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Probing ground and excited states of phospholamban in model and native lipid membranes by magic angle spinning NMR spectroscopy☆

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ABSTRACT

In this paper, we analyzed the ground and excited states of phospholamban (PLN), a membrane protein that regulates sarcoplasmic reticulum calcium ATPase (SERCA), in different membrane mimetic environments. Previously, we proposed that the conformational equilibria of PLN are central to SERCA regulation. Here, we show that these equilibria detected in micelles and bicelles are also present in native sarcoplasmic reticulum lipid membranes as probed by MAS solid-state NMR. Importantly, we found that the kinetics of conformational exchange and the extent of ground and excited states in detergent micelles and lipid bilayers are different, revealing a possible role of the membrane composition on the allosteric regulation of SERCA. Since the extent of excited states is directly correlated to SERCA inhibition, these findings open up the exciting possibility that calcium transport in the heart can be controlled by the lipid bilayer composition. This article is part of a Special Issue entitled: Membrane protein structure and function.

1. Introduction

Membrane proteins exist as ensembles (Boltzmann distribution) of ground and excited states. These states are accessible by small scale atomic fluctuations around the average structure or large scale reorganizations of entire domains. In addition to intrinsic thermal fluctuations, the membrane protein folding energy landscape is shaped by the interactions with the lipid membrane, which have been hypothesized to modulate protein function through allosteric interactions [1]. In the past years, the need to obtain high-resolution membrane protein structures for structural genomics initiatives drove researchers toward high-quality views of average conformations or crystallographic snapshots of membrane proteins in non-native detergent micellar environments. The latter effectively relegated the analysis of conformational fluctuations and protein–lipid interactions to secondary roles. While the structures have given us vivid views of static membrane proteins, our knowledge on how these biological macromolecules transmit the regulatory effects through the membrane at the atomic level is still primordial. It is clear that a complete description of membrane protein function must include: structure, the motions (conformational dynamics), and their interactions with lipid membranes. Magic angle spinning (MAS) NMR [2–12] and oriented solid-state (OSS) NMR [13–18] have evolved as powerful methods to study the structure and dynamics of membrane proteins in native-like lipid bilayers. While the development of these solid-state NMR techniques will be instrumental to determine the structure of large membrane proteins, their major advantage with respect to X-ray crystallography will be to describe membrane proteins as ensemble of structures, including ground and excited states.

In the past years, our group has focused attention on the small membrane protein phospholamban (PLN, 52-residue) that regulates the function of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) in cardiac muscle [19,20]. While apparently simple in its secondary structure content, PLN has a complex dynamics when reconstituted both in micelles and synthetic lipid membranes [21–23]. In its monomeric and pentameric forms, PLN consists of a membrane spanning helix connected to an amphipathic helix (domain Ia) through a short loop [15,16,24] (Fig. 1). The membrane-spanning helix can be divided into a rigid and hydrophobic domain II and a more dynamic and hydrophilic domain Ib, which is positioned at the lipid head group/water interface and unfolds on a μs time scale [21,25]. Domain Ia of PLN is in a pre-existing conformational equilibrium that involves at least four different states and resembles the folding/unfolding proposed for amphipathic helices: detached/

Abbreviations: PLN, Phospholamban; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DPC, dodecylphosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol); MLVs, multi-lamellar lipid vesicles

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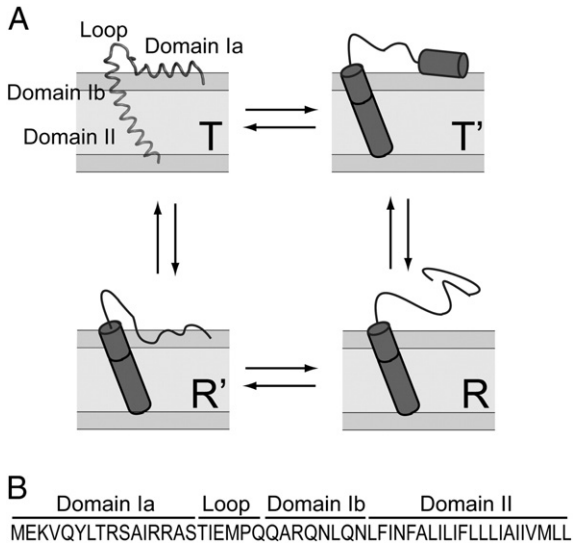

Fig. 1. Conformational equilibria and primary sequence. A.) Conformational equilibria of monomeric PLN. The T state is represented by its high resolution structure (PDB ID: 2KB7) [16]. B.) Primary sequence of AFA-PLN.

unfolded (R), absorbed/unfolded (R′), absorbed/partially folded (T′), and absorbed/fully folded (T) [26–28]. For monomeric and pentameric PLN, the R state has a small population in DPC detergent micelles and mechanically aligned lipid bilayers (<15%) [28]. Although most of the information on the high energy (excited) R, T′ and R′ states was derived from studies in detergent micelles, we found a direct relationship between the population of these excited states and PLN inhibition of SERCA, i.e., the more populated is the R state, the less inhibitory is PLN [26,29]. This structure–function correlation is being exploited to design loss-of-function PLN analogs to be used in gene therapy [30,31].

To understand how PLN's conformational equilibria are modulated by lipid membrane composition, we utilized Magic Angle Spinning (MAS) solid-state NMR in conjunction with solution NMR methods. We show that, although the relative populations of the different states can be tuned by the type and charge of lipids, a conformational equilibrium of PLN is present in all of the membrane mimicking systems analyzed, ranging from dodecylphosphocholine (DPC) micelles and 1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dihexanoyl-sn-glycero-3-phosphocholine (DMPC/DHPC) bicelles to native SR lipid bilayers. These results demonstrate the presence and importance of conformational fluctuations in native cellular membranes.

2. Materials and methods

2.1. Sample preparation

Synthetic lipids and natural egg PC, phosphatidylethanolamine (PE) and phosphatidic acid (PA) lipids were purchased from Avanti polar lipids (Alabaster, AL). Native SR lipids were extracted from rabbit hind leg skeletal muscle using a chloroform:methanol procedure as previously described [32]. All experiments utilized a monomeric PLN mutant with Cys36, 41, and 46 mutated to Ala, Phe, and Ala, respectively (AFA-PLN) [33]. [U-^{13}C, ^{15}N] AFA-PLN was recombinantly expressed in *E. coli* and purified according to Buck et al. [34] [EQN-^{13}C, ^{15}N; A-^{13}C] AFA-PLN was produced by a reversed labeling method, where unlabeled analogs of all other amino acids were added to the growth medium which also contained ^{13}C-glucose and $^{15}NH_4Cl$ [35]. Selectively [^{15}N, ^{13}C] labeled AFA-PLN was synthesized using [^{15}N, ^{13}C] labeled amino acids (Sigma-Aldrich, Isotec, Miamisburg, OH) on a microwave peptide synthesizer (CEM Corporation, Matthews, NC) essentially as previously described [16,33]. The exceptions were that 1% sodium dodecyl sulfate (SDS) was added during cleavage from the resin and the protein was dissolved in 10% SDS prior to HPLC purification, which utilized a 2-propanol/H_2O gradient from 10% to 90% on a C8 column. To avoid oxidation of Met50, we made a conservative substitution to norleucine in the synthetic samples. This substitution had no effect on the inhibitory effect of AFA-PLN on SERCA. The quality of the final synthetic product was assessed by mass spectrometry and solution NMR in DPC micelles. SERCA activity assays were performed as described previously [29,36] to confirm that all recombinantly and synthetically produced AFA-PLN proteins inhibited SERCA.

Isotropic bicelle samples for solution NMR were prepared by dissolving 1 mg of [U-^{13}C, ^{15}N] AFA-PLN in a 174 mg/mL solution of DHPC containing 20 mM MOPS, 100 mM NaCl, and 5% D_2O. The protein/DHPC solution was added to 21.9 mg of lyophilized DMPC lipids, and was followed by several freeze/thaw cycles to form a clear bicellar solution. The pH was adjusted to 7.0 and the 250 μL sample containing a DMPC:DHPC molar ratio (q) of 0.33 was transferred to a 5 mm Shigemi tube. Negatively charged bicelles were prepared identically with the exception that 33% of the DMPC was replaced by 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DMPG).

MAS samples were prepared by dissolving 15 mg of lyophilized lipids in HPLC elution (~70% 2-propanol, ~30% H_2O) containing 1.6 mg AFA-PLN. To this solution, 20 mM MOPS (pH 7.0) and 100 mM NaCl were added based on a final volume of 2 mL. The 2-propanol was then evaporated using nitrogen gas to form AFA-PLN-containing multi-lamellar lipid vesicles (MLVs). The mixture was lyophilized, re-suspended in 2 mL of double distilled H_2O and centrifuged at 200,000 ×*g* to yield a hydrated lipid pellet, which was transferred to a 3.2 mm thin wall rotor MAS rotor. The final samples contained ~60% H_2O and had a lipid:AFA-PLN ratio of ~80:1. For paramagnetic quenching experiments, gadopentetate dimeglumine (Gd^{3+}) (Magnevist; Bayer Shering Pharma, Berlin, Germany) was added to the MAS sample and several freeze/thaw cycles were performed (final Gd^{3+} concentration was 20 mM).

2.2. NMR experiments

All NMR experiments were performed on a Varian (VNMRS) spectrometer operating at a proton frequency of 600 MHz. Solution NMR chemical shifts of AFA-PLN in isotropic bicelles were assigned using two-dimensional HNCO, HN(CO)Cα, and HNCαCβ experiments and were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Chemical shifts in MLVs were assigned using SPC5 single quantum double quantum correlation (SQ-DQ) [40], dipolar assisted rotational resonance (DARR) [37], and refocused Insensitive Nuclei Enhanced by Polarization Transfer [38] (rINEPT) experiments acquired using a ^{1}H/^{13}C 3.2 mm BioMAS Varian probe. Pulse widths corresponding to a flip angle of π/2 were typically 5.5 μs (^{13}C, ^{15}N) and 2.5 μs (^{1}H). Proton TPPM decoupling [39] was applied at $\omega_1/(2\pi) =$ 100 kHz. SQ-DQ experiments were acquired at a spinning rate of 8 kHz, a cross-polarization (CP) time of 1 ms and spectral widths of 100 and 16 kHz in the single and double quantum dimensions, respectively. A 500 μs SPC5 element [40] was used for excitation and reconversion. DARR experiments were acquired with a 200 ms mixing time at a spinning speed of 13.3 kHz and a 1 ms cross-polarization (CP) time. 4000 points with a spectral width of 100 kHz and 30 increments with a spectral width of 13.3 kHz were acquired in the direct and indirect ^{13}C dimensions, respectively. rINEPT experiments were acquired at the same spinning rate with the same number of points and spectral width in the direct ^{13}C dimension and 30 increments with a spectral width of 3.33 kHz in the indirect ^{1}H dimension. Chemical shifts were referenced to the CH_2 signal of adamantane (40.48 ppm). Data were processed by NMRPipe [41] and analyzed with Sparky [42].