How to Prepare Membrane Proteins for Solid-State NMR: A Case Study on the α-Helical Integral Membrane Protein Diacylglycerol Kinase from *E. coli*

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Several studies have demonstrated that it is viable to use microcrystalline preparations of water-soluble proteins as samples in solid-state NMR experiments.[1–5] Here, we investigate whether this approach holds any potential for studying water-insoluble systems, namely membrane proteins. For this case study, we have prepared proteoliposomes and small crystals of the α-helical membrane-protein diacylglycerol kinase (DGK). Preparations were characterised by ^{13}C- and ^{15}N-cross-polarization magic-angle spinning (CPMAS) NMR. It was found that crystalline samples produce better-resolved spectra than proteoliposomes. This makes them more suitable for structural NMR experiments. However, reconstitution is the method of choice for biophysical studies by solid-state NMR. In addition, we discuss the identification of lipids bound to membrane-protein crystals by ^{31}P-MAS NMR.

Introduction

The determination of membrane-protein structures is of undisputed importance. Despite the fact that genes coding for membrane proteins are estimated to represent ~30% of genomes,[6] these proteins make up a tiny proportion of the elucidated structures (~0.5%).[7,8] X-ray crystallography is by far the most successful technique for determining 3D structures of membrane proteins. However, the production of diffracting crystals of membrane proteins often involves decades of accumulated effort. Of the 148 available high-resolution structures of multitopic membrane proteins, only three have been elucidated by NMR and five by electron diffraction; the rest have been derived from X-ray-crystallographic studies.[8]

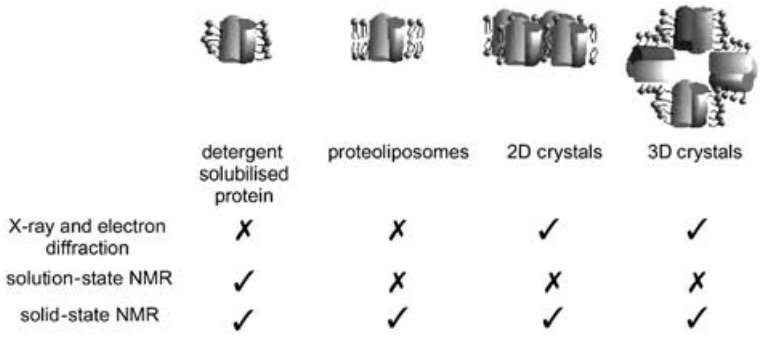

Figure 1. Methods of membrane protein sample preparation and the high resolution structural techniques that can be used to study them. Detergent-solubilised samples must be frozen if they are to be studied with SSNMR.

Solid-state NMR (SSNMR) has already shown that it is capable of yielding structural information on insoluble peptides and proteins. The possibility of a complete de novo structure determination purely based on MAS-NMR recoupling techniques was first demonstrated for small insoluble peptides.[9,10] The techniques have been extended to soluble proteins that have been studied in the solid-state.[11] A great advantage that SSNMR has over other means of determining structures for membrane proteins is that it can be used to study proteins in a plethora of states. Diffraction methods are limited to studying 2D or 3D crystals and solubilised proteins must be used in solution NMR (with detergent or organic solvents) in poor membrane-mimetic environments. SSNMR can utilise all of the afore-mentioned states (frozen in the case of detergent and organic-solvent-solubilised proteins) along with proteins that are reconstituted in lipid bilayers or even aggregated or as fibrils (Figure 1). The ability to study proteins embedded within a native-like lipid environment offers further advantages since it is possible to apply an activity assay directly to the sample being measured. This can prove that the protein of interest is in a native conformation. Furthermore, lipid reconstituted sam-

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ples offer the option of investigating the dynamics of the protein as it binds ligands,[12] negotiates its reaction cycle[13] or responds to changes in the lipid environment.[14]

SSNMR imposes some strict constraints on sample preparation. Firstly, protein must be pure and conformationally homogeneous. However, the greatest hurdle to overcome is that of quantity and concentration. To acquire a good quality 1D ^{15}N spectrum during an overnight measurement requires more than 0.2 μmol of protein. 2D experiments demand even greater quantities in excess of 1 μmol. Given then that the active volume of a standard SSNMR 4 mm MAS-rotor is approximately 50–70 μL, the protein concentration must be in the order of 3–20 mM. This means that 2–10 mg of a small 12 kDa membrane protein has to fit into a MAS rotor. The availability of higher magnetic fields, cryoprobe technology and better polarisation enhancement methods, such as dynamic nuclear polarisation,[15] might significantly increase sensitivity in the future. However, even if the sensitivity is improved by a factor of 10, concentration remains an issue, since the proteins have to be maintained in a natively folded state.

In the case of proteoliposomes, the difficulty of getting a large quantity of protein into the rotor is compounded by the presence of lipids. In our laboratory, protein is routinely reconstituted at molar protein/lipid ratios of 1:100; this is the highest concentration that can be used before one seriously risks aggregation. Furthermore, approximately $^4/_5$ of the volume of the rotor can be taken up by lipids.

There are fortunate exceptions where rather high protein concentrations exist in native membranes, for example, in bacteriorhodopsin (ten lipids per protein in purple membrane patches),[16] the nicotinic acetylcholine receptor (150 lipids per protein in natural membrane),[17] bovine rhodopsin (67 lipids per protein in rod membranes)[18] or the light-harvesting complex. These instances are therefore suitable for advanced NMR studies.[19,20] In general however, the large amount of protein required for SSNMR experiments necessitates an efficient expression and purification system. Furthermore, it is vital that a range of isotope labels can be incorporated into the protein whilst minimizing the risk of label scrambling and dilution. For NMR, *E. coli* and cell free expression systems[21] are highly suitable. Other bacterial systems, such as *Lactococcus lactis*,[22] have also shown promise. It is worth noting that initial screens of sample-preparation conditions will also require a significant amount of protein. Finding ideal reconstitution conditions will require the screening of several methods of removing excess detergent (e.g., dialysis, biobeads, rapid dilution). Numerous other factors, including the initial solubilising detergent, final lipid composition and physical parameters, such as pH and salt concentration, must also be tested.

One means of overcoming the concentration problem is to use crystallised protein samples. Whilst this removes many of the advantages gained from studying proteoliposomes, it can allow the production of highly concentrated and structurally homogeneous samples. Indeed this approach has been taken by several groups that are keen to demonstrate a host of newly developed SSNMR assignment techniques on biological samples.[1–5] Due to the difficulties in obtaining suitable amounts of membrane proteins, these groups have turned to precipitated or microcrystalline preparations of water-soluble proteins for which the 3D structures are known. Examples include lysozyme,[1] streptavidine,[1] ribonuclease A,[1] cytochrome C,[1] ubiquitine[1–4] or, in the case of precipitants, SH3[11] and Crh.[5] These groups have shown that both crystalline and precipitated proteins can yield sharp and well-defined line shapes, a prerequisite for any high-resolution structural investigations. To date, these studies have concentrated on proteins that are known to form diffracting 3D crystals. Therefore, pertinent questions remain: can these techniques be applied to lower-quality crystals, a plethora of which will be produced by a crystallographer during the search for a diffracting crystal? Will these crystals yield the same high-quality spectra as those observed for well-diffracting samples? If so, then applying SSNMR to crystalline preparations of "difficult" macromolecules, such as membrane proteins, could provide a significant new approach to solving 3D structures. Here we aim to address these questions.

In this study, we present work that was carried out on the integral membrane protein diacylglycerol kinase (DGK) from *E. coli*. The protein consists of 121 amino acids that are postulated to form three transmembrane (TM) helices (Figure 2).[23] The final active structure is thought to be a trimer.[24] The protein can be easily over-expressed in *E. coli* to yield 20–30 mg of protein per litre of culture.[25]

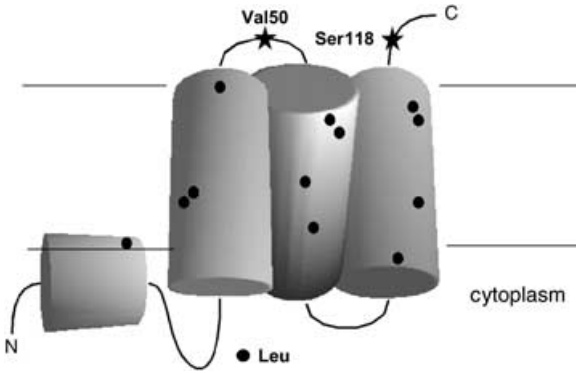

Figure 2. Predicted topology of DGK. Black dots represent the approximate position of the leucine residues. Stars represent the approximate position of Val50 and Ser118 (see text). The SwissProt sequence accession number is P00556.

DGK is typical of many membrane proteins in that it is highly α-helical[23,26] and does not easily form diffracting crystals. Indeed, years of work by crystallographers have produced many crystals of this protein. However, none diffract to a high enough resolution for the determination of 3D structure.[27] Furthermore, unlike other membrane proteins chosen by SSNMR spectroscopists, DGK does not naturally form ordered 2D arrays or exist at very high concentrations in the natural membrane.[28]

Cross-polarization magic-angle spinning (CPMAS) NMR spectroscopy of protein crystals provides well-resolved spectra that allow resonance assignment under solid-state NMR condi-

tions.[2,3] However, to date, nothing has been reported for nondiffracting crystals or membrane proteins. Here we demonstrate that DGK nanocrystals—prepared by using the same precipitation conditions as those required for the growth of nondiffracting microcrystal—make promising subjects for SSNMR based protein-structure determination.

We compare the ^{15}N-CPMAS spectra of specifically labelled residues in crystalline, lipid-reconstituted DGK. Furthermore, we have prepared selectively and extensively (SE) labelled protein for ^{13}C-CPMAS and double-quantum-filtered experiments by using [2-^{13}C] glycerol as the sole carbon source.[29] This labelling scheme results in fewer resonances and hence in an increase in resolution. This is due to a reduction in both the number of overlapping resonances and homonuclear ^{13}C–^{13}C homogeneous line-broadening.[11,29,30]

We demonstrate that SE-labelled, nondiffracting, crystalline protein can be used to acquire high-quality spectra with linewidths that are comparable to previously published SSNMR spectra of diffracting crystals.

Results and Discussion

Amino acid-selective labelling for screening sample preparation conditions

Amino acid-specific labelling of DGK was achieved by expression in a strain of *E. coli* that had lesions in the *avtA*, *ilvE* and *tyrB* genes.[30] These cells are auxotrophic for several amino acids, including leucine. The cultures were grown in a defined medium that contained ^{15}N-labelled leucine along with the remaining unlabelled amino acids. The use of this expression system removes any danger of scrambling or dilution of the label and thus ensures of 100% labelling efficiency. Therefore, all observed resonances can be attributed to the labelled amino acids. This results in just twelve resonances from leucine per protein (Figure 2) and vastly simplifies all spectra. Furthermore, leucine residues are distributed throughout the sequence; therefore, the use of this labelling scheme allows us to probe most of the protein.

There is a considerable reduction in cost when specific labelling, as compared to uniform or SE labelling, is performed. Whilst the latter schemes must be used for structural determination they are not required for screening sample preparation conditions. The fact that labelling with ^{15}N leucine costs about 25 times less than SE labelling makes this strategy an attractive method for initial SSNMR experiments.

Reconstitution for solid-state NMR experiments

DGK was reconstituted into dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) by codissolving lipid and protein in dodecylphosphocholine (DPC), which was later removed by extensive dialysis. The initial molar ratio of lipid to protein, prior to dialysis, was 1:100. The activity of the protein was tested by using a linked-enzyme assay,[31] which showed an activity of 35 μmol min^{-1} mg^{-1} (Figure 3). This value agrees with those previously published for 100% active protein.[31] Decreasing the protein:lipid ratio resulted in lower activities, while increasing it had no effect. Hence it was decided that a 1:100 protein/lipid ratio was optimum. Under these conditions, approximately 5 mg of protein can be placed in a MAS rotor, which is sufficient for high-quality spectra to be collected during an overnight experiment.

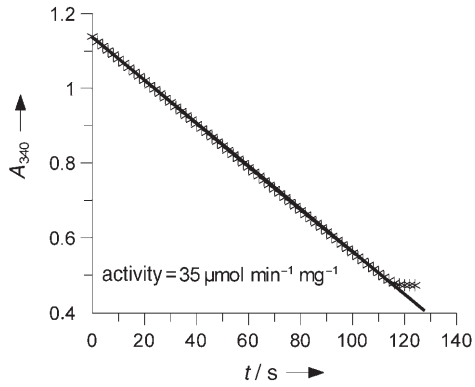

Figure 3. The activity of ATP-dependent turnover of dibutyrylglycerol was monitored by a linked assay. The oxidation of NADH by lactate dehydrogenase was followed by measuring absorption at 340 nm.[21,33] The reaction volume was 105 μL in a 3 mm pathlength cuvette that contained reaction buffer with DGK (0.51 μg mL^{-1}).

Crystallisation for solid-state NMR experiments

Crystallisation conditions were screened and optimised for solid-state NMR requirements by using sitting drop vapour diffusion. It was important to find conditions that produced a large amount of similar crystals while minimizing the amount of aggregates in the preparation; any degree of aggregation complicated subsequent NMR spectra. The best crystallisation was observed when the buffer contained PEG 400 (22.5%) in Na formate buffer (15 mM), NiCl (10 mM) and $MgCl_2$ (15 mM) or $CaCl_2$, pH 4.5, together with NaATP (11.8 mM) in the drop. Crystals generally grew in 2–7 days. Typical crystals are shown in Figure 4. None of the crystals that were grown under these

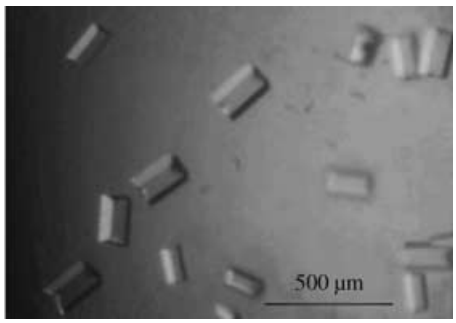

Figure 4. DGK crystals grown by using sitting-drop vapour diffusion in precipitation buffer that consisted of PEG 400 (22.5%), Na-formate (50 mM, pH 3.5), $MgCl_2$ (15 mM), NiCl (10 mM). Drops consisted of protein (4.5 μL, 10 mg mL^{-1}) in DDM (0.5%), Tris (20 mM), NaCl (50 mM, pH 7.0), precipitation buffer (3 μL) and NaATP (1 μL, 100 mM). The nanocrystals were prepared under the same conditions as the crystals shown here and were used for solid-state NMR experiments.

conditions (or under many other conditions) produced X-ray diffraction patterns.[26]

Solid-state NMR requires milligram quantities of small crystals instead of one large individual crystal. Therefore the crystallisation conditions were both scaled and sped up. Millilitres of crystallisation mixture were prepared, and the solution was then evaporated over 2–3 h by using a centrifugal evaporator to yield a fine white powder. It has previously been demonstrated that this method can be used to produce nanocrystals with dimensions less that 100 nm per edge.[1] This study also reported that there was no discernible difference between the ^{13}C NMR spectra of these nanocrystals compared to crystals grown in a more conventional fashion.

^{15}N and ^{13}C-MAS NMR

CPMAS spectra of ^{15}N-leucine DGK were collected at a range of temperatures. The best resolution was obtained at 248 K for the reconstituted sample (Figure 5a) and at 268 K for the crystalline preparation (Figure 5b). The best deconvolution was achieved by fitting six (Figure 5a) or seven (Figure 5b) Lorentzian curves to both spectra. The average peak-width is reduced by about 33% from 30 Hz to 20 Hz when comparing reconstituted with crystallised DGK, respectively. This seems surprising as we can demonstrate the protein to be fully active in the lipid environment. The broader lines are probably caused by increased structural heterogeneity at the temperatures chosen for our experiments. Even very fast sample freezing will result in an ensemble of frozen states, which cause heterogeneous line-broadening, whereas the protein is more constrained in a tightly packed crystal. This might also explain the slightly different chemical-shift distribution of the observed resonances between both samples. Resonances A and B remain unchanged; C appears to split into two peaks (±0.3 ppm); D, E and F are slightly shifted within a range of 0.3–0.5 ppm (Figure 5). Without an assignment no statement can be made about the correct fate of each individual resonance in either sample. However, we assume that these small chemical-shift changes are due to significantly different packing constraints and forces in the lipid bilayer, compared to a 3D crystal, that result in small conformational differences. However, overall signal distribution remains similar and chemical-shift changes are small when compared to those observed in the bacterio-

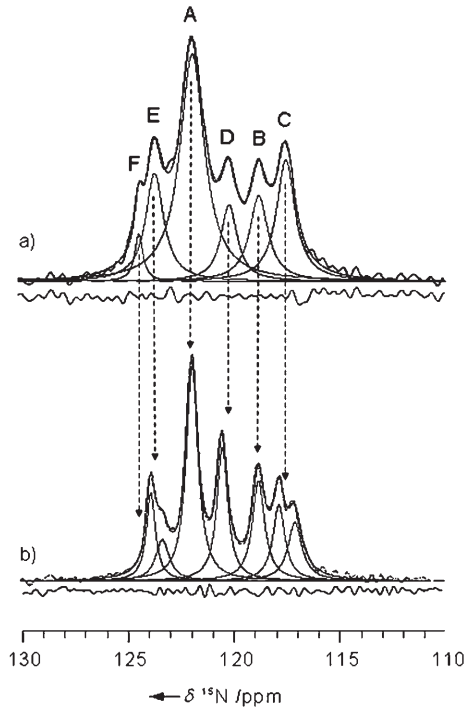

Figure 5. CPMAS spectra of ^{15}N-leucine DGK at 12 kHz of sample spin. a) The reconstituted sample at 248 K can be accurately deconvoluted by a minimum of six Lorentzian curves at 117.6, 118.86, 120.27, 122.01, 123.69 and 124.2 ppm. b) The nano-crystalline sample at 268 K is fitted best to a minimum of seven Lorentzian curves at 117.22, 117.91, 118.87, 120.58, 122.03, 123.4 and 123.9 ppm. Residuals to the fit are shown below the spectra. Cross polarization (CP) experiments were performed at 60.88 MHz ^{15}N-Larmor frequency. Both samples contained approximately 5 mg of protein. Spectra are the sum of 64000 scans.

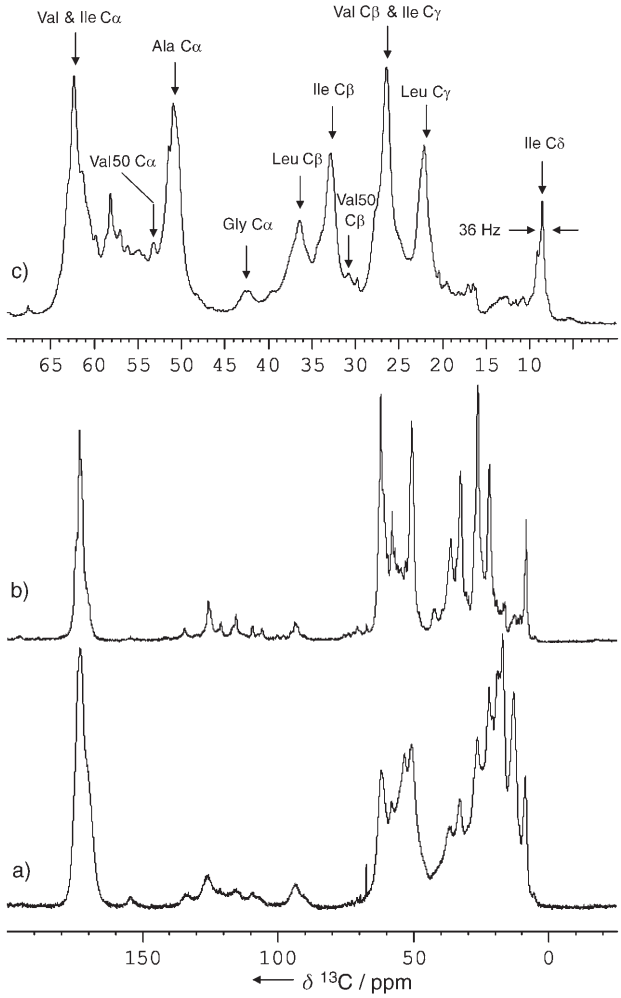

Figure 6. ^{13}C-CP-MAS spectra of a) nanocrystalline U-^{13}C, b) SE ^{13}C-labelled DGK at a sample spin of 12 kHz and 268 K. c) Enlarged region showing side-chain and α-carbon of SE ^{13}C-DGK (see text for further details). Both spectra were obtained at 150.92 MHz ^{13}C-Larmor frequency. The MAS rotor contained approximately 5 mg of protein. Spectra were accumulated over 4000 acquisitions.

rhodopsin ground state, with both *N*- and *O*-like intermediates.[13] Therefore, we are confident that the overall similarity of the spectra suggests that the protein has adopted the same fold in both samples, and hence it is valid to use crystalline samples further.

^{13}C CPMAS spectra of uniformly SE-labelled crystalline DGK samples are compared in Figure 6a and b. SE labelling improves the line widths and simplifies the spectrum remarkably. Figure 6c shows the CP-MAS spectrum of ^{13}C-SE DGK is between −10 and 70 ppm; a region where sharp resonances can be seen with line widths of approximately 36 Hz. These data suggest that the natural line width of individual resonances are good and comparable to the 40 Hz line width reported for crystals of ubiquitin.[1]

The spectrum can be further simplified with double-quantum filtering by using excitation times that are short enough to observe strongly coupled, that is, directly bonded nuclei within one residue only. The SE-labelling scheme results in a well-defined distribution of labels. The only chemically bonded ^{13}C nuclei are the valine α–β, leucine β–γ, isoleucine α–β or β–γ1 carbons. Each isoleucine side chain will contain either one or the other labelling patterns. Hence, only resonances corresponding to these carbons will appear in a DQF spectrum. Double with single-quantum coherences in a 2D experiment were correlated by exciting double-quantum coherences with POST-C7.[32] Thereby, an initial amino-acid selective assignment for the relevant spin pairs in valines, leucines and isoleucines was obtained (Figure 7).[33] In principle, the results agree with assignments of major peaks from liquid-state spectra of detergent-solubilised DGK; these are shown above Figure 6c.[26] It should be noted that the CPMAS spectrum is composed of resonances dominated by 18 alanines, 15 isoleucines, 12 leucines and 15 valines. Together, these make up almost half of the primary sequence. The alanines and glycines indicated in Figure 6c have been identified in DQ-SQ 2D spectra of U-^{13}C DGK (not shown).

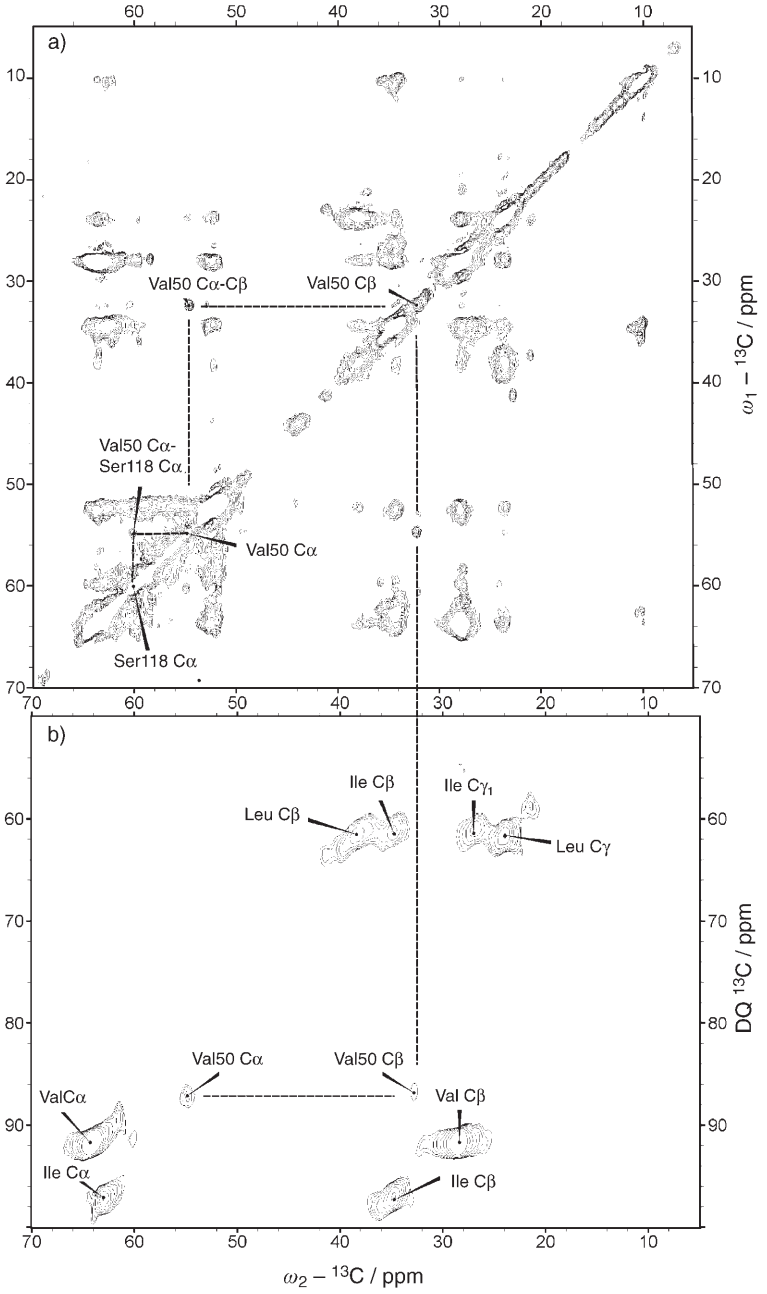

Figure 7. a) ^{13}C-^{13}C PDSD spectrum of SE ^{13}C-DGK at 12 kHz spinning speed, 500 ms mixing time and 268 K correlated with b) ^{13}C DQ-SQ POST-C7 experiment performed on SE-^{13}C DGK (see text for details).

Interestingly, the DQF spectrum (Figure 7) also reveals some peaks that correspond to individual resonances. Double-quantum single-quantum correlation shows that the resonances at 54.7 and 32.45 ppm belong to a pair of strongly coupled nuclei. Due to the labelling pattern, these can only be a Cα–Cβ coupling in Val or Ile. Both resonances appear at significantly different chemical shifts than the rest of the Val and Ile Cα–Cβ pairs—all of which have chemical shifts that are consistent with an α-helical conformation.[34] In comparison the resonance at 54.7 ppm is upfield of the other Cα resonances. It is therefore in a region of the spectrum that is consistent with the reporting nuclei being in a "random coil" or loop conformation.[34] Meanwhile the Cβ resonance at 32.45 ppm is upfield of the bulk of the Ile resonances and downfield of the Val resonance. However, a Cβ resonance in a loop conformation will be found downfield of an equivalent resonance in a helical conformation.[34] Therefore, the Cβ at 32.45 ppm must correspond to a Val residue. Previous studies have shown that the only valine within a loop region of DGK is Val50. Therefore, we can tentatively assign the peaks at 54.7 and 32.45 ppm to Val50 Cα and Cβ, respectively. A clear correlation between Val50 Cα and Cβ can also be seen in a 2D proton-driven spin-diffusion experiment of ^{13}C-SE DGK (Figure 7a). This experiment reveals a number of intra- and inter-residue correlations, which can be used for assignment and structure calculations,

when acquired at different mixing times. For example, Val50 Cα shows a strong cross peak with a Ser Cα.

Resonances belonging to serines (at positions 17, 60, 61, 73, 84, 90, 98, 118) have been identified by using DQ-SQ correlation on uniformly ^{13}C-labelled DGK (not shown). Based on the predicted DGK topology, only Ser118 would be close enough in space to Val50. This tentative assignment has to be confirmed with ^{13}C/^{15}N labelling, but this clear inter-residue Val–Ser correlation illustrates the possibility of obtaining long-range constraints.

Detection of bound lipids in crystals by ^{31}P-CP MAS

A ^{31}P spectrum of nanocrystalline protein, along with a fit to four Lorentzian peaks is shown in Figure 8c. The spectrum is dominated by a large peak at 0.35 ppm, three smaller peaks are evident at 0.52, 0.62 and 0.75 ppm. Since no lipids were added to the protein at any point during the preparation procedure the lipid must have copurifed with the protein.

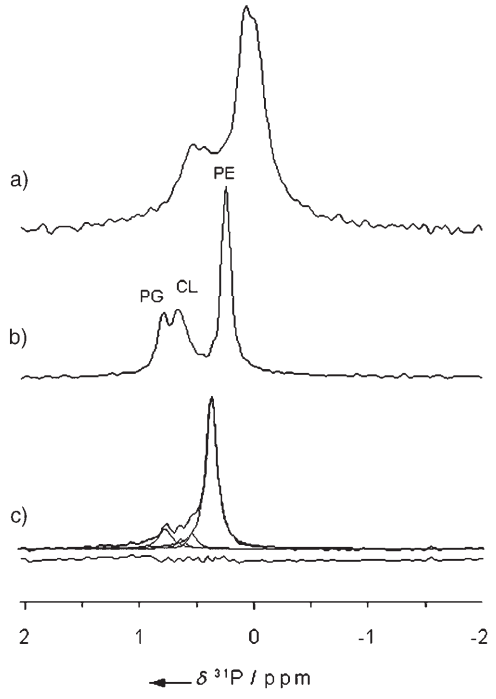

Figure 8. a) Proton-decoupled ^{31}P MAS spectra of *E. coli* lipids, b) DOPE (PE), cardiolipin (CL) and DOPG (PG), mixture at 4:1:1 molar ratio and c) nanocrystalline DGK with four individual Lorentzian curves. ^{31}P measurements were carried out at 242.89 MHz ^{31}P-Larmor frequency, 273 K and at 12 kHz sample spin.

In order to determine whether the protein bound a particular lipid, the ^{31}P spectrum of the crystals was compared to that of liposomes that were obtained from a total *E. coli* lipid extract (Figure 8a) and those that contained 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG) and cardiolipin (the three most abundant lipids in *E. coli* membranes) at a 4:1:1 molar ratio (8:2:1 with respect to ^{31}P spins; Figure 8b). The total extract has a large chemical-shift dispersion; single peaks cannot be resolved. The mixture of lipids gives a well resolved spectrum with all three resonances clearly separated. The differences in linewidth and chemical shift between both spectra are caused by the distribution of several chain lengths of the lipid components and their ratio in the *E. coli* membrane. The four peaks seen in the ^{31}P spectrum of the crystal give linewidths that are comparable to the individual lipids in the mixture shown in Fiure 8b. This suggests that specific lipids are bound to the protein. Furthermore, the relative size of the lipid peaks in the crystal sample suggests that the protein binds one particular lipid with a greater affinity than others. However, identifying the bound lipids is not possible since bound and free forms might have different chemical shifts.

Since the lipid is purified from the bacteria along with the protein, it too will be subject to isotope enrichment. Therefore, resonances derived from the lipid will contribute and complicate any spectra. Thus, information concerning bound lipids is vital when attempting to make resonance assignment. Furthermore, the presence of lipids could be an important factor that governs the crystallisation process. If it is demonstrated that lipids are tightly bound to a membrane protein, it would then be prudent to introduce lipids to the protein during crystallisation trials so as to ensure a homogeneous sample of protein–lipid complexes.

Conclusion

Here we have studied a water-insoluble, multitopic, integral, α-helical membrane protein that does not naturally form ordered arrays or exist in a membrane at high concentrations. Therefore, it represents a more general test case than other systems commonly used as membrane protein models for SSNMR, such as, bacteriorhodopsin.[13,35,36] SSNMR has been shown to provide valuable information when studying systems such as colicin[37,38] or membrane-bound peptides.[39,40,41] Our experiments show that sample preparation contraints can be successfully overcome with multitopic membrane proteins.

We have demonstrated a cost effective, auxotroph based, specific amino acid-labelling scheme that can be used to screen samples in preparation for SSNMR experiments. Furthermore, this labelling scheme could be used to obtain specific amino-acid structural or dynamic information. The application of this labelling scheme shows that nanocrystalline protein samples yield superior spectra when compared to fully active reconstituted protein.

The crystal samples also appear to offer a significant advantage over proteoliposomes with respect to protein concentration. The active volume of the MAS rotor is completely filled by 5 mg of DGK that is reconstituted into DOPC liposomes, at a molar ratio of 1:100. However, 5–10 mg of nanocrystalline protein (as used in the ^{13}C experiments) still leaves room for more sample. Hence, even when resolution is not an issue it might be advantageous to use crystalline samples.

The nanocrystalline samples can be used to obtain high-quality SSNMR spectra of membrane proteins. However, the crystallisation conditions used here were chosen for the homo-

geneity of the samples that they produced, that is, uniformly shaped crystals mixed with little or no precipitate. Therefore, it might prove worth while to screen crystallisation conditions and ascertain whether they have some bearing on the quality of the spectra. Still further improvements to the quality of the spectra can be gleaned from more advanced NMR techniques and technologies, such as *J* decoupling or higher field spectrometers. Nevertheless, present samples give promising spectra compared to those obtained from SSNMR experiments of water soluble proteins, for which full assignments[3,5,42] and one protein structure have been obtained.[21]

We have also shown that SSNMR is a useful technique for demonstrating the presence of strongly bound lipids; an observation that could be important to crystallographers for setting up crystal trials, and NMR spectroscopists for assigning spectra.

Nondiffracting crystals are considerably easier to produce than the high-quality crystals required for X-ray diffraction structural determinations. Sample preparation conditions for 3D crystallisation are also easier to screen than for 2D crystals. For example, this group has produced crystals of a small multidrug transporter from *Mycobacterium tuberculosis* from initial crystal trials.[43] To date, these poor crystals have been of little or no use for diffraction experiments. However, it is apparent that crystalline samples produce excellent line widths in SSNMR experiments. Hence, nondiffracting crystals could be of significance to SSNMR spectroscopy and provide a valuable complementary technique to X-ray crystallography and electron microscopy. Indeed, if the methods suggested here can be generalised, then they represent a significant new approach for investigating membrane-protein structures.

Experimental Section

The CT19 BL(21) auxotrophic *E. coli* strain was a gift from D. Waugh (National Cancer Institute, Frederick, USA), DGK expression plasmid was from C. Sanders (Vanderbilt University, USA) and dibutyrylglycerol (DBG) from P. Booth and A. Seddon (University of Bristol, UK). *n*-Dodecyl β-D-maltoside (DDM), DPC and octyl β-D-glucopyranoside (OG) were purchased from Anatrace (Anatrace (Maumee, OH, USA); Ni-NTA agarose was from QIAgen. All lipids were purchased from Avanti Polar Lipids (Alabaster, Alabama. USA); ^{15}N leucine and [2-^{13}C] glycerol were from Cambridge Isotopes (Cambridge, MA, USA) and all other chemicals from Sigma–Aldrich.

Over-expression and purification of DGK: His-tagged DGK (Swiss-Prot sequence accession number P00556) was over-expressed in either an auxotrophic *E. coli* BL(21) strains that had *ilvE*-, *tyrB*-, *aspC*-, *avtA*-, and *trpB* lesions[12] or BL(21) depending on whether selective labelling was required. To achieve amino-acid specific or SE labelling, cells were grown in either defined media[44] or M9 media with [2-^{13}C] glycerol or ^{13}C-glucose as the sole carbon source, respectively. Growth and expression conditions were carried out as described previously.[25,45] Protein was purified into either DDM or DPC as described.[25,46]

The concentration of DGK was determined at A_{280} by using an extinction coeffiecient of 1.8 mg^{-1} cm^{-1}, as determined from the tryptophan, tyrosine and phenylalanine content of the protein. Protein eluted into DDM was frozen in liquid nitrogen and stored at −80 °C. Protein eluted into DPC was immediately reconstituted into DOPC vesicles. The purity of DGK was judged to be better than 95% by SDS-PAGE.

Reconstitution: DGK was reconstituted as described previously[31] with slight modifications. Briefly, a DOPC/DPC mixture at 1:2 molar ratio was subjected to ~5 freeze thaw cycles until it was clear. DGK in DPC (0.5%) was mixed with DOPC/DPC mixture to give a lipid/protein molar ratio of 1:100. Detergent was removed by extensive dialysis (5–7 days and four buffer changes) against HEPES (50 mM), $MgCl_2$ (10 mM), NaATP (2 mM), EDTA (1 mM), DTT (0.2 mM), pH 7.0. The proteoliposomes were subjected to an enzyme activity assay,[45,46] then frozen in liquid nitrogen and stored at −80 °C.

Activity assay: DGK activity was determined by a linked-enzyme assay, as previously described.[31] In this assay, the DGK reaction is linked to the conversion of NADH to NAD^+ by pyruvate kinase (PK) and lactate dehydrogenase (LDH). DGK activity is thus monitored by the decrease in NADH absorption at 340 nm. LDH/PK were added to the assay buffer and incubated at 30 °C for 5 min prior to the addition of DGK. The change in NADH absorption at 340 nm was monitored by using a Jasco UV/Vis with a path-length of 3 mm and a band-with of 2 nm.

Crystallization procedures: DGK (5–10 mg^{-1} mL^{-1}) in sodium phosphate (50 mM), NaCl (0.3 M), imidazole (250 mM), DDM (0.5%, 9.5 mM), pH 7.5, was buffer exchanged into DDM (0.5%, 9.5 mM), Tris (20 mM), NaCl (50 mM), pH 7.0, by using Amicon 50 kDa cut-off filters in a stirring pressure concentrator. The following precipitation buffers were used to test crystal growth conditions for each batch of protein: Na-formate (50 mM) pH 3.5 or Na-acetate (50 mM), pH 4.5, PEG 400 or 1500 (22.5%, *v/v*), $CaCl_2$ or $MgCl_2$ (15 mM), Zn-acetate or $NiCl_2$ (10 mM). Sitting drops consisted of protein (4.5 μL), precipitation buffer (3 μL) and NaATP (100 mM, 1 μL). Crystals were grown at 25 °C for 2–7 days. The best growth conditions were selected by observing when the most crystals with the least amount of aggregated protein were produced. Nanocrystals were then grown by mixing protein (450 μL) with the appropriate precipitation buffer (300 μL) and NaATP (100 μL, 100 mM). The mixture was rapidly concentrated to ~400 μL in a centrifugal evaporator. The resulting crystals were packed into a 4 mm MAS rotor.

Preparation of liposomes: DOPE, DOPG and 1,1′,2,2′-tetraoleoyl cardiolipin at a molar ratio of 4:1:1 (equivalent to equimolar phosphate) were codissolved in chloroform. The solvent was removed under hard vacuum (~0.05 mbar), overnight. The lipid mixture or *E. coli* lipids were resuspended in HEPES (50 mM), $MgCl_2$ (15 mM), pH 7.0. Unilamellar vesicles were made by extrusion to 100 nm diameter. The resulting liposomes were sedimented and packed into a 4 mm MAS rotor.

Solid-state NMR: All experiments were performed on a Bruker Avance 600 equipped with a 4 mm MAS DVT probe.

Cross polarization (CP) experiments were performed at 60.88 MHz or 150.92 MHz, for ^{15}N and ^{13}C, respectively. An 80–100% ramped proton pulse of 750 μs for ^{15}N and 1.5 ms for ^{13}C was used during the Hartmann–Hahn match. Heteronuclear proton decoupling of typically 70 kHz was applied during a 49 ms acquisition time. The recycle delay time was 1.3 s and 2 s for ^{15}N and ^{13}C, respectively. Spectra were zero filled to 16000 points and referenced externally to the ^{15}N resonance of *N*-acetyl leucine at 128.77 ppm or ^{13}C carbonyl resonance of glycine at 170.03 ppm.

All measurements were carried out at sample spinning of 12 kHz. A number of experiments were performed between 233 and 283 K with 10 K increments. The highest resolutions were achieved at 248 K for the reconstituted sample and 268 K for the crystals. All subsequent measurements were carried out at these temperatures. Temperatures were measured externally; hence the actual sample temperature might be higher due to friction effects produced by fast sample spinning.

Two-dimensional ^{13}C-^{13}C proton driven spin diffusion (PDSD) experiments were recorded by using 500 ms mixing time with 448 increments and 512 acquisitions.

^{13}C double-quantum single-quantum correlation was achieved by using the POST-C7 experiment at 9 kHz spinning speed.[32] Experimental parameters were the same as those for cross polarisation, but with 110 kHz proton decoupling that was applied during 507.8 μs double-quantum excitation and reconversion with 256 increments and 712 scans.

^{31}P measurements were carried out at 242.89 MHz. Spectra were collected by using single-pulse, proton-decoupled MAS experiments. Proton decoupling at approximately 100 kHz was used over the whole acquisition time of 49 ms. Spectra were referenced to 85% phosphoric acid at 0 ppm. All samples were measured at 68 K and 12 kHz sample spinning.

All Spectra were processed by using XWIN-NMR (Bruker BioSpin) and Sparky.[47]

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