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# Uniformly Aligned Full-Length Membrane Proteins in Liquid Crystalline Bilayers for Structural Characterization

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Progress has been made in membrane protein structure determination in lipid bilayer environments.[1] From the complete structure of gramicidin in 1997[1g] to the structure of the M2 transmembrane domain with[2] and without[1e] the antiviral drug amantadine, the structure of MerFt,[1a] etc., there are now ten structures in the Protein Data Bank characterized by aligned-sample solid-state NMR. Recent improvements in RF probe technology[3] and in sample preparation have make it possible here to obtain spectra of uniformly aligned full-length membrane proteins displaying characteristic resonance patterns for their transmembrane (TM) α-helices.[4]

Sample preparation is the key to macromolecular structural characterization, whether it is crystallization for X-ray diffraction or cryo-EM, or homogeneous isotropic samples for solution NMR, or a uniformly aligned sample for solid-state NMR. Here, from monomeric to octameric, from 3.5 to 82 kDa, and from one to three TM helices per monomer we demonstrate uniform protein alignment in hydrated phospholipid bilayers on glass slides.

The helical structure prediction for three proteins is shown in Figure 1. KdpF is a 30 residue protein (33 residues as expressed in *E. coli*) from the *Mycobacterium tuberculosis* genome. This protein appears to be a component of the Kdp $K^+$ transporting complex.[8] It has a single putative TM helix and no predicted 2° structure for the terminal segments. Rv1861 is another putative membrane protein from the *Mtb* genome. It has a predicted ATP or GTP binding site in the N-terminal segment and three TM helices. Rv1861 is adjacent to the genes that code for molybdenum transport and may be involved in this transport function. Nondenaturing PAGE of this protein shows a narrow band at approximately 82 kDa suggesting an octameric state (see Supporting Information). Diacylglycerol kinase (DAGK) from *E. coli* is an extensively studied protein with an experimentally characterized secondary structure as illustrated in Figure 1.[12] In addition to the three TM helices there are two short amphipathic helices thought to be associated with the bilayer interface. Overall, this protein forms a native state trimer and therefore, a nine-helix bundle.

Figure 2 shows the PISEMA spectra of KdpF, Rv1861, and DAGK in liquid crystalline lipid bilayer environments. Uniform helical structures generate patterns of resonances, known as PISA (polar index slant angles) wheels with 3.6 resonances per turn. Because it has been difficult to predict such wheels from crystallographic data in the Protein Data Bank owing to the relatively low resolution of the membrane protein structures and the high sensitivity of the wheels to local structural variations and potential tensor variation (see Supporting Information), it has been questioned in the literature[13] whether PISA wheels would be observed in membrane proteins. Here PISA wheels are clearly observed for two

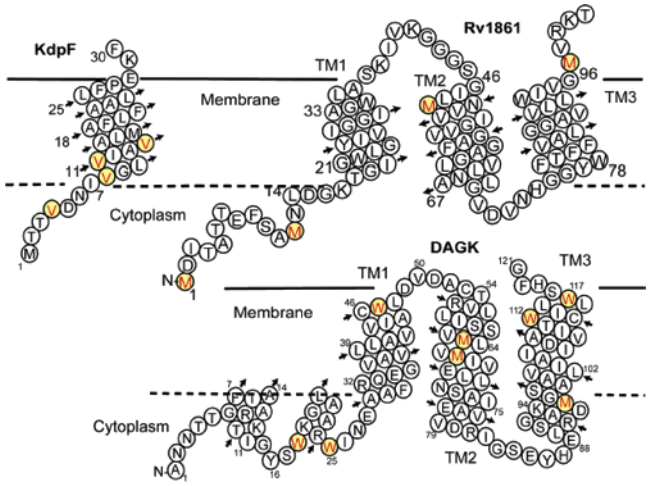


***Figure 1.*** Secondary structure prediction for (A) KdpF and (B) Rv1861 both from *Mtb* and (C) DAGK from *E. coli*.[5] This latter protein is a functional mutant engineered to be a particularly stable trimer.[6] Predictions for KdpF and Rv1861 were performed using TMHMM.[7] The Val, Met, and Trp residues specifically labeled for PISEMA spectra are highlighted.

(KdpF and Rv1861) of the three proteins. We have previously argued that because of the scarcity of strong interhelical interactions and the low dielectric of the membrane interstices that membrane proteins would have very regular helical TM structure.[10] Indeed, the uniformity of helical structures and the unusually short intrahelical hydrogen bonds have been documented,[10,14] meaning that, in general, the helical structure is more uniform[15,16] than observed in water-soluble proteins where approximately 30% of all helical backbone carbonyls accept more than one hydrogen bond. The third protein, DAGK has TM helices displaying small tilt angles such that the PISA wheel, which disappears at a 0° tilt angle, is unresolved in the uniformly $^{15}N$ labeled sample.

For KdpF the three valine residues in the TM helix are in a sequential pattern of $i$ to $i + 3$ to $i + 6$. On the basis of a 100° inter-residue spacing about the helical axis, the connectivity lines between the residues can be drawn on the wheel as in Figure 2B resulting in the assignment of these resonances to residues $Val_8$, $Val_{11}$, and $Val_{14}$. Therefore, not only can the tilt of this helix be characterized as $34 \pm 3°$, but the rotational orientation of the helix is also fixed by this residue specific assignment of the resonances. A fourth Val residue in the N-terminus may be highly dynamic generating a poorly cross-polarized signal and an $^{15}N-^{1}H$ dipolar coupling near 0 kHz.

Rv1861 has three putative TM helices and the PISEMA resonance intensity is distributed over most of the potential spectral area, and yet a characteristic wheel pattern is observed indicating a tilt of $37 \pm 3°$. Since the intensity pattern around the wheel is so strong we anticipate that two of the helices have this same tilt angle.

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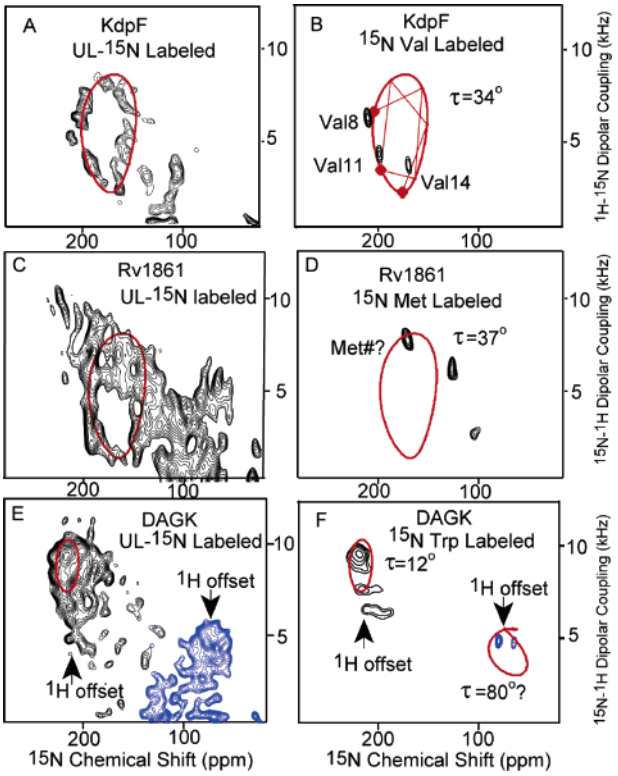


***Figure 2.*** PISEMA spectra of KdpF (A and B), Rv1861 (C and D) and DAGK (E and F) of uniformly $^{15}$N-labeled (A, C, and E) and amino acid specific labeled protein (B, D, and F) expressed in *E. coli* and reconstituted into a mixture of lipids, dimyristoylphosphatidylcholine (DMPC) and phosphatidyl-glycerol (DMPG) in a 4:1 molar ratio. The samples were aligned between glass slides (see Supporting Information). Spectra were obtained with NHMFL low-E probes at 600 MHz except for the $^{15}$N UL KdpF spectrum which was obtained in the UWB 900 MHz magnet. The low-electric field feature was essential for this spectroscopy. Cross polarization contact time was 0.8 ms; an acquisition time of 4 ms with SPINAL$^{9}$ decoupling was applied, and a recycle delay of 6 s was used. To avoid the limitations of the $^{1}$H bandwidth, the spectra of DAGK were obtained in two halves with different offset frequencies. Spectra acquisition time varies from 6 h to 3 days. The PISA wheels were calculated using motionally averaged dipolar ($\nu_{||} = 10.375$ kHz) and chemical shift tensors ($\sigma_{11} = 57$; $\sigma_{22} = 81$; $\sigma_{33} = 228$ ppm).[10,11]

While only three of the four Met resonances are observed, $Met_1$ is likely to be highly dynamic. Of the remaining three Met residues $Met_{49}$ is likely to be part of TM2 and $Met_{97}$ to be part of TM3.

The helical tilt angles for the three TM helices of DAGK are smaller than those for KdpF and Rv1861. Unlike Rv1861 the intensity distribution is at the extremes of the dipolar and chemical shift ranges. All five of the Trp resonances are observed as shown in Figure 2F. As predicted from the secondary structure characterization[12] three of the resonances are in the TM region of the spectrum and two in the amphipathic surface bound region (70−80 ppm). TM3 has two Trp residues and a Met residue and TM2 has two additional Met residues. Shown in Figure 3 are these resonances and theoretical PISA wheels reflecting a 9° and 15° helical tilt angle. The three observed resonances in TM3, $Met_{96}$, $Trp_{112}$, and $Trp_{117}$ would be separated on the PISA wheel by 140° and 160°, respectively. For a regular helical structure these resonances would not be in the same half of the wheel and therefore the helical tilt is likely to be less than 15° and greater than 9° because of the separation of the Trp resonances or 12 ± 3°.

Proteins ranging in molecular weight from a 3.5 kDa monomer to an 82 kDa octamer have been uniformly aligned between glass slides demonstrating the feasibility of preparing full-length membrane protein samples for solid-state NMR structural characterization. Initial PISEMA spectra show that helical structures in membrane proteins can have a very regular structure resulting in resonance patterns known as PISA wheels.

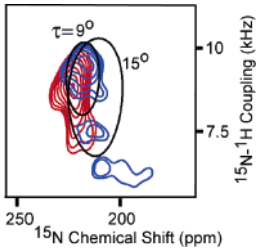


***Figure 3.*** TM region of the PISEMA spectra of $^{15}$N-Trp (blue) and $^{15}$N-Met (red) labeled DAGK. Theoretical 9° and 15° PISA wheels are superimposed on spectra.

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**Supporting Information Available:** Sample preparation information and additional spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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