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Optimization of purification and refolding of the human chemokine receptor CXCR1 improves the stability of proteoliposomes for structure determination

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ABSTRACT

The human chemokine receptor CXCR1 is a G-protein coupled receptor that has been successfully expressed in *E. coli* as inclusion bodies, and purified and refolded in multi-milligram quantities required for structural studies. Expression in *E. coli* enables selective and uniform isotopic labeling with ^{13}C and ^{15}N for NMR studies. Long-term chemical and conformational stability and oligomeric homogeneity of CXCR1 in phospholipid bilayers are crucial for structural studies under physiological conditions. Here we describe substantial refinements in our previously described purification and reconstitution procedures for CXCR1 in phospholipid bilayers. These refinements have led to the preparation of highly purified, completely monomeric, proteoliposome samples that are stable for months at 35 °C while subject to the high power radiofrequency irradiations of solid-state NMR experiments. The principal changes from the previously described methods include: 1) ensure that CXCR1 is pure and homogeneously monomeric within the limits of detection (>98%); 2) monitor and control the pH at all times especially following the addition of TCEP, which serves as a reducing agent but also changes the pH; 3) slowly refold CXCR1 with the complete removal of all traces of SDS using a KCl precipitation/dialysis method; and 4) ensure that the molar ratio between the CXCR1 and the phospholipids does not change during refolding and detergent removal. NMR samples prepared with these protocols yield reproducible results over a period of many months at 35 °C. This purification and refolding protocol is likely to be applicable with minimal changes to other GPCRs as well as other membrane proteins.

1. Introduction

G-protein coupled receptors (GPCRs) are important targets for structural studies. Not only because they represent the largest and most diverse protein family in the human genome [1–3], but also because of their roles as receptors for many therapeutic drugs. Currently, more than one-third of all approved drugs utilize a small fraction of the total number of GPCRs as receptors; currently unused and orphan receptors offer many potential targets for the discovery of new drugs [4–6].

It is essential to determine the three-dimensional structures of GPCRs in their native bilayer environment in order to understand their molecular mechanisms of action, and to lay the groundwork for structure-based drug discovery. However, with only six unique GPCR structures determined at the present time [7–13], structural information about this class of membrane proteins remains sparse. This is largely due to the difficulties encountered in sample preparation for the experimental structure determination of large integral membrane proteins, such as GPCRs with their low expression levels, instability in the presence of detergents, resistance to crystallization of the native proteins in any environment, much less phospholipid bilayers for X-ray diffraction, and their slow overall reorientation in aqueous solution for solution NMR. In contrast, solid-state NMR is well suited for structure determination of proteins in supramolecular assemblies, such as GPCRs in phospholipid bilayers under physiological conditions of temperature, pH, hydration, etc. However, solid-state NMR experiments require extremely high quality and stable samples in order to generate meaningful and reproducible results.

Solubilization-induced instabilities and structural heterogeneities may be limiting factors in the formation of three-dimensional crystals of GPCRs for X-ray diffraction. In response, the X-ray crystallography community has creatively and aggressively tackled this problem through extensive modifications of both the lipid environment and the proteins themselves, using truncation, mutagenesis, and the insertion of an additional protein, generally T4 lysozyme, into the sequence. GPCRs and other membrane proteins are often crystallized

Abbreviations: DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPC, dodecylphosphocholine; GST, glutathione S-transferase; HEPES, 4-(2-hydroxyethyl)-1 piperazineethane-sulfonic acid; HPC, n-hexadecylphosphocholine; MβCD, methyl-β-cyclodextrin; MAS, magic angle spinning; OS, oriented sample; SDS, sodium dodecyl sulfate; TCEP, tris-2-carboxyethylphosphine

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from monoolein in a Lipid Cubic Phase (LCP) that includes several types of detergents and lipids [8,13,14]. However, a major concern is that the crystallization of a membrane protein from monoolein in LCP alters the protein structure [15].

In addition to providing a native, functional environment that is likely to be compatible with the biologically active conformation, there are additional reasons why studying membrane proteins in phospholipid bilayers is preferred over other membrane-mimic environments. For example, many fewer control experiments are required to ensure that the protein is in its native conformation, other molecules such as chemokines, cholesterol, and drugs can be readily added to the samples, and the experiments can be performed over a wide range of relevant temperatures, e.g. 20 °C to 70 °C. However, proteoliposome samples are not without their own concerns; for example, structural changes, which may not be uniform throughout the protein or the sample, may result from the lateral pressure of ~30 nM m^{-1} [16], which acts on membrane proteins embedded in lipid bilayers but not in micelles or isotropic bicelles. While membrane proteins that function as pores or channels may be relatively unaffected, this lateral pressure may have a strong impact on membrane proteins, such as GPCRs, that interact with multiple other proteins and partners in the membrane, and undergo conformational changes as part of their function.

For X-ray crystallography, the lipids and proteins have been highly developed in order to make samples compatible with the available X-ray beams and instrumentation. In contrast, in solid-state NMR, it is the instrumentation and spectroscopic methods that have been highly developed to accommodate the protein and phospholipid bilayers in their native states under physiological conditions. Solid-state NMR is used to study many types of membrane associated peptides and proteins in planar phospholipid bilayers [17]. Besides structure determination, NMR experiments also provide information about protein dynamics, protein–protein interactions, and protein–lipid interactions [18–24]. Sample development has also been important to the success of the NMR experiments [25], since the spectra are sensitive to any changes in conformation or aggregation over the extensive periods of signal averaging required to perform multi-dimensional experiments on these relatively large proteins.

Whether GPCRs are expressed into membranes [26–30] or inclusion bodies [31–33] in bacteria, or are expressed in cell-free systems [34–36], detergents are typically required to solubilize these hydrophobic proteins for isolation and purification. While membrane proteins are stable in their native environment of the liquid crystalline phospholipid bilayer, they often exhibit changes in structure and aggregation when solubilized with detergents. There are an increasing number of reports describing how the structures of membrane proteins in the presence of detergents differ from those in phospholipid bilayers [37–39]. Thus, the use of detergents or other non-native lipids to solubilize or crystallize a membrane protein requires extensive control experiments to demonstrate that the membrane-mimic environment does not perturb the structure and dynamics.

Here we describe improved methods of preparation of GPCR-containing phospholipid bilayers that supersede our previously described methods [40,41]. They provide monodisperse, stable, functional samples suitable for NMR studies. We utilize a member of the rhodopsin-class of GPCRs, the chemokine receptor CXCR1, as an example, but the methods should be generally applicable to many other GPCRs, especially those in this class. Our previous approach to bacterial expression and purification has yielded pure isotopically labeled CXCR1 in multi-milligram quantities [41]. However, although these samples displayed excellent quality in all biochemistry laboratory tests, and yielded high-resolution NMR spectra, they tended to become unstable after one or few days in an NMR spectrometer. This did not provide sufficient time for performing the long-term signal averaging necessary for the three-dimensional experiments that are essential for the assignment of resonances on such a large protein.

2. Materials and methods

2.1. Materials

DMPC, DPC, and HPC were obtained from Affymetrix, Inc. (www.affymetrix.com). DHPC was obtained from Avanti polar lipids, Inc. (www.avantilipids.com). SDS, TCEP, and MβCD were obtained from Sigma-Aldrich (www.sigmaaldrich.com).

2.2. Expression and purification

The cloning, expression, and purification of isotopically labeled CXCR1 were performed as described previously [40, 41]. The DNA sequence coding for human full-length CXCR1 was cloned into the pGEX2a vector containing the fusion partner GST. The fusion protein GST-CXCR1-6His was over-expressed in *E. coli* BL21 using standard M9 minimal media with ^{15}N labeled ammonium sulfate (Cambridge Isotope Laboratories, Inc.; www.isotope.com) as the nitrogen source. The expressed GST-CXCR1-6His polypeptide was predominantly in inclusion bodies (IBs). It was solubilized in the binding buffer containing 1% (w/v) SDS and purified to homogeneity by nickel affinity (Ni-NTA agarose, Qiagen Inc., www.qiagen.com) and size exclusion (HiLoad™ 26/60 Superdex™ 200 pg, GE Healthcare Bio-Sciences AB; www.gelifesciences.com) chromatographies. 0.1% (w/v) TCEP as a reducing agent was added to the buffer in the purification process by nickel affinity chromatography. The fusion protein was cleaved with thrombin in the buffer containing 0.1% (w/v) HPC, and the GST portion was removed from CXCR1-6His immobilized on the nickel affinity column. CXCR1-6His was then eluted from the column with the buffer containing 0.1% (w/v) DPC and 300 mM imidazole. The protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm, and comparing it to the theoretical molar extinction coefficient of 51,980 M^{-1} cm^{-1}.

2.3. Reconstitution

CXCR1 was reconstituted using DMPC. Mixed micelle solutions were prepared by dissolving the DMPC powder with 0.5% SDS in 20 mM HEPES buffer pH 7.3, 50 mM NaCl with a final lipid concentration of 10 mg/ml. Purified monomeric CXCR1 was added to the mixed micelle solution at a protein-to-lipid ratio of 1:10 or 1:5 (w/w) and incubated for 1 h at room temperature.

(1.) *Adsorption to BioBeads.* After incubation, the SDS was removed from the mixture by adsorption to BioBeads (Calbiosorb adsorbent, EMD Chemicals; www.emdchemicals.com). The BioBeads were washed and equilibrated with 20 mM HEPES buffer, pH 7.3, containing no detergent. The required amount of adsorbent for detergent removal was calculated (adsorption capacity for SDS: 94 mg/ml bead slurry) and added directly to the ternary mixture of lipid, detergent and protein. After 2 h of incubation at room temperature under gentle agitation, the mixture was passed over a column with 90 μm filter pore size (Mo Bi Tec, Germany; www.mobitec.com), the detergent-free flow-through was collected, and the proteoliposomes were recovered by ultracentrifugation (145,000 *g*, 2 h, 15 °C, Beckman Ti 70.1 rotor).

(2.) *Complexation to methyl-β-cyclodextrin.* After incubation, the SDS was removed from the mixture by complexation with MβCD. Based on the SDS alkyl-chain length a 1:2 complex between SDS and MβCD was assumed. To remove SDS gently, dialysis tubing (Spectra/Por; MWCO 15,000, Cole-Parmer; www.coleparmer.com) containing the ternary mixture was dialyzed against >200-fold excess volume containing the corresponding amount of MβCD in 20 mM HEPES buffer, pH 7.3 overnight at room temperature. The reconstituted suspension of

proteoliposomes was ultracentrifuged (145,000 *g*, 2 h, 15 °C, Beckman Ti 70.1 rotor), resuspended, and washed with 20 mM HEPES buffer, pH 7.3 to remove any excess MβCD.

(3.) *Co-precipitation with potassium chloride*. After incubation, the SDS was precipitated from the solution by the addition of potassium chloride. To remove SDS gently, dialysis tubing containing the ternary mixture was dialyzed against >200-fold excess volume containing 20 mM HEPES buffer, pH 7.3 overnight at room temperature, followed by >200-fold excess volume of 20 mM HEPES buffer, pH 7.3 containing 20 mM KCl for 6 h. The proteoliposomes were ultracentrifuged (145,000 *g*, 2 h, 15 °C, Beckman Ti 70.1 rotor), resuspended, and washed with HEPES buffer pH 7.3 to remove any residual KCl.

2.4. Lipid analysis

To monitor quantitatively the lipid and detergent content of the proteoliposomes, HPLC analysis using an Evaporative Light Scattering Detection (ELSD) system (Sedex 75; www.sedere.com) was performed. A LiChrosphere 100 NH_2 column (EMD Chemicals) was used as a stationary phase, and the mobile phase was acetonitrile/methanol/0.1 M ammonium acetate pH 4.8, 70:20:10 (v/v). The samples were diluted in the running buffer before being loaded onto the column. After separation on the column, the analytes were evaporated under a stream of N_2, and the non-volatile components were detected by light scattering. The photomultiplier signal is nearly linear to the analyte concentration.

2.5. Bicelle preparation

For solution NMR, isotropic bicelle samples were prepared by adding 400 mM DHPC to the purified protein reconstituted into DMPC liposomes to produce $q = 0.1$ isotropic bicelles. Therefore, proteoliposomes consisting of 1 mg protein reconstituted in 10 mg of DMPC in 20 mM HEPES buffer, pH 7.3 were collected by centrifugation at 300,000 *g* for 2 h at 15 °C. The molar ratio of lipids to protein was 605:1. The resulting supernatant was discarded, and the hydrated proteoliposome pellet was resuspended in 400 μl water with 10% ^2H_2O containing an adequate amount of DHPC in order to produce $q = 0.1$ isotropic bicelles.

The magnetically alignable bilayer samples for OS solid-state NMR spectroscopy were prepared similarly, using higher protein concentrations (4 mg protein and 40 mg of DMPC) and an increased *q* value of 3.2 for DMPC:DHPC bicelles and 5.0 for DMPC:Triton X-100 bicelles with DMPC concentration of 20% (w/v) in a 200 μl volume. The resulting mixture was vortexed thoroughly, and then allowed to equilibrate at room temperature. Upon bicelle formation, proteoliposome becomes a clear, non-viscous solution at 4 °C and forms a gel-like liquid crystalline solution at 30 °C–45 °C. A small, flat-bottomed NMR tube with 5 mm outer diameter (New Era Enterprises, Inc.; www.newera-spectro.com) was filled with 160 μl of the solution using a precooled pipette at 4 °C. The NMR tube was sealed with a tight-fitting rubber cap, pierced with a thin syringe to remove air from the sample and create a tight seal, and used for the stationary solid-state NMR experiments.

2.6. NMR experiments

The solution NMR experiments were performed at 50 °C on a Varian VNMRS 800 MHz spectrometer equipped with 5 mm triple-resonance cold probe and z-axis gradient. $^1H/^{15}N$ HSQC NMR experiments were performed on uniformly ^{15}N labeled samples in $q = 0.1$ isotropic bicelles with a protein concentration of ~60 μM.

The ^{15}N and ^{31}P solid-state NMR spectra were obtained on a Bruker Avance spectrometer with a ^1H resonance frequency of 700 MHz. The homebuilt $^1H/^{15}N$ double-resonance probe had 5 mm inner diameter solenoid coil tuned to the ^{15}N frequency, and an outer MAGC coil tuned to the ^1H frequency [42]. The ^{31}P solid-state NMR spectra were obtained using a homebuilt $^1H/^{31}P$ double-resonance probe equipped with double-tuned scroll coil [43]. The one-dimensional ^{31}P NMR spectra were obtained by direct excitation with a single pulse using a 6 s recycle delay; 64 scans were signal averaged with a 10 ms acquisition time for each spectrum. Continuous wave ^1H decoupling utilized a B_1 radio frequency field strength of 42 kHz during the acquisition period. The one-dimensional ^{15}N solid-state NMR spectra were obtained by spin-lock cross-polarization [44] with a contact time of 1 ms, a recycle delay of 6 s, and an acquisition time of 10 ms. 2048 transients were co-added, and an exponential function corresponding to 50 Hz of line broadening was applied prior to Fourier transformation. The three-dimensional HETCOR/SLF spectrum was obtained with a B_1 field for ^1H during t_1 of 50 kHz, otherwise 45 kHz. The data were zero filled and yielded a $1024 \times 64 \times 64$ real matrix. The 0.33 scaling factor was applied in the ^1H shift dimension.

3. Results and discussion

As described in earlier publications [40,41], we have established an *E. coli* expression system capable of providing the multi-milligram quantities of CXCR1 required for NMR structural studies, which enabled us to obtain initial NMR spectra. The refinements of the preparation procedures described here provide samples that are stable for many months at room temperature, enabling reproducible multidimensional NMR spectra to be obtained. These spectra are used to determine the structures and describe the dynamics of GPCRs. There are several different refinements in the purification and reconstitution procedures. The procedures are now highly reproducible, and essentially all samples prepared in the laboratory are pure, monomeric, and stable for many months.

3.1. CXCR1 monodispersity

The protocol for the preparation of stable samples of CXCR1 in lipid bilayers outlined in Fig. 1 is very efficient. It takes 3 days from the bacterial culture to the final NMR sample: day 1, growth of *E.*

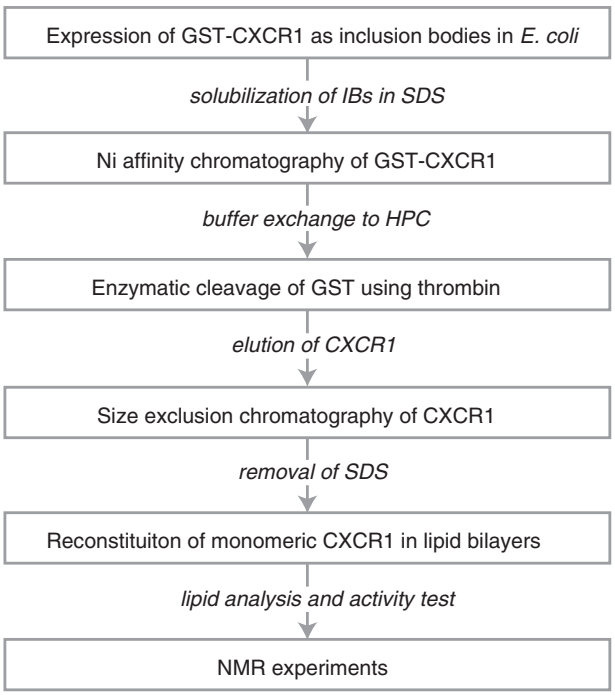

Fig. 1. Schematic flow chart of the expression, purification and refolding procedures of CXCR1 as detailed in the Materials and methods.

coli culture and preparation of IBs; day 2, purification and refolding; day 3, preparation of CXCR1 proteoliposomes and the final NMR sample. The sample purity at each step is monitored by SDS-PAGE (Fig. 2): lane 1, the expression of the fusion protein and its partial purification by isolation of the inclusion bodies; lane 2, the removal of the GST fusion partner following cleavage on the nickel affinity column; and lane 3, the presence of CXCR1 and a few other polypeptides. The final purification by size exclusion chromatography yields pure, monomeric CXCR1, as shown in lanes 4 and 5 of Fig. 2. The protein purity was shown to be >98% by gel quantification analysis (ImageJhttp://rsb.info.nih.gov/ij/).

The CXCR1 samples purified by nickel affinity chromatography that contain a small amount of higher oligomers (Fig. 2, lane 3) must be immediately loaded onto the size exclusion chromatography. We find that trace amount of oligomers (and/or other impurities) apparently facilitate the formation of larger amounts of higher oligomers. As a result any delay between the two chromatography steps significantly reduces the yield of the monomeric protein.

Previously, we added TCEP as a reducing agent during the entire process of the CXCR1 sample preparation in order to improve the sample stability by preventing oligomerization through the formation of non-specific intermolecular disulfide bonds. However, we find that the presence of TCEP in the monomeric form of CXCR1 does not affect the sample monodispersity and therefore TCEP is added only to the buffer in the nickel affinity chromatography. The pH is adjusted immediately following the addition of TCEP.

3.2. Improvement of the stability of CXCR1 proteoliposomes

The most successful strategy for the reconstitution of integral membrane proteins into proteoliposomes is a refinement of our previously described methods [40,41]. The optimized approach relies on the precisely controlled pH and complete removal of the detergent from ternary solutions of protein, lipids and detergents in excess water. The removal of the detergent, which is included initially to solubilize the hydrophobic membrane protein, is essential for forming stable bilayers, and must be performed in a precise and quantitative manner to avoid the formation of protein oligomers. We emphasize that we have found that even the smallest trace of protein oligomers is problematic for the preparation of stable samples. This is not the case for many other proteins, but it certainly is for CXCR1 and presumably other GPCRs and perhaps other relatively large membrane proteins.

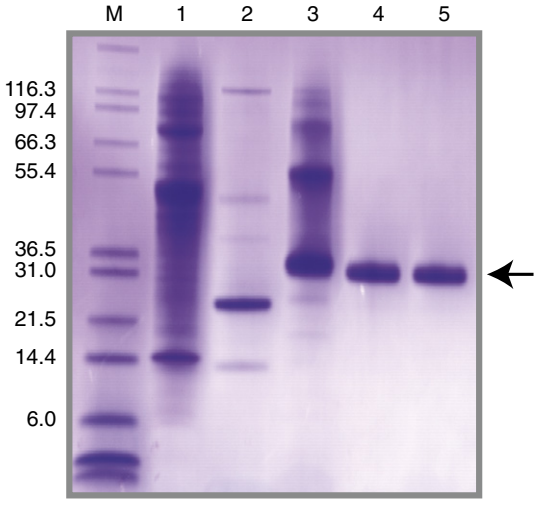

Fig. 2. SDS-PAGE analysis of the purification and refolding procedure: lane 1, inclusion bodies of the GST-CXCR1 fusion protein; lane 2, GST in flow through after thrombin cleavage of fusion protein; lane 3, purified CXCR1 by Ni affinity chromatography; lane 4, purified monomer faction of CXCR1 by size exclusion chromatography; lane 5, monomeric CXCR1 proteoliposome reconstituted in DMPC bilayers. The band at about 31 kDa corresponds to the CXCR1 monomer.

Proteoliposomes were formed from a co-solubilized ternary complex of lipid, protein and detergent. The reconstitution of CXCR1 into phospholipid bilayers by removing the detergent is a key step in the refolding process. CXCR1 purified from bacterial inclusion bodies and reconstituted into proteoliposomes has previously been shown to be functional [41]. The purity and homogeneity of the reconstituted protein is of crucial importance for OS solid-state NMR experiments, since the sample alignment in lipid bilayers is strongly dependent on a precise lipid ratio [45]. The strong interaction of CXCR1 prepared from the proteoliposomes with its physiological ligand interleukin-8 was observed by NMR spectroscopy, which further demonstrates the functionality of CXCR1 proteoliposomes [46].

The final yield of reconstituted protein in liposomes is strongly related to the procedure used to remove the detergent from the ternary protein:lipid:detergent solution, as well as the nature of the phospholipids used during the co-solubilization process. We were able to obtain the best results by using SDS, and improving the method for removal of SDS during the reconstitution of CXCR1 into DMPC phospholipid bilayers. Three different techniques for the removal of SDS were compared: (1) adsorption to BioBeads; (2) complexation with methyl-β-cyclodextrin; and (3) co-precipitation with potassium chloride. We paid particular attention to the quantitative reproducibility and homogeneity of the CXCR1-containing proteoliposomes.

SDS removal by BioBeads is simple and rapid, and provides an alternative to conventional dialysis, especially when dealing with low-CMC detergents. However, batches of CXCR1-containing proteoliposomes produced with this approach varied significantly in their lipid content with a total phospholipid loss of up to 20%. Similar fluctuations were observed using non-recycled adsorbent, which excluded altered affinities due to recycling. The lipid composition was quantitatively and qualitatively monitored by HPLC-ELSD. This approach confirmed the batch wise inconsistency in the protein-containing DMPC liposomes. The reason for the loss of DMPC may be due to non-specific adsorption to BioBeads, which is a known limitation of this method [47]. Although the adsorptive capacity of BioBeads has become more consistent, the total detergent concentration remains difficult to determine. As a result, the total amount of detergent bound to CXCR1 can only be estimated, and this is simply not adequate for the preparation of stable samples for NMR spectroscopy.

The results of gentle reconstitution using either MβCD or KCl to assist the removal of the detergent during dialysis were similar. MβCD or KCl was slowly dialyzed against ternary solutions of protein, lipid, and detergent. While MβCD is applicable to a broad range of detergents containing long acyl chains [48], KCl is only suitable for detergents having sulfate head groups, specifically SDS [49]. In contrast to procedures that used BioBeads, SDS removal by either MβCD or KCl was highly reproducible and yielded proteoliposomes without loss of long chain lipids. Care must be taken, since the rapid reconstitution that results from direct addition of MβCD or KCl to the ternary solution causes protein oligomerization that is readily observable by SDS-PAGE. The timing of the reconstitution was optimized to minimize protein oligomerization. The ternary solution was first dialyzed overnight against >200-fold excess volume of a buffer (20 mM HEPES, pH 7.3) to remove the bulk of the SDS, and then dialyzed against a buffer containing the appropriate amount of MβCD with respect to the sample volume overnight, or 20 mM KCl for 6 h to remove any SDS bound to the proteoliposomes. Although both methods give similar results, we primarily use the KCl co-precipitation method because it is specific to SDS, easier to setup, and less expensive than the MβCD complexation method. In Fig. 3, lipid analysis by HPLC-ELSD clearly shows that all of the SDS is removed (within detection limits) by the KCl co-precipitation method.

GPCRs in phospholipids following the removal of detergents are intermediates in the preparation of protein-containing samples for either solution NMR or solid-state NMR spectroscopy. In one approach, the

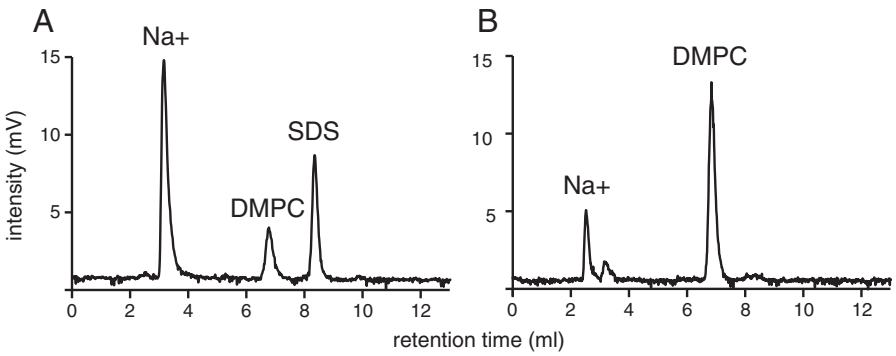

Fig. 3. HPLC-ELSD chromatogram of lipid mixtures in CXCR1 samples. (A) Ternary complex of CXCR1, DMPC and SDS mixtures before reconstitution. (B) CXCR1 proteoliposome after removal of SDS by co-precipitation with KCl.

proteoliposomes are converted to bicelle samples, which are planar bilayer-disks that consist of long chain lipids that form a bilayer in the center, and are flanked by short chain lipids at the edges. The molar ratio of long chain and short chain lipid, the q value, is an important parameter for the alignment properties of bicelles by high magnetic fields. When q is greater than about 2.5, a magnetically aligned bilayer phase is formed. When q is between 0 and about 1.5, isotropic bicelles are formed that undergo relatively rapid and isotropic reorientation in aqueous solution. Although, some membrane proteins have been demonstrated to be functional in bicelle samples [17,24,41,50,51], concern remains about the presence of detergents (DHPC, CHAPSO, or Triton X-100) and the slightly reduced overall order parameters of the bilayer lipids and the protein backbone, which are generally measured to be about 0.85 ± 0.05. In contrast, in proteoliposomes, both the lipids and the protein backbone have an order parameter of 1.0.

We have found that the precise molar ratio of lipid to protein is crucial for stability and function of the protein in proteoliposomes. This ratio must be optimized, and then maintained in order to have stable samples for NMR and other physical experiments. For NMR spectroscopy, which is always limited by sensitivity, it is essential to find the lowest lipid-to-protein ratio in order to achieve the highest NMR sensitivity while the ratio remains in a range for the sample to be stable enough to perform multi-dimensional experiments that may require days of signal averaging.

Any inconsistency in the amount of the long chain lipid after reconstitution affects not only the sample stability, but also the production of bicelles that consist of both long chain and short chain phospholipids (or detergents). Any lipid loss impedes an accurate adjustment of q by the addition of a short chain lipid DHPC [52] or the nonionic detergent Triton X-100 [53], resulting in poor alignment and instability of bicelle samples. The ^{31}P NMR spectra of bicelle samples prepared from two different batches of CXCR1 proteoliposomes in which reconstitution was performed using BioBeads demonstrates how the variation in lipid to detergent ratio affects the magnetic alignment of the bilayers (Figs. 4A and B). By contrast, bicelle samples prepared using the KCl co-precipitation method result in excellent and reproducible alignment in both DMPC:DHPC and DMPC:Triton X-100 bicelles (Figs. 4C and D). The two resolved signals in Fig. 4

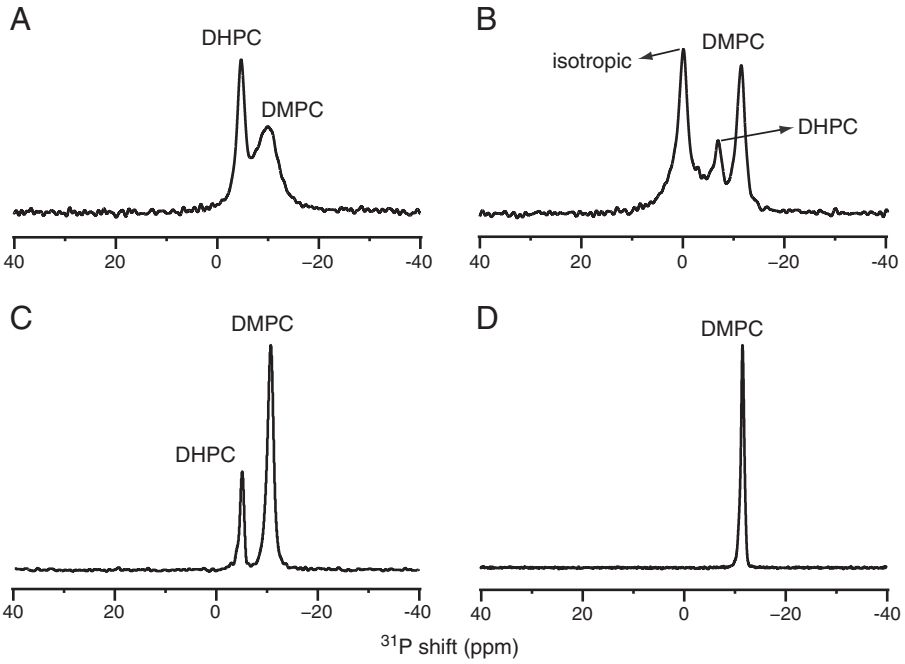

Fig. 4. ^{31}P solid-state NMR spectra of CXCR1 samples aligned magnetically in phospholipid bicelles at 35 °C. (A) DMPC:DHPC, $2.5 < q < 3.2$. (B) DMPC:DHPC, $q < 2.5$. (C) DMPC; DHPC, $q = 3.2$. (D) DMPC:Triton X-100, $q = 5.0$. The samples are prepared from four different batches of CXCR1 proteoliposomes reconstituted by adsorption of SDS to BioBeads (A and B) and by co-precipitation of SDS with KCl (C and D). Appropriate amount of DHPC or Triton X-100 was added with respect to the amount of DMPC in the proteoliposomes without taking into account of the lipid loss after reconstitution. The DMPC and DHPC signals from the aligned bilayers are indicated.

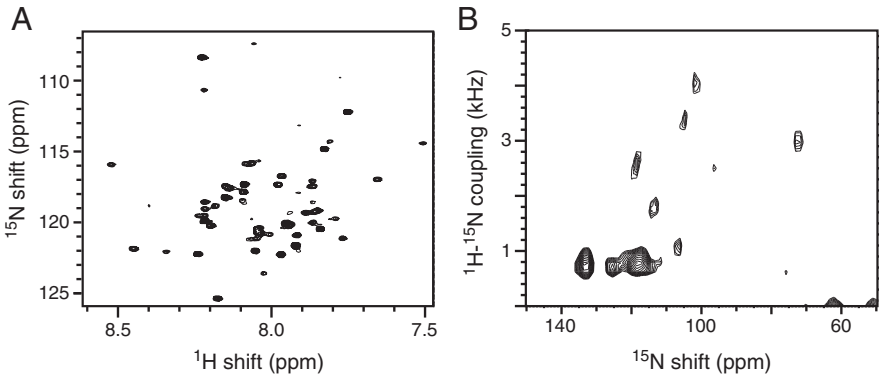

Fig. 5. NMR spectra of uniformly ^{15}N-labeled CXCR1 in lipid environments. (A) Two-dimensional ^{1}H-^{15}N HSQC solution NMR spectrum of CXCR1 in DMPC:DHPC ($q = 0.1$) isotropic bicelles. (B) Two-dimensional ^{1}H-^{15}N dipolar coupling/^{15}N chemical shift plane extracted from the three-dimensional spectrum of CXCR1 aligned magnetically in DMPC:Triton X-100 ($q = 5.0$) bicelles at ^{1}H chemical shift frequency of 14.3 ppm.

correspond to DMPC (−11 ppm) and DHPC (−5 ppm). Since the Triton X-100 does not contain phosphorus, the ^{31}P NMR spectrum consists of a single signal from aligned DMPC in Fig. 4D.

3.3. NMR studies on wild-type CXCR1 in lipid environments

Representative NMR spectra of uniformly ^{15}N labeled CXCR1 samples prepared using gentle KCl co-precipitation are shown in Fig. 5. The ^{1}H/^{15}N HSQC solution NMR spectrum of CXCR1 in $q = 0.1$ DMPC:DHPC isotropic bicelles (Fig. 5A) has homogeneous and reproducible signals, which are from the mobile N- and C-terminal residues of CXCR1 [20]. The signals from the bulk of the residues in the trans membrane helices and the inter-helical loops are not observable in solution NMR spectra because of the slow global reorientation of CXCR1 in isotropic bicelles.

The same sample preparation methods provide excellent samples for solid-state NMR experiments on the protein reconstituted into phospholipid bilayers. A typical two-dimensional separated local field plane extracted from a three-dimensional HETCOR/SLF spectrum of an aligned $q = 5.0$ DMPC:Triton X-100 bicelle sample of uniformly ^{15}N labeled CXCR1 displays single site resolution, which is essential for the resonance assignment and structure determination (Fig. 5B).

It is remarkable that there are no significant changes in either the ^{31}P NMR or ^{15}N NMR spectra of CXCR1 before and after the multiple NMR experiments performed over an 8-day period at 35 °C (Fig. 6). This same sample has been reused over a six month period, and has always yielded reproducible ^{31}P NMR of the phospholipids and ^{15}N NMR spectra of the protein.

4. Conclusion

There is a consensus that the most reliable structural data are obtained from membrane proteins in their native phospholipid bilayer environments under physiological conditions, and that the use of detergents or other non-native lipids in membrane mimics inevitably leads to controversies about possible perturbations of the protein's

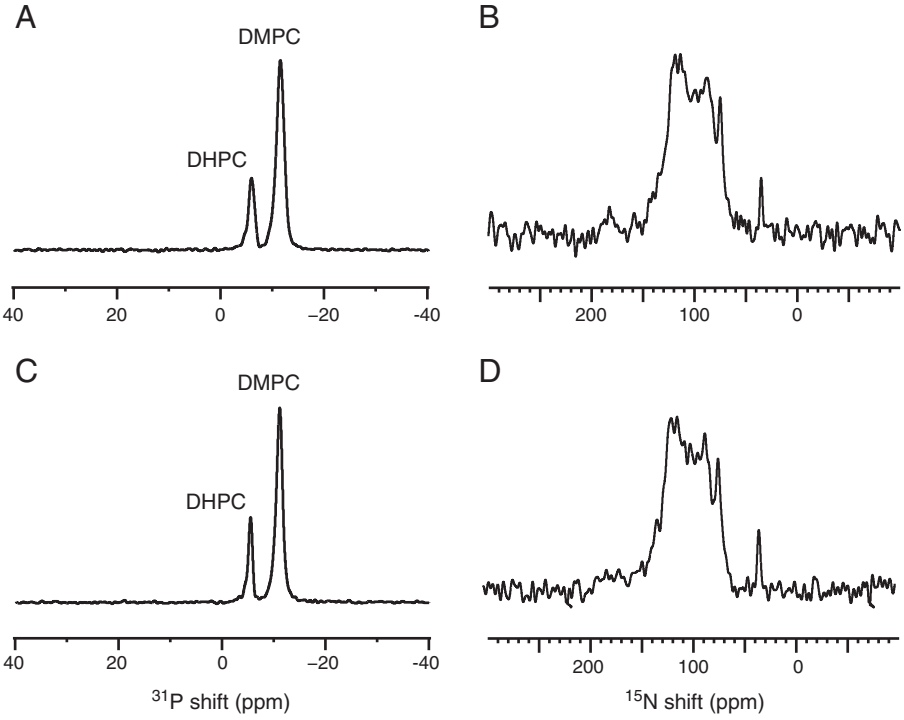

Fig. 6. ^{31}P and ^{15}N solid-state NMR spectra of uniformly ^{15}N-labeled CXCR1 samples aligned magnetically in phospholipid DMPC:DHPC bicelles at 35 °C. (A and B) The spectra were recorded on a freshly prepared sample. (C and D) The spectra were recorded on the same sample after 8 days in the magnet.

structure, dynamics, or functions. Following heterologous expression of a GPCR or other membrane protein as inclusion bodies, refolding of the proteins by reconstituting them into membrane environments is the most crucial step in obtaining functional and stable samples.

The stability of the proteoliposomes is strongly related to the purity and monodispersity of the membrane protein. The concentrated proteoliposomes of integral membrane proteins stabilized in their native environment of liquid crystalline phospholipid bilayers serve as an ideal sample for the structural studies by MAS solid-state NMR methods. Magnetically aligned bilayers produced by addition of DHPC or Triton X-100 to these same proteoliposomes provide enhanced alignment and stability for structural studies by OS solid-state NMR.

These protocols for sample preparation illustrated with CXCR1 should be generally applicable to a wide range of other helical membrane proteins. We have applied these methods to full-length membrane proteins and truncated domains with between one and seven trans membrane helices, including constructs of CXCR1, MerF from the bacterial mercury detoxification system, and p7 from the hepatitis C virus. In all cases, we have seen dramatic improvements in sample stability under the conditions of NMR experiments.

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