Communication

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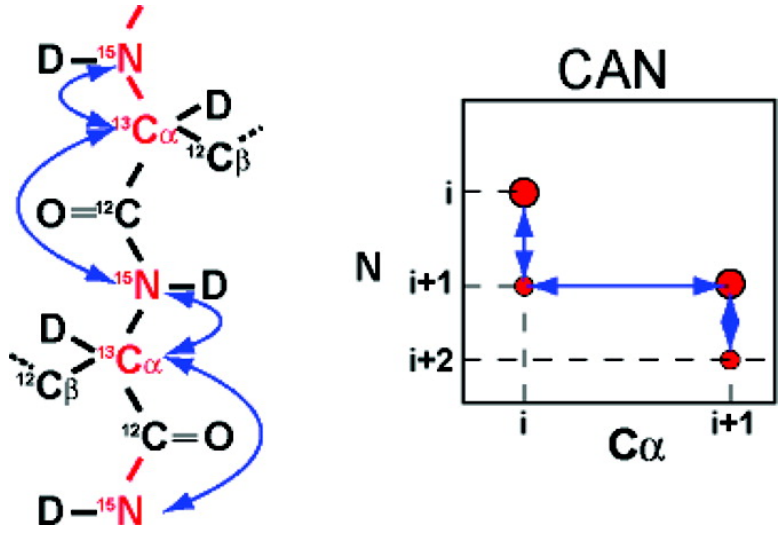

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Alternate $^{13}C-^{12}C$ Labeling for Complete Mainchain Resonance Assignments using Cα Direct-Detection with Applicability Toward Fast Relaxing Protein Systems

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Sequence specific NMR protein assignments typically use uniform $^{13}C/^{15}N$ labeling with ^{1}H-detected triple resonance experiments and have been applied successfully to proteins up to ~80 kDa.[1−5] However, fast relaxation of ^{1}H nuclei hinders the application of ^{1}H-detected experiments for higher molecular weight systems or signals close to paramagnetic centers. To overcome some of these problems ^{13}C-detected experiments have been used, which take advantage of slower relaxation of ^{13}C coherence owing to its lower gyromagnetic ratio. After the pioneering work by Markley and co-workers[6−8] for studies of small diamagnetic proteins, ^{13}C-detected experiments were largely abandoned when the more sensitive ^{1}H detected heteronuclear experiments were introduced. However, increased interest in paramagnetic proteins and the recent arrival of ^{13}C detected cryogenic probes have led to a revival of direct detection experiments.[9−11]

Carbonyl carbon (C′) direct detection has been the preferred choice as this nucleus is coupled only to α carbon (Cα).[9−12] In larger molecular weight systems, particularly at higher magnetic fields, however, carbonyl carbons suffer from fast relaxation because of their large chemical shift anisotropy (CSA). In contrast, Cα nuclei have a small CSA and relax slower if samples are deuterated. Thus, deuterated Cα would be the better nuclei for a ^{13}C detection experiment in large and/or fast relaxing systems.

However, direct Cα detection is complicated because of the scalar couplings with C′ and Cβ causing crowded spectra and reducing sensitivity due to peaks splitting into multiplets. Spectral complexity can be avoided by computational deconvolution of the spectra[13] or by selecting a single component within the split peaks using IPAP or S^3E schemes.[14] While this strategy clearly shows the potential of Cα detection for high molecular weight systems, it requires more complicated pulse programs and/or processing.

Here we present the use of $^{13}C-^{12}C$ alternate labeling in Cα-detected triple-resonance experiments to overcomes one bond $^{13}C-^{13}C$ coupling by isotopic enrichment at alternating carbon sites. This strategy uses an isotopic labeling scheme similar to the procedure established by LeMaster et al.[15,16] It enables alternate $^{13}C-^{12}C$ labeling at most positions by expressing the protein in *E.coli* using either combinations of [2-^{13}C] glycerol and $NaH^{13}CO_3$ or [1,3-^{13}C] glycerol and $NaH^{12}CO_3$ as carbon source. Figure 1A (left) shows the Hα−Cα region in a $^{1}H-^{13}C$ HSQC spectrum of uniformly ^{13}C-labeled SH3 domain and illustrates the spectral complexity in the indirect dimension due to $^{13}C\alpha-^{13}C'$ and $^{13}C\alpha-^{13}C\beta$ couplings. The same protein labeled with [2-^{13}C] glycerol has well resolved singlets indicating that neighboring carbons are not ^{13}C-labeled (Figure 1A right). Only the valines (red arrows) exhibit doublets due to the $^{13}C\alpha-^{13}C\beta$ couplings, as is expected from the metabolic pathways.[15] Figure 1B and 1C report the $^{13}C/^{12}C$ labeling ratios at the Cα positions in the [2-^{13}C] or [1,3-^{13}C] glycerol-labeled SH3 domain. [2-^{13}C] and [1,3-^{13}C] glycerol labeling yielded inverse labeling patterns. As expected from amino acid synthetic pathways, $^{13}C\alpha$-labeling was more abundant with [2-^{13}C] glycerol labeling compared to [1,3-^{13}C] glycerol labeling. Group I residues (red bars in Figure 1B) were more than 80% $^{13}C\alpha$ labeled with [2-^{13}C] glycerol, while group III residues were primary ^{13}C labeled with [1,3-^{13}C] glycerol (green bars in Figure 1B). Group II residues were partially labeled in both labeling schemes. Complete deuteration at Cα sites is critical for taking advantage of reduced dipole relaxation. This was readily achieved by culturing *E.coli* in 100% D_2O media with protonated [2-^{13}C] or [1,3-^{13}C] glycerol (see Supporting Information).

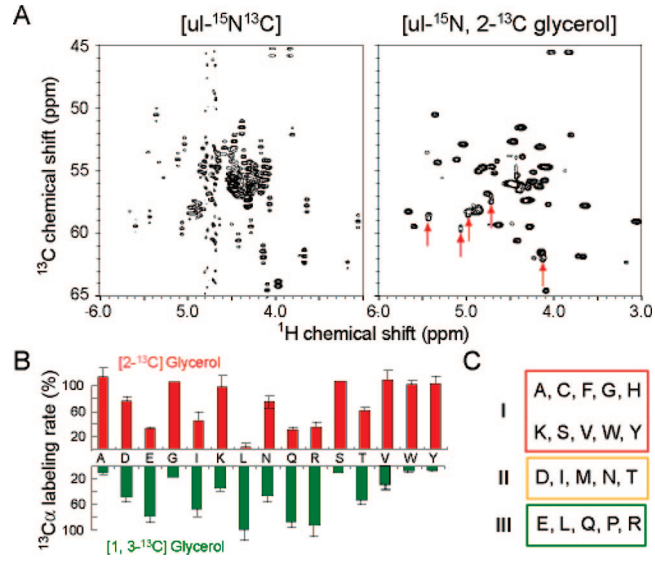

Figure 1. Labeling profile at Cα sites with alternate ^{13}C labeling. (A) The Hα−Cα region in a $^{1}H-^{13}C$ HSQC for uniformly ^{13}C-labeled (left) and [2-^{13}C] glycerol-labeled (right) Nck SH3.1. (B) $^{13}C\alpha$ labeling percentage with [2-^{13}C] (upper) or [1,3-^{13}C] (lower) glycerol (see Supporting Information for detailed procedures). (C) Classification of amino acids based on the $^{13}C\alpha$ labeling percentage (see text for classification criteria). Cys, His, Met, Phe, and Pro are classified on the basis of earlier literature.[15]

A 2D CaN HSQC experiment optimized for the deuterated and alternately ^{13}C-labeled proteins correlates in a straightforward way the chemical shifts of Cα nuclei with the shifts of the two neighboring nitrogen nuclei ($C\alpha^{i}-N^{i}$ and $C\alpha^{i}-N^{i+1}$) (see Supporting Information for pulse program). Thus, it allows sequential linking of the backbone nuclei. The pulse sequence was applied to uniformly $^{2}H^{15}N$ and alternately ^{13}C-labeled Nck first SH3 domain (Nck SH3.1). All experiments were recorded in D_2O to minimize the relaxation of ^{15}N coherence. The measuring time was reasonably

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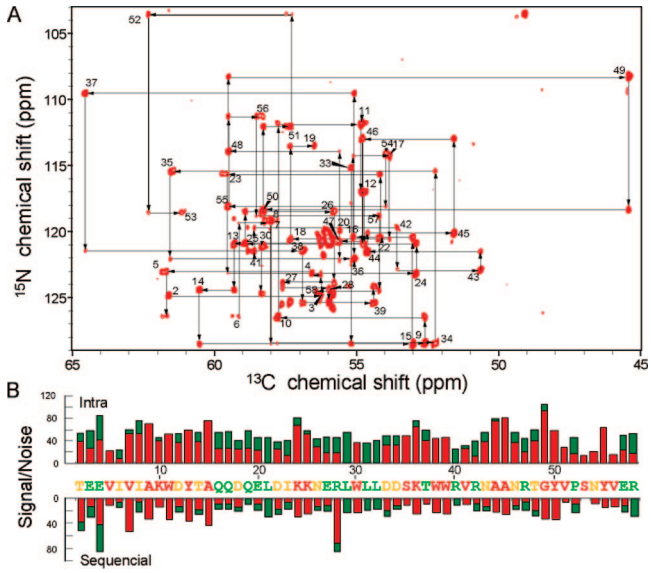

Figure 2. Sequential main chain assignments in a CaN HSQC experiment using Cα direct detection. (A) CaN HSQC spectrum recorded for 1 mM [ul-^{2}H^{15}N, [2-^{13}C] glycerol] Nck SH3.1. Arrows indicate the sequential walk. Note the proline ^{15}N signal (residue 52) is folded from its high frequency position. (B) Signal to noise ratio (S/N) for intraresidue (upper) and sequential (lower) cross peaks. Red and green bars are for samples labeled with [2-^{13}C] and [1,3-^{13}C] glycerol, respectively. Letters are colored according to Figure 1C.

short at 1 mM concentration (20−36 h). As shown in Figure 2A, the larger dispersion of ^{13}C with respect to ^{1}H enabled unambiguous assignment for most of the sites in the protein. As shown in Figure 2B (red bar), [2-^{13}C] glycerol labeling resulted in the observation of 91% of the intraresidue correlations and 86% of the sequential connections. Missing resides (4 Leu and 2 Arg, 1 Gln, and 1 Ser) belong to group III, which has a lower ^{13}C incorporation with [2-^{13}C] glycerol, except for Ser53 as discussed below. On the other hand, 72% of intraresidue and 58% of sequential connections were observed with [1,3-^{13}C] glycerol labeling (green bars), respectively. The smaller number of correlation observed in [1,3-^{13}C] glycerol labeling is because ^{13}C incorporation from the 1, 3 position is less efficient compared to position 2. In total, however, the information from both glycerol-labeling schemes established 100% of all intracorrelations and sequential correlations from the structured region of the protein including proline, except for that between Ser53 Cα and Asn54 N (which became observable when using a 2.5 mM sample, data not shown). For the protonated protein, only 98% and 87% of intracorrelations and sequential correlations are observed, respectively, in experiments for comparable conditions, indicating that full deuteration is a key factor for the Cα detection experiment.

The low intensity of the Ser53−Asn54 cross peak is likely attributed to chemical exchange. Asn54 is not observable in a regular ^{1}H−^{15}N HSQC, and conventional HN detection experiments failed to assign Asn54 N.[17] In contrast, the procedure described here successfully assigned both N and Cα resonance of the residue based on the Asn54 Cα-Tyr55 N and Asn54 Cα-N correlations. This indicates the clear advantage of Cα detection over proton detection experiment at an exchange-broadened site. A further crucial advantage is that prolines can be assigned easily. Pro52 in the Nck SH3.1 is assigned based on the Val51 Cα-Pro52 N and Pro51 Cα-Asn52 N correlations.

To demonstrate the general applicability of this approach, we recorded the same experiments at low temperatures in a viscous buffer to model slower tumbling. In 30% glycerol at 288 K, the Nck SH3.1 exhibited a diffusion coefficient corresponding to a 150 K protein. Even at this condition of slow tumbling, all the correlations assigned in Figure 2 were observed (Supporting Information, Figure 3), which clearly indicates that this approach is applicable to fast-relaxing or large molecular weight systems.

In summary, ^{13}C−^{12}C alternate labeling with deuteration at Cα sites is a key strategy to optimize the resolution and sensitivity in the Cα direct-detection experiment without using any spin-state selective schemes. While we analyze the [2-^{13}C] and [1,3-^{13}C] glycerol-labeled samples separately for clarity, one can also combine the two samples to obtain all the information at once. In this case, however, the Cα labeling rate would be half for all residues. Thus, the CaN experiment optimized with the described labeling strategy can establish main chain assignments in a single experiment and without using protons. Similar data could be obtained from a HMQC-type CaN experiment. In addition, the alternate labeling provides complementary ^{13}C labeling in the C′ position when Cα is not ^{13}C labeled. Thus, it would also enable recording of simple C′N correlated spectra without Cα-C′ coupling, which might be of interest for smaller systems. While the 2D spectrum shown here provided sufficient resolution, one can also apply nonuniform sampling to improve resolution without extending experimental time. In addition, a 3D version of this experiment, which would avoid signal overlap in larger proteins, is under development. In summary, the alternate ^{13}C-labeling strategy has clear advantages for higher molecular weight proteins and/or protein complexes that are inaccessible by proton-detected experiments.

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Supporting Information Available: Detailed experimental procedures including sample preparation, experimental condition, and pulse program. This material is available free of charge via the Internet at http://pubs.acs.org.

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