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Solid-State NMR and Rigid Body Molecular Dynamics To Determine Domain Orientations of Monomeric Phospholamban

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Phospholamban (PLB), a 52-amino acid membrane protein, is the main regulator of Ca-ATPase in cardiac muscle.[1] PLB is thought to assemble into a pentamer and to depolymerize into monomers prior to its inhibitory interaction with Ca-ATPase.[2] The predicted structure of PLB contains three domains: Domain IA, an amphipathic helix (residue 1−20); Domain IB, a hinge (residue 21−30); and Domain II, a transmembrane helix (residue 31−52). It has been hypothesized that PLB's inhibitory activity occurs via intramembraneous interactions between a functionally discrete helical face of Domain II and transmembrane domain 6 of Ca-ATPase, while residues in Domains IA and IB fine-tune the inhibition by interacting with the cytoplasmic domain of the enzyme.[4] This inhibitory interaction is reversed by phosphorylation at S16 and T17 upon adrenergic stimulation of the cardiac myocyte. Failures in this mechanism lead to imbalances in calcium homeostasis, contributing to heart failure and cardiomyopathies[1]. The high-resolution X-ray crystal structure of SERCA1a,[5] combined with electron microscopy of Ca-ATPase/PLB cocrystals,[6] has revealed many interesting features of this complex enzyme, but the structural details of its regulation by PLB remain elusive.

Given its remarkable biological importance and its relatively small size, PLB has been the benchmark used by many theoretical and experimental studies of membrane protein structure and assembly.[7] The three-dimensional structure of PLB is a matter of ongoing dispute. Solution NMR Studies in organic mixtures have shown that PLB adopts an "L-shape" structure with the intervening Domain IA in either a short flexible turn or a β-turn type III conformation.[8,9] In both reported structures, the interhelical angle between Domain II and Domain IB was found to be $68 \pm 23°$. Spectroscopic and modeling studies have also produced two rather different structural models of pentameric PLB. In the first model,[10] PLB is composed of two α-helices connected by a small intervening β-sheet with Domain IA tilted in a range of 50−60°. In the second model,[11] full-length PLB has a continuous α-helix of approximately 40 amino acids with a tilt of $28 \pm 6°$ degrees with respect to the bilayer.

Determining the structure of PLB and its interactions with lipid bilayers is central to understanding its regulatory role. It is likely that the pentameric form of PLB is an inactive state or storage form, while the monomer is the functionally active form in PLB's interaction with the Ca-ATPase[2]. Therefore, we have focused our investigation on AFA-PLB, a highly stable, fully functional PLB monomeric mutant where A36, F41, and A46 have replaced the three corresponding transmembrane cysteine residues.

^{15}N solid-state NMR spectroscopy has emerged as a powerful structural tool for studying the topology of membrane proteins reconstituted in fully hydrated lipid bilayers.[12] This technique relies on measurements of the ^{15}N chemical shift anisotropy of specifically labeled amide sites of the protein backbone, giving precise information about the alignment of the proton−nitrogen chemical bond vectors with respect to the plane of the membrane bilayer.

In this communication, we show that by using a combination of solid-state NMR spectroscopy and rigid body molecular dynamics calculations, we obtained the domain orientations of monomeric PLB in lipid bilayers.

AFA-PLB, with inhibitory function indistinguishable from that of wild-type PLB, was prepared by stepwise Fmoc solid-phase peptide synthesis as described previously.[13] Mechanically oriented PLB samples containing ∼3 mg of PLB in 80 mg of 4:1 mixture of 1,2-dioleyl-*sn*-glycero-3-phosphocholine/1,2-dioleyl-*sn*-glycero-3-phospho-ethanolamine (DOPC/DOPE) were prepared according to the procedure reported previously.[14] All solid-state NMR spectra were acquired at 25 °C using a Chemagnetics CMX-400 MHz spectrometer operating at 400.1 MHz for ^{1}H and equipped with a flat-coil double-resonance ^{1}H/^{15}N probe with the coil dimension 10 mm × 8 mm × 5 mm (Doty Scientific). In oriented samples, the membrane bilayer plane was perpendicular to the magnetic field. The formation of a uniformly oriented lipid bilayer was established using one-dimensional ^{31}P spectroscopy[15] (see Figure 1A).

Figure 1, parts D−F show the one-dimensional solid-state ^{15}N NMR spectra of specifically ^{15}N-labeled PLB in lipid bilayers. The spectrum of unoriented PLB selectively labeled with ^{15}N at position 11 and 36 (Figure 1D) shows the typical powder pattern, characteristic of a statically disordered population.[16] In the spectrum of the oriented sample labeled at positions 11 and 36, we have assigned the resonance at ∼204 ppm (Figure 1E) to A36, which is located in the middle of Domain II. The chemical shift, close to the maximum value for the σ_{33} component of the chemical shift tensor, indicates that the amide bond vector of A36 is perpendicular to the membrane bilayer.[16] On the other hand, the resonance at 80 ppm, which corresponds to a ^{15}N chemical shift of residues aligned parallel to the plane of the bilayer, can be attributed to A11. In the oriented sample labeled on residues A11 and A15 (Figure 1F), we detected two slightly resolved peaks, one at 80 ppm corresponding to A11, and the other at 95 ppm corresponding to A15. Both peaks are indicative of a cytosolic domain of PLB oriented approximately parallel to the plane of the bilayer.

To determine the interhelical angle (θ) between Domains IA and II, we compared our experimental data with various PLB models. Using rigid body molecular dynamics calculations with the program CHARMM, we generated 30 PLB models with different interhelical angles ranging from 0° through 150°. Then, we fitted those structures to our experimental chemical shifts using the program SIMSPEC 1.0.[18] This software rotates the molecular coordinates to find the best match between experimental chemical shifts and model structures. The agreement between PLB models and chemical shifts is reflected by a fitting parameter (or score) χ^2. The program

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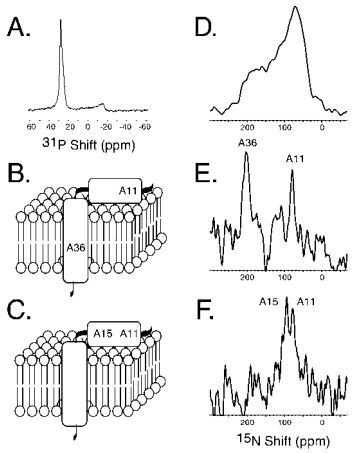

Figure 1. One-dimensional solid-state NMR spectra: (A) ^{31}P NMR spectrum of oriented DOPC/DOPE bilayer. (B, C) Models of PLB with two sites labeled. ^{15}N NMR spectra of PLB in lipid bilayers: (D) ^{15}N powder pattern of A11 and A36 ^{15}N-labeled PLB, (E) oriented bilayers containing A11 and A36 ^{15}N-labeled PLB, (F) oriented bilayers containing A11 and A15 ^{15}N-labeled PLB. The ^{15}N spectra were acquired using the cross-polarization pulse sequence[15] with spin-lock time of 1 ms, a recycle delay of 5 s, and spin-lock field strength of ~40 kHz. The ^{15}N spectra were processed using an exponential function with a line broadening of 300 Hz before Fourier transformation. Chemical shifts were referenced with respect to $(NH_4)_2SO_4$ (27 ppm).

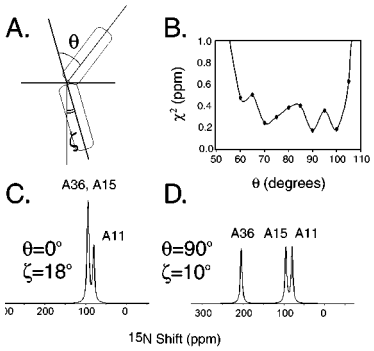

Figure 2. (A) Model of PLB indicating the interhelical angle θ between Domain IA and Domain II. (B) Plot of the χ^2 dependence on the interhelical angle (θ) from the conformers generated using rigid body rotation. C. and D. Simulated ^{15}N solid-state spectra for A11, A15, and A36 for $\theta = 0°$ and $\theta = 90°$, respectively. All the simulations were performed using the magnitudes for the anisotropy tensors reported by Oas et al.[17]

also provides the tilt angle (ζ) of the molecule with respect to the plane of the lipid bilayer (Figure 2A). In particular, using our experimental data for the continuous α-helical model ($\theta = 0°$), the program found a minimum with $\zeta = 18°$ with a high score (or poor agreement) $\chi^2 \cong 35$ ppm. The corresponding simulated ^{15}N spectrum for A11, A15, and A36 reported in Figure 2C does not match our experimental chemical shifts. In contrast, when we fit our data with a structure that has an interhelical tilt of 90°, the simulated spectrum closely reproduces the chemical shifts, with $\zeta = 10°$ and $\chi^2 \cong 0.18$ ppm (compare Figures 1E,F and 2E). Figure 2 shows a plot of χ^2 versus the interhelical angle θ. Although the lowest score was for $\theta = 90°$, two other significant minima are also present at $\theta = 70°$ and 100°. Outside the 60−100° range, the scores become too high, and the program does not give plausible solutions. Clearly, additional NMR frequency data are still needed to determine the exact tilt of the two helices and the conformation of the intervening loop of PLB. Nonetheless, our results rule out the possibility of a continuous α-helical structure for monomeric PLB in lipid bilayers and suggest an interhelical angle between 60° and 100°.

Previous solid-state NMR data on wild-type PLB point toward a continuous helix when the pentamer is formed.[11] Our data demonstrate that the functionally active monomeric AFA-PLB has Domain II oriented approximately perpendicularly to the plane of the bilayers, suggesting that the cytosolic helix lies on the lipid bilayer surface. These new data also suggest that PLB undergoes a substantial conformational change upon its depolymerization, with Domain IA interacting with the lipid bilayers prior to its binding with Ca-ATPase. It is likely that the structural dynamics of PLB involves functionally important transitions among multiple structural states and that PLB's structure is affected by its phosphorylation and by its interactions with other PLB molecules and with Ca-ATPase[2]. Taken together with our recent results,[14] these data represent the first evidence for the topological orientation of these monomeric Ca-ATPase regulatory polypeptides in lipid membranes.

Finally, our combined approach using solid-state NMR spectroscopy and rigid body molecular dynamics calculations can be applied to the determination of other membrane protein topologies once secondary structure elements are known and specific labeling of selected amino acids is accessible.

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