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Structure Determination of Membrane Proteins in Their Native Phospholipid Bilayer Environment by Rotationally Aligned Solid-State NMR Spectroscopy

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CONSPECTUS

One of the most important topics in experimental structural biology is determining the structures of membrane proteins. These structures represent one-third of all of the information expressed from a genome, distinguished by their locations within the phospholipid bilayer of cells, organelles, or enveloped viruses. Their highly hydrophobic nature and insolubility in aqueous media means that they require an amphipathic environment. They have unique functions in transport, catalysis, channel formation, and signaling. Researchers are particularly interested in G-protein coupled receptors (GPCRs) because they modulate many biological processes, and about half of the approximately 800 of these proteins within the human genome are or can be turned into drug receptors that affect a wide range of diseases.

Artwork courtesy of Judith McCabe-Jarvis

Because of experimental difficulties, researchers have studied membrane proteins using a wide variety of artificial media that mimic membranes, such as mixed organic solvents or detergents. More sophisticated mimics include bilayer discs (bicelles) and the lipid cubic phase (LCP), but both of these contain a very large detergent component, which can disrupt the stability and function of membrane proteins. To have confidence in the resulting structures and their biological functions and to avoid disrupting these delicate proteins, the structures of membrane proteins should be determined in their native environment of liquid crystalline phospholipid bilayers under physiological conditions.

This Account describes a recently developed general method for determining the structures of unmodified membrane proteins in phospholipid bilayers by solid-state NMR spectroscopy. Because it relies on the natural, rapid rotational diffusion of these proteins about the bilayer normal, this method is referred to as rotationally aligned (RA) solid-state NMR. This technique elaborates on oriented sample (OS) solid-state NMR, its complementary predecessor. These methods exploit the power of solid-state NMR, which enables researchers to obtain well-resolved spectra from "immobile" membrane proteins in phospholipid bilayers, to separate and measure frequencies that reflect orientations with respect to the bilayer normal, and to make complementary distance measurements. The determination of the structures of several membrane proteins, most prominently the chemokine receptor CXCR1, a 350-residue GPCR, has demonstrated this approach.

1. Introduction

The precisely folded polypeptide backbone of proteins provides the framework for juxtaposition of the side chain atoms and optimal alignment of their orbitals needed to lower the transition state energies, accelerate the reactions, and facilitate the binding to small molecule and macromolecule partners. The three-dimensional structures of proteins are determined by a combination of their sequence of amino acids and surrounding molecular environment. In the 1950s and 1960s, the major focus of attention in structural biology was on globular proteins, following the initial success of X-ray diffraction in

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determining the structures of enzymes,[1] as well as the "honorary" enzymes myoglobin[2] and hemoglobin.[3] It was assumed, and later verified by NMR spectroscopy, that these proteins have essentially the same structures in crystals and in aqueous solution. These initial examples of proteins were selected for structure determination based on a highly restricted set of criteria: their availability from large animals, their solubility in water without aggregation, and their ability to form crystals that yielded interpretable diffraction data from the primitive equipment available at the time. These same properties were essential to obtain the first NMR spectrum of a protein.[4]

To what extent the experience gained from globular proteins pertains to membrane proteins in phospholipid bilayers is an important question addressed in this Account. Anfinsen[5] noted that "The thermodynamic hypothesis states that the three-dimensional structure of a native protein in its normal physiological milieu (solvent, pH, ionic strength, presence of other components such as metal ions or prosthetic groups, temperature, and other) is the one in which the Gibbs free energy of the whole system is lowest; that is, that the native conformation is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given environment. In terms of natural selection of macromolecules during evolution, this idea emphasized that a protein molecule only makes stable, structural sense when it exists under conditions similar to those for which is was selected – the so-called physiological state."

The influence on protein structure of the totality of forces from the environment, in combination with the amino acid sequence of the polypeptide, is nowhere more evident than in biological membranes. The phospholipid bilayer, consisting primarily of hydrated phospholipids, provides an environment exquisitely tuned to support the structures and functions of membrane proteins and bears little resemblance to aqueous solution or crystals.

Amphipathic molecules formed primitive membranes in early living systems in order to maintain the proximity of interacting functional and information-bearing macromolecules, as well as to control the influx and efflux of metabolites from the cell. This is consistent with the hydrophobic effect, as noted by Tanford,[6] according to which "The thermodynamics of biological organization does not involve biosynthesis or other chemical transformations, but focuses solely on where molecules prefer to go after they have been synthesized." Consistent with this, hydrated protein-containing phospholipid bilayers are well-defined structures of biological membranes. The fluid mosaic model of Singer and Nicholson[7] (Figure 1) played a seminal role in organizing experimental and theoretical findings about biological membranes and laboratory-prepared proteoliposomes and illustrates the importance of determining the structure of membrane proteins in phospholipid bilayers.

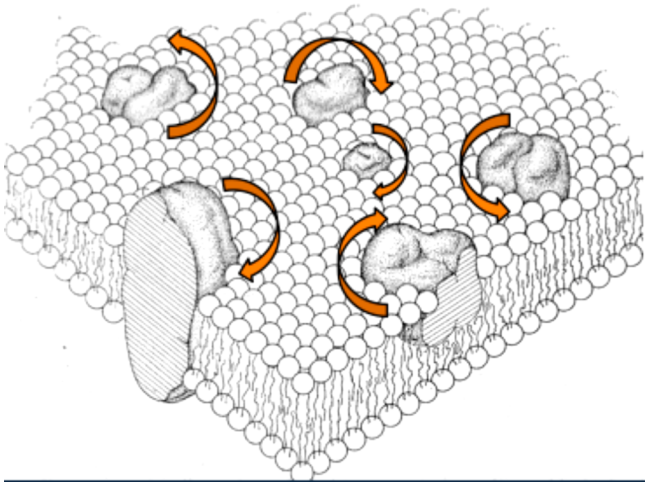

FIGURE 1. Schematic three-dimensional and cross-sectional views of the "fluid mosaic model" of globular membrane proteins that are completely or partially embedded within a lipid matrix. Modified from Singer and Nicholson.[7]

In earlier studies, artificial membrane mimics have often been used to solubilize membrane proteins, including organic solvents, micelles, isotropic and orientable bilayer discs (bicelles),[8] and the lipidic cubic phase[9] as a crystallization medium. With the development of solid-state NMR spectroscopy,[10,11] it is now possible to determine the structures of integral membrane proteins in proteoliposomes under near-native conditions.

The proteoliposome is well suited for solid-state NMR spectroscopy, enabling experimental descriptions of global and local structure and dynamics. However, this sample remains out of reach for other commonly used experimental methods. Although it has taken considerable time and effort to optimize sample preparation and tune the instrumentation and experimental methods for proteoliposome samples, the effort has paid off. Membrane proteins in proteoliposomes are now amenable to structure determination by solid-state NMR.[12,13,18]

Several membrane protein structures have been determined in liquid crystalline phospholipid bilayers by oriented sample (OS) solid-state NMR spectroscopy (e.g., refs 14 and 15). The approach applies the basic principles of single-crystal solid-state NMR in stationary samples to mechanically or magnetically aligned protein-containing phospholipid bilayers. Such samples[16] yield single-line resonances

whose frequencies reflect the angle between the principal value of an anisotropic nuclear spin interaction and a defined external axis. The latest iteration of the method, rotationally aligned (RA) solid-state NMR, combines features of magic angle spinning (MAS) of "powder" samples[17] and OS solid-state NMR spectroscopy. This establishes a completely general method of determining the atomic resolution structures of membrane proteins in liquid crystalline phospholipid bilayers under physiological conditions. The success of RA solid-state NMR has been demonstrated by the structure determination of the chemokine receptor, CXCR1, a 350-residue G-protein coupled receptor (GPCR) with seven transmembrane helices,[13] and truncated[12] and full-length versions of the bacterial mercury transporter MerF[18] with two transmembrane helices.

Methods that rely primarily on orientation-dependent structural restraints are of singular importance. Indeed, all structures of membrane proteins that have been determined to date in liquid crystalline phospholipid bilayers have utilized OS or RA solid-state NMR spectroscopy. Additional measurements, such as, intra- and intermolecular distances, are useful, and complementary binding and functional measurements can be made utilizing a wide variety of solid-state experiments on aligned and unoriented samples.

2. Comparison of Approaches for Protein Structure Determination

Crystallographic methods, applied to single crystals of proteins, were developed using globular proteins as the initial examples.[2,3] They also served as the initial examples for the development of solution NMR spectroscopy.[4]

In contrast, membrane proteins remain highly problematic for both X-ray crystallography and solution NMR spectroscopy. This reflects the differences between globular and membrane proteins; membrane proteins are hydrophobic and insoluble in water, are dominated by a single type of secondary structure, whether α-helix or β-sheet, and require a phospholipid bilayer environment. This makes crystallization impossible and halts the overall reorientation of the protein, making it behave like a solid rather than a liquid.

As a result, experimental structure determination of membrane proteins has lagged behind that of globular proteins in several important ways, particularly sample preparation and structural accuracy. In large part, this reflects the structural distortions that arise from the presence of detergents and other agents used in crystallography and from the highly curved nature of detergent micelles used in solution NMR spectroscopy. The presence of excess monomeric detergent molecules has the potential of destabilizing the extramembrane domains of membrane proteins. Crystallization has a greater potential for causing structural distortions in membrane proteins because of the presence of non-native environments. A recent review describes the many structural distortions observed for membrane proteins in artificial sample conditions.[19]

Finally, the substantial truncations and mutations introduced in membrane proteins to induce crystallization can produce significant alterations in structure, function, and dynamics.[18] For example, the addition of an entire foreign protein in the middle of a membrane protein polypeptide sequence has become a popular method of facilitating crystallization.[20]

Most protein samples are prepared by heterologous expression, and large changes in sequence can be readily introduced. In globular proteins studied by both X-ray crystallography and solution NMR spectroscopy, comparisons among mutated proteins have led to insights into protein structure, folding, and function that could not be obtained from studying wild-type proteins alone. Most single site mutations of proteins cause minor, but sometimes critical, changes in conformations and functions. Some larger alterations in sequence, such as truncations of mobile, unstructured N- and C-terminal segments are thought of as rather benign alterations that leave the basic structure and functions of a globular protein intact. However, we have found that these seemingly modest changes can cause dramatic rearrangements in the structure of membrane proteins.[18]

Recently it has become possible to determine the structures of membrane proteins in liquid crystalline phospholipid bilayers under physiological conditions through the use of solid-state NMR spectroscopy. References 12−15 and 18 show that incorporation of the protein in bilayer membranes avoids the distorting effects of non-native environments and also that the proteins adopt a single, stable conformation.

3. The Special Properties of the Phospholipid Bilayer Membrane

Most discussions about the inception of life following the formation of Earth about 4.5 billion years ago are focused on molecules that evolved into the polymers that store information and carry out chemical processes. However, the emergence of membrane-like barriers to separate these molecules from the larger, diverse environment was a prerequisite for the formation of self-replicating systems.

The prebiotic amphiphiles that assembled to form a barrier[21] may bear some resemblance to the amphiphilic compounds used for solubilization and crystallization of membrane proteins. Subsequently the coevolution of proteins and phospholipid was required to arrive at contemporary biological membranes with their many unique functions. For the purposes of structure determination, prebiotic amphiphiles provide a starting point in the search for detergents capable of solubilizing hydrophobic membrane proteins. Simply put, both X-ray crystallography[22] and solution NMR spectroscopy[23] experiments on samples that utilize detergents rather than phospholipids are several billion years behind the times.

Phospholipids found in biological membranes consist of an amphipathic or charged polar headgroup attached to two long hydrocarbon chains through a trisubstituted glycerol backbone. A wide range of head groups, chain lengths, number of double bonds, and other characteristics are found in various organisms and organelles. In water, phospholipids have negligible concentrations due to their insolubility and, instead, self-assemble to form extended bilayer membranes, with the polar head groups exposed to water and the hydrophobic hydrocarbon chains facing the membrane interior. On the molecular scale, these bilayers are infinitely large in two dimensions, but only two molecules thick in the third dimension, as illustrated in Figure 1. Intramolecular motions add to the complexity of the membrane; in addition to their definitive gel to liquid crystalline phase transitions, different lipids have different gradients of motion from the surface to the center of the bilayer.[24] Both the phospholipids and the proteins undergo fast rotation diffusion about the bilayer normal, as illustrated schematically in Figure 1, as well as translational diffusion in the plane of the bilayer.

4. Structure Determination of Membrane Proteins by Solid-State NMR

Particularly relevant to membrane proteins is NMR's strict dependence on time scales of molecular motions. This enables high-resolution solid-state NMR to be used to decouple, recouple, and otherwise manipulate the nuclear spins of the membrane proteins. In solid-state NMR, the relevant time scales are determined by the frequency breadths of the static powder patterns associated with the anisotropic chemical shift and dipolar coupling interactions. Broadly speaking, large-amplitude motions that occur more rapidly than $\sim 10^6$ Hz cause substantial motional averaging, and those that occur less frequently than $\sim 10^2$ Hz do not affect the spectra.

OS solid-state NMR has the principal advantage of deriving orientation restraints directly from the measurement of frequencies of single-line resonances.[25,26] The restraints are used both for determining the protein structure and for precisely characterizing its orientation relative to the direction of mechanical alignment, the applied magnetic field, or the normal of the lipid bilayer membrane, depending on the sample and the experiment being performed. With this background, it is readily understood how uniaxially aligned samples can be analyzed in the same framework as single crystal samples.

The evolution of RA solid-state NMR from the methods of OS and MAS solid-state NMR depends on a crucial feature of membrane proteins: they undergo fast rotational diffusion about the bilayer normal in liquid crystalline bilayers. RA solid-state NMR eliminates the need for macroscopic sample alignment and improves the speed, accuracy, and precision of membrane protein structure determination. Among the advantages over conventional OS solid-state NMR, the combination of methods enables the application of triple-resonance experiments to uniformly $^{13}C/^{15}N$-labeled samples, which improves the sensitivity of the measurements through the use of ^{13}C (instead of ^{15}N) detection, permits the implementation of systematic assignment schemes among proximate sites, and provides signals from essentially all backbone and side chain sites for complete three-dimensional structure determinations.

Mechanical rotation of the sample about the magic angle relative to the magnetic field averages out the interfering $^{13}C-^{13}C$ homonuclear couplings in fully $^{13}C/^{15}N$-labeled proteins. Through the use of the appropriate recoupling pulse sequences,[27−30] it is possible to measure the orientation-dependent frequencies from the motionally averaged powder patterns for the $^1H-^{13}C\alpha$ and $^1H-^{15}N$ amide heteronuclear dipolar couplings (DC), as well as the $^{13}C\alpha$ (CA), $^{13}C'$ (CO), and ^{15}N (N) amide chemical shift anisotropies (CSA). Complementary information about the dynamics of backbone and side chain sites is also available. Following their initial demonstrations in the development of high-resolution solid-state NMR of proteins, MAS solid-state NMR and OS solid-state NMR methods have progressed, for the most part, along separate paths. They are reunited and fully integrated through the rotational alignment that results from the rapid diffusion of membrane proteins around the bilayer normal.

The unification of the methods is represented by the equivalence between the single-line chemical shift frequency observed from a uniaxially oriented bilayer sample

and the parallel edge of a rotationally averaged powder pattern from an unoriented bilayer sample.[31] McLaughlin and co-workers first demonstrated this property in phospholipids using ^{31}P NMR.[32] Similarly, Griffin and co-workers demonstrated that ^{13}C′-labeled bacteriorhodopsin in unoriented phospholipid bilayers undergoes rotational diffusion around the bilayer normal and that lowering the sample temperature slows the diffusion sufficiently to enable the static powder pattern to be observed.[33] We obtained analogous results for a transmembrane helical polypeptide labeled with ^{15}N at a single backbone amide site.[31] In combination with the experiments of McLaughlin and Griffin, these results demonstrate that a general feature of both the phospholipids and proteins is that they undergo rotational diffusion around the bilayer normal fast enough (>10^6 Hz) to motionally average the static CSA and DC powder patterns to axially symmetric powder patterns with reduced frequency spans. Motional averaging of powder patterns was observed in some of the earliest solid-state NMR experiments.[34,35] In principle, the same experiment is repeated for the resolved sites of isotopically labeled membrane proteins; the sign and breadth of the powder patterns from individual sites reflect the angle between the principal axis of the relevant spin-interaction tensor and the bilayer normal.[31]

5. Structure Determination of the Mercury Transporter MerF

We have determined the structures of both MerFt,[12] a 60-residue truncated construct corresponding to the integral membrane core of the protein, and MerF,[18] the full length 81-residue protein, in several lipid environments, including phospholipid bilayers, enabling useful comparisons to be made.

In structure determinations of membrane proteins by RA solid-state NMR, it is essential to verify that the protein undergoes rapid rotational diffusion about the bilayer normal and that this rotation can be switched between the slow and fast limits by changing the temperature (typically between 10 and 25 °C in protein-containing DMPC bilayers). As shown in Figure 2, we monitored the effect of temperature on the ^{13}C′ CSA powder pattern of uniformly ^{13}C/^{15}N-labeled MerFt in DMPC proteoliposomes.[12] This powder pattern is particularly convenient for demonstrating that a helical membrane protein is undergoing fast rotational diffusion, since the static powder pattern is highly asymmetric with a large frequency span. For transmembrane helices a large fraction of the CO bonds are approximately parallel to the bilayer normal (i.e., the axis of motional averaging); thus, when the protein undergoes fast rotational diffusion, the ^{13}C′ powder pattern becomes axially symmetric and is significantly narrowed. MerFt in DMPC bilayers undergoes fast rotational diffusion at temperatures above ~17 °C. As a result, the ^{13}C′ powder pattern obtained at 25 °C for uniformly ^{13}C/^{15}N-labeled MerFt in liposomes is fully rotationally averaged (Figure 2C), while the powder pattern obtained at 10 °C spans the full width of the static spin interaction with no evidence of motional averaging (Figure 2D). These results verify that the sample is well behaved for structural studies by RA solid-state NMR.

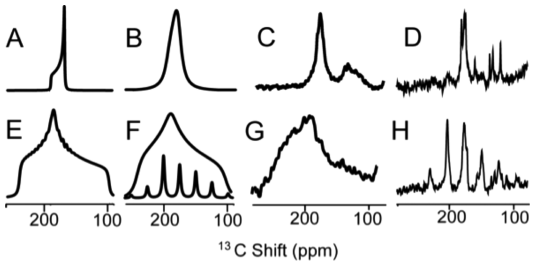

FIGURE 2. ^{13}C solid-state NMR spectra of uniformly ^{13}C/^{15}N-labeled MerFt in proteoliposomes. The majority of the resonance intensity shown in the expanded region, centered near 175 ppm, is from ^{13}C′ backbone sites. The spectra in panels C and G were obtained at 25 °C where the protein undergoes fast rotational diffusion. The spectra in panels D and H were obtained at 10 °C where the protein is immobile on the time scale of a static ^{13}C′ chemical shift powder pattern (~10^5 Hz). The simulated spectra are for a single ^{13}C′ group in a transmembrane helix aligned approximately parallel to the lipid bilayer normal. Panels B and F are spectra for a static protein. Panels A and E are spectra for a protein undergoing fast rotational diffusion about the bilayer normal. The experimental spectra in G and H were obtained from samples undergoing slow (5 kHz) magic angle spinning. The corresponding simulated spectra in E and F confirm that a family of sidebands is observable in the absence of rotational diffusion of the membrane protein.

Because of the limited resolution among resonances in helical membrane proteins, multidimensional multiple-resonance experiments are routinely employed. Typically, the first set of MAS solid-state NMR experiments is used to obtain two-dimensional ^{13}C/^{13}C dilute-spin-exchange and ^{13}C/^{15}N heteronuclear correlation spectra. As shown in Figure 3A, some resonances can be resolved in a two-dimensional spectrum of a relatively small membrane protein with two transmembrane helices. More typically, the initial two-dimensional spectra confirm the integrity and sensitivity of the samples and serve as building blocks for the families of three-dimensional spectra used to make resonance assignments and the measurements of rotationally averaged powder patterns that provide the orientation-dependent frequencies for calculation of the protein structure.

Complete ^{13}C/^{15}N labeling of a protein enables direct transfers of magnetization through dipole−dipole couplings

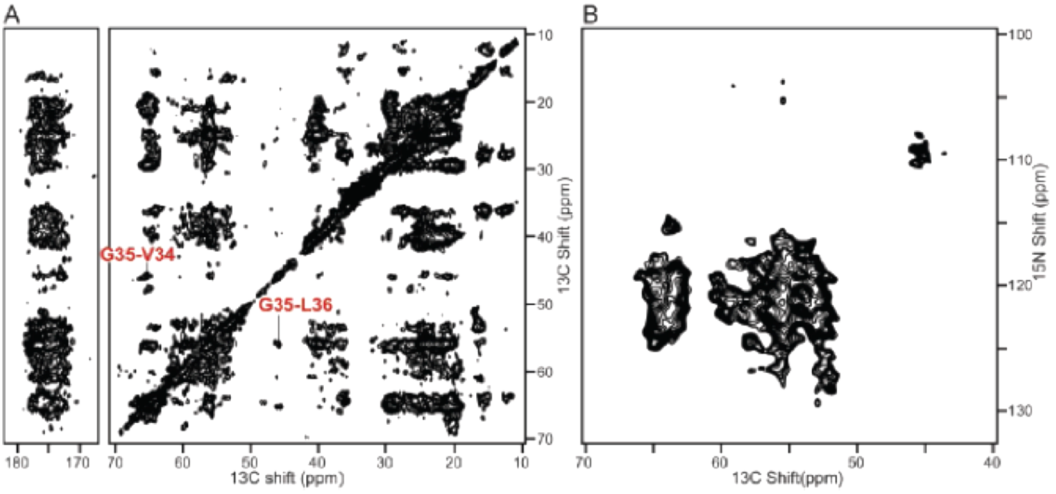

FIGURE 3. Two-dimensional magic angle spinning spectra of uniformly $^{13}C/^{15}N$-labeled MerFt in DMPC proteoliposomes at 10 °C. (A) $^{13}C/^{13}C$ homonuclear spin-exchange correlation spectrum. (B) $^{13}C/^{15}N$ heteronuclear correlation spectrum. The experiments were performed at 750 MHz under conditions of 11.11 kHz MAS.

in the backbone. This provides a mechanism for correlating the isotropic chemical shift frequencies of directly bonded ^{13}C and ^{15}N nuclei. Backbone walks that involve the correlation of inter- and intraresidue resonances are not currently feasible on static, aligned samples because of interference from the network of $^{13}C-^{13}C$ homonuclear DCs. However, in MAS solid-state NMR experiments, it is feasible to assign the backbone resonances of a membrane protein in unoriented proteoliposomes by utilizing triple-resonance experiments. The protein does not have to undergo fast rotational diffusion for this step, and the sensitivity is generally improved at lower temperatures because of more favorable relaxation times.

The rotationally averaged $^{1}H-^{15}N$ DC and ^{15}N CSA powder patterns associated with each isotropic resonance are characterized with three-dimensional MAS solid-state NMR recoupling experiments. The two-dimensional $^{1}H-^{15}N$ DC/^{13}C shift separated local field (SLF) spectrum shown in Figure 4A contains resonances from all of the MerFt ^{13}CA sites. Although there is considerable spectral overlap, an individual resonance assigned to Leu31 can be identified as marked in the figure. Figure 4B,C contains two-dimensional $^{1}H-^{15}N$ DC/^{13}C shift SLF planes selected from a three-dimensional spectrum at the ^{15}N shift frequency corresponding to Leu31. $^{1}H-^{13}C$ heteronuclear DCs were measured in similar three-dimensional experiments.

Since we previously verified that the measurement of orientation-dependent frequencies from the parallel edges of the recoupled, motionally averaged, axially symmetric powder patterns are identical to those measured from the single line resonances observed in aligned samples,[31] the measured spectroscopic parameters could be used to calculate the three-dimensional structure of MerFt. The computational methods used to determine protein structures from orientation restraints obtained from OS solid-state NMR experiments with stationary, aligned samples are equally applicable to the frequencies measured from the edges of the rotationally averaged powder patterns. The $^{1}H-^{15}N$ and $^{1}H-^{13}CA$ DC restraints and ^{15}N CSA restraints are sufficient to determine the orientation of the associated peptide plane relative to the axis of alignment and yield the three-dimensional structure of the protein.

We have determined the high-resolution structure of the full-length 81-residue mercury transporter,[18] MerF, which is shown in Figure 5. This enables the structure of the full-length protein to be compared with the previously studied N- and C-terminal truncated 60-residue version, MerFt, whose structure has been determined in micelles, bicelles, and bilayers. For direct comparison, samples of both the truncated and full-length forms of MerF have been prepared as protein-containing liquid crystalline phospholipid bilayers. The structure of the full-length protein reveals large conformational differences compared with the truncated protein. The N-terminal truncation results in a nearly 90° shift in the helix axis at a distance that is ~10 residues away from the truncation site (Figure 6). Relatively small distortions near the truncation site would not be surprising. Instead, this structure serves as an example where a seemingly innocuous truncation of N- and C-terminal residues shown to be mobile and unstructured in micelles has an enormous effect on a membrane protein. This suggests that caution be employed in the use of truncated membrane proteins for structural analysis.

The structure of full-length MerF shows that the two mercury-binding sites are both located on the

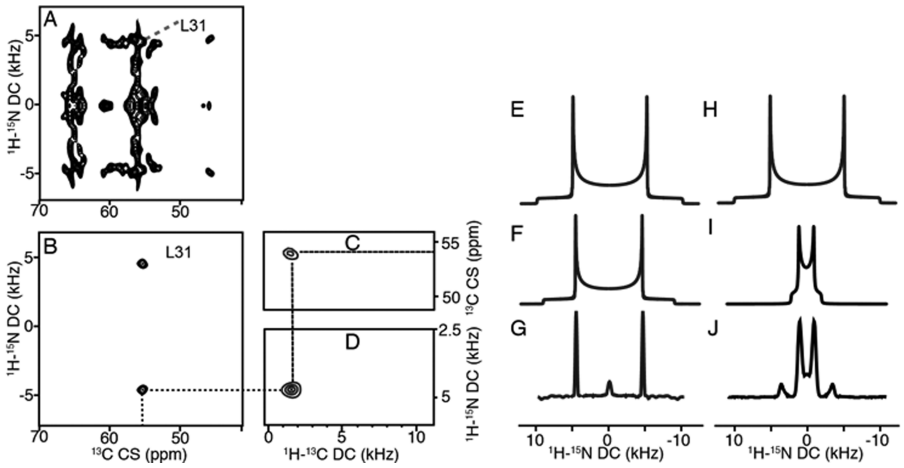

FIGURE 4. Examples of spectroscopic data for residue L31 obtained from two- and three-dimensional NMR spectra under the same conditions as in Figure 3 except at 25 °C. (A) Two-dimensional ^{1}H−^{15}N DC/^{13}C shift SLF spectrum. (B) Two-dimensional ^{1}H−^{15}N DC/^{13}C shift SLF spectral plane from a three-dimensional spectrum selected at the ^{15}N isotropic chemical shift frequency of 118.6 ppm. (C) ^{1}H−^{13}C DC/^{13}C shift plane obtained at the same ^{15}N isotropic chemical shift frequency of 118.6 ppm as panel B. (D) ^{1}H−^{13}C DC/^{1}H−^{15}N DC correlation plane obtained at a ^{13}C shift frequency of 54.6 ppm. All three spectral planes are associated with residue L31; the dashed line traces the correlation among the resonance frequencies. (E, F, H, I) Simulated Pake-doublets for a static peptide bond. (E). Rigid powder pattern for ^{1}H−^{15}N dipolar coupling constant of 10.5 kHz. (F) Motionally averaged powder pattern for ^{1}H−^{15}N dipolar coupling constant of 9.4 kHz. (G) Experimental one-dimensional ^{1}H−^{15}N dipolar slice obtained from three-dimensional SLF experiment for ^{13}C and ^{15}N shifts at 54.8 and 118.6 ppm, respectively. (H) Rigid powder pattern for ^{1}H−^{13}C$_{\alpha}$ dipolar coupling constant of 22.68 kHz. (I) Motionally averaged powder pattern for ^{1}H−^{13}C$_{\alpha}$ dipolar coupling constant of 7.6 kHz. (J) Experimental one-dimensional ^{1}H−^{13}C dipolar slice obtained from three-dimensional SLF experiment for ^{13}C and ^{15}N shifts at 54.8 ppm and 118.6 ppm respectively.

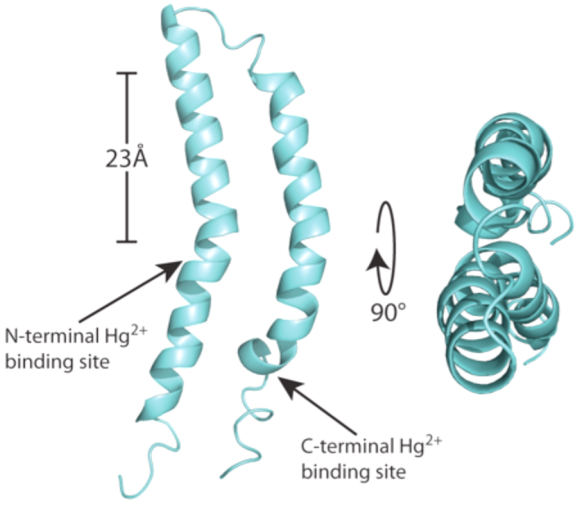

FIGURE 5. Ribbon drawing of the three-dimensional structure of MerF. The two proximal mercury-binding sites are labeled with arrows. The scale bar corresponds to the 23 A thickness of the hydrocarbons in the lipid bilayers. Reproduced from ref 18. Copyright 2013 American Chemical Society.

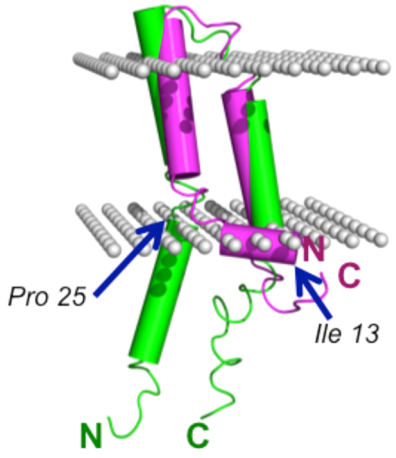

FIGURE 6. The large conformational rearrangement of the N-terminal domain between the truncated (MerFt) construct (in magenta color) and the full-length (MerF) construct (in green color). The MerFt structure and MerF structure are aligned on the transmembrane region, and the N-terminal domains between the two structures show an orientation difference of close to 90°. Reproduced from ref 18. Copyright 2013 American Chemical Society.

cytoplasmic side of the protein, and their close proximity suggests possible contact between them. This is the first information directly pertinent to describing the mechanism for transporting Hg(II) across the membrane bilayer to the enzyme mercuric reductase, which reduces it to Hg(0).

6. Structure Determination of the Chemokine Receptor CXCR1

The method of RA solid-state NMR was used to determine the three-dimensional structure of CXCR1, a class-A G-protein coupled receptor (GPCR).[13] This is the only structure of this chemokine receptor; it is a member of the largest

class of integral membrane proteins responsible for cellular signal transduction and the largest class of drug receptors. When the chemokine interleukin-8 (IL-8) is released, its binding to CXCR1 triggers intracellular signaling pathways that result in neutrophil migration to the site of inflammation.[36] Despite its importance, the molecular mechanism of CXCR1, like that of other chemokine receptors, is poorly understood. This is due in large part to the limited structural information that is available.

Previous studies suggest an activation mechanism that involves N-terminal residues and extracellular loops of CXCR1. Our previous NMR studies[37] provided additional evidence for such a two-site extracellular binding mechanism and also indicated that IL-8 binding to the receptor's N-terminus is mediated by the membrane, underscoring the importance of the phospholipid bilayer. The NMR structure of CXCR1 has many similarities but some significant differences when compared with the crystal structure of CXCR4.[38]

After verifying that CXCR1 in proteoliposomes undergoes rapid rotational diffusion about the bilayer normal, the majority of data used to resolve, assign, and measure isotropic chemical shift frequencies from N, Cα, C′, and Cβ sites of the structured residues of CXCR1 were obtained from ^{13}C-detected three-dimensional, triple-resonance experiments. A total of 97% of the backbone resonances for residues 20−325 of CXCR1 were assigned. The ^{15}N and ^{13}C signals from the mobile N- and C-termini (residues 1−19 and 326−350) are missing from the spectra, which is consistent with our observation of the corresponding signals in solid-state NMR experiments designed to detect only signals from mobile sites, as well as our previous analysis of local and global motions by CXCR1. Three-dimensional ^{13}C-detected SLF experiments were used to measure the ^{1}H−^{15}N DC and ^{1}H−^{13}Cα DC frequencies that provide orientation restraints for structure determination. The protein backbone structure was calculated by a molecular fragment replacement approach. An initial structural model was generated using a set of molecular fragments generated with CS-Rosetta. This initial model was refined first with the experimental restraints using the implicit membrane potential of Rosetta and then by restrained simulated annealing using Xplor-NIH.

The three-dimensional structure of CXCR1 shown in Figure 7 has the consensus fold of a GPCR, with seven transmembrane helices connected by three extracellular loops (ECL1−ECL3) and three intracellular loops (ICL1−ICL3). The experimentally measured ^{1}H−^{15}N DC and ^{1}H−^{13}Cα DC values correlate remarkably well with those calculated from the refined protein structure.

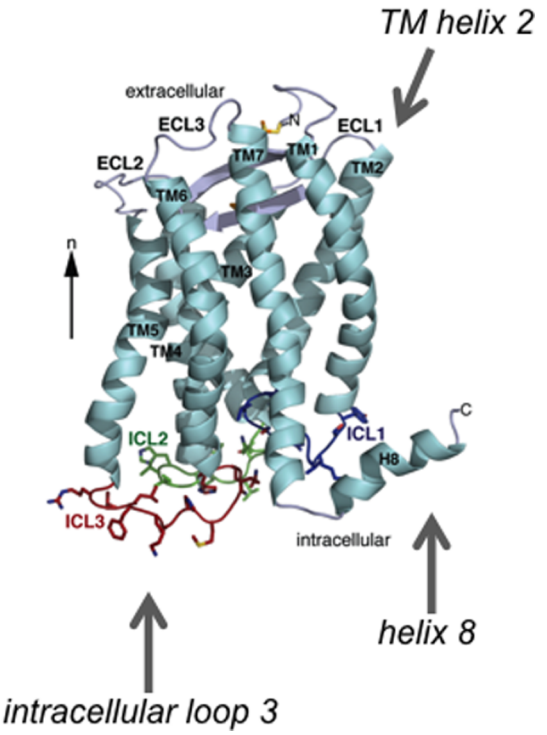

FIGURE 7. Three-dimensional structure of CXCR1 in phospholipid bilayers under physiological conditions. Backbone representation of CXCR1 showing helices (TM1−TM7 and H8) in aqua, extracellular loops (ECL1−ECL3) in gray, and intracellular loops in blue (ICL1), green (ICL2), and red (ICL3). Note that ICL3, which is essential for activity, has a well-defined tertiary structure. Disulfide bonded cysteine pairs (C30−C277; C110−C187) are shown as sticks.

The CXCR1 helices are well-defined by the spectroscopic data. The intracellular loops of GPCRs are critical for G-protein interactions. Our sample of CXCR1 is fully active with respect to both G-protein activation and chemokine binding, and its three intracellular loops are structurally well-defined. Notably, ICL3, which is important for CXCR1 coupling to G-proteins and involved in calcium mobilization, chemokine-mediated migration, and cell adhesion, extends from Thr228 to Gln236, connecting helices TM5 and TM6, which are both one turn shorter than the corresponding helices in CXCR4. ICL3 protrudes into the cytoplasm where it is available for G-protein binding.

7. Summary of New Findings from Studying Membrane Proteins in Phospholipid Bilayers

As the accuracy and precision of the structures determined by solid-state NMR spectroscopy improve, the perturbations caused by removal of the protein from the bilayer environment, nonphysiological conditions, and major alterations to

the protein sequences become more apparent.[19] The stage is set for solid-state NMR to become the dominant method for describing membrane proteins. Already, the membrane protein structures determined by NMR in phospholipid bilayers are the "gold standard" for comparisons to structures determined in other environments and by other methods.

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BIOGRAPHICAL INFORMATION

Stanley Opella obtained his doctoral degree in 1974 with O. Jardetzky and H. M. McConnell at Stanford University. He was a postdoctoral fellow in the laboratory of J. S. Waugh at MIT in 1975 and 1976. He joined the faculty of the Department of Chemistry at the University of Pennsylvania in 1976. He moved to the University of California, San Diego, in 2000 where he is Distinguished Professor of Chemistry and Biochemistry. His research aims to develop and apply NMR spectroscopy to the study of proteins in supramolecular complexes, especially membranes.

FOOTNOTES

The authors declare no competing financial interest.

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