta$ and $C_\beta-C_\gamma$ bonds. The agreement between the OS packing values and the deuterium line shapes favors the packing model proposed by Hubbard and Herzk and is consistent with cysteine reactivity studies of Thomas and co-workers, which showed that Cys41 is unreactive to sulfhydryl reagents.[25] Distance measurements using solid-state NMR methods[26,27] are in progress to establish specific helix−helix contacts in the pentamer on the basis of knowledge of the rotational orientation of the phospholamban helices and motion of the specific sites in the helix−helix interfaces.

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Supporting Information Available: ^{2}H NMR spectra of samples at 36 and −25 °C (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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