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Labeling strategies for ^{13}C-detected aligned-sample solid-state NMR of proteins

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ABSTRACT

^{13}C-detected solid-state NMR experiments have substantially higher sensitivity than the corresponding ^{15}N-detected experiments on stationary, aligned samples of isotopically labeled proteins. Several methods for tailoring the isotopic labeling are described that result in spatially isolated ^{13}C sites so that dipole–dipole couplings among the ^{13}C are minimized, thus eliminating the need for homonuclear ^{13}C–^{13}C decoupling in either indirect or direct dimensions of one- or multi-dimensional NMR experiments that employ ^{13}C detection. The optimal percentage for random fractional ^{13}C labeling is between 25% and 35%. Specifically labeled glycerol and glucose can be used at the carbon sources to tailor the isotopic labeling, and the choice depends on the resonances of interest for a particular study. For investigations of the protein backbone, growth of the bacteria on [2-^{13}C]-glucose-containing media was found to be most effective.

1. Introduction

The majority of aligned-sample solid-state NMR studies on proteins immobilized in supramolecular complexes, such as virus particles or membranes, have relied on ^{1}H/^{15}N double-resonance experiments [1]. There are several advantages to this approach, including the relative ease and low cost of labeling all nitrogen sites in proteins obtained by expression in bacteria [2]. Solid-state NMR experiments on uniformly ^{15}N labeled proteins are straightforward because they have no nitrogen atoms directly bonded to other nitrogen atoms in either backbone or side chain sites. As a result, there is no need to implement homonuclear ^{15}N decoupling at any stage in the pulse sequences, including during the direct acquisition of ^{15}N signals, and the requisite heteronuclear decoupling is accomplished by irradiation of the ^{1}H resonances. It is feasible to make accurate measurements of ^{1}H chemical shift, ^{15}N chemical shift, and ^{1}H–^{15}N heteronuclear dipolar coupling frequencies for individual sites, as well as to detect ^{1}H–^{1}H and weak ^{15}N–^{15}N homonuclear couplings using a wide variety of multi-dimensional NMR experiments.

However, there are two disadvantages to the ^{1}H/^{15}N double-resonance approach: it is restricted to the amide nitrogen sites in the polypeptide backbone and the few nitrogen-containing side chain sites, and the direct detection of ^{15}N signals has low sensitivity because of its low gyromagnetic ratio. Both of these issues can be addressed by implementing ^{1}H/^{13}C/^{15}N triple-resonance experiments on proteins labeled with both ^{13}C and ^{15}N. The sensitivity can be improved by detecting ^{13}C signals because its gyromagnetic ratio is about 2.5 times larger than that of ^{15}N, and there is the opportunity to obtain spectroscopic data from nearly all backbone and side chain sites. The design of triple-resonance experiments for stationary samples is considerably different than that for magic angle spinning (MAS) experiments. In this article, we describe progress towards the development of isotopic labeling schemes compatible with direct detection of ^{13}C signals in ^{1}H/^{13}C/^{15}N triple-resonance experiments on stationary aligned samples [3–10] by examining a wide range of ^{13}C labeling approaches [11] over an array of fractions of dilutions, ranging from 15% to 100% of all sites in the proteins, and [2-^{13}C]-glucose in addition to the complementarily labeled glycerols for labeling through metabolic pathways. Magnetically aligned filamentous bacteriophage particles are used as the samples to demonstrate the influence of the labeling patterns on solid-state NMR spectra.

In single-contact spin-lock cross-polarization experiments on single crystals of peptides and aligned samples of proteins, we have observed a fourfold improvement in the signal-to-noise ratio when ^{13}C magnetization is detected compared to ^{15}N magnetization for individual labeled sites in the same samples under equivalent experimental conditions [7]. As is the case for ^{15}N labeling of proteins expressed in bacteria, 100% uniform labeling of all carbons sites with ^{13}C is straightforward when completely labeled carbon sources, such as $^{13}C_6$ glucose or $^{13}C_3$ glycerol, are used in the growth media. However, in protein samples where all of the carbon sites are labeled with ^{13}C, the homonuclear dipole–dipole couplings among the dense network of bonded and nearby ^{13}C nuclei

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present significant complications in solid-state NMR of stationary samples. Indeed, one of the principal advantages of high-speed magic angle spinning solid-state NMR experiments is that the homonuclear dipole–dipole couplings among the ^{13}C sites are greatly attenuated. In contrast, in stationary samples, the ^{13}C homonuclear couplings must be dealt with in order to obtain high-resolution spectra and to realize the sensitivity advantages of ^{13}C detection.

There are two approaches to dealing with the homonuclear ^{13}C–^{13}C dipole–dipole couplings in stationary aligned samples. The first is to apply multiple-pulse homonuclear decoupling sequences to the ^{13}C nuclei [12]. We have demonstrated the efficacy of this approach in the indirect dimensions of multi-dimensional experiments that enable ^{13}C chemical shift frequencies to be measured; however, these experiments were performed with ^{15}N-detection in the direct dimension [4]. The detection of signals in the windows of multiple-pulse sequences rarely leads to optimal sensitivity because of the filtering limitations associated with the short periods of time available for sampling the signals. The second approach is to tailor the pattern of isotopic labeling so that the ^{13}C labeled sites of interest are sufficiently isolated from other ^{13}C nuclei to eliminate the need for homonuclear decoupling.

The isotopic labeling schemes described in this article generate samples with spatially isolated ^{13}C sites so that dipole–dipole couplings among the ^{13}C are minimized, thus eliminating the need for homonuclear ^{13}C–^{13}C decoupling in either indirect or direct dimensions of one- or multi-dimensional NMR experiments that employ ^{13}C detection. The isotopic precursors utilized for tailored labeling of proteins expressed in bacteria range from specifically labeled two-, three-, or six-carbon molecules to random fractionally labeled growth media prepared from algae grown in the presence of a defined mixtures of ^{12}C and ^{13}C carbon dioxide. The coupling among ^{13}C nuclei is avoided either as a result of the alternate site pattern of metabolic incorporation [13–15] or because of the low statistical probability of two ^{13}C nuclei being bonded to each other. Here, results from uniform ^{13}C labeling and metabolically tailored $^{13}C_\alpha$ labeling based on [2-^{13}C]-glucose, [2-^{13}C]-glycerol, or [1,3-^{13}C]-glycerol are compared to fractional ^{13}C labeling. Two-dimensional projections of triple-resonance solution-state NMR spectra are used to characterize the labeling patterns. The filamentous bacteriophages fd, which infects *Escherichia coli*, and Pf1, which infects *Pseudomonas aeruginosa*, are used for the experimental demonstrations. The structural forms of the major coat proteins are immobilized and aligned along with the virus particles by the magnetic field for solid-state NMR experiments, and the membrane-bound forms of the same proteins are solubilized in detergent micelles for solution NMR experiments. The bacteriophage samples provide direct insights into the spectroscopic effects of the ^{13}C labeling schemes at different protein backbone sites in solid-state NMR experiments., They have been used in complementary magic angle spinning studies [16,17].

2. Results

Filamentous bacteriophage samples were obtained from bacterial cultures grown on algal-based media containing various fractions of ^{13}C labeled nutrients, or on minimal media supplemented with [2-^{13}C]-glucose, [2-^{13}C]-glycerol, or [1,3-^{13}C]-glycerol. In this article, our main focus is on the ^{1}H–$^{13}C_\alpha$ sites that are uniformly distributed throughout the protein backbone, separated from each other by three bonds, and have one directly bonded hydrogen (except for glycine and proline). At the intersection of peptide planes, the associated ^{1}H chemical shift, ^{13}C chemical shift, and ^{1}H–^{13}C dipolar couplings provide valuable structural constraints, and enable direct comparisons of sensitivity and resolution between ^{15}N- and ^{13}C-detected experiments.

In the absence of homonuclear decoupling, only proteins labeled such that the $^{13}C_\alpha$ sites are isotopically isolated from other ^{13}C, i.e., bonded to $^{12}C_\beta$ and $^{12}C_O$ (Fig. 1A), will yield solid-state NMR spectra with high resolution and sensitivity in stationary samples. The presence of ^{13}C in either or both adjacent carbon sites (Fig. 1B) will result in broadened signals due to the presence of unresolved ^{13}C–^{13}C dipolar couplings. The probability of having an isolated $^{13}C_\alpha$ site can be calculated from the individual probabilities of having $^{13}C_\alpha$, $^{12}C_\beta$, and $^{12}C_O$ present in the same polypeptide with random ^{13}C labeling at various fractions. As shown in Fig. 1D, the maximum probability occurs near a labeling ratio $p = {}^1/_3$. Because the maximum is broad and both natural abundance ^{13}C and the enrichment of ^{13}C in other proximate sites are potential complications, we prepared custom algal media with ^{13}C percentages of 15%, 25%, 35%, and 45%, as marked by arrows in Fig. 1D, in order to experimentally determine the optimal isotopic composition for solid-state NMR spectroscopy on stationary aligned samples of proteins.

The isotope labeling patterns of the protein samples were analyzed using solution NMR spectra. In general, the proteins were uniformly labeled to the predicted extent in all carbon sites in both *E. coli* and *P. aeruginosa* when grown on the algal-based media. One-dimensional ^{1}H-decoupled, ^{13}C solution NMR spectra report on the ^{13}C labeling at all sites, including the carbonyl and aromatic ring carbons that are not directly bonded to hydrogens. However, only a detailed analysis of individual sites reveals whether labeled sites are isotopically isolated. Since *E. coli* is the host for fd bacteriophage, its major coat protein represents the labeling that occurs in this bacterium. Solution NMR spectra of the membrane-bound form of fd coat protein in micelles are shown in Fig. 2. The comparison of the spectrum obtained on a 25% uniformly labeled sample (Fig. 2A) and that from a sample labeled from media containing [2-^{13}C]-glucose (Fig. 2B) demonstrates that there is a greater extent of ^{13}C labeling in the C_α region (45–65 ppm) and reduced labeling in the aliphatic (0–50 ppm), aromatic (110–170 ppm), and carbonyl (165–185 ppm) regions in the [2-^{13}C]-glucose labeled sample.

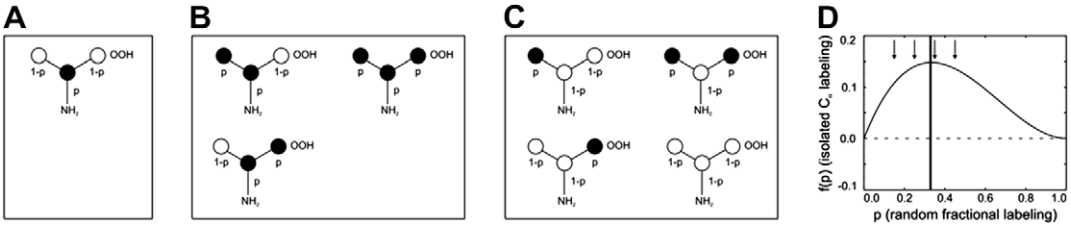

Fig. 1. Analysis of fractional uniform ^{13}C labeling at the C_α sites in the polypeptide backbone. (A) Schematic chemical structure of an isolated $^{13}C_\alpha$ site bonded only to ^{12}C that would contribute a high resolution NMR signal. (B) Schematic chemical structure of a non-isolated $^{13}C_\alpha$ site bonded to $^{13}C_\beta$ and/or $^{13}C_O$ sites that would contribute a broadened or undetectable NMR signal. (C) Schematic chemical structure of an unlabeled $^{12}C_\alpha$ site that would not contribute a signal. (D) The probability (p) of occurrence of an isolated $^{13}C_\alpha$ sites that would contribute high resolution NMR signals is predicted by the function $f(p) = p(1-p)^2$. The arrows denote the experimentally tested labeling ratios of 15%, 25%, 35%, and 45%.