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"Solid-State NMR of Membrane Proteins"

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These are preliminary lecture notes, intended only for distribution to participants.

Summary of Lectures by Stanley J. Opella on Solid-State NMR of Membrane Proteins

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Currently used methods in structural biology are able to address only a small portion of the biological functions and structures within the cell. This is significant, because the great success of the two commonly used methods of determining protein structures, x-ray crystallography and multidimensional solution NMR spectroscopy, has resulted in so much attention being focused on the soluble, globular proteins of the cytoplasm and periplasm. Even though 30 – 40 % of the expressed proteins found in the complete genomes that are now available are membrane proteins, only a very small percentage of the proteins in the Protein Data Bank are from this class, probably around 0.1%. The few membrane proteins that have been crystallized from detergent solutions have tended to be large complexes that already exist as highly ordered arrays in the cell membranes, for example the photosynthetic reaction center (Garavito et al, 1996). These structures are quite valuable in showing the principal structural features of membrane proteins, which are likely to also exist in the smaller proteins of greatest interest in biomedical research.

A general approach to NMR studies of membrane proteins was presented (Opella, 1997). This approach recognizes the current limitations in the methods of structural biology, in particular the need to study relatively small, tractable examples of membrane proteins to facilitate the development of new methods and to explore the structure and dynamics of these proteins at atomic resolution and the complete absence of crystallographic results on this class of proteins. Therefore, a range of membrane peptides and proteins with between about 25 and 200 residues are being investigated with parallel studies of micelle and bilayer samples. In both cases the correlation time problem must be confronted directly. With careful sample preparation micelles can be prepared that give reasonable quality multidimensional solution NMR data, but the correlation times are always relatively slow compared to equivalent globular proteins. More significantly, proteins in bilayers are completely immobilized on NMR timescales (milliseconds) and give essentially no signals in conventional solution NMR experiments. Fortunately, high resolution solid-state NMR spectroscopy was developed in the late 1960s and early 1970s for studies of single and poly-crystalline materials, and these same methods are well suited for immobile proteins in biological supramolecular structures such as membranes.

The first major step in solid-state NMR spectroscopy is represented by the WaHuHa multiple-pulse experiment developed by Waugh, Huber, and Haeberlen (1968) at M.I.T. This four pulse sequence designed using the principles of coherent averaging theory narrows resonances broadened by homonuclear dipolar interactions, most importantly among the highly abundant 1H spins in a sample, while preserving the chemical shift differences among the resonances. This experiment had an enormous influence on the field, since it showed for the first time that it was possible to do high resolution NMR experiments on solid samples. However, because of the low magnetic fields available at the time, there were relatively few practical uses for multiple-pulse NMR in chemical and biochemical applications.

In the mid-1970s higher field magnets were becoming available and 13C NMR experiments were becoming of increasingly important in solution NMR spectroscopy. In this context, the second major breakthrough in solid-state NMR spectroscopy was also developed in the Waugh laboratory at M.I.T. (Pines et al, 1973). Double-resonance methods were used to obtain dilute-spin (13C, 15N) spectra with high sensitivity by cross-polarization from the abundant 1H spins, and high resolution was obtained through acquisition of the dilute-spin free induction decays under conditions of 1H decoupling.

Both the homonuclear multiple-pulse and heteronuclear double-resonance experiments yielded high resolution solid-state NMR spectra where the resonance properties were determined solely by the chemical shift interaction. For a number of practical reasons, not the least of which is the difficulty of observing signals during the "windows" of a multiple-pulse sequence, the double-resonance methods have been much more popular among chemists and biochemists. A slide was shown illustrating the dramatic effects on the broad spectra of a model 15N labeled peptide. 1H decoupling alone revealed the 15N chemical shift powder pattern of the amide site, which has a frequency span of about 170 ppm (Wu et al, 1995). An additional step is needed to obtain high resolution among individual atomic sites. Magic angle sample spinning allows the use of powder samples, giving a single narrow line at the isotropic resonance position. This has been widely employed for analysis of chemicals, polymers, and other materials. In biochemical applications some of the information averaged by the magic angle spinning needs to be retained by the addition of extra pulses in the sequences that interfere with the averaging of all spatial and angular

information. This is an active area of research (Smith, 1993). High resolution spectra can be obtained with a second type of sample manipulation, and that is sample orientation. The best known and characterized way to orient samples is to form single crystals. However, a single axis of orientation, when parallel to the direction of the applied magnetic field, is sufficient to yield single line resonances from all sites in a polymer, including uniformly isotopically labeled proteins. Fortunately, membrane proteins can be readily oriented by reconstituting them into lipid bilayers and placing them onto thin glass slides. The proteins are completely immobilized by their interactions with the phospholipids in multilayers and can be highly oriented, with linewidths similar to those observed in single crystals of model peptides (Marassi et al, 1997).

The chemical shift interaction is anisotropic, since the observed resonance frequency varies with the orientation of the site with respect to the direction of the applied magnetic field. The breadth of the powder pattern shows the full range of available chemical shift frequencies, while its shape represents a statistical average of all orientations present in the disorder sample. All of the intensity is concentrated into a single resonance frequency in an oriented sample, which contributes to a large gain in sensitivity with these samples.

In relatively small peptides and proteins (25 – 50 residues) it is possible to prepare samples with only one or a few labeled sites. Because of the characteristic orientations of the principal elements of the amide 15N chemical shift tensor, qualitative descriptions of the arrangements of helical segments in the bilayers can be made. For example, once it is established that the secondary structure is helical, then the 15N shift frequency can be diagnostic if the helix axis is trans-membrane or in the plane of the bilayer. As the number of labeled sites increases, the one-dimensional chemical shift spectra of oriented proteins in bilayers become more complex. In some cases, it is possible to identify substantial intensity associated with trans-membrane and in-plane helical components, but more generally there are resonances with frequencies over the full range of the powder pattern from loops and other types of structures.

The use of uniformly isotopically labeled samples is an essential step in the development of a general method for determining the structures of membrane proteins by solid-state NMR spectroscopy. Thus, higher dimensional spectroscopy is needed to obtain the necessary resolution,

and the additional frequency measurements used to actually determined the orientations of the peptide planes relative to the direction of the applied magnetic field, and the plane of the bilayer. Uniform 15N labeling of proteins is particularly advantageous because this single nucleus has three associated spin-interactions, including the 1H chemical shift, 1H-15N heteronuclear dipolar coupling, and 15N chemical shift. Further, since each 15N is located in the peptide linkages of the protein backbone separated from each other by two carbon atoms, it maintains its basic characteristics as a dilute-spin nucleus without strong homonuclear couplings.

The separated local field experiment, also developed at M.I.T, separates the frequencies of the chemical shift and heteronuclear dipolar spin interactions along two separate axes in two-dimensional spectra (Waugh, 1976). This is extremely valuable, increasing resolution and giving direct measurements of two orientationally dependent frequencies for each amide site. The PISEMA (polarization inversion spin exchange at the magic angle) experiment is a high resolution version of the original separated local field experiment (Wu et al, 1994). While the linewidths in the chemical shift dimension remain the same, approximately 3 ppm in a well oriented protein sample, in both experiments, there is a dramatic decrease in the linewidths in the dipolar dimension. This decrease in linewidth reflects the ability of the flip-flop Lee-Goldburg pulse sequence (Lee and Goldberg, 1965) to decouple the hydrogens from each other.

PISEMA spectra of uniformly 15N labeled membrane proteins with 200 residues show many resolved resonances. However, they still have overlapping regions, especially where the N-H bond vectors are perpendicular to the direction of the applied magnetic field. Three-dimensional solid-state NMR correlation experiments provide a large boost in resolution by using the 1H chemical shift as a third frequency axis (Ramamoorthy et al, 1995). The initial results are very promising and we expect to be able to completely resolve the spectra off uniformly 15N labeled membrane proteins with more than 200 residues using the current generation of the NMR spectrometers with magnets corresponding to a 1H resonance frequency of 700 MHz. We anticipate that substantially larger membrane proteins can be studied with the next generation of magnets with 1H resonance frequency of 900 MHz.

Since hundreds of resolved resonances can be observed in the spectra of uniformly 15N labeled membrane proteins, the next essential step is to be able assign them to specific residues in the protein. Several different assignment methods are being developed. Short-range distances between nearby 1H sites as well as between nearby 15N sites can be detected with homonuclear spin-exchange experiments, analogous to NOE experiments used in solution NMR spectroscopy. For helical proteins, this offers the possibility of assigning sequentially up the backbone from a known starting location. More general approaches, involving uniform labeling with 13C and 15N offer the possibility for assignment through directly bonded atoms in the protein backbone.

Micelle samples have several important roles in structural studies of membrane proteins. One is that it is possible to determine the structures of smaller membrane proteins in micelles by solution NMR methods, providing much needed structural information (Almeida and Opella, 1997). Further, these structures can then be used to check the validity of the initial structures being determined by solid-state NMR spectroscopy. This is significant, because we do not have crystal structures of these molecules available to check the results.

Even though the proteins being investigated by solution NMR methods are relatively small with 25 – 125 residues, they present spectroscopic problems because of their relatively slow correlation times in the micelle environments. It has been necessary to prepare peptides with as few as 25 residues by expression for uniform labeling with 15N and double labeling with 13C and 15N for solution NMR studies in micelles. And proteins larger than about 50 residues require extensive uniform 2H labeling as well. The main problem is making resonance assignments with triple-resonance methods when the relaxation times are very short.

The best characterized example of a membrane protein structure determined in a micelle is the fd coat protein (Almeida and Opella, 1997). The protein was shown to have a hydrophobic trans-membrane helix and an in-plane amphipathic helix separated by a loop region with complex structural and dynamic properties. The loop regions between helices in membrane proteins are problematic in all of the methods, since they tend to have very high temperature factors in the few x-ray crystal structures available. In the case of fd coat protein, the structural studies were

supplemented with relaxation measurements which corroborated the finding of flexible regions in the loop and terminal regions of the protein.

The loop between the two helices in fd coat protein provides an outstanding opportunity to investigate the roles of molecular dynamics in biological functions, since this protein has a well established function of assembling into virus particles as it is extruded from the cell membrane. The secondary structure of the protein changes little, but there is a major rearrangment of the helices with the short amphipathic helix going from an orientation approximately perpendicular to the hydrophobic helix to one nearly parallel to it, forming a continuous helix throughout the length of the protein. Solid-state NMR studies of the coat protein in virus particles and on membrane bilayers show this structural rearrangement in striking detail.

The M2 segments of ion channel proteins such as the acetylcholine receptor are important model systems for the development and application of NMR spectroscopy to membrane proteins. These 25 residue peptides with the same sequences as found in the conserved segments of the intact proteins self-assemble in membrane bilayers and function as ion channels. We have determined the structures of the peptides in micelles by solution NMR spectroscopy and in bilayers by solid-state NMR spectroscopy. The comparisons show the peptide to have the same structure in both membrane environements. However, only the solid-state NMR data are able to show that the peptide is tilted in the membrane bilayers (Opella et al, 1997).

NMR studies of membrane proteins are proceeding at a rapid pace. The main area in need of further development is general assignment procedures. The initial results show that it will be possible to resolve, and potentially, determine the structure of proteins with 200 or more residues. As more examples are completed, solid-state NMR of oriented systems will establish itself as a third independent method of protein structure determination alongside x-ray crystallography and multidimensional solution NMR spectroscopy.

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