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Isotopic Labeling in Solid-state NMR of Proteins

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Introduction

Isotopic labeling has played a crucial role in the development of solid-state NMR spectroscopy as a method for determining the structures of proteins that reside in membranes, aggregates, or other types of assemblies. With the realization that one-third of the protein sequences in a genome correspond to intrinsic membrane proteins, there has been a surge of interest in the further development and routine application of solid-state NMR to membrane proteins. Oriented sample methods are particularly well-suited for membrane proteins because of the highly asymmetric properties of phospholipid bilayers.[1]

The availability of a wide variety of labeled amino acids, carbon and nitrogen sources, as well as bacterial growth media are essential for the preparation of samples of isotopically labeled proteins. There are three main approaches to isotopic labeling of proteins: specific, selective, and uniform labeling, and all are used in solid-state NMR studies of proteins. Most of the effort has been focused on structure determination; however, these same samples can be used to characterize the dynamics of backbone and side chain sites. An important advantage of NMR spectroscopy over other methods of studying proteins is its ability to describe both the structure and motions of individual sites.

Specific vs. Selective Isotopic Labeling

Specific isotopic labeling refers to the placement of a single label (e.g., ^{2}H, ^{13}C, or ^{15}N) in a specified location in a polypeptide. Nearly always, specific labeling is accomplished by incorporating a labeled amino acid through chemical synthesis, which restricts this approach to relatively small polypeptides, although recent advances in synthetic methods have made it possible to specifically label an 81-residue membrane protein for NMR studies.[2] In favorable cases, specific labeling can be done biosynthetically if the expressed protein sequence contains only a single copy of an amino acid, and, in some cases, it can be arranged by site-directed mutagenesis. Generally, specific labeling is employed in situations where the spectroscopic experiments provide very limited or no opportunities for resolution among sites. In the initial stages of development of solid-state NMR of proteins, isotopic labeling had to carry most of the burden of the experiments. For example, a single ^{15}N label at a specific site provides the sensitivity needed to observe signals, distinguishes the signals from background, assigns the resonance to a specific site in the protein, and provides the spin-interactions that are the sources of the frequencies and other spectroscopic parameters that are measured.

The initial application of solid-state NMR to an aligned protein was performed by biosynthetically labeling the indole nitrogen of the sole tryptophan sidechain in the protein with ^{15}N.[3] This enabled the ^{15}N NMR spectrum of an unoriented powder sample to be used to verify that the site did not undergo motional averaging on the NMR timescales and, by comparison, to demonstrate the dramatic spectral

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simplifications that enabled the direct measurement of frequencies and splittings that provide orientation constraints as input for structure determination. The most comprehensive study performed with specific labeling through chemical synthesis is the structure determination of the ion channel peptide gramicidin in phospholipids bilayers.[4]

Nevertheless, while specific labeling was crucial in the initial stages of development, and remains useful as a tool, it is inefficient for comprehensive studies because of the requirement for the separate preparation of many samples. As the instrumentation and experimental methods have become more powerful, it has become feasible to utilize samples where the proteins are labeled in multiple sites.

Selective labeling refers to the biosynthetic incorporation of a single type of labeled amino acid with all of the others unlabeled. For example, if a protein has eleven leucines, then only those eleven residues will be labeled, and the labels provide sensitivity and improve resolution. As with specific labeling, the low natural background, so detrimental to the sensitivity of ^{13}C and ^{15}N, became an advantage because signals from only the labeled sites are observed in the spectra. Selective labeling is widely used to assist resonance assignments in both solution NMR and solid-state NMR experiments; however, it has special significance in solid-state NMR of aligned samples where the mapping of protein structure onto the patterns of resonances in the spectra from selectively labeled samples serves as a method to simultaneously assign resonances and determine structure.[5]

Combinations of specific and selective labeling are essential for the solid-state NMR methods used to measure specific distances between strategically placed pairs of nuclei in unoriented samples of proteins, including rotational resonance[6] and REDOR[7].

Uniform Labeling

Uniform labeling refers to biosynthetic labeling of all carbon, nitrogen, or hydrogen sites with stable isotopes. Uniform labeling of proteins with ^{15}N is particularly convenient because of the strategic locations of nitrogens in the backbone and the absence of homonuclear couplings due to the intervening carbons.[8] It is also possible to replace all of the carbons with ^{13}C, although in this case the spectroscopy has to deal with the network of couplings among bonded carbons. Similarly, all of the hydrogens can be replaced with deuterons in order to attenuate the dipolar couplings among the hydrogens that often present complications and difficulties in the experiments.

Until recently, the vast majority of solid-state NMR studies have been performed with specific or selective isotopic labeling; however, this was accomplished at a considerable cost in flexibility. For example, when many resonances are resolved in a spectrum from a uniformly labeled protein, assignments can be readily made by comparisons with spectra from selectively labeled samples. But this is not possible if adequate resolution is present only in spectra of selectively labeled samples. Uniform labeling has the effect of shifting the experimental burdens from the isotopic labeling to the spectroscopy, which is fundamentally more powerful and flexible and now capable of yielding completely resolved spectra of membrane proteins in phospholipids bilayers.[9]

Uniform labeling with ^{13}C and/or ^{15}N is readily accomplished by growing bacteria in media containing ^{13}C labeled glucose and/or $^{15}NH_4Cl$ and opened up many avenues for NMR studies of proteins. Concurrent advances in molecular biology meant that a wide variety of prokaryotic and eukaryotic proteins could be expressed in high yields in *E. coli* grown on appropriately labeled media. This started with systematic methods for making resonance assignments in solution NMR spectra of proteins[10] and enabled the acquisition of the extensive chemical shift data that contributes to the constraints available for structure determination. It now includes magic angle sample spinning solid-state NMR of insoluble aggregates[11, 12] and polycrystalline proteins[13]. In some cases, there are advantages to random fractional labeling with ^{13}C.

Combining Uniform and Selective Labeling

A common use of selectively labeled samples in both solution NMR and solid-state NMR studies of proteins is to compare spectra from uniformly and selectively labeled samples. This enables the resonances from one type of amino acid to be identified by inspection. Although limited in scope, this information is valuable because it is a reliable way of resolving ambiguities in alternative assignment schemes, and while isotopic scrambling occurs in some cases, it is generally readily recognized and can be taken into account.

The comparison of two-dimensional solid-state NMR spectra of uniformly and selectively ^{15}N labeled samples is the basis for an approach to simultaneous resonance assignment and three-dimensional structure determination of membrane proteins in lipid bilayers. This method utilizes proteins aligned in the field of the NMR magnet and relies on Pisa Wheels (polarity index slant angle) to first, obtain shotgun-style resonance assignments and structure assembly of isolated polypeptide segments of the protein, and second, to assemble the segments in their correct order and obtain the full three-dimensional structure.[5] Pisa Wheels are circular patterns of resonances in two-dimensional PISEMA (polarization inversion spin exchange at the magic angle) spectra that mirror the helical wheels of membrane protein helices in oriented bilayers.[14, 15] This approach short-circuits the laborious process of obtaining complete sequential assignments, greatly accelerating the process of structure determination.

Backbone and Side-chain Dynamics

Deuteration can be used in two distinct ways. ^{2}H can be a very effective specific or selective label, as more commonly employed with ^{13}C or ^{15}N, and the quadrupolar interaction provided by this spin one nucleus can be used in solid-state NMR experiments to describe both aliphatic and aromatic side-chain dynamics.[16] The second way for deuteration to be used in solid-state NMR experiments is similar to the approach used in solution NMR of larger proteins because of the beneficial effects of dilution of the larger number of nearby hydrogens in a protein.

Future Prospects

Based on the success of isotopic labeling in solution NMR[17] and in solid-state NMR as summarized briefly in this article, one can look forward to the implementation of even more elegant isotopic labeling schemes that will complement the development of instrumentation and experimental methods for NMR studies of proteins.

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