4 Studies on the transmembrane domain of phospholamban using rotational resonance and Magic angle oriented sample spinning (MAOSS) NMR spectroscopy.

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The structure of the membrane spanning domain of the cardiac contractility regulating peptide phospholamban (PLBs20)1 is being investigated in the membrane using solid state NMR spectroscopy. Local secondary structure has been probed using rotational resonance (RR)2 NMR by measuring the distance between NMR sensitive isotopes e.g. 13C, placed i and i+4 residues apart. Additionally, a new method MAOSS3 NMR, is being applied to PLB52 in oriented membranes. This method markedly enhances the resolution of the proton spectrum, allowing individual resonances to be used as a marker for the protein/lipid mole ratio, as well as providing orientational information on the peptide in the membrane from analysis of the 13C spinning sideband pattern.

Phospholamban is a small (Mr=6.123k) membrane spanning peptide localised in the cardiac sarcoplasmic reticulum (SR). This pentameric peptide has two major biological functions; (i) it serves as a selective Ca2+ ion channel in the membrane bilayer and (ii) it serves as an important regulator, in an inhibitory association, of Ca2+-ATPase. The N-terminal charged hydrophilic domain (1-31) extends into the cytoplasmic region and contains two sites for phosphorylation (Ser16 and Thr17) which may be important for its inhibitory association with the Ca2+-ATPase. The hydrophobic carboxyl domain (32-52) traverses the lipid bilayer, reputedly as an α-helix, and may be involved in oligomerisation4. Ca2+-ATPase is a large (Mr=110k) membrane protein also found in the SR and as the major protein component of skeletal and cardiac cells5. Its function is to establish and preserve the Ca2+ ion gradient across biological membranes. The contraction - relaxation cycle of the heart is controlled by cytosolic levels of Ca2+ ions in cardiac muscle and in order for muscle cells to relax after contraction, a rapid pumping of Ca2+ ions from the cytosol to the lumen of the SR is required. β-adrenergic stimulated phosphorylation of phospholamban, by protein kinases, causes it to dissociate from the Ca2+-ATPase, alleviating its inhibitory interaction and restoring the Ca2+-ATPase pumping action. The detailed molecular interactions between Ca2+-ATPase and phospholamban in the SR are not well defined.

Solid state magic angle spinning (MAS) NMR is emerging as an effective high-resolution method for studying integral membrane proteins directly in the lipid bilayer without the use of solvents or detergent micelles6. NMR sensitive isotopes enhance sensitivity and, by virtue of their position, permit the conformation, orientation and dynamics of functionally active membrane peptides to be studied.

Here we describe MAS-NMR experiments involving the transmembrane fragment of phospholamban52 synthesised by solid phase peptide synthesis, incorporating cysteine-alanine substitutions and 13C isotopes at Cα-Phe20 and Cα-Ala31. Phospholamban52 was reconstituted into 1,2-dimyristoylglycerol-3-phosphocholine (DMPC) bilayers with an initial lipid:peptide mole ratio of 20:1. Reconstitution was achieved by co-dissolving the lipid and peptide in a 50:50 CHCl3/CH3OH mix, drying to a thin film, resuspending in phosphate buffer (10mM H2PO4, 50mM NaCl, pH7) and centrifuging to a pellet (123,000g, 4°C, 1 hr).

In the first set of experiments the 13C cross-polarisation (CP) MAS-NMR spectrum of reconstituted phospholamban52 at different temperatures was recorded. Low temperatures are required to reduce sufficiently the dynamics of the peptide in the membrane and to allow synchronisation of the 1H and 13C spins for CP. Spinning at 223K was found to be the best temperature at which the 13C carbonyl and Cu resonances were resolved.

The distance between the isotopic spin pair, from computer simulations of the transmembrane domain as a α-helix, was calculated to be 5.14Å. This distance will enable the dipolar coupling and subsequently the internuclear distance to be deduced from RR experiments (Figure 1).

![Figure 1. RR experiment where the Cu resonance was selectively inverted and the spinning speed set to n=2 resonance condition (ω0 = 5440Hz). The mixing time was varied from 1ms-50ms. Off-resonance condition was recorded ω0 = 4900Hz with a mixing time of 50ms. All spectra recorded at 223K.](image)

The intensity of both resonances is monitored as a function of mixing time during which magnetisation is allowed to exchange between the spin pair. Preliminary results show a slight but measurable decay after 50ms which reflects a certain degree of coupling. Interpretation of this change, by generation of a magnetisation exchange curve, will enable the spin pair distance to be calculated.

The second set of experiments involved orientating the constituted peptide onto glass plates and then applying MAS methods. This technique MAOSS, dramatically increases the spectral resolution at much lower spinning speeds (Figure 2).

![Figure 2. The 1H MAOSS NMR spectra of phospholamban52 in aligned DMPC bilayers.](image)

The high-resolution spectrum at 3000Hz allows the individual lipid resonances to be assigned and be used to calculate the lipid:peptide mole ratio from the aromatic resonances of the peptide. This was found to be 22:1. For the less abundant 13C nuclei spinning at low speed produced spinning sidebands from which valuable orientational information can be obtained.

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