Structural and dynamic analysis of pathogenic modifications in cardiac sarcoplasmic reticulum proteins involved in Ca$^{2+}$ transport

A Thesis

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Abstract

Calcium signaling pathways are essential for the coordination of contraction and relaxation in cardiac muscle. Disruption of cardiac calcium cycling by pathogenic modifications in calcium transport proteins leads to a variety of cardiomyopathies including dilated cardiomyopathy and arrhythmias. The following thesis summarizes the structural and dynamic characterization of key regulatory proteins involved in calcium release and reuptake in the sarcoplasmic reticulum (SR). Calmodulin (CaM), a calcium-sensing protein that regulates its cellular targets based on the level of calcium in the cell, mediates calcium release from the SR via the homotetrameric calcium release channel, ryanodine receptor (RyR). The CaM-RyR complex has been a challenging structural target due to the size and complexity of the RyR. By applying a combination of solution and solid-state NMR techniques we have begun to develop a molecular model for CaM’s regulation of the RyR and how this regulation is disrupted by pathogenic modifications such as oxidation and mutation. Disruptions in calcium reuptake to the SR due to mutations in the small transmembrane protein, phospholamban (PLN), result from dysregulation of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). These PLN mutations are primarily associated with the development of dilated cardiomyopathy and by applying solution and solid-state NMR techniques we have begun to develop a model for how changes in PLN's structure and dynamics correlate to the dysregulation of SERCA. Together, the structural and dynamic studies outlined in this thesis provide further insights into the correlations between protein structure and function and the crucial roles CaM and PLN play in cardiac function.
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List of Abbreviations

NMR – Nuclear Magnetic Resonance
ssNMR – Solid-State NMR
CP – Cross-polarization
DARR – Dipolar Assisted Rotational Resonance
INEPT – Insensitive Nuclei Enhanced by Polarization Transfer
TOBSY – Total Through Bond Correlation Spectroscopy
HSQC – Heteronuclear Single Quantum Coherence
NOE – Nuclear Overhauser Effect
CD – Circular Dichroism
DPC – dodecylphosphocholine
DMPC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DHPC – 1,2-dihexanoyl-sn-glycero-3-phosphocholine
PLN – Phospholamban
AFA-PLN – Monomeric Phospholamban
SERCA – Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
PKA – Protein Kinas A
ATP – Adenosine triphosphate
CaM – Calmodulin
RyR – Ryanodine Receptor
Chapter 1: Background and Significance

1. Cardiac physiology and the molecular mechanism of Ca\textsuperscript{2+} cycling

   Cardiac contraction and relaxation is dependent on an intricate cycle of ion transport that propagates electrical signals throughout the heart and coordinates the contraction of individual cardiomyocytes.\textsuperscript{2} The heart’s ability to maintain this precise synchronization of contraction and relaxation is based on the integration of the nervous and muscular tissue, and the connection of cardiomyocytes via intercalated disks. The structure of ventricular cardiomyocytes is stabilized by a basement membrane composed of type IV collagen, laminin, fibronectin, and proteoglycans.\textsuperscript{6} This membrane provides a protective barrier for cardiomyocytes as well as an anchoring site for the collagen matrix that forms the extracellular space and exterior structure of the cardiomyocyte network. The exterior of the individual cardiomyocytes is formed by the sarcolemma, a lipid bilayer interspersed with integrins that anchor the membrane to the extracellular collagen matrix and

![Figure 1.1: Structure of a ventricular myocyte.](image)

Depolarization of the sarcolemma triggers a localized Ca\textsuperscript{2+} influx through the L-type Ca\textsuperscript{2+} channels, initiating calcium-induced calcium release from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR) on the junctional SR. The increasing level of intracellular Ca\textsuperscript{2+} stimulates the formation of cross-bridges between the myosin thick filaments and actin in the thin filaments allowing the sarcomeres to contract. Ca\textsuperscript{2+}-reuptake to the SR via SERCA is mediated by the transmembrane protein, phospholamban (PLN).
assist in propagating the contractile shortening to adjacent myocytes. The sarcolemma also contains a variety of ion channels, pumps, and signal receptors and the localization of these membrane proteins within the sarcolemma relates to their function. Parts of the sarcolemma form intercalated disks which depending on their location and composition act as fascia adherens, desmosomes, or gap junctions. Gap junctions facilitate the propagation of electrical currents and signaling molecules between cardiomyocytes due to the close proximity of the myocyte membranes (typically less than 2 nm apart) and the presence of six-subunit integral membrane channels called connexons. These gap junctions are stabilized by desmosomes, which provide structural stability through anchoring interactions with intermediate filaments. While the gap junctions propagate electrical signals between cardiomyocytes, the fascia adherens provides a mechanical coupling that transmits contractile force from myocyte to myocyte. The sarcolemma also contains key structural invaginations in the membrane surface called transverse, or T, tubules. These T-tubules contain a high concentration of ion channels and places these channels in close proximity to intracellular organelles such as the sarcoplasmic reticulum (SR) to facilitate signaling between the extra- and intracellular space.

The electrical signals that are critical for pacing the contractile behavior of the heart are initiated by the sinoatrial node. The action potential initiated in the sinoatrial node is propagated throughout the heart, passing through the atrioventricular node which generates a slight delay between the contraction of atrial and ventricular cardiomyocytes. In ventricular myocytes, the action potential consists of five phases – a rapid depolarization followed by a brief repolarization, a plateau phase, rapid repolarization and the resting potential. The resting membrane potential is primarily maintained by the passive diffusion of K\(^+\) ions through the K\(^+\) rectifier, a current that is balanced by the efflux of Na\(^+\) ions, resulting in a resting membrane potential of approximately -80 mV for most cardiomyocytes. The rapid depolarization of this resting membrane potential changes the
permeability of the membrane to specific ions by the voltage-dependent stimulation of ion channels. The rapid depolarization phase of the action potential results from activation of the fast Na\(^+\) channel which transports Na\(^+\) ions into the cell and generates a positive membrane potential.\(^6\) This rapid depolarization activates the L-type Ca\(^{2+}\) channels (Figure 1.1), causing localized increases in the cytosolic Ca\(^{2+}\) concentration.\(^10,11\) The ‘driving force’ of this localized influx of Ca\(^{2+}\) is stimulated by the brief repolarization period following depolarization, where the fast Na\(^+\) channel is inactivated and Cl\(^-\) ions are brought into the cell as K\(^+\) is extruded.\(^6\) The increase in Ca\(^{2+}\) influx induced by this brief repolarization phase generates a plateau in the membrane potential which transitions into a rapid repolarization due to K\(^+\) influx and returns the membrane potential back to the resting potential.\(^2,11\)

**Figure 1.2: Structure of a mammalian ryanodine receptor.** Structure of the RyR at 4.8 Å, figure from Zalk \textit{et al.} (2015).\(^12\) a) Mesh rendering showing the channel axis. b) Cartoon representation of RyR1. Panels (c-e) show color coded models of the RyR’s structural regions at different orientations to the membrane: N-terminal domain (blue); SPRY1, SPRY2, and SPRY3 (cyan); clamp region and phosphorylation domain (salmon); calstabin (yellow); bridge solenoid scaffold (green); core solenoid (red); C-terminal and transmembrane domains (orange); EF hand Ca\(^{2+}\)-binding sites (purple). Panel (c) shows the side view of the RyR, panel (d) shows a top-down view of the cytoplasmic domain, and panel (e) shows a bottom-up view from the lumen of the SR.
In ventricular cardiomyocytes the electrical events of the action potential are translated to mechanical contraction through Ca\(^{2+}\) signaling in excitation-contraction coupling. The localized Ca\(^{2+}\) influx through the voltage-gated L-type Ca\(^{2+}\) channels causes the Ca\(^{2+}\) concentration around the terminal cisternae of the SR to increase to 10-20 μM (Figure 1.1).\(^9,11\) The terminal cisternae of the SR contains a high concentration of the tetrameric ryanodine receptor (RyR, Figure 1.2) Ca\(^{2+}\)-release channel (approximately a 1:10 ratio between L-type Ca\(^{2+}\) channels and RyR channels) which sense the change in Ca\(^{2+}\) level and release a burst of Ca\(^{2+}\) ions from the SR, increasing the local Ca\(^{2+}\) concentration to 200-400 μM.\(^6\) This increased level of Ca\(^{2+}\) diffuses throughout the cardiomyocyte to stimulate contraction at the sarcomeres.

The sarcomere consists of interdigitating thick and thin filaments that slide past each other to contract or relax the sarcomere depending on the level of Ca\(^{2+}\) present in the cell.\(^6\) The thin filament is composed primarily of actin and decorated with troponin and tropomyosin in a 7:1:1 molar ratio.\(^13\) Tropomyosin’s binding interaction with actin is mediated by troponin-T and in the absence of Ca\(^{2+}\), tropomyosin blocks the binding site for myosin from the thick filament preventing a physical link between myosin and actin.\(^6\)

When the intracellular Ca\(^{2+}\) concentration increases, Ca\(^{2+}\) binds to the N-domain of troponin-C generating a conformational change and increasing the binding affinity between troponin-C and troponin-I. These shifts pull troponin-I off its actin binding site. This in turn shifts the troponin/tropomyosin complex into the grooves of the actin filament and away from the myosin binding site. The subsequent binding of myosin to the actin filament to form a cross-bridge is a cooperative binding interaction as myosin binding pushes the troponin/tropomyosin complex further into the groove and increases Ca\(^{2+}\) binding at the neighboring troponin-C sites.\(^6,10\) The type and strength of contraction generated by the actin-myosin cross-bridge in ventricular myocytes is modulated by regulatory path-
ways in the heart that respond to both internal and external cues such as disease states, or activity and stress levels.\textsuperscript{2,14-17}

![Figure 1.3: Structure of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase.} The proposed structural model for Mg\textsuperscript{2+}'s facilitation of Ca\textsuperscript{2+} coordination in the E1 state. Figure from Espinoza-Fonseca et al. (2014)\textsuperscript{18}. From left to right, the Ca\textsuperscript{2+}-free E2 state (PDB 2AGV), the proposed Mg\textsuperscript{2+}-bound E1 state (PDB 3W5B), and the Ca\textsuperscript{2+}-bound E1 state (PDB 1SU4). The N domain is shown in green, the P domain in blue, the A domain in red, and the transmembrane (TM) domain in gray.

In addition to stimulating sarcomere contraction, the level of calcium present in the cytosol determines the duration and strength of the contraction. In order for the heart muscle to relax, the intracellular calcium concentration must be reduced. The bulk of intracellular calcium is sequestered back into the sarcoplasmic reticulum for the next contraction cycle via the cardiac isoform of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA), SERCA2a, while the remaining Ca\textsuperscript{2+} is extruded from the cell via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and the sarcolemmal Ca\textsuperscript{2+}-ATPase.\textsuperscript{6,19} SERCA is a P-type ion channel that transports approximately two Ca\textsuperscript{2+} ions per ATP hydrolyzed by coupling the conformational changes caused by ATP hydrolysis in the N-domain to the movement of Ca\textsuperscript{2+} through its transmembrane helices (Figure 1.3).\textsuperscript{20} As the concentration of calcium in the cytoplasm drops back to nanomolar levels, SERCA’s transport activity is modulated by the transmembrane proteins, phospholamban (PLN, in ventricular tissues; Figure 1.4)
and sarcolipin (SLN, in atrial tissue). In addition to post-translational modifications to SERCA that directly affect its Ca$^{2+}$ transport activity and often lead to disease states,

**Figure 1.4: Conformational states of PLN.** Top, monomeric T (left, PDB 2KB7) and R (right, PDB 2LPF) states of PLN. Key residues for the cytoplasmic domain's interaction with the membrane – V4, L7, A11, and A15 – are shown in the T state, while the site for β-adrenergic control via PKA phosphorylation (Ser16), is shown for both the T and R state. Bottom, the structures for the pentameric form of PLN in the unphosphorylated (left, PDB 2KYV) and pS16 (right 2M3B) form. The insertion angle of the transmembrane domain of the monomeric units is shown for each structure relative to the membrane normal and indicated by the dashed lines. Residues in the Leu-Ile zipper for the pentameric forms are shown in blue for the unphosphorylated pentamer and green for the pS16 pentamer.

SERCA's regulation by PLN in ventricular cardiomyocytes provides sensitivity to β-adrenergic pathways in the cell.$^{14,15,21-24}$ PLN is a small, transmembrane protein with four domains: a cytoplasmic region that contains domain Ia, the loop, and domain Ib which links the cytoplasmic region and transmembrane domain II.$^{25}$ Upon phosphorylation at Ser16 by protein kinase A (PKA), PLN's inhibition of SERCA's calcium transport is relieved and calcium can be returned to the SR. PLN's conformational equilibrium is relat-
ed to its ability to modulate SERCA activity via a mechanism that is still incompletely un-
derstood.

2. Pathogenic modifications in Ca\textsuperscript{2+} cycling proteins

2.1 Pathogenic modifications in calmodulin

CaM is a 16.7 kDa cytosolic protein that acts as a calcium sensor and with many of its cellular targets, CaM regulatory effects change depending on the concentration of calcium present in the cell.\textsuperscript{9} This sensitivity to calcium is derived from CaM’s four EF-hand motifs. CaM’s overall structure consists of \(\alpha\)-helical N- and C-terminal domains connected by a flexible linker, and each of CaM’s domains contains paired EF-hand mot-
tifs allowing CaM to bind a total of four Ca\textsuperscript{2+} ions (Figure 1.5A).\textsuperscript{26,27} The coordination of calcium by these EF-hands stimulates CaM’s structural transition from the Ca\textsuperscript{2+}-free (apo) to the Ca\textsuperscript{2+}-bound (holo) state. The EF-hands (I and II) in the N-lobe have a low micromolar affinity for Ca\textsuperscript{2+}, while EF-hand III and IV in the C-lobe have a high nanomolar affinity.\textsuperscript{28} Each EF-hand consists of the consensus helix-loop-helix pattern, and the varied sensitivity of each of CaM’s EF-hands is due to the varied amino acid content in the 12 residue loop that provides the residues for the pentagonal bipyramidal chelation of Ca\textsuperscript{2+}.\textsuperscript{29,30} The most common amino acid for Ca\textsuperscript{2+} coordination in the EF-hand is aspar-
tic acid and the presence of this residue at the first position of the loop is 100% con-
served across EF-hand motifs.\textsuperscript{30} Aspartic acid is also well represented at other chelating positions in the loop, with a 76%, 52%, and 32% probability of occurring at the 2\textsuperscript{nd}, 3\textsuperscript{rd}, and 5\textsuperscript{th} positions. EF-hands I and II in CaM’s N-lobe follow this pattern with aspartic acid at the 1\textsuperscript{st} through 3\textsuperscript{rd} positions in EF-hand I and the 1\textsuperscript{st}, 2\textsuperscript{nd}, and 5\textsuperscript{th}, positions in EF-hand II. This pattern is repeated in the C-lobe, however the other chelating positions contain varied residues between the two domains, with the N-lobe EF-hands favoring threonine, and the C-lobe favors a mix of residues.\textsuperscript{31}
The ability of the EF-hands to coordinate calcium is crucial for CaM’s regulatory function. This is emphasized by the recent identification of more than ten mutations in CaM that are associated with various cardiac arrhythmias (Figure 1.5B).\textsuperscript{32-36} Primarily local-

\textbf{Figure 1.5: Pathogenic modifications in calmodulin.} (PDB 1CLL) \textbf{A)} Four Ca\textsuperscript{2+}-binding sites in CaM. Sites I (orange) and II (yellow) in the N-lobe and sites III (light blue) and IV (dark blue) in the C-lobe. \textbf{B)} Mutations in CaM associated with cardiac arrhythmia and sudden cardiac death shown in green. \textbf{C)} CaM contains nine methionine (Met) residues (shown in red), four in each lobe and one in the linker.
ized to CaM’s C-lobe, seven of the mutations (D96V, N98S, N98I, D130G, D132E, D134H and Q136P) occur in residues that are directly involved in Ca\textsuperscript{2+} coordination. Each mutation has varied effects on CaM’s structural and functional behavior, but multiple mutations have been identified to alter the duration and intensity of the calcium transients in cardiomyocytes in addition to having altered binding affinities for crucial ion channels regulated by CaM.\textsuperscript{32,33,37,38} One cellular target that experiences aberrant regulation from multiple mutants is the RyR, which has sparked renewed interest in understanding the mechanism of CaM’s binding and regulation of the RyR on an atomic level.\textsuperscript{38} A method for the characterization of CaM’s binding to the RyR via solid-state NMR is outlined in Chapter 6 of this thesis.

The conserved hydrophobic core and target binding clefts created by the EF-hand motifs are also crucial to CaM’s ability to regulate its cellular targets.\textsuperscript{26} In addition to mutation, the packing of these hydrophobic cores, and as a result CaM’s Ca\textsuperscript{2+}-binding and regulatory function, can be disrupted by oxidation of CaM’s methionine residues. CaM contains a total of nine methionine residues spread throughout the N-lobe, C-lobe, and linker (Figure 1.5C).\textsuperscript{39} All of these methionine residues are susceptible to oxidation, and eight play key roles in maintaining a hydrophobic core in the N- and C-lobes that is exposed when CaM transitions from the apo to the holo state (M37, M52, M72, and M73 in the N-lobe; M110, M125, M145, and M146 in the C-lobe).\textsuperscript{40-42} The role of two of these methionine residues, M52 and M125, in maintaining CaM’s hydrophobic core during the transition to the Ca\textsuperscript{2+}-bound state and the effects of oxidation at these sites on CaM’s structure and dynamics is explored in Chapter 2 of this thesis.
2.2 Phospholamban mutations associated with dilated cardiomyopathy

As SERCA is responsible for the bulk of Ca\(^{2+}\) removal from the cytosol for cardiac relaxation, any disruption in SERCA regulation has the potential to cause devastating effects on cardiac function.\(^{43,44}\) Genetic screening in cases of hereditary dilated cardiomyopathy has identified six mutations in PLN – R9C, R9H, R9L, R14del, R25C, and L39stop – that disrupt SERCA’s ability to transport calcium (Figure 1.6; Table 1.1).\(^{45}\) In patients with DCM, the heart exhibits morphological changes in the size of the left ventricle, which can progress to enlargement of other chambers of the heart as well (Figure 1.7).\(^{46}\) Due to the enlargement of the left ventricle and thinning of the ventricle wall, the heart can no longer pump blood efficiently.

**Figure 1.6: Pathogenic mutations in PLN.** Mutations in PLN associated with cardiac pathologies mapped to PLN’s primary sequence. Mutations in domain Ia (blue) include R9C, R9H, R9L, and R14del, the R25C mutation occurs in domain Ib (red), and the L39stop mutation occurs in the transmembrane domain II (green). The mutations are mapped, from left to right, to the pentameric (PDB 2KYV), T (PDB 2KB7), R (PDB 2LPF), and proposed B states of PLN.
The six mutations in PLN appear to alter SERCA’s regulation via distinct mechanisms that are still under investigation. The first mutation, R9C, results from a missense mutation that is inherited in an autosomal dominant fashion and causes a severe form of DCM that begins by 20-30 years of age.\textsuperscript{46} Functionally, R9C acts as a weak inhibitor of SERCA activity and studies in mouse models indicated that wild-type PLN may be able to outcompete R9C for binding to SERCA. The greatest controversy with the R9C mutant rests in its ability to respond to β-adrenergic regulation. While \textit{in vivo} studies have provided ambiguous results, \textit{in vitro} assays with both peptide and full-length monomeric forms of R9C indicated that this mutant’s ability to be phosphorylated at Ser16 by PKA is dependent on its oxidative state and may depend on structural or dynamic disruptions to PKA’s binding site.\textsuperscript{47} The effects of oxidation on R9C’s structure and function are also of interest due to the elevated levels of reactive oxygen species that are often associated with the progression of DCM.\textsuperscript{48} Further characterization of the structural and dynamic effects of the Arg9Cys mutation are outlined in Chapter 5, and continuing these studies to probe the effects of these changes on R9C’s ability to respond to PKA and suscepti-

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<td>Domain Ia</td>
<td>Weak inhibitor</td>
<td>DCM</td>
</tr>
<tr>
<td>R9L</td>
<td>Domain Ia</td>
<td>--</td>
<td>Heart failure</td>
</tr>
<tr>
<td>R9H</td>
<td>Domain Ia</td>
<td>--</td>
<td>Heart failure</td>
</tr>
<tr>
<td>R14del</td>
<td>Domain Ia</td>
<td>Constant inhibitor</td>
<td>DCM</td>
</tr>
<tr>
<td>R25C</td>
<td>Domain Ib</td>
<td>Constant inhibitor</td>
<td>Ventricular arrhythmia and DCM</td>
</tr>
<tr>
<td>L39stop</td>
<td>Domain II</td>
<td>SERCA is uninhibited</td>
<td>DCM</td>
</tr>
</tbody>
</table>
bility to oxidation have the potential to provide significant insights into the molecular mechanism that leads to DCM.

While R9C is known to be a weak inhibitor of SERCA, the mechanism by which the remaining mutations at Arg9, R9H and R9L, generate milder phenotypes of heart failure is incompletely understood. Structural predictions of PLN’s cytoplasmic domain done with these mutations indicate they may induce structural changes in PLN.\textsuperscript{49}

![Figure 1.7: Cardiac structure in dilated cardiomyopathy.](image)

The L39stop mutation generates a PLN-null phenotype and may be inherited in both a homozygous and heterozygous fashion with concurrent effects on the severity of DCM.\textsuperscript{50} Patients that are homozygous for the mutation exhibit a severe, very early onset form of DCM while patients that are heterozygous don’t present with DCM until 20-30 years of age.

The R14del mutation is one of the best characterized mutations in PLN. This mutation leads to variable DCM phenotypes that may be linked to a gender-dependent penetrance where males are more affected by R14del’s constant inhibition of SERCA activity.\textsuperscript{51-53} R14del’s behavior as a constant inhibitor of SERCA may be due to the dis-
ruption of phosphorylation by PKA. Kinetic assays with a peptide spanning the cytoplasmic domain of R14del PLN and the catalytic domain of PKA demonstrated both a reduced binding affinity for the R14del peptide and slower phosphorylation kinetics for the phosphorylation reaction at Ser16. These results are most likely due to R14del’s disruption of the R-R-X-S/T-Φ (Φ is a hydrophobic residue) recognition sequence for PKA in PLN’s cytoplasmic domain. This insensitivity to β-adrenergic regulation coupled with the structural and dynamic changes observed in R14del PLN using solid-state NMR support hypotheses that PLN’s conformational state may play a role in its regulation of SERCA.

The most recently identified mutation associated with DCM is the R25C mutation in PLN’s domain Ib. This mutant is associated with ventricular arrhythmias that progress to the DCM phenotype. Like R14del, R25C may act as a ‘super-inhibitor’ of SERCA, and exhibits an ability to decrease the level of Ca²⁺ reuptake to the SR at micromolar concentrations of Ca²⁺. Rat cardiomyocytes that overexpressed R25C also exhibited a high level of spontaneous aftercontractions relative to cardiomyocytes containing wild-type PLN. Unlike R14del, R25C appears to be susceptible to phosphorylation at both Thr17 and Ser16, and phosphorylation at Ser16 appears to promote R25C’s dissociation from SERCA. The application of solid-state NMR techniques to characterize the structural and dynamic effects of the R25C mutation on PLN’s conformational equilibrium is described in Chapter 5.

3. Probing protein structure, dynamics, and ligand binding by Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is a valuable research technique for the characterization of protein structure and dynamics. In addition, NMR has many applications for characterizing protein-ligand binding interactions on an atomic level. As
a small, soluble protein, CaM is ideally suited for solution NMR characterization, and Chapter 2 outlines the application of NMR techniques in the atomic level characterization of the effects of pathogenic modifications on CaM’s structure, dynamics, and ability to respond to Ca$^{2+}$. CaM’s conformational flexibility and the structural plasticity that enables it to adapt to multiple binding targets makes NMR an ideal technique for characterizing CaM’s structure and dynamics.

The characterization of membrane proteins by NMR is a significant challenge due to the presence of transmembrane domains that are accustomed to the hydrophobic core of a lipid bilayer in order to maintain their structural integrity. For smaller peptides and transmembrane domains, solution NMR experiments utilizing detergent micelles and isotropic bicelles to maintain protein structure are a viable solution as shown in Chapter 5 for the analysis of the pathogenic R9C and R25C mutations in PLN.

For larger membrane proteins, protein-protein complexes, and for proteins like PLN whose structure and dynamics are sensitive to their lipid environment as shown in Chapter 3, solid-state NMR (ssNMR) provides an alternative method for probing protein structure and dynamics. The work outlined in Chapters 3-6 primarily focuses on the application of magic angle spinning (MAS) ssNMR for the characterization of membrane proteins and protein-protein complexes. ssNMR provides an advantage for the structural

Figure 1.8: Sample preparation in Magic Angle Spinning (MAS) solid-state NMR. Sample preparation methods for reconstituted MAS samples.
characterization of membrane proteins as it allows samples to be prepared in reconstituted lipid vesicles (Figure 1.8) as for PLN in Chapters 3-5, or even in biologically extracted lipids as in the samples of CaM in complex with the RyR in Chapter 6. Maintaining this lipid environment allows the structure and dynamic information obtained from solid-state NMR to be as biologically relevant as possible.

3.1 \([^{1}\text{H}, ^{15}\text{N}]\) HSQC as an amide fingerprint

The chemical shift, or the observed resonance frequency for nuclei, provides a sensitive measure of the local chemical environment. For an isotopic solution state sample, the chemical shift can be described by:

\[
v(\text{Hz}) = \gamma B_0 \left(1 - \frac{\sigma}{2\pi}\right)
\]  

where \(\sigma\) represents the shielding constant which describes the contribution of secondary or local magnetic fields to the local magnetic field experienced by a nucleus. These secondary magnetic fields arise from the electron motions induced by the presence of the strong external magnetic field generated by the NMR magnet, and provide a unique chemical environment for the nucleus. In order for chemical shift values to be compared across spectrometers with different magnetic field strengths, chemical shift values are typically reported using the value parts per million (ppm) in relation to a reference signal from a standard molecule:

\[
\delta = \frac{v - v_{\text{ref}}}{\omega_0} \times 10^6
\]  

where \(\delta\) represents the chemical shift value in ppm, and \(\omega_0\) represents the Larmor frequency of the spectrometer as defined by:

\[
\omega_0 = -\gamma B_0
\]
The Larmor frequency is dependent on the strength of the magnetic field \((B_0)\) and the gyromagnetic ratio \((\gamma)\) of the nuclei being characterized.\(^{59}\)

Since the chemical shift of nuclei is dependent on their local chemical environment, and proteins have repeating structural characteristics based on the structure of amino acids and the common secondary and tertiary folding patterns, chemical shift databases containing predicted chemical shift ranges for each nuclei in an amino acid have been developed. An example of these common chemical shift ranges for the \(^{13}\)C nuclei in amino acids is shown in Figure 1.9, and illustrates how chemical shift values change based on the local chemical environment. Since many carbonyl \(^{13}\)C nuclei experience

![Figure 1.9: \(^{13}\)C chemical shift ranges for amino acids. Common chemical shift ranges for the Cα, Cβ, Cy, Cδ, Cζ, and carbonyl atoms for amino acids mapped from the statistics provided by the Biological Magnetic Resonance Data Bank.](image-url)
similar chemical environments in the protein backbone, carbonyl chemical shifts fall in a
similar chemical shift range from 165-195 ppm.\textsuperscript{60} However due to variations in second-
ary structure and the primary sequence of individual proteins, the local environment for
each amino acids carbonyl group will be slightly different, meaning that an Ala residue at
one position in the protein may have a different carbonyl chemical shift than a second
Ala residue at a different position in the protein.

| Table 1.2: J coupling constants\textsuperscript{59} |
|-----------------|---------|
| $^{1}J_{\text{NH}}$ | ~91 Hz  |
| $^{1}J_{\text{N}C_{\alpha}}$ | ~7-11 Hz |
| $^{1}J_{\text{NC}}$ | ~4-9 Hz  |
| $^{1}J_{\text{NCO}}$ | ~15 Hz   |
| $^{1}J_{\text{CaCO}}$ | ~55 Hz   |
| $^{1}J_{\text{CH}}$ | ~140 Hz  |
| $^{1}J_{\text{CaC}_{\beta}}$ | ~35 Hz   |

The [$^{1}\text{H},^{15}\text{N}$] heteronuclear single quantum coherence (HSQC) experiment pro-
vides an amide fingerprint of the protein backbone and sidechain by correlating protons
($^{1}\text{H}$) to their directly bonded $^{15}\text{N}$.\textsuperscript{59} This is accomplished by using an insensitive nuclear
enhanced polarization transfer (INEPT) to transfer magnetization from $^{1}\text{H}$ to $^{15}\text{N}$ using
scalar coupling.\textsuperscript{4} Since the bonding interaction between different types of nuclei gener-
ates unique scalar (J) coupling constants (Table 1.2), this INEPT transfer can be opti-
mized to specifically select the protons that are bound to $^{15}\text{N}$. Once the magnetization is
transferred to $^{15}\text{N}$, the magnetization is allowed to evolve in the presence of the $^{1}\text{H}$
chemical shift effects, refocused, and then transferred back to $^{1}\text{H}$ for detection (\textbf{Figure
1.10A}). This experiment yields 2D spectra with one axis representing the $^{1}\text{H}$ chemical
shift and the other representing the $^{15}\text{N}$ chemical shift. Each peak in the 2D plot repre-
sents an amide group in the protein (\textbf{Figure 1.10B-C}). Since the $^{1}\text{H}$, and $^{15}\text{N}$ chemical
shift values are sensitive to their chemical environments as previously described, it is possible to use the \([^{1}H, ^{15}N]\) HSQC experiment to track conformational changes in the protein backbone resulting from ligand binding or other changes in the chemical envi-

**Figure 1.10: \([^{1}H, ^{15}N]\) Heteronuclear Single Quantum Correlation (HSQC) experiment.** A) Pulse sequence for the gradient enhanced \([^{1}H, ^{15}N]\) HSQC as described in Zhang et al. (1994)^1. B) Sample of a protein backbone (black) and sidechains (gray) showing the N-H amide groups probed by the HSQC experiment in blue. C) \([^{1}H, ^{15}N]\) HSQC overlay of CaM\textsuperscript{WT} in the apo (dark blue) and Ca\textsuperscript{2+}-bound (light blue) states showing the changes in chemical shift caused by the changes in chemical environment induced by Ca\textsuperscript{2+} binding.
3.2 Characterizing backbone dynamics via $[^1\text{H}, {}^{15}\text{N}]$ heteronuclear NOE

Just as changes in the chemical shifts for the amide backbone can be used to trace conformational changes in response to ligand binding, the fast (ps-ns) dynamics of the amide backbone can be used to describe changes in the dynamic behavior of a protein (Figure 1.11). This dynamic behavior can be measured using the Nuclear Overhauser Effect (NOE), which recognizes that dipolar-coupled spins do not relax independently of each other and the NOE effect on the NMR spectra is determined by the strength of the dipolar, or through space, coupling between two nuclei. The $[^1\text{H}, {}^{15}\text{N}]$ heteronuclear NOE experiment (Figure 1.12) works by acquiring one NOE experiment with saturation on $^1\text{H}$ to provide the NOE enhancement, and one experiment without.
The relative intensities for each residue are then compared for the two spectra and provide a residue-specific measure of the fast backbone dynamics in the protein. These experiments are applied to great effect in Chapter 2 to characterize the backbone dynamics of CaM in response to mutation and ligand binding.

3.3 Application of magic angle spinning (MAS) solid-state NMR for characterizing membrane protein dynamics

The structural and dynamic analysis of membrane proteins and their supramolecular complexes is complicated by the need to maintain a biologically relevant sample environment that reflects the complexity of the biological membrane. While smaller membrane proteins may be amenable to solution NMR analysis using micelles and iso-
tropic bicelles as shown for PLN in Chapter 5, these systems are limited in their ability to replicate the lipid bilayer and by the size limit of solution NMR. In addition, many membrane proteins are sensitive to their lipid environment and alter their structure and dynamics in response to their lipid environment as demonstrated for PLN in Chapter 3. The ability of solid-state NMR (ssNMR) to accommodate lipid vesicle and bilayer sample conditions provides an advantage for structural and dynamic studies by providing conditions that are as close to biological conditions as possible. ssNMR is also advantageous for large proteins and protein-protein complexes as it doesn’t have the same size limitations as solution NMR. In solution, samples are able to tumble freely. The rate of this

Figure 1.13: Effect of spinning speed on resolution in MAS ssNMR. Figure from Evans (1995) highlighting the effects of decoupling and spinning speed on the sensitivity and resolution of MAS ssNMR spectra using crystalline glycine as a standard. a) $^{13}$C spectra without proton decoupling. b) Spectra with proton decoupling. c) Spectra with both proton decoupling and cross-polarization. Panels d-f) Spectra with cross-polarization and MAS spinning speeds of 1 kHz, 3 kHz, and 5 kHz respectively. g) Spectra with cross-polarization and sideband suppression with a spinning speed of 5 kHz.
tumbling, or correlation time \( (\tau_c) \), affects the relaxation of the magnetization.\(^1\) The larger the molecule, the larger the molecule or more viscous the solution the slower the tumbling and the more efficiently the magnetization will relax. Currently, the upper size limit for solution NMR is around 50 kDa, making ssNMR techniques essential for the characterization of the SERCA-PLN and CaM-RyR binding interactions.

One significant challenge in ssNMR is the effects of dipolar, or through space, coupling on line broadening.\(^6\) In solution, the ability of molecules to tumble freely causes the dipole-dipole interactions to average out.\(^1\) Under solid-state conditions, the molecular motions are more restricted so the dipole interactions are unable to average to zero causing line broadening. There are multiple techniques that can be used to minimize this effect including decoupling and spinning the sample to mimic the effects of tumbling.\(^6\) Decoupling suppresses the interactions between nuclear spins by applying high powered pulses at the frequency of the spins being decoupled. Spinning the sample at the ‘magic angle’ of 54.7\(^\circ\) to the bulk magnetic field drives the contribution of dipolar interactions to zero (Figure 1.8). Figure 1.13 shows an application of these techniques for a sample of crystalline glycine. The first panel shows the spectra of glycine with the broad lines caused by dipole-dipole interactions. Introducing decoupling into the pulse sequence (Figure 1.13b) provides some resolution between the peaks but at a level that would be insufficient for the analysis of larger molecules. Spinning the sample at increasing frequencies (Figure 1.13d-f) provides increasing resolution between the peaks and provides spectra with linewidths that allow for the distinction between individual peaks. Using these techniques as a foundation for MAS ssNMR it is possible to apply a variety of pulse sequences to probe different dynamic states of proteins as described in Chapters 3 through 6.
Chapter 2: NMR studies show that Met125 is essential to maintaining the structural integrity of calmodulin’s C-terminal domain

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2.1 Abstract

Calmodulin (CaM) is a ubiquitous, Ca$^{2+}$-sensitive regulatory protein that interacts with numerous targets. In both skeletal and cardiac muscle, CaM binds and regulates the ryanodine receptor (RyR), a 2.3 MDa, homotetrameric channel responsible for releasing Ca$^{2+}$ from the sarcoplasmic reticulum into the cytoplasm to initiate muscle contraction. Due to its high methionine content, CaM is highly susceptible to oxidation by reactive oxygen species (ROS) produced by normal metabolism. Elevated ROS also contribute to muscle degeneration in aging. Oxidized CaM has a decreased ability to regulate RyR function, thus contributing to the degeneration of muscle contraction. It has been shown that a point mutation in CaM (M125Q) can mimic the functional effects of methionine oxidation on CaM’s regulation of RyR. Here, we used solution NMR spectroscopy and circular dichroism to investigate the structure and dynamics of CaM$^{M125Q}$ and compare with that of oxidized wild-type CaM (CaM$^{WT}$). We found that the M125Q mutation causes a dramatic unfolding of the C-terminal lobe of CaM, preventing the formation of a hydrophobic cluster of residues in the proximity its EF-hand Ca$^{2+}$ binding sites. Furthermore, we found that the NMR fingerprint of CaM$^{M125Q}$ is similar to that of partially oxidized CaM$^{WT}$, suggesting that the structural changes in CaM caused by oxidative insult blunts CaM’s ability to regulate the RyR. This may be a potent mechanism that promotes the decay of muscle function in aging.
2.2 Introduction

Calmodulin (CaM) is a 16.7 kDa protein that converts intracellular $[\text{Ca}^{2+}]$ into functional signals for a multitude of targets and is directly involved in cardiac muscle contractility\(^9\). Due to its high content of Met residues, CaM is also susceptible to oxidative stress, which alters its ability to properly transduce $\text{Ca}^{2+}$ signals\(^63\). CaM’s primarily α-helical structure consists of two globular domains (lobes) tethered by a flexible linker region that allows CaM to bind to a variety of cellular targets.\(^{27}\) Changes in cytoplasmic $\text{Ca}^{2+}$ levels lead to modulation of both CaM’s conformation and regulatory properties shifting the population from the $\text{Ca}^{2+}$-free (apo) to the $\text{Ca}^{2+}$-bound (holo) state. CaM’s $\text{Ca}^{2+}$ sensitivity stems from the cooperatively paired EF-hand motifs in the N- and C-terminal lobes. Each lobe contains two EF-hand motifs allowing CaM to bind a total of four $\text{Ca}^{2+}$ ions\(^{26,27}\). The N-terminal lobe contains EF-hands I and II with a low $\text{Ca}^{2+}$ affinity (~10 μM) while the C-terminal lobe EF-hands III and IV have a ~10-fold higher $\text{Ca}^{2+}$ affinity\(^{28}\). Eight of the nine Met residues play a key role in stabilizing CaM’s $\text{Ca}^{2+}$ binding pockets and participate in the structural changes and hydrophobic packing that enables CaM to bind to a variety of cellular targets\(^{54}\).

In addition to the hydrophobic environment around the Met residues, the EF-hand motifs contain paired aromatic residues in helices 1 and 4 that shift from nearly antiparallel orientation in the $\text{Ca}^{2+}$-free (apo) state to nearly perpendicular in the $\text{Ca}^{2+}$-bound state\(^{40,65-69}\). The stacking of these aromatic residues and stabilization of hydrophobic interactions throughout CaM’s domains play a key role in the cooperativity of $\text{Ca}^{2+}$ binding\(^{40}\). CaM’s structural transition from the apo to $\text{Ca}^{2+}$-bound state exposes a hydrophobic cleft formed around eight of CaM’s nine Met residues\(^{40-42}\). In both the N- and C-terminal domains, these hydrophobic clefts contain a cluster of four Met residues that are able to adopt multiple conformations, facilitating CaM’s conformational flexibility in binding to a variety of target proteins\(^{70,71}\). The first hydrophobic core (in the N-lobe)
includes Met37, Met52, Met72, and Met73, while the second (in the C- lobe) contains Met110, Met125, Met145, and M146. The remaining Met residue, Met77 is more solvent exposed and resides in the linker region between the two lobes. The role of these methionines as well as the hydrophobic cores, in mediating CaM’s interaction with target peptides has been established through a variety of structural studies\textsuperscript{29,42,72,73}. It has been found that selective Met mutations in CaM to selenomethionines or other non-natural amino acids significantly alters CaM’s binding affinity for several targets\textsuperscript{72,74-76}. In particular, site-specific Met to Leu mutations have been exploited to identify the role of individual Met residues in binding target peptides\textsuperscript{77}.

Functionally, oxidation of CaM’s Met residues has been shown to alter CaM’s ability to regulate its target proteins\textsuperscript{77-79}. Met oxidation is a reversible biological process that plays an important role in a variety of signaling and regulatory pathways. Reactive oxygen species (ROS) that generate the bulk of this oxidation are produced as a result of normal metabolism, however, under conditions of oxidative stress proteins become non-specifically oxidized, generating changes in cellular function\textsuperscript{80-82}. While some forms of oxidized CaM are selectively degraded by the 20S proteasome\textsuperscript{83,84}. CaM oxidation may also be reversed by methionine sulfoxide reductase (Msr) and may play a role in cellular signaling. It has been found that all nine methionines in CaM are susceptible to oxidation both \textit{in vivo} and \textit{in vitro}\textsuperscript{80,83,85,86}. Interestingly, the C-terminal Met145 and Met146 are more susceptible to oxidation than Met 77 despite the increased solvent exposure of the latter\textsuperscript{80,83,85,86}.

Spectroscopic studies of CaM oxidation have indicated that oxidation disrupts CaM’s $\alpha$-helical secondary structure\textsuperscript{86,87}. Since selective oxidation of CaM’s methionines is difficult to achieve experimentally, several groups resorted to studying site specific mutations that mimic the effects of oxidation. Particular attention has been devoted to the M125Q mutant, as this residue is crucial for CaM recognition and regulation of the
A recent fluorescence and EPR study suggested that M125Q mimics oxidation, affecting CaM's Ca$^{2+}$-dependent structural shift (close-to-open transition) and altering its interactions with the RyR. Here, we analyzed the structural and dynamic features of CaM$^{M125Q}$ using circular dichroism (CD) and high-resolution solution NMR spectroscopy. We found that this single mutation destabilizes the structure of the C-terminal domain of CaM into a molten globule. Remarkably, we found that partially oxidized CaM displays a structural fingerprint similar to CaM$^{M125Q}$. By comparing CaM$^{M125Q}$ with the equivalent N-terminal mutation, CaM$^{M52Q}$, we found that the C-terminal lobe is more susceptible to unfolding than the N-terminal lobe. These results provide further insight into the structural and functional consequences of site-directed oxidation in CaM, with particular relevance to dysregulation of the RyR.

2.3 Materials and Methods

CaM$^{WT}$, CaM$^{M125Q}$, and CaM$^{M52Q}$ were expressed using BL21(DE3) E. coli in LB and M9 media and purified using phenylsepharose CL-4B resin purchased from Sigma Aldrich as previously described. NMR samples were uniformly labeled using $^{15}$NH$_4$Cl from Sigma Aldrich and $^{13}$C-D-glucose from Cambridge Isotope Laboratories Inc. $^{13}$C/$^{15}$N Met selectively labeled samples of CaM$^{WT}$ for NMR were prepared using established protocols. Briefly, CaM was expressed in M9 media containing an excess of unlabeled amino acids and 0.4 mg/mL of uniformly $^{13}$C/$^{15}$N labeled Met purchased from Sigma Aldrich. All NMR samples were prepared by buffer exchange using Amicon Centrifugal Filter Units with a MWCO of 3 kDa. NMR buffers contained 20 mM imidazole, 100 mM KCl, 1 mM NaN$_3$, and either 1.5 mM EGTA (apo) or 6 mM CaCl$_2$ (Ca$^{2+}$-bound) at pH 6.5. All NMR spectra were obtained on Varian 600 MHz, Bruker 850 MHz, and Bruker 900 MHz spectrometers maintained by the Minnesota NMR Center. Assignments of apo and Ca$^{2+}$-bound CaM$^{WT}$ were determined using triple resonance
HNCACB\textsuperscript{90} and CBCA(CO)NH\textsuperscript{91} experiments in combination with the PINE server developed by the NMR Facility at Madison\textsuperscript{92-94}. Assignments obtained using the PINE server were compared to manual assignments and checked using PINE-SPARKY\textsuperscript{93}. TOCSY\textsuperscript{95,96} and NOESY-HSQC spectra were used to resolve ambiguities in assignments. NMR data was processed and analyzed using NMRPipe\textsuperscript{97}, Sparky\textsuperscript{93}, and NMR-View. The resonance assignments correspond well with the previously published data by Urbauer\textit{et al.}\textsuperscript{92}.

NMR Ca\textsuperscript{2+} titrations were performed by adding CaCl\textsubscript{2} from stock solutions to apo CaM\textsuperscript{WT}, CaM\textsuperscript{M125Q}, and CaM\textsuperscript{M52Q} containing 1.5 mM EGTA. Changes in CaM’s response to Ca\textsuperscript{2+} were tracked using [\textsuperscript{1}H-\textsuperscript{15}N] HSQC and [\textsuperscript{1}H-\textsuperscript{15}N] Heteronuclear NOE experiments\textsuperscript{5}. To track the oxidation of CaM\textsuperscript{WT} in response to 50 mM H\textsubscript{2}O\textsubscript{2} as a function of time, a series of [\textsuperscript{1}H-\textsuperscript{15}N] HSQC spectra were collected consecutively for 14-21 hours and the changes in chemical shift tracked using NMRView’s titration analysis software.

Chemical shift changes between CaM\textsuperscript{WT} and the CaM\textsuperscript{M52Q} and CaM\textsuperscript{M125Q} mutants were calculated using the following equation\textsuperscript{98}:

$$\Delta\delta_{\text{combined}} = \sqrt{(\delta_{1H,WT} - \delta_{1H,Mutant})^2 + \left(\frac{\delta_{15N,WT} - \delta_{15N,Mutant}}{5}\right)^2}$$ (1)

where $\Delta\delta_{\text{combined}}$ is the difference in chemical shift relative to CaM\textsuperscript{WT}. The $\Delta\delta_{\text{combined}}$ for CaM\textsuperscript{M52Q} and CaM\textsuperscript{M125Q} were mapped to structures of Ca\textsuperscript{2+}-free (PDB 1DMO\textsuperscript{99}) and Ca\textsuperscript{2+}-bound (PDB 1CLL\textsuperscript{100}) in PyMOL\textsuperscript{101}. [\textsuperscript{1}H, \textsuperscript{15}N] heteronuclear NOE values for CaM\textsuperscript{WT}, CaM\textsuperscript{M52Q}, and CaM\textsuperscript{M125Q} were determined by taking the ratio between peak intensities in the saturated ($I_{\text{sat}}$) and unsaturated ($I_{\text{unsat}}$) as previously described\textsuperscript{5,98}:

$$[1H, 15N] \text{NOE} = \frac{I_{\text{sat}}}{I_{\text{unsat}}}$$ (2)

The uncertainty in the NOE measurement ($\sigma_{\text{HN-NOE}}$) was determined for each residue as previously described\textsuperscript{5,98}.
\[ \sigma_{HN-NOE} = \left[ ^1H, ^{15}N \right] NOE \times \sqrt{\left( \frac{\sigma_{Isat}}{T_{sat}} \right)^2 + \left( \frac{\sigma_{Iunsat}}{T_{unsat}} \right)^2} \]  

where \( \sigma_{Isat} \) and \( \sigma_{Iunsat} \) represent the baseline noise levels in the saturated and unsaturated spectra respectively.

CD spectra were recorded from 280 to 200 nm using a JASCO J-815 spectrophotometer coupled with a data processor through the University of Minnesota Biophysical Technology Center. Spectra were recorded digitally and fed through the data processor for signal averaging and base line subtraction. Spectra were recorded at 25°C in a buffer containing 2 mM HEPES, 50 mM NaCl, and 1 mM DTT at pH 7.4 in a quartz cuvette with a path length of 1 mm. Apo samples for CD were prepared in the presence of 2 mM EDTA whereas Ca\(^{2+}\)-bound samples were prepared with 6 mM CaCl\(_2\). Spectra were recorded at 20 nm/min and were signal-averaged six times. The time-dependent oxidation of apo and Ca\(^{2+}\)-bound CaM\(^{WT}\) in response to 50 mM H\(_2\)O\(_2\) was monitored at 222 nm for five hours.

2.4 Results

2.4.1 M125Q mutation causes unfolding in CaM’s C-terminal domain. We first analyzed the effects of the M125Q mutation on CaM’s secondary structure using CD spectroscopy. The dichroic profiles show a significant loss of \( \alpha \)-helical secondary structure upon introduction of the M125Q mutation (Supplementary Figure 2.S1) corresponding to a 12%-14% decrease in \( \alpha \)-helical structure based on the decrease in signal intensity at 208 and 222 nm. To identify the domains affected by the mutation, we expressed and purified U\(^{15}\)N CaM\(^{WT}\) and CaM\(^{M125Q}\) and analyzed their amide backbone fingerprint with NMR spectroscopy. The comparison of the \([^1H-^{15}N]\) HSQC spectra for apo CaM\(^{WT}\) and CaM\(^{M125Q}\) indicates that the Met to Gln mutation causes dramatic changes in the protein
fingerprint. Upon close inspection, we found that significant chemical shift changes are localized to the C-terminal domain of CaM (Figure 2.1, Supplementary Figure 2.S2); while the resonances for the dynamic linker and the N-terminal lobe remain essentially unperturbed. In fact, the resonances assigned to CaM’s N-terminal domain and linker region consistently overlay between the two spectra, while a few residues in the C-terminal domain of M125Q move toward the 8-9 ppm region of the $^1$H dimension relative to CaM$^{WT}$, suggesting a shift towards a disordered conformation for those residues. In addition, several resonances of the C-terminal are significantly broadened. These dramatic changes observed in the spectrum suggest that the C-terminal domain undergoes localized structural destabilization and increased molecular motions upon mutation; whereas the N-terminal domain remains essentially unperturbed. To test whether Ca$^{2+}$ binding would restore the native folding of the C-terminal domain, we carried out parallel titrations with both CaM$^{WT}$ and CaM$^{M125Q}$ and followed the amide fingerprint using $[^1$H, $^{15}$N] HSQC experiments (Supplementary Figure 2.S3). In the NMR spectra, CaM has a very distinct response to Ca$^{2+}$ binding. In the apo state, the resonances are broader and clustered in the middle of the spectrum, reflecting the dynamic nature of the N and C domains in the absence of Ca$^{2+}$. Upon the addition of Ca$^{2+}$, the residues in CaM$^{WT}$ progressively move toward a fully Ca$^{2+}$ saturated form that exhibits sharp, well-dispersed peaks. In their Ca$^{2+}$-free states, the position of the resonances of CaM$^{WT}$ and CaM$^{M125Q}$ are very similar, with localized chemical shift changes in the vicinity of the mutation site. Upon binding Ca$^{2+}$, several amides of the N-terminal domain of CaM$^{M125Q}$ display chemical shifts corresponding to a partially Ca$^{2+}$-bound state, whereas the corresponding resonances of CaM$^{WT}$ show a fully bound state (Figure 2.1D, Supplementary Figure 2.S2). In contrast, the residues belonging to the N-domain of CaM$^{M125Q}$ did not achieve the same Ca$^{2+}$-bound conformation as CaM$^{WT}$ at the same Ca$^{2+}$ saturation conditions, indicating that the M125Q mutation may disrupt at least one, if not both, of the Ca$^{2+}$-
binding sites in the C-terminal domain. Overall, the chemical shift analysis supports the hypothesis that the M125Q mutation causes a selective unfolding of the C-terminal globular domain with disruption of the Ca$^{2+}$ binding sites and a subsequent increase in conformational dynamics. Importantly, the addition of Ca$^{2+}$ does not seem to reverse the unfolding of the mutant’s C-terminal domain.

To further test this hypothesis, we carried out [$^1$H-$^{15}$N] heteronuclear NOE experiments, monitoring the protein’s backbone dynamics in the ps-ns time scale (Figure 2.2)\(^5\). As expected from previous studies, apo and Ca$^{2+}$-bound CaM$^{\text{WT}}$ exhibit backbone NOE values between 0.2 and 0.9 (Supplementary Figure 2.S4). Accordingly, CaM’s linker region and C-terminal domain exhibit slightly lower NOEs, indicating that these regions undergo faster structural dynamics than the N-terminal domain. Upon addition of Ca$^{2+}$, holo CaM$^{\text{WT}}$ shows a significant decrease of internal motions, reflecting the stabilization of CaM’s structure caused by Ca$^{2+}$ binding and hydrophobic interactions in the EF hand motifs\(^{29,103}\). Upon increasing the Ca$^{2+}$ concentration to reach the complete holo state, the conformational dynamics are gradually reduced for these regions. In contrast to the CaM$^{\text{WT}}$ experiments, the [$^1$H-$^{15}$N] heteronuclear NOE spectrum for the apo CaM$^{\text{M125Q}}$ shows two distinct behaviors. While all of the resonances of the N-terminal domain show positive enhancement upon saturation of the $^1$H resonances, the majority of residues belonging to the C-terminal domain show negative enhancements. This behavior is typical of small unfolded peptides or intrinsically disordered domains and supports the significant unfolding of the C-terminal domain’s secondary and tertiary structure as a result of the mutation. Upon increasing [Ca$^{2+}$], we observed some changes in the amide fingerprint (i.e. peak doubling and broadening). However, the dynamic behavior of the C-terminal lobe did not change substantially, indicating that this domain remains essentially unfolded and unable to bind Ca$^{2+}$ with high affinity. In contrast, the resonances
associated with the N-terminal domain undergo structural transitions typical of CaM\textsuperscript{WT}, reaching the full holo state at [Ca\textsuperscript{2+}] > 400 μM.

2.4.2 \textit{CaM\textsuperscript{M52Q} exhibits divergent behavior to CaM\textsuperscript{M125Q}.} To understand whether a mutation similar to M125Q destabilizes the N-terminal domain, we engineered the M52Q (CaM\textsuperscript{M52Q}) mutant. CaM\textsuperscript{M52Q} has been previously studied in the context of its binding to the RyR under reducing conditions\textsuperscript{104}. M52 is situated in the N-terminal domain and belongs to the hydrophobic cluster of residues in a location equivalent to M125 in the C-terminal domain. Unlike the M125Q mutation, M52Q does not disrupt CaM’s structure and displays the same Ca\textsuperscript{2+} response as CaM\textsuperscript{WT} (Figure 2.1, Supplementary Figure 2.S5). In fact, the N-terminal domain of CaM\textsuperscript{M52Q} appears to retain Ca\textsuperscript{2+} with greater affinity than CaM\textsuperscript{WT} and transitions to a fully Ca\textsuperscript{2+}-bound state without sampling the denatured states exhibited by CaM\textsuperscript{M125Q} (Supplementary Figures 2.S3 and 2.S5). In contrast to both CaM\textsuperscript{WT} and CaM\textsuperscript{M125Q}, CaM\textsuperscript{M52Q} also exhibits lower thermostability and a decreased ability to recover its native structure following thermal denaturation indicating that this mutation disrupts the N-terminal domain’s ability to refold following denaturation (Figure 2.3). The analysis of CaM\textsuperscript{M52Q} confirms that despite the sequence similarity and conserved structural elements between CaM’s N- and C-terminal domains, the N-terminal domain exhibits greater stability and Ca\textsuperscript{2+} affinity relative to the C-terminal domain as previously reported\textsuperscript{77}.

2.4.3 \textit{CaM\textsuperscript{M125Q} and CaM\textsuperscript{M52Q} exhibit hallmarks of partially oxidized forms CaM\textsuperscript{WT}.} It has been reported that both CaM\textsuperscript{M125Q} and oxCaM\textsuperscript{WT} show a drastically reduced affinity for the RyR, while CaM\textsuperscript{M52Q} exhibits only a slight shift in RyR binding and regulatory function\textsuperscript{77,88}. To understand the structural basis for the functional similarity between CaM\textsuperscript{M125Q}, CaM\textsuperscript{M52Q}, and oxCaM\textsuperscript{WT}, we analyzed them by CD and compared the NMR fingerprint of these mutants with that of CaM\textsuperscript{WT} upon treatment with 50 mM H\textsubscript{2}O\textsubscript{2}. The time course of the CD spectra obtained upon incubation of apo CaM\textsuperscript{WT} with H\textsubscript{2}O\textsubscript{2} shows
a progressive loss of $\alpha$-helical secondary structure, with a 57% decrease in the signal at 222 nm relative to unoxidized CaM$^{\text{WT}}$ (Figure 2.4). This is in agreement with previous studies showing that CaM$^{\text{WT}}$ causes a significant loss of $\alpha$-helical secondary structure$^{86,105,106}$. With NMR, we carried out parallel studies following the time dependence of the chemical shifts in the protein fingerprint for both the Ca$^{2+}$-free and Ca$^{2+}$-bound forms of CaM$^{\text{WT}}$. After addition of H$_2$O$_2$, apo CaM shows incremental shifts of the resonances corresponding to the C-terminal domain within 10 minutes, while resonances corresponding to the residues in the CaM's N-terminal domain begin to change chemical shifts only after 6 hours of H$_2$O$_2$ exposure (Supplementary Figure 2.S6). Significant chemical shift changes are apparent for Met146 and Met145, which is consistent with both in vivo and in vitro assays reporting the sensitivity of these sites to oxidation in the absence of Ca$^{2+}$ $^{86,107}$. During the course of the experiment, some of the Met residues show gradual and linear changes in their chemical shifts (fast exchange), while other show the presence of multiple conformational states under slow exchange. Within 2 hours of H$_2$O$_2$ exposure, Met125 begins to broaden out together with other residues in the C-terminal domain, indicating the probable transition towards a molten globule state similar to the spectra of CaM$^{M125Q}$ (Supplementary Figure 2.S6A). Following complete oxidation, the $^1$H chemical shifts in both the N- and C-terminal domain residues of CaM$^{\text{WT}}$ collapse toward the 8-9 ppm range, indicating that the protein structure is largely denatured (Figure 2.5). The complete oxidation of CaM corresponds to the plateau of the intensity of the resonances and was confirmed by MALDI.

In the presence of saturating Ca$^{2+}$, the structural stability of hydrophobic interactions in the EF hand motif appear to confer a protective effect in preserving CaM's secondary structure. While all nine of CaM's Met residues are still susceptible to oxidation in the presence of Ca$^{2+}$, instead of adopting a fully denatured structure as seen in the apo form, oxCaM$^{\text{WT}}$ retains its $\alpha$-helical structure and shifts to a different conformation (Fig-
ure 2.4, Supplementary Figure 2.S7). As with the apo form, Met145 and Met146 are highly susceptible to oxidation and are the first methionines to be completely oxidized upon exposure to H$_2$O$_2$. The remaining C-terminal Met residues are also rapidly oxidized on a timescale similar to their oxidation in the absence of Ca$^{2+}$. The N-terminal residues however, are oxidized more quickly in the presence of Ca$^{2+}$ than in the apo form. This is particularly true for Met72, 73, and 77 which become more solvent exposed upon Ca$^{2+}$ binding (Figure 2.6)$^{29,99}$. The ability of Ca$^{2+}$ binding to rescue $\alpha$-helical structure is also demonstrated by the addition of Ca$^{2+}$ to the apo oxCaM$^\text{WT}$, inducing a transition in the structure to that of Ca$^{2+}$-bound oxCaM$^\text{WT}$ (Figure 2.5).

2.5 Discussion

The C-terminal domain of CaM was identified as a sensor for oxidative processes. In particular, the M125Q mutation located in the hydrophobic cluster of the C terminus has been used to mimic the ROS insults on CaM and muscle aging. This position is rather sensitive as the M125Q mutation reduces CaM binding to smooth muscle myosin light chain kinase, CaM-dependent protein kinase Ila, and CaM-dependent protein kinase IV$^{108}$. Moreover, it has been shown that the M125Q mutation weakens the interactions with the RyR in a Ca$^{2+}$-dependent manner. Recent EPR experiments focusing on the conformational equilibrium between closed to open conformations of CaM$^\text{WT}$ and CaM$^\text{M125Q}$ showed that the populations of these states are significantly affected by this single mutation. These studies also showed that more dramatic changes are observed when CaM is completely oxidized by treatment with 50 mM H$_2$O$_2$ for 24 hours. However, EPR studies did not reveal the complete effects of the single mutation on the overall structure of CaM. Indeed, NMR spectroscopy identifies melting in the C-terminal lobe that disrupts the EF-hands (III and IV) and the high-affinity binding of Ca$^{2+}$. The changes in CaM's structure, target regulation, and Ca$^{2+}$ binding because of the M125Q mutation
are most likely the result of disruptions in the hydrophobic interactions of CaM’s C-lobe and the resulting conformational transition of the C-lobe towards a molten globule state. Remarkably, almost identical behavior is observed for the C-lobe of CaM upon exposure (>2 h) to 50 mM H₂O₂. In the holo CaM, Met125 is surrounded by hydrophobic residues that contribute to the hydrophobic surface that is involved in CaM’s binding to several target proteins (Figure 2.6). The mutation of Met to Gln introduces a bulkier and charged amino acid that prevents the formation of the hydrophobic core that holds together the C-terminal lobe. While Met144 and Met145 cause small changes in the structure upon oxidation, the oxidation of Met125 to Met sulfoxide destabilizes the tertiary interactions within the hydrophobic cluster formed by Leu105, Met109, Met125, Val121, and Leu116.

Recently, it has been shown that the oxidation of Met to Met-sulfoxide in the proximity of aromatic residues such as Phe results in a stabilization of the inter-residue interactions, leading to a stabilization of the secondary structure interactions. The authors showed that this phenomenon takes place in a fragment of CaM spanning residues 136-146. Here, we show that in the absence of aromatic residues the modification of the Met thioether to a sulfoxide within the hydrophobic cluster of the C-terminal lobe causes a significant destabilization of the tertiary structure that melts into a molten globule. Since the C-lobe is central for the binding to RyR and a variety of kinases, the dramatic unfolding of this domain may explain the loss of affinity of CaM for these targets as well as the similarity between CaM^{M125Q} and oxCaM^{WT}. Therefore, the main role of Met125 is to maintain the structural integrity of CaM’s C-lobe, providing a rationale for why oxidation at Met residues, such as Met125, causes such large changes in CaM’s structure and disruptions in the regulation of CaM’s cellular targets. This new role for Met125 is further supported by the comparison of CaM^{M125Q} to partially oxidized forms of CaM^{WT}, suggesting that ROS cause the oxidation of CaM, thereby perturbing its struc-
ture so that it can no longer bind its targets and is tagged for selective degradation by the 20S proteasome\textsuperscript{83,84}.

2.6 Conclusions

In conclusion, we have established a direct correlation between the unfolding of the C-terminal domain, caused either by the M125Q mutation or by oxidation, and the functional effects of these modifications toward CaM targets such as RyR\textsuperscript{88} and smooth muscle myosin light chain kinase, CaM-dependent protein kinase IIa, and CaM-dependent protein kinase IV\textsuperscript{108}. These studies emphasize the importance of the C-terminal domain of CaM in target recognition and binding.

2.7 Acknowledgements

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Figure 2.1: Met to Gln mutations alter CaM’s conformation in a domain specific manner.  

A) [\textsuperscript{1}H, \textsuperscript{15}N] HSQC overlay of CaM\textsuperscript{WT} (black) and CaM\textsuperscript{M52Q} (blue) in the absence of calcium. Chemical shift changes (Supplementary Figure 2B) between WT and M52Q were mapped to the NMR structure of calcium-free CaM (PDB 1DMO).  

B) [\textsuperscript{1}H, \textsuperscript{15}N] HSQC overlay of CaM\textsuperscript{WT} (black) and CaM\textsuperscript{M52Q} (blue) in the presence of calcium. Chemical shift changes (Supplementary Figure 2C) between WT and M52Q were mapped to the crystal structure of calcium-bound CaM (PDB 1CLL).  

C) [\textsuperscript{1}H, \textsuperscript{15}N] HSQC overlay of CaM\textsuperscript{WT} (black) and CaM\textsuperscript{M125Q} (green) in the absence of calcium. Chemical shift changes (Supplementary Figure 3B) were mapped to PDB 1DMO.  

D) [\textsuperscript{1}H, \textsuperscript{15}N] HSQC overlay of CaM\textsuperscript{WT} (black) and CaM\textsuperscript{M125Q} (green) in the presence of calcium. Chemical shift changes (Supplementary Figure 3C) were mapped to PDB 1CLL. NMR spectra were acquired on a Bruker 900 MHz spectrometer at 25\degree C.
Figure 2.2: M125Q mutation disrupts the hydrophobic packing in CaM’s C-terminal domain. A-C) $^{1}H, ^{15}N$ Heteronuclear NOE spectra of WT (top), M52Q (middle) and M125Q (bottom) in the absence of calcium. D-F) $^{1}H, ^{15}N$ Heteronuclear NOE spectra of WT (top), M52Q (middle) and M125Q (bottom) following titration with 1 M CaCl$_2$ to a final Ca$^{2+}$ concentration of 6 mM. Residues with positive values are shown in black and residues with negative values are shown in red. Spectra were acquired on a Bruker 900 MHz spectrometer at 25°C.
Figure 2.3: Gln variants exhibit distinct melting patterns compared to CaM\textsuperscript{WT}. A) CD melting profiles for CaM\textsuperscript{WT} (black), CaM\textsuperscript{M52Q} (blue), and CaM\textsuperscript{M125Q} (green) acquired from 25°C to 95°C. B) CD refolding profiles for CaM\textsuperscript{WT} (black), CaM\textsuperscript{M52Q} (blue), CaM\textsuperscript{M125Q} (green) acquired from 95°C to 25°C. All spectra were acquired at 222 nm in the presence of 2 mM EDTA. Spectra were normalized to the initial $\alpha$-helical signal at 222 nm.
Figure 2.4: Ca²⁺ protects against structural degradation from oxidation. A) [¹H, ¹⁵N] HSQC overlay of apo ¹³C/¹⁵N Met labeled CaM<sup>WT</sup> before (black) and after (red) exposure to 50 mM H₂O₂. B) CD spectra at 222 nm tracking the progressive loss of secondary structure in apo CaM<sup>WT</sup> upon oxidation by 50 mM H₂O₂. C) [¹H, ¹⁵N] HSQC overlay of Ca²⁺-bound ¹³C/¹⁵N Met labeled CaM<sup>WT</sup> before (black) and after (red) exposure to 50 mM H₂O₂. D) CD spectra at 222 nm tracking the level of α-helical secondary structure present in the Ca²⁺-bound state upon oxidation by 50 mM H₂O₂.
Figure 2.5: Ca$^{2+}$ rescues secondary structure in fully oxidized CaM. Left: [$^{1}$H, $^{15}$N] HSQC of fully oxidized apo CaM. Right: [$^{1}$H, $^{15}$N] HSQC overlay of fully oxidized Ca$^{2+}$-bound CaM (black) and fully oxidized Ca$^{2+}$-free (apo) CaM with the addition of 6 mM CaCl$_{2}$. Spectra were acquired on a Bruker 900 MHz spectrometer at 25°C.
Figure 2.6: Hydrophobic interactions stabilize CaM’s EF hand domains. Top: N and C-terminal domains of Ca^{2+}-bound CaM (PDB 1CLL) with Met residues shown in yellow, Phe residues shown in green and the mutation sites 52 and 125 shown in red. Bottom: Ca^{2+} binding alters the solvent exposure of CaM’s Met residues (red; PDB 1DMO and 1CLL).
Supplementary Figure 2.S1: CD show a loss in α-helical secondary structure for CaM$^{\text{M125Q}}$. Overlay of CD spectra for CaM$^{\text{WT}}$ (black) and CaM$^{\text{M125Q}}$ (green) in the absence of Ca$^{2+}$. Spectra were acquired at 25°C.
Supplementary Figure 2.S2: M125Q mutation perturbs the C-terminal domain in both the absence and presence of calcium. A) Left, full $[^1\text{H}, \, ^{15}\text{N}]$ HSQC overlay of CaM$^{\text{WT}}$ (black) and CaM$^{\text{M125Q}}$ (green) in the absence of calcium. Right, full $[^1\text{H}, \, ^{15}\text{N}]$ HSQC overlay of CaM$^{\text{WT}}$ (black) and CaM$^{\text{M125Q}}$ (green) in the presence of calcium. B) Combined change in amide chemical shifts between CaM$^{\text{WT}}$ and CaM$^{\text{M125Q}}$ in the absence of calcium. C) Combined change in amide chemical shifts between CaM$^{\text{WT}}$ and CaM$^{\text{M125Q}}$ in the presence of calcium. NMR spectra were acquired on a Bruker 900 MHz spectrometer at 25°C.
Supplementary Figure 2.S3: Mutation of Met to Gln alters CaM's response to Ca$$^{2+}$$ in a domain-specific manner. Overlay of [$^1$$^H$$,$$^{15}$$N$$]$$ HSQC spectra tracking the calcium response of CaM$$^{WT}$$ (A), CaM$$^{M52Q}$$ (B), and CaM$$^{M125Q}$$ (C) from the apo to calcium-saturated state. Titrations were acquired on a Bruker 900 MHz spectrometer at 25°C and samples of apo CaM (red) in the presence of EGTA were titrated with 1 mM CaCl$_2$ to free calcium concentrations of 409.6 μM (orange), 1.6 mM (yellow), 2.5 mM (green), and 6 mM (blue).
Supplementary Figure 2.S4: Met to Gln mutations induce localized changes in amide backbone dynamics. $[^1\text{H}, ^{15}\text{N}]$ heteronuclear NOE values calculated for CaM$^{\text{WT}}$, CaM$^{\text{M52Q}}$, and CaM$^{\text{M125Q}}$ in the absence (top) and presence (bottom) of calcium. Spectra for $[^1\text{H}, ^{15}\text{N}]$ heteronuclear NOE calculations were acquired on a Bruker 900 MHz spectrometer at 25°C.
Supplementary Figure 2.S5: M52Q mutation perturbs the N-terminal domain in the absence of calcium. A) Left, full [¹H, ¹⁵N] HSQC overlay of CaM<sup>WT</sup> (black) and CaM<sup>M52Q</sup> (blue) in the absence of calcium. Right, full [¹H, ¹⁵N] HSQC overlay of CaM<sup>WT</sup> (black) and CaM<sup>M52Q</sup> (blue) in the presence of calcium. B) Combined change in amide chemical shifts between CaM<sup>WT</sup> and CaM<sup>M52Q</sup> in the absence of calcium. C) Combined change in amide chemical shifts between CaM<sup>WT</sup> and CaM<sup>M52Q</sup> in the presence of calcium. NMR spectra were acquired on a Bruker 900 MHz spectrometer at 25°C.
Supplementary Figure 2.S6: Oxidation completely denatures Ca\textsuperscript{2+}-free CaM.  

A) \([^{1}H, ^{15}N]\) HSQC overlay of U\textsuperscript{13}C/\textsuperscript{15}N apo CaM\textsuperscript{WT} before (black) and after (red) exposure to 50 mM H\textsubscript{2}O\textsubscript{2}.  

B) CD profile showing decrease in \(\alpha\)-helical secondary structure in apo CaM\textsuperscript{WT} following exposure to 50 mM H\textsubscript{2}O\textsubscript{2}.  

C) \([^{1}H, ^{15}N]\) HSQC overlay of \(^{13}C/^{15}N\)-Met labeled apo CaM\textsuperscript{WT} (red) with U\textsuperscript{13}C/\textsuperscript{15}N apo CaM\textsuperscript{WT} (black) showing the assignments for the nine Met residues in CaM.  

D) Top, overlay of \([^{1}H, ^{15}N]\) HSQC spectra tracking the progressive change in chemical shift for apo CaM\textsuperscript{WT} following exposure to 50 mM H\textsubscript{2}O\textsubscript{2}. Bottom, change in intensity for each Met residue in apo CaM\textsuperscript{WT} illustrating the increased susceptibility of C-terminal Met residues to oxidation. All NMR spectra were acquired on a Bruker 900 MHz spectrometer at 25\textdegree C. CD spectra were acquired at 25\textdegree C.
Supplementary Figure 2.S7: Ca$^{2+}$ protects against structural denaturation from oxidation.  

**A** $[^1H, ^{15}N]$ HSQC overlay of U-$^{13}C/^{15}N$ Ca$^{2+}$-bound CaM$^{WT}$ before (black) and after (red) exposure to 50 mM H$_2$O$_2$.  

**B** CD spectra showing the limited decrease in α-helical secondary structure following exposure to 50 mM H$_2$O$_2$.  

**C** $[^1H, ^{15}N]$ HSQC overlay of U-$^{13}C/^{15}N$ labeled Ca$^{2+}$-bound CaM$^{WT}$ (black) with $^{13}C/^{15}N$ Met labeled Ca$^{2+}$-bound CaM$^{WT}$ (red) showing the assignment for CaM’s nine Met residues.  

**D** $[^1H, ^{15}N]$ HSQC overlay tracking the progressive oxidation of Met labeled CaM$^{WT}$ following exposure to 50 mM H$_2$O$_2$. NMR spectra were acquired on Bruker 850 and 900 MHz spectrometers at 25°C. CD spectra were acquired at 25°C.
Chapter 3: Probing the Conformationally Excited States of Membrane Proteins via $^1$H-detected MAS Solid-State NMR Spectroscopy

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3.1 Abstract

Proteins exist in ensembles of conformational states that interconvert on various motion- nal time scales. High-energy states of proteins, often referred to as conformationally excited states, are sparsely populated and have been found to play an essential role in many biological functions. However, detecting these states is quite difficult for conventional structural techniques. Recent progress in solution NMR spectroscopy made it possible to detect conformationally excited states in soluble proteins and characterize them at high resolution. As for soluble proteins, integral or membrane-associated proteins populate different structural states often modulated by their lipid environment. Solid-state NMR spectroscopy is the method of choice to study membrane proteins as it can detect both ground and excited states in their natural lipid environments. In this work, we apply newly developed $^1$H-detected $^{15}$N-HSQC type experiments under moderate magic angle spinning speeds to detect the conformationally excited states of phospholamban (PLN), a single-pass cardiac membrane protein that regulates Ca$^{2+}$ transport across SR membrane. In its unbound state, the cytoplasmic domain of PLN exists in equilibrium between a T state, which is membrane bound and helical, and an R state, which is membrane detached and unfolded. The R state is important for regulation of the sarcoplasmic reticulum Ca$^{2+}$-ATPase, but also for binding to protein kinase A. By hybridizing $^1$H detected solution and solid-state NMR techniques, it is possible to detect and resolve the amide resonances of the R state of PLN in liquid crystalline lipid bilayers. These new methods can be used to study the conformationally excited states of membrane proteins in native-like lipid bilayers.
3.2 Introduction

NMR is a rapidly evolving technique for chemical, biochemical, and biophysical studies of macromolecules in solution, semi-solid, and solid states. The most exciting frontier for NMR is to investigate membrane protein structures under physiological conditions, which has been very challenging for X-ray spectroscopy. Currently, both solution and solid-state NMR techniques are being used for studying membrane proteins. However, solution NMR is still limited to studying membrane proteins in detergent micelles, isotropic bicelles or in select cases, nanodisks, none of which are able to accurately mimic the composition of biological membranes. On the other hand, solid-state NMR (ssNMR) does not have a protein size limitation and is ideal for studying membrane proteins in native-like lipid membranes. To maintain their tertiary fold and mimic proper functional conditions, membrane proteins need to be reconstituted in lipid membranes. Hydration, pH, temperature, lipid composition, as well as lipid-to-protein ratios, are crucial parameters to maintain transmembrane protein functional integrity.

As with all biomacromolecules, membrane proteins exist as ensembles of low and high conformational energy states. Biological responses to stimuli such as ligand binding, post-translational modifications, and changes in ionic or pH conditions skew the conformational equilibrium toward active or inactive states. Often, biological activity is carried out by a high-energy conformational state (i.e., excited states) that is only sparsely populated under normal physiological conditions. As both X-ray crystallography and cryoEM trap the structure of membrane proteins in defined, low energy basins, they cannot detect conformationally excited protein states. Using nuclear spin relaxation experiments, solution NMR experimentalists are able to detect protein and oligonucleotide excited states and determine their structures. The presence of conformationally excited states is inferred through the analysis of protein motions. Although similar experiments have been proposed in ssNMR for microcrystalline protein prepara-
tions, where solution-like properties of the spectra allow for measurement of $T_{1p}$ relaxation times, these methods are not readily applicable to membrane proteins within phospholipid membrane bilayers. In fact, the most important experiments for structure determination of membrane proteins are based on cross polarization (CP) techniques that rely on strong dipolar couplings (DC) for polarization transfer. Although these approaches are now being combined with novel multi-dimensional acquisition methods, they fail to detect dynamic regions that are likely to encode for conformationally excited states.

The conformational plasticity of membrane proteins directly influences the magnitude of the orientational-dependent NMR interactions such as DC and chemical shift anisotropy (CSA). As a result, dynamic regions of membrane proteins are insensitive to CP-based NMR techniques. Certain regions of membrane proteins are also exposed to aqueous environments and undergo fast motions, thereby dramatically scaling down the dipolar interactions. However, the conformational dynamics increase $T_2$ relaxation times of mobile residues enabling the application of through-bond INEPT (Insensitive nuclei enhanced by polarization transfer) experiments. For instance, Baldus and coworkers have demonstrated the use of through-bond $^{13}$C and $^{15}$N detected refocused INEPT (RINEPT) experiments under MAS conditions for studying the cytoplasmic domain of the membrane protein phospholamban (PLN). Similarly, $^{13}$C detected RINEPT and CP experiments were respectively used for studying mobile and rigid domains of cartilage. This approach has also been used for studying the dynamic regions of cytochrome-b5 using $^{15}$N detected RINEPT experiment in oriented solid state NMR. However the intrinsic low sensitivity of $^{13}$C and $^{15}$N nuclei dramatically increases the experimental times.

Recent progress in fast MAS experiments has enabled $^1$H detection with dramatic sensitivity enhancement. Perdeuterated sample preparations of crystalline proteins combined with $^1$H detected fast MAS methods are now being routinely used for
structure determination. However, these methods have limited applications for membrane proteins reconstituted in phospholipid membrane mimetic systems. In this work we show that $^1$H detected ssNMR can be used for studying dynamic regions of fully protonated membrane proteins at moderate MAS rates. We show that $^1$H-detected $^{15}$N HSQC-type MAS experiments are able to map the mobile residues of the conformationally excited state (R-state) of PLN under moderate spinning speed conditions. PLN is a 52 residues membrane protein that comprises an inhibitory transmembrane domain (domain Ib and domain II) and a regulatory domain (domain Ia), connected by a short, flexible loop (Figure 3.1A). PLN binds and regulate the sarcoplasmic reticulum Ca$^{2+}$-ATPase, or SERCA, decreasing its apparent affinity for Ca$^{2+}$ ions in a reversible manner. Protein kinase A recognizes and phosphorylates PLN at Ser16 in domain Ia, reversing this inhibition. Previous solution and solid-state NMR as well EPR (Electron Paramagnetic Resonance) experiments from our group and others have shown that PLN’s regulatory region in the absence of SERCA undergoes a conformational equilibrium between an ordered T state (helical) and a disordered R state (unfolded and membrane detached). The population of the R state was detected as weak peaks from the dipolar assisted rotational resonance (DARR) experiments that mainly reported on the T state. The application of the new refocused-INEPT heteronuclear single quantum coherence (RI-HSQC) pulse sequences enabled us to probe the R state of PLN with an average of eight-fold sensitivity enhancement compared to $^{15}$N-detected INEPT-HETCOR experiments. We also show that the sensitivity of these experiments is further enhanced by the simultaneous detection of cosine and sine modulated chemical shift coherences with a new experiment called SERI-HSQC.

These new methods are applicable to a wide range of membrane proteins and complexes that display structural dynamic regions, allowing researchers to characterize their importance in biological function.
3.3 Materials and Methods

Monomeric PLN (PLN\textsuperscript{AF}) and R14 deletion PLN (PLN\textsuperscript{R14del}) were expressed in BL21(DE3) \textit{E.coli} and purified using affinity chromatography and HPLC according to the published procedures.\textsuperscript{154} Purified PLN was lyophilized and stored at -20 °C. Protein reconstitution in lipid membranes and MAS sample preparations followed the previously published protocols.\textsuperscript{148} Lipids for MAS samples were dried down under nitrogen and lyophilized once after resuspension in water to remove trace organic solvent. The lyophilized lipids were reconstituted in 2 mL of reconstitution buffer consisting of 20 mM HEPES (pH 7.0), 100 mM KCl, 1 mM MgCl\textsubscript{2}, 5% (v/v) glycerol, and 0.02% (w/v) NaN\textsubscript{3}. The lipids were solubilized using 25% C\textsubscript{12}E\textsubscript{8}, with 50 μL of detergent per 10 mg of lipid. PLN was solubilized in 25% C\textsubscript{12}E\textsubscript{8}, using approximately 25 μL per 1 mg of protein, and was added to the reconstituted lipid preparations. Following a brief incubation, BioBeads\textsuperscript{®} SM-2 were added in a 30-fold (w/w) excess over detergent and allowed to stir for 3 hours at room temperature. The BioBeads\textsuperscript{®} were removed via filtration through a 25 gauge needle, and the lipid vesicles pelleted by centrifugation at 100,000xg for 30 minutes at 4°C. The pelleted vesicles were resuspended in 2 mL of the reconstitution buffer and centrifuged at 350,000xg for 20 hours at 4°C. The resulting proteoliposomes were packed into 3.2 mm Bruker/Agilent MAS rotors using a series of centrifugation steps as previously described.\textsuperscript{155} The final samples contained 0.5 to 2 mg of PLN reconstituted in neutral DMPC liposomes or charged lipid mixtures containing 4:1:1 or 4:1:3 ratios of DMPC:DOPE:ePOPC, where ePOPC is a positively charged lipid. All lipids were purchased through Avanti Polar Lipids\textsuperscript{®} and used without further purification.

All of the solid-state NMR experiments were acquired at the Minnesota NMR center using Bruker or Agilent spectrometers operating at a $^1\text{H}$ Larmor frequency of 700 MHz equipped with 3.2 mm probes with reduced RF heating technology.\textsuperscript{156} All of the
spectra were processed with 30 Hz line broadening, and 20k x 10k zero filling using NMRPipe, and analyzed using Sparky. For all ssNMR experiments, \( t_2 \) acquisition time was set to 100 ms for both \( ^1H \) and \( ^{15}N \) detections, 80 t1 points with 5 kHz t1 spectral width, and a recycle delay of 3 seconds. The PLN\(^{AFA}\) ssNMR spectra shown in Figure 2, and 8 were respectively acquired on Agilent spectrometer with 512 and 64 scans per t1 increment; whereas all of the remaining data were acquired on Bruker spectrometer. The 90° pulse lengths for \( ^1H \), \( ^{13}C \), and \( ^{15}N \) were set to 3, 6, and 6 μs, respectively. For \( ^1H \) or \( ^{15}N \) heteronuclear decoupling, the WALTZ-16 sequence was used with the RF amplitude set to 10 kHz. PLN\(^{R14del}\) spectra were acquired with 64 scans per each t1 increment. Water suppression in the HSQC pulse sequence was obtained from a continuous presaturation pulse during the recycle delay with RF amplitude set to 200 Hz. For both RI-HSQC and SERI-HSQC, spin-lock pulses with phases x and y were used with RF amplitude of 30 kHz and \( \tau_1 \) set to 200 to 300 ms. The RI-HSQC spectra of PLN\(^{AFA}\) in DMPC lipids was acquired with 1024 scans, whereas 600 and 400 scans were respectively used for mixed lipid samples 4:1:1 and 4:1:3 (Figure 6B). The heat induced at higher MAS rates was monitored by the water frequency of the \( ^1H \) spectrum, and compensated accordingly by lowering the sample temperatures. The spectra were indirectly referenced to the \( CH_2 \) resonance of adamantine sample at 40.48 ppm using the relative gyromagnetic ratio of \( ^{15}N \) and \( ^1H \). Solution NMR HSQC spectrum of PLN\(^{AFA}\) in isotropic bicelles was recorded on 600 MHz spectrometer, using 128 scans and 64 t1 increments with a total experimental time of 6 hrs.

3.4 Results

3.4.1 Ground and excited states of phospholamban

In membranes, PLN undergoes a conformational equilibrium between an ordered T state (ground state) and a dynamic R state (conformationally excited state) as represented in
Figure 3.1A. Our studies using PLN variants show that the population of these states can be shifted by phosphorylation, R14 deletion or by single site mutations.\textsuperscript{16,47,55,148,161}

At room temperature, it is possible to simultaneously observe both ground and excited states in slow exchange for selected resonances of the cytoplasmic domain using $^{13}$C detected CP or INEPT based experiments. However, the resulting $^{13}$C spectra are quite complex to analyze and typically require selective labeling.\textsuperscript{148} Figure 3.1B shows the $^{15}$N signatures for the backbone amides of the T and R states. These spectra were acquired using $^{15}$N detection. While the more rigid residues of the transmembrane and membrane bound cytoplasmic domains are mapped by CP experiments, the more dynamic residues are observable using the relatively long J-coupling evolution periods ($\sim$10 ms) of the INEPT-based HETCOR experiment that selects for mobile residues with long T$_2$ relaxation time.\textsuperscript{135} Therefore, in the CP spectrum it is possible to identify both transmembrane and membrane-bound cytoplasmic residues. On the other hand, the INEPT-HETCOR experiment probes only cytoplasmic residues of the excited R state that undergoes fast conformational dynamics. A comparison of the 1D $^{15}$N signal intensities at 25°C for CP and INEPT- HETCOR spectra (Figure 3.1B, 3.1C) shows a significant difference in the relative R state population for the PLN$^{\text{AFA}}$ and PLN$^{\text{R14del}}$ variants reconstituted in neutral or fractionally charged mixed lipids. Figure 3.1C clearly demonstrates that while PLN$^{\text{AFA}}$ in DMPC and 4:1:1 (DMPC:DOPE:ePOPC) mixed lipids exists primarily in the T state and is more sensitive to the CP-based experiment, deletion of R14, or increasing the percentage of positively charged lipids(4:1:3 mixed lipids), pushes the equilibrium towards the R state and increases the sensitivity of the INEPT-HETCOR experiment on these samples. Due to lower protein concentrations, CP and INEPT-HETCOR spectra of PLN$^{\text{AFA}}$ were acquired using 10k to 50k scans. All the samples were acquired with identical experimental parameters. The integrated intensity of the CP and INEPT-HETCOR spectra were measured between 100 and 140 ppm at the same noise level. Although it
is difficult to estimate the absolute R-state population, relative integrated intensity of INEP-HETCOR with respect to CP indicates significant change in the relative R state population (Figure 3.1C). The latter is due to the decreased electrostatic interactions between the cytoplasmic domain and the membrane with the deletion of R14 or the introduction of positively charged lipids that shift the cytoplasmic domain toward the unfolded state due to an increase in PLN’s conformational dynamics. The 2D INEPT-HETCOR spectra of PLN\textsuperscript{AFA} and PLN\textsuperscript{R14del} reconstituted in zwitterionic DMPC lipids are shown in Figure 3.2A. The number of peaks for PLN\textsuperscript{R14del} is higher than that of PLN\textsuperscript{AFA}, indicating more residues transition from the rigid, membrane bound T state to the more dynamic R state. For both samples, we observed significant variation in relative peak intensities as well as line widths between 45 to 105 Hz indicating different time scale motions of residues. Figure 3.2B shows the solution NMR HSQC spectrum of PLN\textsuperscript{AFA} reconstituted in neutral isotropic bicelles. Due to the longer correlation times of isotropic bicelles, immobile transmembrane residues are either weak or undetectable in this spectrum. Unlike in isotropic bicelles, PLN\textsuperscript{AFA} reconstituted in proteoliposomes shows very few peaks with significantly broader resonances (Figure 3.2A, top left), indicating a higher degree of conformational heterogeneity. This emphasizes that the motions of PLN\textsuperscript{AFA} cytoplasmic domain reconstituted in proteoliposomes are quite different from those in isotropic bicelles.

3.4.2 \textsuperscript{1}H-detected MAS solid-state NMR for probing excited states of membrane proteins. Although the \textsuperscript{15}N-detected experiments are able to probe the R state confirmation for different PLN samples, we sought to boost sensitivity by using \textsuperscript{1}H-detected HSQC experiments (Figure 3.3). A significant challenge for ssNMR is the suppression of the water signal in the \textsuperscript{1}H detected spectra. While this is no longer a problem for solution NMR, where the probes are equipped with gradient coils and the water signal is easily dephased, commercially available ssNMR probes are not equipped with gradient pulse
technology and deuterium spin-lock circuitry to compensate for the drift of the $B_0$ field. In order to achieve reasonable water suppression, we used two modified pulse sequences, HSQC (Heteronuclear Single Quantum Coherence) and RI-HSQC (Refocused INEPT - Heteronuclear Single Quantum Coherence) that are similar to the solution NMR ‘out-and-back’ experiments. For these HSQC-type experiments, the polarization transfer starts from $^1\text{H}$ to $^{15}\text{N}$ followed by $^{15}\text{N}$ $t_1$ evolution period and then is transferred back to $^1\text{H}$ for acquisition ($t_2$). Figure 3.3A shows the INEPT-based HSQC pulse sequence, where a presaturation pulse is applied on the water resonance during the recycle delay. The second example of a pulse sequence utilizing $^1\text{H}$ detection is reported in Figure 3.3B. This experiment uses a refocused INEPT sequence to transfer the polarization from $^1\text{H}$ to $^{15}\text{N}$ which is then stored along the z-direction while suppressing (scrambling) the water magnetization using multiple pulses for a time period of $\tau_1$ ($\sim$200 to 300 ms). After water suppression, a $90^\circ$ pulse is applied on $^{15}\text{N}$ and is followed by a $t_1$ evolution period. A second refocused INEPT period is then used to transfer the $^{15}\text{N}$ polarization back to $^1\text{H}$ followed by $t_2$ acquisition period. The SERI-INEPT (sensitivity enhanced RI-HSQC) sequence shown in Figure 3.3C utilizes simultaneous detection of cosine and sine modulated chemical shift coherences to enhance the overall sensitivity and is explained further below.

Figure 3.4 shows the first increment of the $^{15}\text{N}$ and $^1\text{H}$ detected spectra of PLN$^{R_{14}\text{del}}$ in DMPC lipids at 25°C using 12 kHz MAS rate and 64 scans. These spectra were obtained using HSQC, RI-HSQC, and INEPT-HETCOR pulse sequences with $t_1$=0. Although the 1D HSQC shows superior water suppression, the exchange between $^{15}\text{N}$ and water during the presaturation period lowers the signal intensity by $\sim$ 25% in comparison to RI-HSQC. On the other hand, the $^{15}\text{N}$ 1D spectrum obtained from INEPT-HETCOR has nearly ten times lower signal than the corresponding amide $^1\text{H}$ signal obtained from RI-HSQC. Unlike the HSQC and INEPT-HETCOR experiments, the duration
of the RI-HSQC experiment is relatively longer, due to the water suppression period ($\tau_1$) during which the $^{15}$N magnetization is stored along the z-direction, and an additional $2\tau$ period for in-phase coherence transfer from $^{15}$N to $^1$H. In spite of the longer duration of the RI-HSQC experiment, the gain in sensitivity is very significant with respect to both $^{15}$N INEPT-HETCOR and $^1$H HSQC.

To further enhance the sensitivity, we incorporated an additional $\tau$ period in the RI-HSQC sequence prior to $t_2$ acquisition according to the sensitivity enhancement scheme by Rance and co-workers.\textsuperscript{164,165} The resultant pulse sequence, namely, sensitivity enhanced (SE) RI-HSQC (Figure 3.3C) simultaneously transfers both cosine and sine modulated $^{15}$N coherences to $^1$H and acquires the signals in a phase-sensitive mode by switching the $\phi_1$ phase of $^1$H pulse. The SE element nearly doubles the signal, whereas the RMS noise increases by 41 % giving a theoretical sensitivity gain of 41%.

Figure 3.5 shows the 2D spectra of PLN\textsuperscript{R14del} reconstituted in DMPC lipids obtained from INEPT-HETCOR, HSQC, RI-HSQC, and SERI-HSQC experiments using 80 $t_1$ increments and 64 scans per increment. All the spectra were processed using 30 Hz line broadening in both dimensions. $^1$H and $^{15}$N line widths of resolved peaks are in the range of 45 to 105 Hz. The spectra of RI-HSQC, and SERI-HSQC were drawn at the same noise level, whereas the INEPT-HETCOR and HSQC spectra were multiplied by 8 and 1.3 times due to lower peak intensities. The average sensitivity of the 2D RI-HSQC is 10 times greater compared to the 2D INEPT-HECOR. In other words, a $^{15}$N detected INEPT-HETCOR experiment would require 100 times ($10^2$) more experimental time compared to RI-HSQC. As expected from the 1D spectrum, presaturation lowers the sensitivity of the 2D HSQC spectrum, causing some of the peaks in the 2D spectrum to have lower intensity with respect to the RI-HSQC. Interestingly, we also found that the $^1$H line widths of HSQC are slightly narrower by 10 to 20 Hz compared to RI-HSQC.
Most likely the narrower line widths in HSQC are due to presaturation pulse that eliminates the contribution of chemical exchange with water. The sensitivity of RI-HSQC is further enhanced by using SERI-HSQC, where the average sensitivity gain is 31% with respect to the RI-HSQC spectrum (Figure 3.5 bottom right panel).

Figure 3.6A demonstrates the sensitivity comparison of $^1$H- and $^{15}$N-detected spectra of PLN$^{AFA}$ reconstituted in neutral DMPC lipids. In this case, the sensitivity of the 1D HSQC using presaturation is almost three times lower than the RI-HSQC spectrum. On the other hand, the 1D-HSQC shows dramatic signal enhancement for side chain resonances between 6 and 7.5 ppm, suggesting a possible increase of the magnetization due to NOE transfer taking place during presaturation. Figure 3.6B shows the comparison $^1$H detected 2D RI-HSQC spectra of PLN$^{AFA}$ reconstituted in DMPC or DMPC:DOPE:ePOPC lipid mixtures. The overall pattern of different spectra looks similar with small changes in peak positions or few additional peaks. Also, weak peaks of PLN$^{AFA}$ were observed (between 112 to 117 ppm in the $^{15}$N dimension) in mixed lipids samples, that were broadened when reconstituted DMPC lipids indicating more conformational heterogeneity. In fact these peak positions may correspond to Ser10, Ser16 and Thr17 residues (based on solution NMR assignment, Figure 3.2B) that were missing in the $^{15}$N detected experiments (Figure 3.2A, top left) due to lower sensitivity. This also confirms that serine and threonine residues are less sensitive to through-bond polarization transfer indicating intermediate time scale motion.

3.4.3 Effect of magic angle spinning rate on sensitivity and resolution: The spinning speed can influence the appearance of the NMR spectra and high spinning speeds may in certain cases affect the sample stability. In order to understand the effect of spinning speed on sensitivity and resolution, we recorded the $^1$H spectra using the RI-HSQC sequence at various MAS rates (Figure 3.7). While at 0 KHz broad peaks were observed, but the sensitivity was tremendously improved even at 5.5 kHz spinning speed.
In fact for both the samples of PLN$^{AFA}$ and PLN$^{R14del}$ reconstituted in DMPC lipids, the gain in sensitivity is about 15 to 20% higher at 12 kHz compared to 5.5 kHz MAS rate. Whereas from 12 to 15 kHz, the gain in sensitivity is only about 2 to 5%. Figure 3.8 shows a comparison of the 2D RI-HSQC spectra of PLN$^{AFA}$ in DMPC lipids at different spinning speeds. This indeed demonstrates that mobile residues in membrane proteins can be studied with optimal sensitivity and resolution using moderate spinning speeds via $^1$H detection.

3.5 Discussion

Both in vitro and in vivo studies indicate that in the monomeric form, PLN$^{AFA}$ adopts an L-shaped conformation with the cytoplasmic domain undergoing a conformational equilibrium between T (bound) and R (free) states. The T state is helical and adsorbed on the surface of the bilayer, with the hydrophobic side chains pointing toward the interior of the membrane and the hydrophilic residues pointing toward the bulk solvent. On the other hand, the conformationally excited R state is membrane detached and unfolded. SERCA preferentially binds the R state with a helical conformation in the transmembrane domains and an extended conformation of the cytoplasmic domain.$^{148,166}$ Functional studies and mutations show that this conformational equilibrium is central to maintaining SERCA’s Ca$^{2+}$ transport within a physiological window.$^{16,55}$ In our previous studies, we showed that the population of dynamic R state, which can be biased by mutations, plays a major role in SERCA inhibition.$^{16,148}$ Additionally, the R state is the one selected by protein kinase A for phosphorylation.$^{167}$ Therefore, this high-energy conformational state plays a central role in PLN’s regulatory function.

The R state of PLN$^{AFA}$ was first detected by Baldus and co-workers using $^{13}$C-detected through-bond correlations. Here, we show that $^1$H-detected $^{15}$N HSQC type experiments can probe dynamic regions of membrane proteins and provide higher sensitivity than $^{15}$N-detected INEPT-HETCOR experiments. We demonstrated this with PLN’s
amide backbone fingerprint, which displayed a sensitivity increase up to ten times higher than the corresponding $^{15}$N-detected INEPT-HETCOR experiments. Though these new experiments will replace the highly insensitive $^{15}$N detected experiments, it is important that new probes are developed containing gradients and lock circuitry since a significant hurdle is presented by the loss of sensitivity due to water exchange with backbone amide groups when applying presaturation of the water magnetization. Unlike solution NMR where the presaturation field strength is only about 50 Hz, we had to use ~200 Hz to cover larger spectral regions around the water frequency so that presaturation is still effective in spite of the $B_0$ frequency drift. However for insensitive samples, depending on the magnet drift, it is recommended to acquire multiple data sets with $^1$H offset correction, which can then be combined for signal averaging. On the other hand, water suppression in RI-HSQC was obtained by applying a phase switched RF pulses on $^1$H, while storing the $^{15}$N magnetization along the z-direction. Therefore water suppression in RI-HSQC and SERI-HSQC is insensitive to slight water frequency changes caused by the magnet drift. Water suppression can be avoided by reconstituting the protein in fully deuterated buffer. Recently, this approach was successfully used for studying mobile regions of Anabaena sensory rhodopsin using $^{13}$C HSQC experiments at moderate MAS rates.$^{168}$ However, this method is limited to non-exchangeable aliphatic protons.$^{168}$ On the other hand, the RI-HSQC, and SERI-HSQC (Figures 3.3B and C) pulse sequences can be applied on fully protonated samples with 100 % H2O, for studying labile amide protons. Interestingly, the loss of signal in the HSQC due to presaturation is higher for PLN$^{AFA}$ compared to PLN$^{R14del}$. This indicates different $^N$H-H$_2$O exchange rates of cytoplasmic domain in two different lipid systems. In fact, such experiments were used to study the exchange rates of globular proteins using solution NMR.$^{169}$

Although current experiments were demonstrated using a regular MAS setup (without gradient and field lock channels) we anticipate a further gain in sensitivity and
resolution with these technological advancements. In fact, double resonance ($^1$H-$^{13}$C) HR-MAS probes that are now routinely being used for metabolomics are equipped with gradient channels and deuterium spin-lock. While triple resonance HR-MAS probes could be promising for the detection of flexible domains or excited states of membrane proteins, they are limited to lower RF powers and MAS rates of 5 to 8 kHz.

3.6 Conclusions

In this study, we have shown that solid-state NMR is a unique method for probing ground and excited states of membrane proteins. Combination of CP and INEPT-based experiments were used to demonstrate the relative affinity of T and R states of PLN samples reconstituted in hydrated proteoliposomes. $^1$H detected HSQC type experiments can be used under moderate MAS rates for mapping the excited states of membrane proteins or mobile residues of membrane proteins in fully hydrated lipid bilayers. These methods are demonstrated on PLN variants reconstituted in proteoliposomes, using commercial ssNMR probes without pulsed field gradients. Efficient water suppression was achieved by using RI-HSQC and detecting the amide protons with an average sensitivity enhancement of up to 10 times in comparison to $^{15}$N detection. Incorporation of a sensitivity-enhanced element in the pulse sequence further enhances the sensitivity up to 40% using SERI-HSQC. Presaturation of water resonances in the HSQC pulse sequence leads to amide proton signal loss due to water exchange. In fact, the comparison of HSQC and RI-HSQC spectral intensity can be used for understanding water-protein interactions. Although these methods were demonstrated for 2D $^{15}$N-$^1$H correlation experiments, it provides a basis for the development of 3D sequential correlation experiments for membrane proteins.
3.7 Acknowledgements

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Figure 3.1: (A) Phospholamban conformational equilibrium between T (PDB 2KB7) and R (PDB 2LPF) states. (B) $^{15}$N CP, and INEPT-HETCOR spectra of various PLN samples reconstituted in neutral DMPC or mixed DMPC, DOPE, and positively charged ePOPC lipid vesicles. (C) Relative sensitivity of INEPT-HETCOR spectra normalized with respect to CP is shown for each of the PLN samples reflecting the change in R-state population. Due to the complex interactions during CP and INEPT, this plot represents a relative estimation of the R state with respect to the T state.
Figure 3.2: (A) Two-dimensional $^{15}$N detected INEPT-HETCOR spectra that map the dynamic cytoplasmic domain of phospholamban membrane proteins (PLN$^{AFA}$ and PLN$^{R14del}$) reconstituted in DMPC lipid vesicles. (B) Solution NMR HSQC spectrum of PLN$^{AFA}$ reconstituted in isotropic bicelles, showing the assignment of cytoplasmic residues.
Figure 3.3: Two-dimensional $^1$H-$^{15}$N correlation experiments for mapping the dynamical regions of membrane proteins using MAS solid-state NMR at moderate spinning speeds. Pulse sequences for HSQC (A), Refocused INEPT (RI) HSQC (B), and sensitivity enhanced (SE) RI-HSQC (C), with $\tau$ value set to 5.4 ms ($=1/2J_{NH}$). For HSQC water suppression is obtained from a presaturation pulse during the recycle delay, whereas in RI-HSQC and SERI-HSQC RF spin locks during $\tau_1$ period ($\sim$250 ms) purges the water signal. For all pulse sequences, a two-step phase cycle was used by switching the $\phi$ and receiver phases. For HSQC and RI-HSQC $\tau_1$ states acquisition was obtained by altering the phase of $\phi$ pulse between x and y, whereas for SERI-HSQC Rance-Kay mode $\tau_1$ acquisition was obtained by altering the $\phi_1$ phase between x and $-x$. 
Figure 3.4: (A) Comparison of $^1$H 1D spectra of PLN$^{R14del}$ in DMPC lipids, obtained from one pulse experiment, and 1$^{st}$ increment (t1=0) of $^{15}$N HSQC and RI-HSQC. Efficient water suppression was obtained in HSQC and RI-HSQC displaying the amide proton spectra of protein. The net signal intensity in H$^N$ region is about 25% lower for HSQC compared to RI-HSQC. (B) $^{15}$N 1D spectrum obtained from INEPT-HETCOR experiment with t1=0. The number of scans for acquiring HSQC, RI-HSQC, and HETCOR was set to 64. The sensitivity enhancement of RI-HSQC with respect to INEPT-HETCOR was approximately 10 times. Note that for the one pulse $^1$H spectrum the receiver gain was lowered 10 times to avoid signal truncation.
Figure 3.5: Two-dimensional $^{15}$N-$^1$H correlation spectra of PLN$^{R14del}$ in DMPC lipids, obtained from $^{15}$N detection using INEPT-HETCOR, and $^1$H detection using HSQC, RI-HSQC and SE-RI-HSQC pulse sequences. All the spectra were acquired using 64 scans and 80 t1 increments. INEPT-HETCOR and HSQC spectra shown at 8 and 1.3 times higher noise floor. The relative integrated intensity of INEPT-HETCOR, HSQC, RI-HSQC, and SE-RI-HSQC between the spectral regions 7.5 and 9 ppm ($^1$H) and 113 and 133 ppm ($^{15}$N) is respectively 1.0:6.9:10.2:13.7.
Figure 3.6: (A) HSQC, RI-HSQC and HECTOR 1D spectra of PLN$^{\text{AFA}}$ in DMPC lipids, obtained from 2D pulse sequences with $t_1=0$. (B) Two-dimensional $^{15}$N-$^1$H correlation spectra of PLN$^{\text{AFA}}$ in various lipid compositions, obtained from $^1$H detected RI-HSQC shown in figure 2B.
**Figure 3.7:** One-dimensional RI-HSQC spectra of PLN^{R14del} and PLN^{AFA} samples reconstituted in DMPC lipids. MAS rates were varied from 0 to 15.5 kHz. For both the samples, similar sensitivity enhancement was obtained as a function of MAS rate.
Figure 3.8: $^1$H detected RI-HSQC spectra of PLN$^{AFA}$ reconstituted in DMPC lipids, recorded from the pulse sequence of figure 2B at various spinning speeds. All the spectra were drawn at same noise level showing the relative sensitivity.
Chapter 4: $^1$H-detected MAS Solid-State NMR Experiments Enable the Simultaneous Mapping of Rigid and Dynamic Domains of Membrane Proteins

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4.1 Abstract

Magic angle spinning (MAS) solid-state NMR (ssNMR) spectroscopy is emerging as a unique method for the atomic resolution structure determination of native membrane proteins in lipid bilayers. Although $^{13}$C-detected ssNMR experiments continue to play a major role, recent technological developments have made it possible to carry out $^1$H-detected experiments, boosting both sensitivity and resolution. Here, we describe a new set of $^1$H-detected hybrid pulse sequences that combine through-bond and through-space correlation elements into single experiments, enabling the simultaneous detection of rigid and dynamic domains of membrane proteins. As proof-of-principle, we applied these new pulse sequences to the membrane protein phospholamban (PLN) reconstituted in lipid bilayers under moderate MAS conditions. The cross-polarization (CP) based elements enabled the detection of the relatively immobile residues of PLN in the transmembrane domain using through-space correlations; whereas the most dynamic region, which is in equilibrium between folded and unfolded states, was mapped by through-bond INEPT-based elements. These new $^1$H-detected experiments will enable one to detect not only the most populated (ground) states of biomacromolecules, but also sparsely populated high-energy (excited) states for a complete characterization of protein free energy landscapes.
4.2 Introduction

Solid-state NMR (ssNMR) is the method of choice for probing structure, dynamics, chemistry, and ligand binding of microcrystalline, membrane-bound, and fibrillar proteins with atomic resolution \(^{111,129,130,170}\). Sensitivity and resolution of the resonances in multidimensional NMR spectra are fundamental requirements for protein structural analysis. Recent reviews have illustrated the most important achievements in this field \(^{112,127,171,172}\). These advancements are due to developments in high-field magnet technology, probe design, isotopic-labeling schemes, improved sample preparation techniques, and modern pulse sequences. However, the sensitivity and resolution of the ssNMR spectra still limit the routine application of these techniques for membrane proteins, where lipids are essential in sample preparations to maintain native conditions.

In recent years, our group has been redesigning the classical MAS ssNMR experiments for resonance assignment and distance determination in biological solids, boosting both sensitivity and resolution \(^{128,155,173,174}\). Toward this goal, we developed a new class of experiments called Polarization Optimized Experiments (POE) that utilize orphan spin operators to generate up to eight multi-dimensional NMR spectra from one pulse sequence \(^{173,175}\). These experiments are performed using commercial ssNMR probes for bio-solids and require only one receiver. POE methodology exploits simultaneous cross-polarization (CP) and long-lived \(^{15}\)N polarization of isotopically labeled proteins for generating multiple acquisitions. Recently, POE have been extended to \(^1\)H detection under moderate or fast MAS conditions using perdeuterated sample preparations to further improve their sensitivity \(^{147,176-179}\).

As for soluble proteins, integral or membrane-associated proteins populate different structural states modulated by their lipid environment as well as conformational dynamics \(^{122,148,180}\). Conformational dynamics can promote proteins’ to alternate states, characterized by high conformational energy, often referred to as conformationally excited
states. These states are sparsely populated and have been found to play an essential role in many biological functions \(^{120,148,180}\). However, detecting these lowly populated dynamic states using \(^{13}\)C detected experiments and conventional CP-based ssNMR pulse sequences is quite challenging. In fact, fast motions of protein segments can dramatically scale down anisotropic NMR interactions such as chemical shift anisotropy and dipolar couplings \(^{133,135,137}\). These effects increase the coherences lifetime (\(T_2\)), and have enabled the detection of mobile domains using solution-NMR type pulse sequences under moderate spinning speeds \(^{57,168}\). Following a similar strategy, we recently described \(^1\)H detected through-bond correlation experiments for studying conformationally excited states of membrane proteins under moderate MAS conditions using fully protonated samples reconstituted in hydrated proteoliposomes \(^{181}\). These experiments were successfully applied to phospholamban (PLN), a cardiac membrane protein that regulates calcium transport across the sarcoplasmic reticulum (SR) membrane by interacting with the sarcoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (SERCA) \(^{148}\). We demonstrated that a sensitivity enhancement of up to ten times can be achieved via \(^1\)H-detected \(^{15}\)N HSQC type MAS experiments. Notably, these highly sensitive \(^1\)H detected experiments allowed us to detect weakly populated (less than 5%) excited states of phospholamban in various lipid mixtures \(^{181}\). Therefore, a complete structural analysis of the different functional states of membrane proteins may require the application of both INEPT and CP-based experiments \(^{126,134}\).

In this work, we report a new strategy for \(^1\)H detected experiments on membrane proteins. We showed that it is possible to acquire both INEPT- and CP-based 2D correlation spectra in a single experiment. Specifically, we were able to image both N-H\(^N\) and C\(\alpha\)-H\(^N\) fingerprints detecting both the ground and the excited states of PLN, speeding up the characterization of the conformational energy landscape for membrane proteins.
4.3 Material and Methods

Uniformly $^{13}$C/$^{15}$N labeled monomeric PLN (PLN$^{\text{AF}}$) was expressed as a fusion protein with maltose binding protein in BL21(DE3) E. coli and purified as reported previously. The final sample consisting of about 2 mg of PLN was reconstituted in 1:100 protein to lipid ratio with DMPC liposomes and packed into a 3.2 mm Agilent MAS rotor using a series of centrifugation steps. All of the solid-state NMR experiments were acquired at the Minnesota NMR center using an Agilent ssNMR spectrometer operating at a $^1$H Larmor frequency of 600 MHz and equipped with 3.2 mm MAS probe with reduced RF heating technology. MAS rate of 12 kHz was used in all the experiments with a recycle delay of 3 seconds and 50 kHz spectral width in the $t_2$ dimension. For immobile regions of membrane proteins that are detectable via CP-based experiments, the transverse relaxation times ($T_2$) are relatively short. For this reason, the $t_1$ and $t_2$ evolution periods are relatively short for the cpHSQC compared to the INEPT-based riHSQC experiment. In our experiments, the number of $t_1$ increments for riHSQC and cpHSQC were set to 80 and 40, with a dwell time of 200 $\mu$s that gives maximum $t_1$ evolution periods of 16 and 8 ms, respectively. Two loops were applied (n=2, see Figures 4.1B and C) during the $t_1$ evolution of cpHSQC, where each $t_1$ loop with 40 increments was processed separately and then added for obtaining the final cpHSQC spectrum. A similar strategy was previously applied to obtain dual acquisition in the DUMAS and MEIOSIS methods. The $t_2$ acquisition time was set to 30 and 20 ms for riHSQC and cpHSQC experiments, respectively.

The 90$^\circ$ pulse lengths for $^1$H, $^{13}$C, and $^{15}$N were set to 2.5, 7, and 7 $\mu$s, respectively. Water suppression was obtained from phase switched spin-lock pulses with phases x and y with RF amplitude of 30 kHz and duration ($\tau_1$) set to 200 to 250 ms. For riHSQC experiments, the $\tau$ value was set to 5.4 ms (1/2 $J_{NH}$), and during $t_2$ acquisition $^{15}$N het-
eronuclear decoupling was obtained using the WALTZ-16 sequence with the RF amplitude set to 10 kHz. For cpHSQC, during $^1$H-$^{13}$C or $^{15}$N-$^1$H CP transfer $^{13}$C, $^{15}$N and $^1$H RF amplitudes were set to 35, 35 and 59 kHz respectively, while the $^1$H RF amplitude was linearly ramped from 80 to 100%. The optimal Hartmann–Hahn (HH) contact time for $^1$H-$^{15}$N or $^1$H-$^{13}$C CP was found to be 500 to 1000 μs at different temperatures, whereas the length of $^1$H-$^{15}$N CP prior to acquisition was set to 200 μs for selective N-H$^N$ transfer. During $^{15}$N or $^{13}$C $t_1$ evolution, TPPM decoupling was applied on $^1$H with 100 kHz RF amplitude. For the specific CP transfer from $^{13}$Cα ($^{13}$CO) to $^{15}$N, 100 kHz CW decoupling was applied on $^1$H, whereas $^{15}$N RF amplitude was set to $(5/2)\cdot\omega_r$ (~29.5 kHz), and $^{13}$C RF amplitude was set to $(3/2)\cdot\omega_r$ (~18.6 kHz) and $(7/2)\cdot\omega_r$ (~41 kHz) for $^{13}$Cα and $^{13}$CO specific CP, respectively. The specific CP was implemented with an adiabatic ramp ($\Delta \sim 1.6$ kHz and $\beta = 0.5$ kHz), and the contact times for CAN and CON transfers were set to 3.0 and 3.3 ms, respectively. During $t_2$ $^1$H acquisition of cpHSQC experiments a window-PMLG (phase modulated Lee-Goldberg) was applied on the $^1$H channel. The window acquisition parameters were optimized on a standard U-$^{13}$C, $^{15}$N NAVL (N-acetyl-Valine-Leucine) sample, where the $^1$H line widths were found to be between 120 to 150 Hz. In each cycle of window-PMLG, a detection period of 1.8 μs was inserted between 1.3 and 0.8 μs delay periods that account for receiver and probe ringing, followed by m5m PMLG sequence with RF amplitude of 100 kHz and length 14 μs. All the spectra were processed using NMRPipe and analyzed using Sparky.

4.4 Results

When applied to a given membrane protein, the riHSQC and cpHSQC pulse sequences map N-H$^N$ backbone fingerprints of dynamic and immobile resides, respectively. The $^1$H detected riHSQC pulse sequence consists of water suppression ($\tau_1$) and $t_1$
evolution periods that are sandwiched between two $^1$H-$^{15}$N refocused INEPT periods (Figure 4.1A). In the cpHSQC pulse sequence (Figure 4.1B), on the other hand, water suppression and $t_1$ evolution periods are sandwiched between two $^1$H-$^{15}$N CP periods followed by $^1$H detection under window PMLG sequence. The window PMLG sequence improves the $^1$H line widths significantly by suppressing homonuclear dipolar couplings. A significant drawback of window acquisition is the reduced sensitivity due to limited detection points. However, under fast MAS conditions, it is possible to recover both sensitivity and resolution.

To make the best of nuclear spin polarization and reduce the experimental time, we combined riHSQC and cpHSQC pulse sequences into single experiments. We propose two new schemes (Figures 4.1C and D) that include two acquisition periods per each scan and record riHSQC and cpHSQC in first and second acquisitions, respectively. In the pulse sequence represented in Figure 4.1C, the polarization is initially transferred from $^1$H to $^{13}$C using CP followed by a 90° pulse that creates $^{13}$C z-magnetization. The riHSQC pulse sequence is then applied on $^1$H and $^{15}$N channels while storing the $^{13}$C polarization along z-direction. After the first acquisition, the carbonyl magnetization is transferred to $^{15}$N via specific-CP followed by a $t_1$ evolution period. A short $^1$H-$^{15}$N CP ($\sim$ 150 to 200 $\mu$s) is then applied for selective polarization transfer from the $^{15}$N nuclei to the directly bonded amide protons followed by $^1$H detection during the second acquisition. Note that during riHSQC sequence and its $t_1$ evolution, the $^{13}$CO z-spin operator undergoes $T_1$ relaxation. In order to keep the relaxation constant for all $t_1$ increments, a delay of (CT-$t_1$) is applied at the end of first acquisition. Figure 4.1D shows another version of the pulse sequence, where the riHSQC is acquired during the first acquisition followed by a 90° pulse on $^{13}$C that creates $^{13}$CO direct polarization that is used for recording cpHSQC during the second acquisition. In conventional cpHSQC (Figure 4.1B), the polarization follows the H-N-H$^N$ pathway; whereas for the cpHSQC acquired in second ac-
quisition the polarization follows the H-CO-N$_{13}$H and CO-N$_{15}$H pathways, respectively (Figures 4.1C and 4.1D).

We applied the $^1$H detected HSQC experiments on U-$^{13}$C,$^{15}$N labeled phospholamban (PLN), a 52-residue membrane protein that regulates the SR Ca$^{2+}$-ATPase. PLN comprises an inhibitory transmembrane domain (domain Ib and domain II) and a regulatory domain (domain Ia), connected by a short, flexible loop. Figure 4.2 compares the sensitivity of riHSQC and cpHSQC 1D spectra acquired using single and dual acquisition methods, respectively. The sensitivity for the riHSQC is almost identical between the two methods; whereas the sensitivity of cpHSQC acquired using the dual acquisition method is $\sim$50% lower compared to single acquisition sequence. The sensitivity achieved in ‘n’ scans is $\sqrt{n}$, so the sensitivity in ‘n/4’ scans is equal to $\sqrt{n/4}$ or 0.5$^*$ $\sqrt{n}$ which is equal to half of the sensitivity. In other words, the cpHSQC acquired separately would require about one fourth of the number scans ($\sim$25% more experimental time), hence, the dual acquisition sequences result in a time saving of $\sim$25%. Indeed, a loss of signal is expected for the cpHSQC sequence as the additional polarization transfer pathways as well $^{13}$C $T_1$ relaxation reduces the initial $^{15}$N polarization prior to the $t_1$ evolution period. Nevertheless, the cpHSQC spectrum is acquired along with riHSQC with almost no additional time. This sensitivity comparison is also shown for the 2D spectra in Figure 4.3. The riHSQC of Figure 4.3A and cpHSQC of Figure 4.3B were acquired in 23.52 and 6.1 hours, respectively, whereas Figure 4.3C shows the simultaneous acquisition of both the spectra using 24.08 hours. Integrated intensity of riHSQC and cpHSQC were found to be almost similar between Figures 4.3A, B, and C, with a 25% time saving using the dual acquisition. Previous solution and solid-state NMR as well electron paramagnetic resonance (EPR) experiments from our group and others have shown that PLN’s regulatory region in the absence of SERCA undergoes a conformational equilibrium between an ordered T state (helical) and a disordered R state (unfolded and mem-


brane detached). The red envelop of peaks correspond to the most rigid resonances (T-state + inhibitory domain) of the protein, while the resonances in blue correspond to the most dynamic region of the protein (domain Ia and loop) which correspond to the disordered R-state (see Figure 4.3C). The schematic of T and R-state equilibrium is shown in Figure 4.3D (PDB structures 2KB7 and 2LPF), where the domains Ia, Ib and loop regions are highlighted in blue.

The pulse sequences for simultaneous acquisition $^{15}$N-edited riHSQC and cpHSQC of Figures 4.1C and 4.1D, can also be extended to $^{13}$C edited experiments. Figure 4.4A shows another example where the first acquisition records $^{15}$N-edited sensitivity enhanced riHSQC (SE-riHSQC) experiment; whereas second acquisition utilizes a direct C\textalpha polarization for CA(N)H\textsuperscript{N} correlation via CA-N and N-H polarization transfer periods. The 2D $^{15}$N-edited riHSQC and $^{13}$C\textalpha edited cpHSQC spectra of PLN are shown in Figure 4.4B. Note that the $^{1}$H resolution in the $^{15}$N-edited cpHSQC (Figures 4.3) is similar to that of the $^{13}$C-edited cpHSQC (Figure 4.4B). The t\textsubscript{2} for the detection of the amide protons is identical in both spectra. However, for PLN the chemical shift breath for the $^{13}$C\textalpha resonances is ~12 ppm, which is much larger than the corresponding $^{15}$N dimension (~3 ppm). Therefore, the spectrum shown in Figure 4.4B ($^{13}$C\textalpha-edited cpHSQC) has better overall resolution. While the current experiments were implemented at 12 kHz spinning, we anticipate that the application of dual acquisition riHSQC-cpHSQC can further enhance the sensitivity at higher spinning speeds.

To further understand the dynamics of these structural regions, we carried out the ri-HSQC-cpHSQC experiments at different temperatures, i.e., -20, 2, 25, and 37 °C (Figure 4.5) using the pulse sequence of Figure 4.1C. While the chemical shifts of the peaks attributed to the T-state remain essentially unchanged, the R-state peaks gradually shift towards higher fields as a function of temperature. Note that a complete unfolding of the
cytoplasmic domain occurs only by removing the transmembrane domain and analyzing a peptide corresponding to residues 1-23 of PLN. From the linear behavior of the chemical shifts as a function of the temperature, it is possible to deduce a single thermal unfolding process as previously reported for PLN using chemical unfolding. The latter is further supported by our earlier studies on PLN using $^{13}$C detected DARR and rINEPT experiments.

**4.5 Discussion**

Historically, INEPT and CP pulse sequences represented the basic building blocks of solution and solid-state NMR experiments, respectively. While the ssNMR of proteins predominantly requires the use of CP-based experiments, recent studies have shown that the dynamic regions of membrane proteins as well as for some protein fibrils, INEPT-based experiments are crucial for complete structural mapping. Baldus and co-workers have demonstrated the use of $^{13}$C and $^{15}$N detected CP and refocused INEPT experiments under moderate MAS conditions for studying several membrane proteins including PLN. Recently, Ramamoorthy and co-workers have utilized the hybridization of CP and INEPT pulse sequences for sensitivity enhancement of oriented as well as MAS samples.

There is growing evidence that sparsely populated states play a significant role in biology. PLN is a classic example of such phenomenon, where the dynamic R-state binds and regulates SERCA, decreasing its apparent affinity for Ca$^{2+}$ ions in a reversible manner. Recently, we have also shown that the population of R-state can be dramatically changed by both mutations in PLN and lipid composition. The high sensitivity of $^1$H detection can detect both ground and sparsely populated conformationally excited states simultaneously and make it possible to have a semi quantitative estimate of the conformational equilibrium and the energy landscape of PLN. Although the coexistence
of T and R-state were already demonstrated in our previous publications using $^{13}$C detected experiments$^{57,148}$, the complete assignment of R state residues was not possible due to a low sensitivity of the samples. The highly sensitive $^1$H-detected methods will enable the acquisition of 3D sequential correlation experiments for mapping of the R-state residues of the cytoplasmic region. Recently, we have demonstrated the quantification of relative populations of the T and R states using $^{15}$N detection (1D CP and rINEPT experiments) $^{181}$. In principle, $^1$H detection (1D cpHSQC and rHSQC experiments) could be used for estimating these populations. However, the length of the $^1$H detected experiments is twice that of $^{15}$N detected experiments and the spin dynamics is different due to the strong $^1$H-$^1$H dipolar interactions, preventing an accurate quantification. The latter is exacerbated by the long water suppression element that is necessary for $^1$H detected experiments. While for crystalline and amyloid proteins, water suppression is usually achieved using short spin lock periods ($\tau_1$) of 50 to 100 ms, in our experiments $\tau_1$ needs to be set to 200 to 250 ms due to the higher water content of lipid-based membrane protein samples. Also, the $^1$H line widths of cpHSQC experiments at 12 kHz MAS are relatively broad compared to crystalline proteins in spite of using homonuclear window-PMLG decoupling. We anticipate that the application of these pulse sequences under fast MAS conditions (40 to 60 kHz) can further improve the line widths as well as sensitivity. Although the current experiments were demonstrated using a regular MAS setup (without gradient and field lock channels) a further gain in both sensitivity and resolution will be possible thanks to these technological advancements.

4.6 Conclusions

In conclusion, we have developed a new $^1$H-detection strategy for simultaneous acquisition of CP- and INEPT-based out-and-back experiments on membrane proteins, which resulted in approximately 25% time saving. We successfully tested these new
pulse sequences on fully protonated U-$^{13}\text{C},^{15}\text{N}$ labeled PLN in proteoliposomes using 12 kHz MAS spinning rate. These new methods provide the basis for the development of 3D sequential correlation experiments for detecting and characterizing both ground and excited states of membrane proteins embedded in biologically relevant lipid membrane models.

4.7 Acknowledgments

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Figure 4.1: Pulse sequences for two-dimensional $^1$H-detected cpHSQC and riHSQC two-dimensional experiments for mapping N-H$^N$ backbone fingerprint of ground and excited states of proteins. (A) riHSQC, (B) cpHSQC pulse sequences using single acquisition. (C) and (D) show new pulse sequences for simultaneous acquisition of riHSQC and cpHSQC in first and second acquisitions respectively. The t1 evolution was used with two loops (n=2) for cpHSQC prior to second acquisition. For all the pulse sequences, a two-step phase cycle was used by switching the $\phi$ and receiver phases, t1 states acquisition was obtained by altering the phase of $\phi$ pulse between x and y.
Figure 4.2: Phospholamban amide $^1$H spectra obtained from riHSQC and cpHSQC experiments by using the pulse sequences of Figure (4.1) with t1 set to zero. (A) and (B) were respectively acquired using riHSQC and cpHSQC shown in Figure 4.1A and B. Each of the two spectra in (C) and (D) were simultaneously acquired by using the pulse sequences of Figures 4.1C and D respectively.
Figure 4.3: 2D N-H\textsuperscript{N} correlation spectra of phospholamban membrane protein obtained from the pulse sequences of Figure 4.1. The spectra in (A) and (B) were acquired separately using the pulse sequences of Figure 4.1A and B respectively. (C) 2D spectra ri-HSQC (blue) and cpHSQC (red) were simultaneously acquired using the pulse sequence of Figure 4.1D. The riHSQC spectrum maps the dynamic cytoplasmic residues of phospholamban, whereas the relatively immobile transmembrane and juxtamembrane residues are mapped by the cpHSQC spectrum. (D) Structures of PLN in the T (2KB7) and R (2LPF) states.
Figure 4.4: (A) Pulse sequence for simultaneous acquisition of INEPT-based N-H\textsuperscript{N} and CP-based CA(N)H\textsuperscript{N} correlation experiments acquired in first and second acquisitions respectively. A sensitivity enhanced riHSQC was used for first acquisition, whereas a direct \textsuperscript{13}C\alpha polarization was used for CA(N)H\textsuperscript{N} experiment during the second acquisition. (B) \textsuperscript{15}N-edited SE-riHSQC and \textsuperscript{13}C\alpha-edited cpHSCQ spectra of phospholamban obtained from the pulse sequence of (A).
Figure 4.5: Simultaneous acquisition of riHSQC and cpHSQC spectra of phospholamban using the pulse sequence of Figure 4.1D at different temperatures. The relative peak positions of cpHSQC spectra remain similar, whereas the riHSQC peaks are gradually shifted as a function of temperature indicating the change in structural dynamics.
Chapter 5: Effects of the Arg9Cys and Arg25Cys Mutations on Phospholamban’s Conformational Equilibrium in Membrane Bilayers

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5.1 Abstract

Approximately, 70% of the Ca\(^{2+}\) ion transport into the sarcoplasmic reticulum is catalyzed by the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), whose activity is endogenously regulated by phospholamban (PLN). PLN comprises a TM inhibitory region and a cytoplasmic regulatory region that harbors a consensus sequence for cAMP-dependent protein kinase (PKA). The inhibitory region binds the ATPase, reducing its apparent Ca\(^{2+}\) binding affinity. \(\beta\)-adrenergic stimulation activates PKA, which phosphorylates PLN at Ser 16, reversing its inhibitory function. Mutations and post-translational modifications of PLN may lead to dilated cardiomyopathy (DCM) and heart failure. PLN's cytoplasmic region interconverts between a membrane-associated T state and a membrane-detached R state. The importance of these structural transitions on SERCA regulation is emerging, but the effects of natural occurring mutations and their relevance to the progression of heart disease are unclear. Here we use solid-state NMR spectroscopy to investigate the structural dynamics of two lethal PLN mutations, R9C and R25C, which lead to DCM. We found that the R25C mutant enhances the dynamics of PLN and shifts the conformational equilibrium toward the R state confirmation, whereas the R9C mutant drives the amphipathic cytoplasmic domain toward the membrane-associate state, enriching the T state population. The changes in membrane interactions caused by these mutations may explain the aberrant regulation of SERCA.
5.2 Introduction

Phospholamban (PLN) is a central regulator of cardiac contractility\textsuperscript{188}. This 52-amino acid single-pass membrane protein forms a complex with the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA), handling 70-90\% of the cytoplasmic Ca\textsuperscript{2+} ions\textsuperscript{9}. In fact, the SERCA/PLN complex is responsible for Ca\textsuperscript{2+} reuptake into the sarcoplasmic reticulum (SR), regulating the diastolic phase of heart muscle\textsuperscript{9,189,190}. When unphosphorylated, PLN binds SERCA via intramembrane interactions and reduces its apparent Ca\textsuperscript{2+} affinity\textsuperscript{191}. Upon β-adrenergic stimulation, cAMP-dependent protein kinase A phosphorylates PLN at Ser16, reversing its inhibitory effects and augmenting cardiac contractility\textsuperscript{9}. Protein phosphatase 1 dephosphorylates PLN, reestablishing its basal inhibitory activity\textsuperscript{192}.

Functional and sequence analyses show that PLN structure is divided into two regions: a cytoplasmic (regulatory) region consisting of domain Ia (residues 1-16), a loop region (residues 17-22), and domain Ib (residues 23-30), and a transmembrane (TM) inhibitory region that includes domain II (residues 31-52)\textsuperscript{192}. In cell and reconstituted membrane systems, PLN exists in equilibrium between a monomeric and an oligomeric state\textsuperscript{193,194}. The homopentamer is arranged in a pinwheel topology\textsuperscript{193,195}. Although initial studies suggested that the pentamer functions as a Ca\textsuperscript{2+} or Cl\textsuperscript{-} channel, more recent studies revealed that oligomerization is a mechanism for storage of active PLN monomers, playing a key role in SERCA regulation\textsuperscript{196,197}. Structural studies in lipid membranes show that upon de-oligomerization, PLN’s helical transmembrane (TM) domain changes its tilt angle with respect to the lipid bilayer by approximately 5 degrees with a slight rotation of the azimuthal angle\textsuperscript{193,198}. The amphipathic cytoplasmic region, on the other hand, samples a range of conformations, including two major states: a T state, more populated, helical, and membrane associated, and an R state, more dynamic, membrane detached, and unfolded\textsuperscript{57,148,199}. By correlating biophysical data with functional assays and site-directed mutagenesis, we discovered that the T-to-R equilibrium regulates the extent
of SERCA inhibition by PLN\textsuperscript{98}. In fact, by shifting the conformational equilibrium toward the R state, we were able to generate PLN loss-of-function (LOF) mutants that mimic the effects of Ser16 phosphorylation by PKA\textsuperscript{16,55}. When bound to SERCA, the T-to-R equilibrium is further shifted towards a SERCA-bound (B) state\textsuperscript{193,198,200}. While the T and R states are inhibitory, the B state is non-inhibitory. Functional assays carried out on a truncated version of PLN lacking the cytoplasmic region, support this regulatory model\textsuperscript{148}.

In the past years, several pathological mutations or post-translational modifications have been identified in patients diagnosed with familial dilated cardiomyopathy (DCM). The first human mutation identified was R9C (PLN\textsuperscript{R9C}), located in domain Ia of the regulatory region\textsuperscript{46}. This autosomal dominant, missense mutation at nucleotide 25 causes the typical DCM phenotype, with a severe dilation of the left ventricle and decreased contractile function of the heart muscle\textsuperscript{201}. The later sequencing of R9H and R9L mutations in DCM patients further emphasized the importance of PLN’s position 9 for SERCA regulation\textsuperscript{202}. More recently, Kranias and co-workers identified another mutation of PLN at Arginine 25 (PLN\textsuperscript{R25C}). As for the R9C, the arginine 25 was mutated into a cysteine with cardiotoxic effects associated with SERCA super-inhibition and arrhythmia\textsuperscript{58}. To date, the molecular mechanisms for dysregulation of Ca\textsuperscript{2+} transport by these mutants are still unclear.

In an effort to elucidate the structural basis of the PLN\textsuperscript{R9C} and PLN\textsuperscript{R25C} cardiotoxicity, we investigated the effects of these single-site mutations on the conformational equilibrium of PLN in lipid bilayers using solid-state NMR techniques. We found that these mutants affect substantially the conformational equilibrium of PLN. Specifically, the R9C mutation shifts PLN’s conformational equilibrium toward the membrane associated T state, supporting earlier conclusions that increased hydrophobicity at position 9 of domain Ia can be used as a predictor for the development and progression of DCM\textsuperscript{202}. In
contrast, the R25C mutant enhances the R state population, shifting the equilibrium of the cytoplasmic region of PLN toward more dynamic ensemble. The structural and topological changes of these mutants in the membrane are interpreted in the light of our proposed regulatory model and represent a first step to explain the different manifestations in DCM patients carrying these two mutations.

5.3 Material and Methods

5.3.1 Protein expression and purification. The R9C and R25C mutation was cloned onto the pMal c2E AFA-PLN and PLNWT background using the QuikChange II mutagenesis kit (Agilent). Uniformly $^{13}\text{C}/^{15}\text{N}$ labeled AFA-PLN, AFA-PLN$^{\text{R9C}}$, and AFA-PLN$^{\text{R25C}}$ were expressed in BL21(DE3) E. coli, while selectively $^{13}\text{C}$ and $^{15}\text{N}$ Ile labeled samples were expressed using the ML12 auxotroph line in $^{14}\text{N}$ M9 media following previously described protocols$^{203}$. Fifty milligrams of $^{13}\text{C}/^{15}\text{N}$ Ile was added per 500 mL of growth media, along with fifty milligrams each of unlabeled leucine, valine, and tyrosine to fulfill the requirements of the auxotroph line. The only modification with AFA-PLN$^{\text{R9C}}$ and AFA-PLN$^{\text{R25C}}$ was that expression was limited to four hours. Purification followed the previously described protocol for maltose binding protein (MBP) tagged PLN, except that the cleaved AFA-PLN$^{\text{R9C}}$ in SDS was incubated at 55°C during HPLC purification with a C4 column (Agilent) while AFA-PLN$^{\text{R25C}}$ was incubated at 45°C. AFA-PLN$^{\text{R9C}}$ and AFA-PLN$^{\text{R25C}}$ were also purified in the presence of TCEP-HCl (pH 7.3) to prevent oxidation.

5.3.2 Solution NMR in isotropic bicelles. Samples for solution NMR experiments were prepared in isotropic bicelles with a q ratio of 0.33 using DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine) and DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) purchased from Avanti Polar Lipids. Chloroform stocks for DHPC and DMPC were aliquoted into glass tubes and dried down under nitrogen. The dried film was reconstituted in water, flash frozen in liquid nitrogen and lyophilized overnight. The lyophilized lipids
were reconstituted in buffer containing 20 mM HEPES (pH 7.0), 2.5% glycerol, 100 mM KCl, 1 mM MgCl₂, and 0.02% NaN₃. The samples of AFA-PLN⁹⁹C and AFA-PLN⁸⁵C also contained 10 mM TCEP. Lyophilized PLN was solubilized in the reconstituted DHPC and then added to the DMPC to form the bicelle. All the spectra in isotropic bicelles were acquired at the Minnesota NMR center using an Agilent 600 MHz spectrometer operating a 5mm triple resonance cryoprobe at 25°C.

5.3.3 Magic Angle Spinning (MAS) ssNMR. MAS samples of AFA-PLN, AFA-PLN⁸⁵C and AFA-PLN⁹⁹C were prepared in ²H DMPC (1,2-dimyristoyl-d₅₄-sn-glycero-3-phosphocholine, Avanti Polar Lipids®) at a protein to lipid ratio of approximately 1:100 following previously described protocols ⁵⁷. Briefly, ²H DMPC suspended in chloroform was dried down under nitrogen flux. The dried lipids were reconstituted in a buffer containing 20 mM HEPES, 50 mM KCl, 2.5 mM MgCl₂, and 1 mM NaN₃ at pH 7. Samples containing AFA-PLN⁹⁹C and AFA-PLN⁸⁵C were prepared in the presence of 10 mM TCEP. Lyophilized uniformly or selectively labeled AFA or mutant PLN was reconstituted in 1% C₁₂E₈, prior to mixing with the ²H DMPC. The reconstituted sample was incubated with Biobeads® to remove the C₁₂E₈ (60 mg Biobeads®/mg C₁₂E₈).

All of the solid-state NMR experiments were acquired at the Minnesota NMR center using an Agilent ssNMR spectrometer operating at a ¹H Larmor frequency of 700 MHz and equipped with 3.2 mm MAS probe with reduced RF heating technology. MAS rate of 12 kHz was used in all the experiments with a recycle delay of 3 seconds and 50 kHz spectral width in the t₂ dimension. The 90° pulse lengths for ¹H, ¹³C, and ¹⁵N were set to 2.5, 7, and 7 μs, respectively. The direct dimension (t₂) acquisition time was set to 100 ms for INEPT-HETCOR and riHSQC experiments, and 20 ms for CP and DARR experiments. The ¹H detected riHSQC 2D spectra were acquired with 80 t₁ increments with 5 kHz t₁ spectral width. Whereas ¹³C detected 2D DARR spectra were acquired with 256 t₁ increments with 33 kHz t₁ spectral width using 100 ms DARR mixing period. The
Hartmann–Hahn (HH) contact time for $^1\text{H}-^{13}\text{C}$ CP was set to 1 ms, during $t_1$ and $t_2$ periods TPPM decoupling was applied on $^1\text{H}$ with 100 kHz RF amplitude. The 1D CP and INEPT-HETCOR $^{15}\text{N}$ spectra of Figure 5.1A were acquired using 2k to 10k scans. All the samples were acquired with identical experimental parameters. The relative R-state populations (Figure 5.1B) were obtained by measuring the integrated intensities of CP and INEPT-HETCOR spectra between 100 and 140 ppm at the same noise level, where the CP intensity was normalized to 100$^{204}$. The $^1\text{H}$ detected $^{15}\text{N}$ HSQC spectra of Figure 5.2A were obtained from riHSQC pulse sequence. Water suppression was obtained from phase switched spin-lock pulses with phases x and y with RF amplitude of 30 kHz and duration ($\tau_1$) set to 200 to 250 ms. The $\tau$ value was set to 5.4 ms ($1/2$ $J_{\text{NH}}$), and during $t_2$ acquisition $^{15}\text{N}$ heteronuclear decoupling was obtained using the WALTZ-16 sequence with the RF amplitude set to 10 kHz. All the spectra were processed using NMRPipe and analyzed using Sparky$^{93,205}$.

5.4 Results

The NMR experiments were carried out on the monomeric state of PLN, where the three transmembrane (TM) cysteines are mutated into Ala, Phe, and Ala residues to disrupt the pentameric assemblies. This mutant (AFA-PLN) has identical activity to PLN$^{\text{WT}}$ and eliminates the monomer-pentamer equilibrium, simplifying our spectroscopic analysis and data interpretation. To analyze the changes in the structural dynamics of PLN’s cytoplasmic domain, we recorded both cross-polarization (CP) and insensitive nuclei enhancement by polarization (INEPT) based solid-state NMR experiments on AFA-PLN and its R9C and R25C mutants. Figure 5.1A shows the $^{15}\text{N}$ signatures of the backbone amides for AFA-PLN$^{\text{AFA}}$, AFA-PLN$^{\text{R9C}}$, and AFA-PLN$^{\text{R25C}}$ reconstituted in zwitterionic DMPC lipid bilayers. While the CP-based experiment detects the most rigid residues
of membrane proteins, the INEPT-based HETCOR experiment images the more dynamic residues. In fact, for the INEPT experiments, we generally use relatively long $J$-coupling evolution periods (~10 ms), which select for mobile residues with long transverse relaxation time ($T_2$)\textsuperscript{135}. As a result, the CP spectra map both TM and membrane-associated residues; whereas the INEPT-HETCOR spectra image residues corresponding to the R state undergoing fast motions. Although it is difficult to estimate the absolute R-state population, we utilized the relative integrated intensities from the INEPT-HETCOR peaks with respect to the corresponding CP experiments to obtain a semi-quantitative estimate of the R state population for the three different AFA-PLN variants. By comparing the histogram of the relative populations (Figure 5.1B), it is possible to appreciate a significant difference in the relative R state for AFA-PLN\textsuperscript{R9C} and AFA-PLN\textsuperscript{R25C} mutants with respect to AFA-PLN. In the R9C mutant, the population of the R state is significantly reduced relative to that of AFA-PLN (approximately one tenth). In contrast, the R state population in the R25C mutant is approximately 7 times higher than that of AFA-PLN. To further validate the shift in populations for these mutants, we carried out 2D riHSQC MAS ssNMR experiments in proteoliposomes. Figure 5.2A shows the HSQC spectra of AFA-PLN, AFA-PLN\textsuperscript{R9C}, and AFA-PLN\textsuperscript{R25C}. While the fingerprints for both AFA-PLN and AFA-PLN\textsuperscript{R9C} show significantly broader peaks indicative of a higher degree of conformational heterogeneity, the R25C resonances are notably narrower with higher relative peak intensities. These data support the conformational shift toward the R state caused by the R25 mutation in domain Ib, and at the same time, reveal its highly dynamic nature for the cytoplasmic region. Similar HSQC patterns were observed for AFA-PLN and AFA-PLN\textsuperscript{R25C} reconstituted in isotropic lipid bicelles using solution NMR (Figure 5.2B). Due to the longer correlation times of isotropic bicelles, the more rigid TM residues are either weak or undetectably broad; whereas the peaks corresponding to the dynamic cytoplasmic region display higher intensities. The favorable
relaxation conditions in isotropic bicelles made it possible to assign the majority of these peaks, which correspond to the 1-20 region of AFA-PLN, encompassing domain Ia and the dynamic loop. The heteronuclear correlated spectra are more resolved in the isotropic bicelles than in the proteoliposomes, suggesting that the dynamics of AFA-PLN in different membrane-mimetic systems occur on different time scales.

To analyze the secondary structure adopted by the cytoplasmic domains in proteoliposomes, we carried out $^{13}$C-$^{13}$C TOBSY and DARR experiments on all three PLN variants. The TOBSY experiment correlates aliphatic intra-residue carbon peaks of dynamic regions, transferring the polarization through $^{13}$C-$^{13}$C resonances via $J$-couplings; whereas the DARR experiment utilizes dipolar coupling based polarization transfer that is sensitive to immobile transmembrane and membrane attached cytoplasmic residues. As expected from the analysis of the $^{15}$N-$^1$H HSQC spectra, the TOBSY spectra for both AFA-PLN and AFA$^{R9C}$ display only a few internuclear correlations, due to the restricted motions of the cytoplasmic domain, i.e., lower R state population. In contrast, the TOBSY spectrum of the R25C mutants shows a significantly higher number of correlations that enabled us to assign 16 out of the 22 residues encompassing the cytoplasmic region. Note that the $^{13}$C resonances in this spectrum have chemical shifts identical to those in the solution NMR experiments carried out in isotropic bicelles$^{166,206}$. The chemical shift index (CSI) obtained from the CA-CB correlations (Figure 5.3B) shows a residual helical secondary structure the R25C resonances. As reported previously$^{98}$, the chemical shifts of the resonances in the cytoplasmic domain represent a weighted average between the population of the completely unfolded state of PLN and its helical membrane-adsorbed T state; therefore, the R25C mutation still possess a significant fraction of the membrane adsorbed helical conformation. Figure 5.4 shows residue-specific changes as probed by DARR and TOBSY experiments for the different PLN mutants, emphasizing the T-to-R state equilibrium. Due to spectral overlap, only Ala, Ser,
and Thr residues are resolved (the full 2D spectra are reported in the Supporting Information, Figure S5.1). It is apparent that the cytoplasmic Ala residues (A11, and A15) are in slow conformational exchange between T and R states (Figure 5.4)\textsuperscript{148}. These two populations are apparent for AFA-PLN and AFA-PLN\textsuperscript{R25C}, however, the R-state peaks are missing or undetectable for the R9C mutant, due to the low population of the R state. For R25C mutant, Ser and Thr residues located in the short loop connecting domains Ia and Ib are essentially missing in the DARR spectrum, but they are present in the TOBSY spectrum, again, indicating their higher mobility. The same residues are present although with much lower intensities in the corresponding experiments for AFA-PLN and AFA-PLN\textsuperscript{R9C}. Taken all together, these observations demonstrate that the loop region is more mobile for the AFA-PLN\textsuperscript{R25C} than AFA-PLN and AFA-PLN\textsuperscript{R9C} mutants.

5.5 Discussion

Our previous studies unveiled the importance of PLN’s conformational equilibrium in the regulatory cycle of SERCA\textsuperscript{148}. We found that PLN adopts three different conformational states (T, R, and B) and their relative populations determine the extent of inhibition of SERCA. We also discovered that post-translational phosphorylation of PLN at Ser16 by cAMP-dependent protein kinase shifts the conformational equilibrium of the SERCA/PLN complex, augmenting the apparent affinity for Ca\textsuperscript{2+} and muscle contractility\textsuperscript{148}. Phosphorylation does not detach PLN from the ATPase; rather its cytoplasmic domain rearranges its SERCA-bound conformation upon phosphorylation\textsuperscript{148,207}, with PLN acting as a subunit\textsuperscript{208}. These structural changes are mostly localized in the cytoplasmic region of PLN, while the TM domain of the protein remains essentially unperturbed\textsuperscript{207}. Mutagenesis studies carried out by our group showed that it is possible to modulate SERCA function by engineering single or double mutations in the short intervening loop between domain Ia and the juxtamembrane domain Ib\textsuperscript{16,55} that affect the
folding of the cytoplasmic mutations. Remarkably, a single mutation (P21G) nearly mimics the dynamic state of Ser16 phosphorylated PLN\textsuperscript{16,55}. Parallel EPR and NMR studies also showed that the nature as well as the composition of lipid bilayers affects PLN’s conformational equilibrium\textsuperscript{57,98}, whose existence has been detected even in native lipids extract from the sarcoplasmic reticulum membrane\textsuperscript{57,209}. The folding-unfolding mechanism of the cytoplasmic region probably plays an important role for PLN interactions with other binding partners\textsuperscript{210}. For instance, we found that the unfolding of domain Ia, which contains the consensus sequence for protein kinase, is kinetically important for kinase recognition of PLN and high efficiency of phosphoryl transfer\textsuperscript{149,167,211}.

Based on these data, we hypothesized that the dysfunctional folding and unfolding mechanism of the PLN’s cytoplasmic domain is also involved in the progression of cardiac disease, and that structural disruptions of the amphipathic helix of domain Ia may have effects on SERCA regulation. In fact, Young and co-workers have proposed that the higher hydrophobicity caused by the R9C mutation has a direct effect on regulation of SERCA\textsuperscript{202}. In our first attempt to characterize the structural effects of this mutant, we performed solution NMR spectroscopy using DPC micelles as a membrane mimetic environment (Figures S5.2-S5.4). Unfortunately, this membrane-mimetic system revealed its considerable limitations. Specifically, solution NMR did not detect any substantial difference in the structure and dynamics of PLN cytoplasmic domain caused by the R9C mutation. In contrast, our structural and dynamic NMR analysis in lipids highlights substantial changes caused by both R9C and R25C mutations, suggesting that the DPC micelle is an inadequate system to investigate the complexity of PLN’s regulatory mechanism. In particular, solid-state NMR experiments in lipid membranes show that the R9C mutation shifts the conformational equilibrium toward the T state, the inhibitory and membrane associated conformation. Moreover, functional assays carried out in the presence of SERCA show that the R9C mutant is partially inhibitory, while kinetic phos-
phorylation assays reveal slower kinetics of phosphorylation by protein kinase A\textsuperscript{47}. The latter can be explained by a stronger association of the regulatory region of PLN with the membrane bilayer, rendering the phosphorylation site virtually inaccessible to the kinase\textsuperscript{47}. Also, Ha et al.\textsuperscript{47} showed that R9C pentamer is unable to regulate SERCA’s activity, due to the formation of disulfide bridges between the protomers, a situation that is exacerbated under oxidizing conditions\textsuperscript{47,212}.

On the other hand, the R25C mutant has been associated with super-inhibition of SERCA and Ca\textsuperscript{2+} transport, and unlike the R9C mutant, gives rise to sarcoplasmic Ca\textsuperscript{2+} leaks and causes arrhythmogenesis under stress conditions\textsuperscript{58}. The R25C mutation is located in the juxtamembrane domain I\textsubscript{b}, which is more dynamic than the TM domain II\textsuperscript{57,213}. In the wild-type PLN, Arg25 interacts electrostatically with the lipid membrane head groups\textsuperscript{193,198}, anchoring the protein to the membrane. The cysteine mutation abolishes these interactions, causing a structural rearrangement of the lipid-protein interactions, which leads to topological changes of the TM domain, with a concomitant increase of the R state population. The super-inhibitory character of the R25C mutant can be probably explained by an increase of affinity between SERCA and PLN and a possible topological rearrangement within the inhibitory complex with SERCA. On the other hand, the increased population of the R state might affect the interactions with other binding partners, resulting in dysfunctional effects such as arrhythmogenesis or increase in the frequency of Ca\textsuperscript{2+} sparks and waves that were not observed in the R9C mutations. Additional structure-function correlations need to be carried out to test these hypotheses. While these studies provide a first structural look at the effects of R9C and R25C mutations on PLN’s interactions with lipid bilayers, only the characterization of the binding interactions with SERCA will provide more definitive insights into how these pathogenic mutations influence Ca\textsuperscript{2+} cycling.
5.6 Conclusions

In conclusion, we used a combination of solid-state NMR techniques to analyze the effects of two deadly mutations of PLN that lead to heart disease. Both CP and INEPT-based experiments show that these mutations affect PLN’s conformational equilibrium in opposite directions. The R9C mutation shifts the equilibrium toward the membrane-associated T state, which prevents phosphorylation and causes its LOF character; whereas the R25C mutants shifts the equilibrium toward the unfolded R state, which preferentially binds SERCA\textsuperscript{166} and might interfere with the recognition mechanism of PLN by its multiple binding partners. Functionally, both mutants result in similar phenotypes; however, this new structural evidence suggests that the interactions with lipid bilayers also contribute to dysregulation of SERCA and make this mutant less responsive to β-adrenergic regulation \textit{in vivo}.

5.7 Acknowledgements

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5.8 Supplementary Material and Methods

5.8.1 Solution-state NMR in DPC micelles. Solution NMR samples were prepared with a buffer containing 20 mM Na2HPO4 (J.T. Baker), 120 mM NaCl (Malinkrodt), 0.1% NaN3, and 300 mM dodecylphosphocholine (DPC) (Avertec) and protein concentration of ~1 mM. All heteronuclear single quantum coherence (HSQC) spectra were collected on a Varian INOVA spectrometer operating at a $^1$H Larmor frequency of 600 MHz prior to
collection of other NMR spectroscopic data. Resonance assignments were performed as previously described using a combination of three-dimensional \[^1\text{H} - ^{15}\text{N}\] TOCSY-HSQC and NOESY-HSQC. Heteronuclear steady-state NOE spectra were collected using the established pulse sequence based on Farrow et al. with a spectral width of 6000 Hz in the direct proton dimension and a 3 s presaturation period on the proton frequency. $R_1$ values were determined from $T_1$ measurements collected with a series of relaxation delays of $10^\ast$, 20, 40, 180, 300, 500, 800, and 1000 ms, and $R_2$ values from $T_2$ spectra collected with relaxation delays of $10^\ast$, 30, 50, 70, 90, 110, and 150 ms, where the asterisk indicates that the experiment was repeated to estimate the experimental error. The $T_1$ and $T_2$ values were obtained by fitting the peak intensities to a single exponential decay.

**5.8.2 Structure calculation for AFA-PLN^{R9C}.** The chemical shift index was determined by taking the difference in the measured $^1\text{H}_\alpha$ chemical shifts for PLN and those for a random coil. NOE constraints were obtained from $^{15}\text{N}$ edited NOESY-HSQC experiments collected with a mixing time of 150 ms. NMR spectra were processed using NMRPipe (Delaglio et al., 1995) and analyzed using NMRView software (Merck, Whitehouse Station, NJ, 3). Structure calculations were performed using Xplor-NIH software starting from an extended structure and initial temperature of 6000K, with a generation of 30 total structures. A total of 426 NOEs were classified in “strong” (1.0–2.8 Å), “medium” (1.6–3.4 Å), and “weak” (3.2–5.0 Å). The covalent geometry of the conformers generated was determined using PROCHECK_NMR. AFA-PLN^{R9C} was fit using 426 NOE constraints along with dihedral angles obtained from TALOS values back-calculated from the $\text{H}_\alpha$ chemical shift.
Figure 5.1: (A) $^{15}$N spectral mapping of T- and R-states of AFA-PLN$^{\text{AFA}}$, AFA-PLN$^{\text{R9C}}$, and AFA-PLN$^{\text{R25C}}$ using CP and INEPT solid-state NMR experiments respectively. (B) Relative sensitivity of R-state confirmation is obtained by normalizing the integrated intensity with respect to CP spectra.
Figure 5.2: $^{15}$N-HSQC spectra of AFA-PLN$^{AFA}$, AFA-PLN$^{R25C}$, and AFA-PLN$^{R9C}$ reconstituted in proteoliposomes (A), and isotropic lipid bicelles (B). The spectra in (A) and (B) were obtained from solid-state, and solution-NMR techniques, respectively.
Figure 5.3: $^{13}$C-$^{13}$C TOBSY solid-state NMR spectra for dynamic R-state of AFA-PLN samples reconstituted in DMPC proteoliposomes. (B) Chemical shift index of residues 2 to 17 using $\alpha$ and $\beta$ peak positions.
Figure 5.4: Alanine, Serine and Threonine spectral regions of DARR and TOBSY spectra of AFA-PLN\textsuperscript{AFA}, AFA-PLN\textsuperscript{R25C}, and AFA-PLN\textsuperscript{R9C}. DARR and TOBSY respectively map the immobile T-state and dynamic R-state.
Figure 5.S1: Solid-state NMR $^{13}\text{C}, ^{13}\text{C}$ DARR spectra of PLN$^{\text{AFA}}$, PLN$^{\text{R25C}}$, and PLN$^{\text{R9C}}$. 
Figure 5.S2: The backbone conformation of PLN’s cytoplasmic domain is conserved with R9C mutation. A) [\(^1\)H, \(^{15}\)N] HSQC overlay of monomeric (AFA-PLN) and pentameric PLN (black) with AFA-PLN\(^{R9C}\) (red). B) Combined chemical shift perturbation (\(\Delta \delta\)) showed a significant difference only at the mutation site, residue 9, for both AFA-PLN\(^{R9C}\) (top panel) and PLN\(^{R9C}\) (middle panel). The NOE map (lower panel) for the monomer also shows a typical pattern of \((i, i+4)\) for the helical regions.
Figure 5.S3: Internal dynamics are conserved with R9C mutation. A) Proton-proton connectivities assigned from $^{15}\text{N}$-edited NOESY-HSQC spectra. Strong, medium, and weak connectivities are represented by thick, medium, and thin bars respectively. Note the pattern of $d\alpha_N(i, i+4)$ connectivities which are indicative of two helical segments in the protein. B) Change in $1\text{H}$ chemical shift plotted for each helical segment of PLN. C) Internal protein dynamics measurements for monomeric AFA-PLN$^{R9C}$ (black) in DPC micelles. Measurements for AFA-PLN are plotted for comparison (from Metcalfe et al. 213).
Figure 5.S4: Structure of monomeric AFA-PLN<sup>R9C</sup> in DPC micelles. PLN’s structural domains are indicated: domain Ia (blue), loop (orange), domain Ib (red), and domain II (green). The view down domain Ia is shown with the membrane represented by the black line and shaded region. Left panel: Alignment of the ensemble of the lowest 20 structures for AFA-PLN<sup>R9C</sup> based on NMR structural restraints. Right panel: Averaged structural model for AFA-.
Chapter 6: Application of solution and solid-state NMR to calmodulin’s binding interaction with the ryanodine receptor
6.1 Introduction

Calmodulin (CaM) binding to the ryanodine receptor (RyR) provides essential regulation of Ca$^{2+}$ flow for cardiac contraction. The large size (>2.3 MDa) of the RyR has made atomic-level structural analysis difficult. In its functional homo-tetrameric form (Figure 1.2), the RyR is regulated by binding interactions with multiple small proteins and signaling molecules, as well as post-translational modifications that modulate the RyR's structure-function relationship in a way that is incompletely understood. Previous NMR, x-ray crystallography, FRET, and EPR studies on CaM's binding interaction with the RyR have primarily focused on CaM's binding to a RyR peptide that spans one of three originally proposed binding sites for CaM in the primary sequence of the RyR. These peptide models show CaM adopting a closed conformation with CaM's two globular domains wrapping fully around the peptide (Figure 6.1). However this structure may be altered by the bulk of the full-length RyR. This hypothesis is supported by recent FRET trilateration experiments that challenge the wrapped structure model by demonstrating a more open and dynamic structure for CaM bound to the RyR.

By applying a combination of solution and solid-state NMR techniques it is possible to characterize both CaM's structural and functional response to Ca$^{2+}$ and how CaM's response to Ca$^{2+}$ exerts different regulatory effects when CaM is bound to the RyR. Solution [$^1$H, $^{15}$N] HSQC experiments provide an amide fingerprint of free CaM for comparison to the RyR-bound state. In addition to [$^{13}$C, $^{13}$C] DARR and INEPT-based experiments to probe the dynamics of CaM in complex with the RyR, the recent developments in $^1$H-detected ssNMR pulse sequences provide an amide fingerprint that can be directly compared to the solution state [$^1$H, $^{15}$N] HSQC spectra. ssNMR indicates that residues in CaM sample both static and dynamic regimes when bound to the RyR. In addition, experiments with $^{13}$C/$^{15}$N methionine labeled CaM have demonstrated that CaM's nine methionine residues are largely immobilized by binding to the RyR.
6.2 Materials and Methods

Protein expression and purification: Uniformly $^{13}\text{C}/^{15}\text{N}$ labeled CaM was expressed in BL21(DE3) *E. coli* and purified based on its Ca$^{2+}$ binding using phenylsepharose CL-$4B$ resin (Sigma) as previously described. Preparation of CaM$^{\text{WT}}$ with Ser/Ile and Thr/Ala/Gly reverse unlabeling to aid in the assignment of the ssNMR spectra was optimized from previously established protocols for expression in BL21(DE3) *E. coli* using uniformly $^{13}\text{C}/^{15}\text{N}$ labeled M9 media prepared using $^{15}\text{NH}_4\text{Cl}$ (Sigma), $^{13}\text{C}$-D-glucose (Cambridge Isotopes) and an excess of the unlabeled amino acids. CaM$^{\text{WT}}$ with Ser/Ile reverse unlabeling was expressed in the presence of 1 mg/mL of non-isotopically labeled L-serine and L-isoleucine (Sigma) at 37°C for two hours. Due to the high degree of overlap in *E. coli*’s metabolic pathways for serine and isoleucine, the unlabeled amino acids were added to the M9 media 30 minutes prior to induction with IPTG to limit scrambling of the isotopic labels. CaM$^{\text{WT}}$ with Thr/Ala/Gly reverse unlabeling was expressed in the presence of 1 mg/mL of non-isotopically labeled L-threonine and L-alanine (Sigma) for 8 hours at 30°C. The combinations of unlabeled amino acids used for each sample and their expression conditions are outlined in Table 6.1 and were selected to provide the greatest amount of assignment information with the least amount of spectral overlap. Selectively $^{13}\text{C}/^{15}\text{N}$ Met labeled CaM$^{\text{WT}}$ for Met assignment was expressed in BL21(DE3) *E. coli* based on previously established protocols. Briefly, $^{13}\text{C}/^{15}\text{N}$ Met (Sigma) was added to M9 media prepared with $^{14}\text{NH}_4\text{Cl}$ and $^{12}\text{C}$-D-glucose at a concentration of 0.2 mg/mL along with 0.9 mg/mL of unlabeled Lys, Thr, and Ile, and 0.6 mg/mL of unlabeled Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Leu, Phe, Pro, Ser, Trp, Tyr, and Val.

SR preparation: Heavy sarcoplasmic reticulum (HSR) used in NMR sample preparation was obtained from pig skeletal muscle (PelFreeze) and extracted following previously established methods. Briefly, homogenized muscle tissue was run through a series
of centrifugation, and filtration steps to remove extraneous connective tissue and cellular material, leaving a crude SR mixture containing proteins and lipids from both the light and heavy SR. The heavy SR was further isolated using a 5-20% sucrose gradient. Fractions containing the RyR were identified using a 4-20% SDS-PAGE gel, and were then flash frozen in liquid nitrogen and stored at -80°C.

**Solution NMR sample preparation:** Uniformly $^{13}$C/$^{15}$N, selectively $^{13}$C/$^{15}$N Met, and reverse unlabeled CaM$^{WT}$ for solution NMR was exchanged into sample buffers containing: 20 mM imidazole, 100 mM potassium chloride, 6 mM calcium chloride ($\text{Ca}^{2+}$-bound) or 1.5 mM EGTA (apo), and 1 mM sodium azide at pH 6.5.

**MAS sample preparation:** Heavy SR for NMR sample preparation was thawed on ice, and solubilized in a dropwise manner in a buffer containing 100 mM KCl, 20 mM PIPES (pH 7.0 using KOH), 5 mM glutathione (reduced; GSH), 0.1 mg/mL BSA, 1 mM DTT, and 1x protease inhibitors (Roche cOmplete tablets) to a final concentration of 4 mg/mL HSR. The solubilized SR was then homogenized and the endogenous CaM stripped from the sample by incubation with 600 nm CaMBP, a competitive binding peptide that spans the CaM binding site in myosin light chain kinase (MLCK), for 90 minutes at 37°C. In the absence of the stripping step, the isotopically labeled CaM does not outcompete the endogenous CaM to a level that can be observed by ssNMR (Figure 6.2). Removing the endogenous CaM leaves the CaM binding sites on the RyR open for occupation by the isotopically labeled CaM. Following incubation with CaMBP, the homogenate was spun down at 40,000 rpm for 25 minutes at 4°C and the supernatant removed. The pellets were resuspended and homogenized in 500 μL of isotopically labeled CaM in the $\text{Ca}^{2+}$-bound solution NMR buffer. Following incubation with the isotopically labeled CaM for 60 minutes at 37°C, the sample was centrifuged for 25 minutes at 40,000 rpm and 4°C. The resulting pellet was packed into a 3.2 mm rotor for solid-state NMR acquisition.
MAS ssNMR: \([^{13}\text{C},^{13}\text{C}]\) DARR\(^{223}\) spectra were acquired on a Bruker 900 MHz spectrometer through the National High Magnetic Field Laboratory (NHMFL) in Tallahassee, FL using their 105 mm wide bore probe equipped for 3.2 mm rotors. Mixing periods of 20, 50, 100, and 200 ms were used during acquisition. \([^{1}\text{H},^{13}\text{C}]\) INEPT\(^{224}\), and \([^{13}\text{C},^{13}\text{C}]\) TOBSY\(^{225}\) were acquired on an Agilent 600 MHz spectrometer, while \(^{1}\text{H}\)-detected \([^{1}\text{H},^{15}\text{N}]\) INEPT and HSQC experiments\(^{181,218,224,226}\) were acquired using an Agilent 700 MHz spectrometer through the Minnesota NMR Facility at the University of Minnesota. Both the 600 and 700 MHz Agilent spectrometers were equipped with 3.2 mm MAS probes with reduced RF heating technology. All NMR experiments were processed and imaged using NMRPipe\(^{97}\) and SPARKY\(^{93}\).

6.3 Results and Discussion

6.3.1 Initial solid-state NMR acquisition on CaM in heavy SR indicates both static and dynamic behavior.

CaM samples bound to the RyR in heavy SR extract exhibit both static and dynamic behavior. \([^{13}\text{C},^{13}\text{C}]\) DARR spectra exhibit strong correlations at a variety of mixing times, indicating that a number of CaM’s residues exhibit static behavior for both intra- and inter-residue correlations (Figure 6.3) In particular, the carbonyl region exhibits a high level of variation between different mixing times, indicating that the protein backbone may exhibit a number of inter-residue correlations on slightly different timescales. In addition to the static interactions probed by DARR, CaM also exhibits highly dynamic residues as indicated in the \([^{1}\text{H},^{13}\text{C}]\) INEPT and \([^{13}\text{C},^{13}\text{C}]\) TOBSY shown in Figure 6.4. Interestingly, both the TOBSY and the 20 ms DARR exhibit clear cross peaks corresponding to serine and threonine residues (~57 ppm), however these peaks are absent or broadened out at higher DARR mixing times. Since many of CaM’s 16 serine and threonine residues are in close proximity to the Ca\(^{2+}\) binding sites, these residues may
represent interesting targets for modeling CaM’s structural interaction with the RyR in response to Ca\(^{2+}\).

**6.3.2 Application of selective labeling in solid-state CaM-RyR samples.**

In order to elucidate which residues in CaM are exhibiting static or dynamic behavior the solid state spectra needs to be assigned. To begin residue assignment, reverse unlabeling of specific residues was identified as the most accessible strategy due to concerns over the loss of sensitivity resulting from selective labeling of specific amino acids. To determine which residues were the most accessible for reverse unlabeling, the possible residue types for the cross peaks of the DARR and TOBSY spectra were determined based on the typical \(^{13}\)C chemical shift ranges for amino acids (**Figure 1.9**). While carbon chemical shifts alone were insufficient to assign the peaks as a single residue type, it did indicate that the greatest clarification was needed between serine, threonine, alanine, leucine, isoleucine, and valine resonances. To provide more efficient identification multiple amino acids were unlabeled per sample in order to provide assignment for the maximum number of peaks without the concern of significant overlap in chemical shift for the residues selected. Therefore, the reverse unlabeled samples covered serine and isoleucine (12 residues total), as well as threonine, alanine and glycine (33 residues total; **Figure 6.4**). In addition to being key residues to resolve due to overlaps in chemical shift, many of these residues occur at key points in CaM’s EF hand structure and have the potential to provide direct insight into behavior of CaM’s globular domains and the structure-dynamic patterns surrounding the Ca\(^{2+}\)-binding sites.\(^{31}\)

While yielding the high expression levels characteristic of CaM\(^{WT}\) growths, initial expression in the presence of unlabeled Ser and Thr exhibited a high level of scrambling, or the exchange of isotopic labels between amino acids via metabolic intermediates (**Figure 6.6**). This is most likely the result of the unlabeled amino acids in the media...
being completely consumed during protein expression, resulting in a mix of uniformly
\(^{13}\text{C}/^{15}\text{N}\) labeled and reverse unlabeled protein being produced. All of the scrambling ob-
erved in the preparation of the selectively unlabeled samples is consistent with the
metabolic overlap of amino acids in \textit{E. coli} (Figure 6.6). Both serine and threonine readi-
ly scramble to a number of different amino acids, accounting for the extensive scram-
bling observed in the initial sample preparations.

Multiple strategies were applied to limit the rate of scrambling during protein ex-
pression. The concentration of unlabeled amino acids in the M9 media was increased
from 0.2 mg/mL to 1 mg/mL, and the expression temperature and time was also adjust-
ed to slow consumption of the unlabeled amino acids. Protein expression in the pres-
ence of 1 mg/mL unlabeled threonine and alanine at 30\(^\circ\)C for 8 hours generated protein
with complete reverse unlabeling at Thr, Ala, and Gly (Figure 6.7). Successful expres-
sion of reverse unlabeled Ser and Ile was achieved using 1 mg/mL of unlabeled serine
and isoleucine added to the isotopically \(^{13}\text{C}/^{15}\text{N}\) labeled M9 media 30 minutes prior to
induction and with protein expression limited to 2 hours. While expression of reverse un-
labeled protein was successful in both cases, the modifications to the standard \textit{CaM}^{WT}
expression protocol resulted in a lower yield of protein that may make it difficult to apply
these growth protocols to other proteins, such as \textit{CaM}’s pathogenic mutants, that exhibit
lower expression levels compared to \textit{CaM}^{WT}.

NMR acquisition on the reverse unlabeled samples exhibited four-fold lower sen-
sitivity than the uniformly \(^{13}\text{C}/^{15}\text{N}\) labeled samples due to the reduced levels of \(^{13}\text{C}\) and
\(^{15}\text{N}\) present in the sample. To determine the resonance frequencies for Ser, Thr, Ala, Ile,
and Gly residues in the solid-state spectra, \([^{13}\text{C}, \,^{13}\text{C}]\) DARR, \([^{13}\text{C}, \,^{13}\text{C}]\) TOBSY, and \([^{1}\text{H},
^{13}\text{C}]\) INEPT were acquired on both the Ser/Ile and Thr/Ala/Gly unlabeled samples. The
increased sensitivity of the \(^{1}\text{H}\)-detected ssNMR pulse sequences also permitted acquisi-
tion of \(^{15}\text{N}\)-edited experiments as described in section 6.3.3.
Comparison of uniformly and reverse unlabeled $[^1\text{H}, ^{13}\text{C}]$ INEPT, $[^{13}\text{C}, ^{13}\text{C}]$ TOBSY, and $[^{13}\text{C}, ^{13}\text{C}]$ DARR spectra clearly identify resonances that correspond to the unlabeled amino acids (Figure 6.8). The $[^1\text{H}, ^{13}\text{C}]$ INEPT for the Thr/Ala/Gly unlabeled sample shows loss of signal for 4-6 peaks in the Ala Cβ range between 6-10 ppm, 3-4 peaks in the Ala Cβ and Thr Cγ range around 20 ppm, and more than four peaks in the Thr Cα, Ala Cα, and Gly Cα regions from 40-50 ppm. The high degree of overlap in the $[^1\text{H}, ^{13}\text{C}]$ INEPT from 40-50 ppm makes it difficult to distinguish the Cα groups for Thr, Ala, Gly, and Ser and Ile. This is not entirely surprising due to the overlap in chemical shift range for the Cα and Cβ resonances of these amino acids, but makes it difficult to resolve which residues these peaks represent and will require further experiments to fully resolve those resonances. The assignment of residues in the $[^{13}\text{C}, ^{13}\text{C}]$ DARR spectra exhibited similar concerns for the carbonyl region due to the high degree of overlap in the carbonyl chemical shift range for different amino acids (Figure 1.9).

ssNMR acquisition on the $^{13}\text{C}/^{15}\text{N}$ Met labeled CaM in complex with the RyR confirmed previous mutagenesis studies that indicated CaM’s methionine residues facilitate binding to the RyR. While 1D $^{15}\text{N}$ cross-polarization spectra that acquires signal from all of the methionines in CaM showed distinct signal, the INEPT-based 1D spectra probing dynamic methionine residues showed limited signal (Figure 6.9). $[^{13}\text{C}, ^{13}\text{C}]$ DARR spectra probing the static backbone and sidechains resonances in methionine at two different mixing times also showed distinct peaks confirming that the methionine residues are largely immobilized by CaM’s binding to the RyR. This is consistent with solution NMR results acquired in the present of RyRp, which indicate that all of CaM’s methionine residues except M146 undergo a change in their chemical environment upon binding the peptide. However, the chemical shift values observed in $^{13}\text{C}$-edited solution NMR experiments do not appear to correlate with the $^{13}\text{C}$ chemical shifts observed in the ssNMR experiments indicating that while the methionine residues are involved in binding
both the peptide (RyRp) and the full-length RyR they may not sample the same conformational states for both binding interactions.

6.3.3 Applying $^1$H detected pulse sequences to enhance sensitivity of $^{15}$N acquisition.

Canonical solid-state NMR pulse sequences have acquisition set on the insensitive nuclei as in the HETCOR pulse sequence for a [$^1$H, $^{15}$N] INEPT. However the development of new INEPT-based pulse sequences that incorporate 1H-detection have significantly enhanced the sensitivity of acquisition. Of the three main $^1$H-detected pulse sequences available (see Chapter 3), the RI-HSQC provided the greatest sensitivity enhancement. Interestingly, the 1D spectra acquired using the pulse sequence for the presaturation HSQC, showed complete ablation of the protein signal indicating that the dynamic regions of CaM imaged in the INEPT-based spectra are highly accessible to water.

An additional advantage to the $^1$H detected $^{15}$N experiments is their ability to be compared directly to the [$^1$H, $^{15}$N] HSQC spectra obtained on solution state samples of free CaM (not bound to the RyR). Figure 6.10 shows the overlay of Ca$^{2+}$-bound CaM from solution state NMR with the ssNMR spectra of Ca$^{2+}$-bound CaM in complex with the RyR. There are distinct differences in the amide fingerprints of the two spectra indicating that CaM undergoes a significant conformational change when binding the RyR. As assignment of the solid-state spectra is completed it will also be possible to calculate the changes in chemical shift between individual residues, providing the foundation for calculating a structural model of CaM in complex with the full-length RyR.
<table>
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<th>Amino Acids</th>
<th>Amount of Amino Acid Added</th>
<th>When Amino Acids were Added</th>
<th>Expression Temperature (°C)</th>
<th>Expression Time (hours)</th>
<th>Scrambling Present</th>
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<tr>
<td>Ser/Ile</td>
<td>100 mg/500 mL With M9</td>
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<td>4-5</td>
<td>Yes</td>
<td></td>
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<tr>
<td></td>
<td>500 mg/500 mL With M9</td>
<td>37</td>
<td>4-5</td>
<td>Yes</td>
<td></td>
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<tr>
<td></td>
<td>500 mg/500 mL 30 min before induction</td>
<td>37</td>
<td>2</td>
<td>No</td>
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<tr>
<td>Thr/Ala</td>
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<tr>
<td></td>
<td>500 mg/500 mL With M9</td>
<td>30</td>
<td>8</td>
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</tbody>
</table>
Figure 6.1: Peptide bound structure of calmodulin. PDB 2BCX. Structure of CaM in complex with the RyR peptide (Ry Rp) spanning residues 3614-3643 of the RyR. Pathogenic mutations in CaM are indicated in green.
Figure 6.2: ssNMR spectra show the absence of isotopically labeled CaM\textsuperscript{WT}. A) 1D $[^1\text{H}, ^{13}\text{C}]$ INEPT showing lipid signals from the HSR. B) 2D $[^{13}\text{C}, ^{13}\text{C}]$ DARR spectra with a mixing time of 100 ms showing the absence of cross peaks from $^{13}\text{C}$-$^{13}\text{C}$ correlations in the protein backbone and sidechains. C) 1D $[^1\text{H}, ^{15}\text{N}]$ INEPT showing the lack of $^{15}\text{N}$ signal from the protein backbone and sidechains.
Figure 6.3: Different DARR mixing times indicate variable dynamics in CaM binding to the RyR. \([^{13}\text{C},^{13}\text{C}]\) DARR spectra with mixing times of 20 ms (red), 50 ms (gold), 100 ms (green), and 200 ms (blue) acquired on a 900 MHz spectrometer at 25°C with a spinning speed of 10 kHz. Each mixing time shows distinct differences in the cross peak intensities and position, particularly in the carbonyl range.
Figure 6.4: $[^{13}\text{C},^{13}\text{C}]$ INEPT and TOBSY indicate dynamic regions in CaM. $[^{1}\text{H},^{13}\text{C}]$ INEPT (top) and $[^{13}\text{C},^{13}\text{C}]$ TOBSY (bottom) spectra acquired on a 600 MHz spectrometer at 25°C with a spinning speed of 10 kHz.
Figure 6.5: Distribution of reverse unlabeled residues in Ca\textsuperscript{2+}-bound CaM\textsuperscript{WT}. (PDB 1CLL) Top panel identifies the location of the four serine and eight isoleucines targeted for unlabeling. The bottom panel indicates the location of the twelve threonine, ten alanine, and eleven glycine residues.
Figure 6.6: *E. coli* metabolic pathways provide insight into scrambling. Simplified scheme of the overlap between amino acids in *E. coli* metabolism. The unlabeled amino acids added to the growth media for the CaM NMR sample preparations are indicated in red.
Figure 6.7: [$^{1}$H, $^{15}$N] HSQC confirms reverse unlabeling in CaM$^{\text{WT}}$. Overlay of uniformly $^{13}$C/$^{15}$N labeled CaM$^{\text{WT}}$ (black) with CaM$^{\text{WT}}$ with reverse unlabeling at the Thr, Ala, and Gly residues (blue). Panels A-F show zoomed regions of the spectra highlighting the positions of Thr, Ala, and Gly residues.
Figure 6.8: Selective unlabeling identifies amino acid type in $[^1H, ^{13}C]$ INEPT. Overlay of uniformly $^{13}C/^{15}N$ labeled WT CaM (black) with Thr/Ala/Gly unlabeled WT CaM (blue, top panel) and Ser/Ile unlabeled WT CaM (blue, bottom panel). $C\alpha$ and $C\beta$ chemical shift ranges for Ser, Thr, Ile, Ala, and $C\alpha$ peaks in Gly from 40-50 ppm are outlined in gray. $C\beta$ peaks for Ala (5-12 ppm) are outlined in red, $C\beta$ and $C\gamma$ peaks from Thr and $C\beta$ peaks from Ala are outlined in green, and Ile's $C\delta$ and $C\gamma$ peaks (5-28 ppm) are outlined in purple. Spectra were acquired on a Varian 600 MHz spectrometer at 25°C.
Figure 6.9: Met residues appear to be immobilized by CaM<sup>WT</sup>'s binding to the RyR.

A) Solution [<sup>1</sup>H, <sup>15</sup>N] HSQC overlay of <sup>13</sup>C/<sup>15</sup>N Met labeled CaM<sup>WT</sup> in the presence (red) and absence (black) of RyRp. Solution NMR spectra were acquired on a Bruker 900 MHz spectrometer at 25° C. B) Solid-state NMR spectra of <sup>13</sup>C/<sup>15</sup>N Met labeled CaM<sup>WT</sup> bound to the full-length RyR. Top: 1D <sup>15</sup>N cross-polarization (CP) and INEPT spectra. Bottom: 2D [<sup>13</sup>C, <sup>13</sup>C] DARR spectra with mixing times of 20 ms (left) and 50 ms (right). 1D solid-state spectra were acquired on a Varian 600 MHz spectrometer while 2D spectra were acquired on a Bruker 900 MHz spectrometer. All spectra were acquired at 25° C.
Figure 6.10: $^1$H detected [$^1$H, $^{15}$N] INEPT spectra. Overlay of solution state [$^1$H, $^{15}$N] HSQC spectra of Ca$^{2+}$-bound CaM in the absence of the RyR (black) with ssNMR spectra of CaM bound to the RyR (blue).
Conclusion

The structural and dynamic characterization of proteins provides valuable insight into their function and how their ability to bind and regulate other proteins is disrupted by pathogenic modifications. The characterization of CaM and PLN using solution and solid-state NMR techniques has provided key insights into how oxidation and mutation perturb the structure and dynamics of these proteins leading to dysregulation of their cellular targets. These key structural studies, and in particular the investigation of large protein complexes, are made possible by the continued development of new pulse sequences in NMR (such as those described in Chapters 3 and 4) that enhance sensitivity and resolution. The continued application of these NMR techniques has the potential to provide atomic-level insights into the mechanism behind calcium transport in cardiomyocytes, and how disruptions in calcium transport lead to disease states.

The characterization of oxidative insults in CaM’s methionine residues (Chapter 2) and the resulting structural perturbations provide key insights into CaM’s dysregulation and altered binding to the RyR in disease states. Many cardiomyopathies are associated with elevated levels of reactive oxygen species (ROS) that lead to oxidation of proteins like CaM. As our solution and solid-state NMR studies (Chapter 6) indicate that CaM’s methionine residues are largely immobilized by CaM’s binding to the RyR, these residues may be key sites for CaM’s regulatory interactions with the RyR. While previous studies have indicated that the hydrophobic environments around CaM’s methionine residues are involved in binding to CaM’s cellular targets and that mutation or oxidation of specific methionines can alter CaM’s binding to the RyR, the application of NMR studies in the presence of the full-length RyR have the potential to describe why these changes occur on an atomic level. The indication that CaM samples multiple dynamic regimes upon binding to the RyR also provides a foundation for understanding how CaM exerts
different regulatory effects on the RyR in response to calcium, and how mutations and pathogenic modifications can perturb these dynamic regimes in disease states.

The structural and dynamic characterization of mutations in PLN associated with dilated cardiomyopathy has also provided further insights into PLN’s regulation of SERCA. Previous studies established that the dynamics of PLN’s cytoplasmic domain is highly sensitive to mutation and that tuning these dynamics has therapeutic potential for restoring cardiac function by tuning SERCA’s calcium transport activity\textsuperscript{55,57,148}. Further studies have also identified DNA and RNA aptamers that have the potential to tune the regulatory interaction between PLN and SERCA. By identifying how the R9C and R25C mutations alter PLN’s conformational equilibrium, and as a result perturb PLN’s regulation of SERCA and response to \( \beta \)-adrenergic regulation, it is possible to build a molecular model describing how these mutations lead to dilated cardiomyopathy and what therapeutic intervention can provide the most targeted response to restore cardiac function.

Coupling structural and dynamic studies such as those outlined in this thesis with protein function provides atomic-level insights into the development of disease states. By employing a combination of solution and solid-state NMR techniques it is possible to characterize not only individual proteins, like CaM and PLN, but to characterize how these proteins interact with their cellular targets, building a full model of how these proteins fit into calcium signaling pathways. The critical role of calcium transport in cardiac contraction and relaxation, and the devastating impact of single mutations or pathogenic modifications on cardiac function make understanding the molecular etiologies for these disease states crucial for the development of new, more targeted therapies.
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