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*Article*

# Solid-state NMR spectroscopy of 10% $^{13}$C labeled ubiquitin: spectral simplification and stereospecific assignment of isopropyl groups

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**Abstract**

We describe the simplification of $^{13}$C–$^{13}$C correlation spectra obtained from a microcrystalline protein sample expressed on a growth medium of 10% fully $^{13}$C labeled glucose diluted in 90% natural abundance glucose as compared to a fully labeled sample. Such a labeling scheme facilitates the backbone and side-chain resonance assignment of Phe, Tyr, His, Asp, Asn, Ile, Lys and Pro and yields an unambiguous stereospecific assignment of the valine Cγ1, Cγ2 $^{13}$C resonances and of Leucine Cδ2.

## Introduction

The assignment and structure determination of microcrystalline and non-crystalline solid proteins by MAS-NMR spectroscopy has recently become feasible but severe limitations still exist (Pauli et al., 2001; Castellani et al., 2002; Böckmann et al., 2003; Igumenova et al., 2004a, b; Heise et al., 2005; Siemer et al., 2005; Zech et al., 2005). Sequence specific resonance assignment is typically achieved using 2D and 3D triple-resonance experiments, e.g. NCOCA, NCACB and N(CO)CACB. The introduction of a third dimension significantly helps to resolve ambiguities but remaining overlap and reduced sensitivity due to additional coherence transfer steps are bottlenecks which limit the applications. Strategies to simplify spectra and reduce signal overlap are therefore desirable, especially for the assignment of larger proteins. The advantages of using non-random $^{13}$C/$^{15}$N labeling schemes based on specifically labeled precursors like glycerol or succinic acid have already been demonstrated (LeMaster and Kushlan, 1996; Castellani et al., 2002; Gammeren et al., 2005). In this contribution, we explore the application of a different labeling approach, namely 10% non-random labeling to a solid protein.

The specific labeling approach discussed here does not only simplify the $^{13}$C spectra, but can also be used for the stereo-specific assignment of the isopropyl groups of valine and leucine residues. Related approaches to stereospecific assignment using $^{1}$H–$^{13}$C correlation spectroscopy and the analysis of $^{13}$C–$^{13}$C scalar couplings are well established in liquid-state NMR (Senn et al., 1989; Szyperski et al., 1992). The availability of stereospecific assignment for isopropyl groups and for methylene protons is known to improve both the accuracy and precision of 3D structures (Driscoll et al., 1989; Güntert et al., 1989; Havel, 1991). Stereospecific assignment also leads to a faster convergence of the simulated annealing protocols by reducing the number of local minima and makes floating assignments (or explicit atom

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swapping) in the structure determination procedure (Folmer et al., 1997) unnecessary. The same considerations are, of course, valid for structure determination from solid-state NMR data.

In the following, we describe the stereospecific assignment of the $^{13}C$ resonances from isopropyl groups of Val and Leu in the solid state using a 10% $^{13}C$ labeled microcrystalline ubiquitin sample. In addition we discuss the spectral simplifications in the 10% sample for aiding backbone and side-chain assignment.

## Materials and methods

### *Sample preparation*

10% $^{13}C$/100% $^{15}N$ labeled ubiquitin was obtained from VLI Research Inc. (Malvern, PA, USA) as a customer expression. Uniformly $^{13}C/^{15}N$ labeled ubiquitin was expressed and purified as described previously (Sass et al., 1999). Microcrystals of the protein have been grown at 4 °C in sitting drops with identical precipitant as described by Igumenova et al. (2004a). Crystals were harvested after 10–14 days and centrifuged into 2.5 mm rotors. Rotor caps were glued in to prevent water evaporation. One rotor contained typically 5–6 mg protein.

### *MAS NMR experiments*

All spectra were acquired on a Bruker DRX600 wide-bore spectrometer operating at a static field of 14.09 T using a 2.5 mm triple-resonance T3 MAS probe (Chemagnetics/Varian), at a spinning frequency of 12 kHz and a sample temperature of −5 °C. The temperature was calibrated with a $Sm_2Sn_2O_7$ sample (Grimmer et al., 1997). The $^{1}H$ and $^{13}C$ $\pi/2$ pulse was set to 2.5 $\mu$s. For adiabatic passage cross polarization (Hediger et al., 1995), average $B_1$-field strengths of 72 and 60 kHz were used on $^{1}H$ and $^{13}C$, respectively, with a variation on the $^{13}C$ channel of $\pm 6$ kHz, and contact time of 1.5 ms. In the PDSD experiment, decoupling of $^{1}H$ during $t_1$ and $t_2$ was achieved with SPINAL64 (Fung et al. 2000) at a field strength of 100 kHz. TPPI was used for phase-sensitive detection in the indirect dimension. The PDSD spectrum of the 10% $^{13}C$/100% $^{15}N$ labeled ubiquitin was recorded using a mixing time of 500 ms, a spectral width of 50 kHz in both dimensions, 512 points in $t_1$, a recycle delay of 3.0 s and 128 scans per increment resulting in a total measurement time of 64 h. The PDSD spectrum of the uniformly $^{13}C/^{15}N$ labeled ubiquitin was recorded using a mixing time of 30 ms, a spectral width of 30 kHz in both dimensions, 750 points in $t_1$, a recycle delay of 2.6 s and 128 scans per increment resulting in a total measurement time of 66 h. Data were processed using XWINNMR (Bruker Biospin). Squared sine-bell window functions with a 90° phase shift were applied in both dimensions. Spectra were referenced externally to DSS using the $^{13}C$ adamantane methylene peak as described previously (Igumenova et al., 2004a). $^{13}C$ and $^{15}N$ chemical shifts have been deposited in the BioMag ResBank under accession number 7111.

## Results and discussion

### *Expected labeling pattern*

Known biochemical pathways for *E. coli* (Kanehisa et al., 2004; Keseler et al., 2005) were used to predict the $^{13}C$ distribution in proteins expressed on a growth medium of 10% fully $^{13}C$ labeled glucose diluted in 90% natural abundance glucose. Pathways exemplified for Val and Leu are given in the supplementary material. The expected distribution of isotopes for the individual amino-acid residues is summarized in Figure 1, where carbon fragments originating from the same glucose molecule are indicated in the same color. Ideally, these carbons are either all $^{13}C$ labeled, or are all $^{12}C$ with some $^{13}C$ statistically distributed at natural isotopic abundance. In the case of Gly, Ser, Cys, Ala, His, Phe, Tyr, Trp, Val and Leu (group I in Figure 1) well-defined labeled blocks are expected. For Glu, Gln, Arg, Pro, Asp, Asn, Met, Thr, Ile and Lys (group II in Figure 1), whose synthesis involves the citrate cycle, a mixture of different labeling patterns is expected with statistical weights as indicated in Figure 1. It should, however, be noted that the involvement of the citrate cycle in the biochemical synthesis of those amino acids (group II) depends on the bacterial strain and the conditions of expression. As a consequence, the statistical weights of the different labeling patterns can vary.

### *Stereospecific assignment of isopropyl groups*

The $^{13}C$ signals from the two prochiral $CH_3$ groups in Val and Leu can be distinguished by the

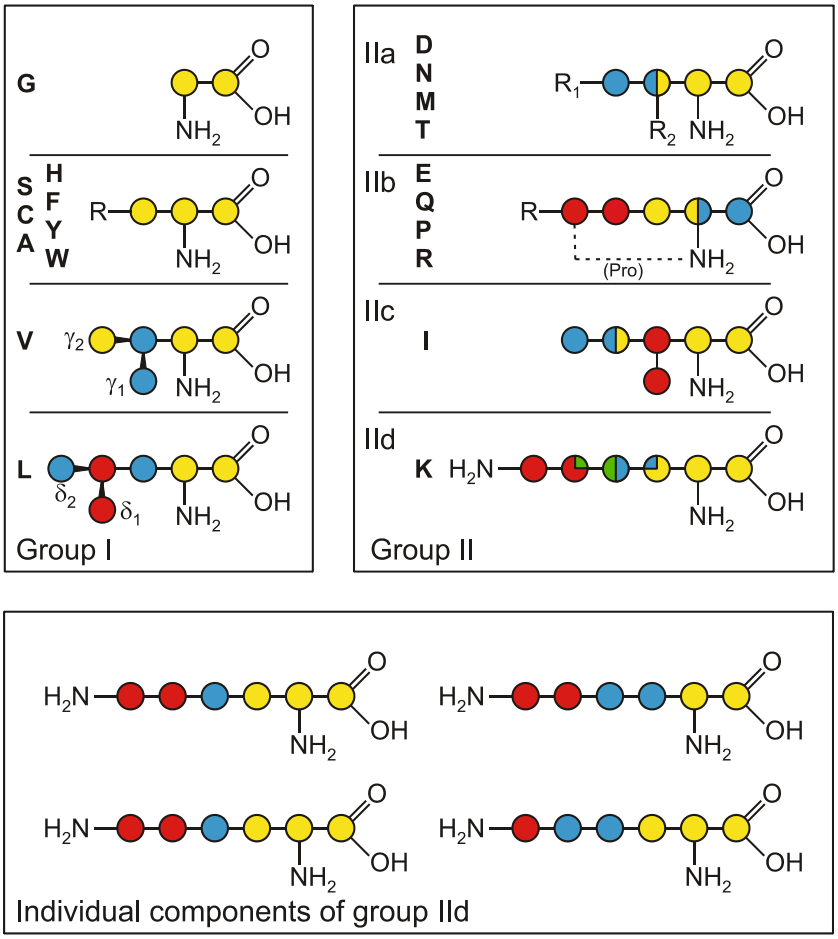


*Figure 1.* $^{13}$C enrichment-scheme for the individual amino acids as obtained by protein expression in *E. coli* growing on a medium with 10% uniformly $^{13}$C labeled glucose. Symbols of the same color mark carbons atoms originating from the same glucose molecule. The carbons marked with a given color are either all labeled or all at natural abundance. In group II, the statistical weight of the various labeling patterns is indicated. In the case of Asp, Asn, Met and Thr (group IIa) the $^{13}$C$\beta$ is, with a 50% probability, part of a $^{13}$C$\beta$–$^{13}$C$\gamma$ pair and in the remaining 50% is included in a $^{13}$C–$^{13}$C$\alpha$–$^{13}$C′ three spin system.

presence or absence of a $^{13}$C neighbor: Valine C$\beta$ and C$\gamma$1 originate from the same glucose and labeling appears in pairs, the same is true for valine C$\alpha$, C′ and C$\gamma$2. In leucine, C$\beta$ and C$\delta$2 origin from one glucose molecule, the same holds for C$\gamma$ and C$\delta$1. Thus, in a $^{13}$C–$^{13}$C correlation spectrum, strong Val C$\beta$–C$\gamma$1, C$\alpha$–C$\gamma$2 and C′–C$\gamma$2, but weak C$\beta$–C$\gamma$2, C$\alpha$–C$\gamma$1 and C′–C$\gamma$1 cross-peaks are expected. In the case of Leu, strong C$\beta$–C$\delta$2 and C$\gamma$–C$\delta$1, but weak C$\beta$–C$\delta$1 and C$\gamma$–C$\delta$2 cross-peaks are expected.

A proton-driven spin-diffusion spectrum (PDSD) with a mixing time of 500 ms was recorded. Such spectra are expected to show cross-peaks between all $^{13}$C resonances within one amino-acid residue, while inter-residue cross-peaks are weak due to the isotopic dilution (only 10% overall labeling). The PDSD spectrum of a 10% $^{13}$C/100% $^{15}$N labeled sample of ubiquitin is shown in Figure 2a. For comparison, the spectrum of a uniformly $^{13}$C/$^{15}$N labeled sample, taken with a mixing time of 30 ms, is shown in Figure 2b. Extensions of the regions of interest are displayed in Figures 3–5. The resonance assignment for microcrystalline ubiquitin was taken from reference (Igumenova et al., 2004a), except for the stereospecific assignment of the isopropyl groups of Val and Leu, the aromatic rings and a few additional resonances.

For valine, always one of the two C$\beta$–C$\gamma$i, C$\alpha$–C$\gamma$i and C′–C$\gamma$i resonances is found to be significantly more intense than the other (Figure 3). This observation immediately yields an unambiguous

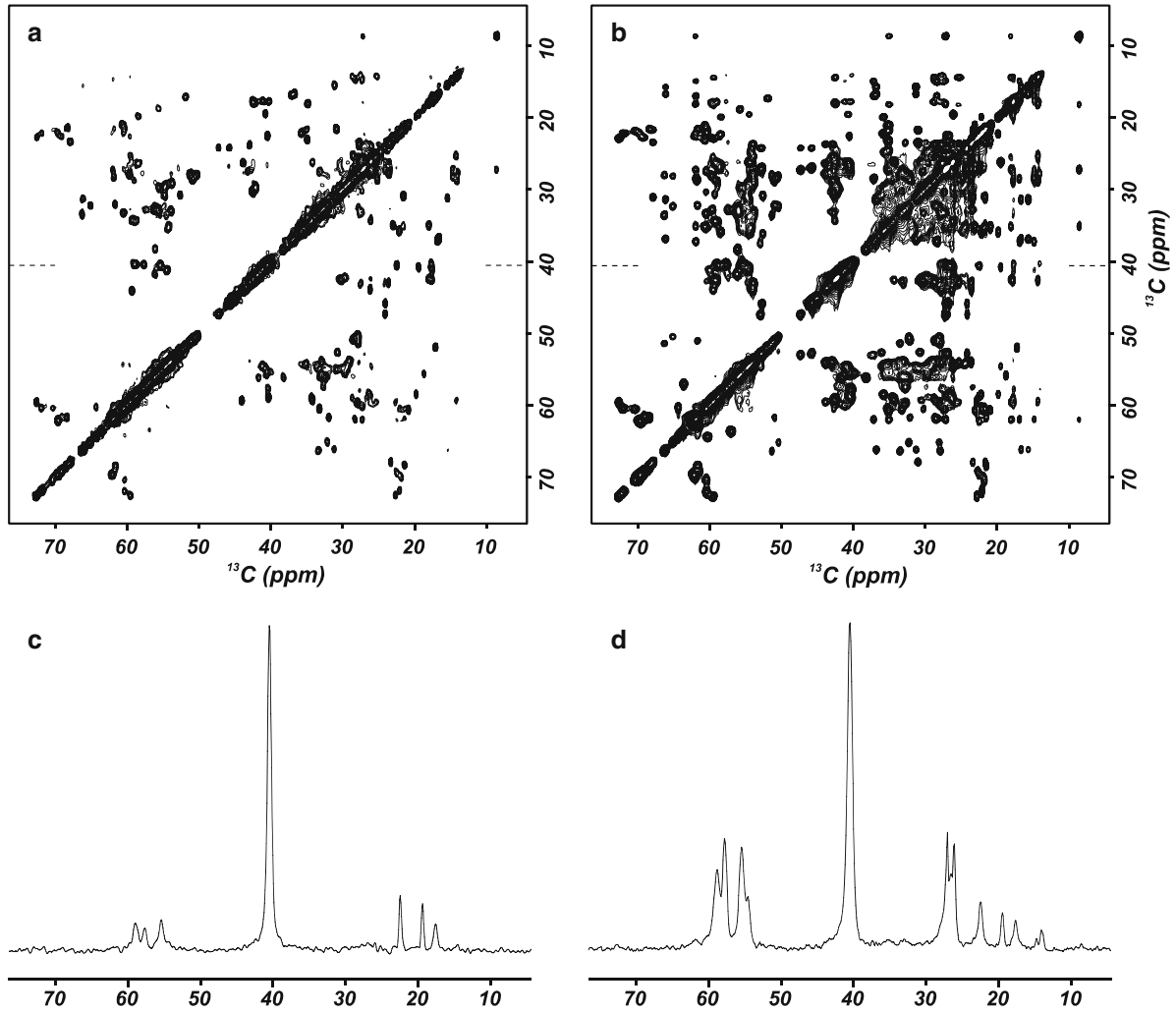


*Figure 2.* Proton-driven spin-diffusion (PDSD) spectrum of 10% $^{13}$C/100% $^{15}$N labeled (a) and uniformly $^{13}$C/$^{15}$N labeled ubiquitin (b). Mixing times were 500 ms for the 10% $^{13}$C and 30 ms for the 100% $^{13}$C sample. Both spectra were recorded in ~65 h using ~6 mg ubiquitin crystals. Cross sections of spectra (a) and (b) at 40.5 ppm, as indicated with dashed lines, are shown in (c) and (d), respectively.

stereospecific assignment of all valine Cγ1 and Cγ2 chemical shifts (supplementary material, Table S1). The resonances from V70 were found to be less intense than the signals from V5, V17 and V26, possibly as a consequence of dynamical processes. The C′–Cγi cross-peaks are generally weak in the fully labeled sample, probably because the intensity is distributed over many cross-peaks in such a multi-spin system.

Leucine Cβ–Cδ2 cross-peaks are shown in Figure 4 and feature all expected correlations. Signals of L8, L71 and L73 are, as in spectra from fully $^{13}$C/$^{15}$N labeled samples, not observed, probably due to dynamic effects. Since Leu Cγ and Cδ1 chemical shifts are similar, the Cγ–Cδ1 cross-peaks are close to the diagonal and therefore difficult to observe in PDSD spectra. An unambiguous assignment of Leu Cδ2 chemical shifts is, however, easily obtained from the data in Figure 4 and is reported in the supplementary material.

### *Simplification of backbone and side-chain resonance assignment*

The 10% $^{13}$C labeling leads to a reduction of the number of Cα–Cβ cross-peaks in $^{13}$C–$^{13}$C correlation spectra and a comparison between the spectra of 10% and fully labeled material in

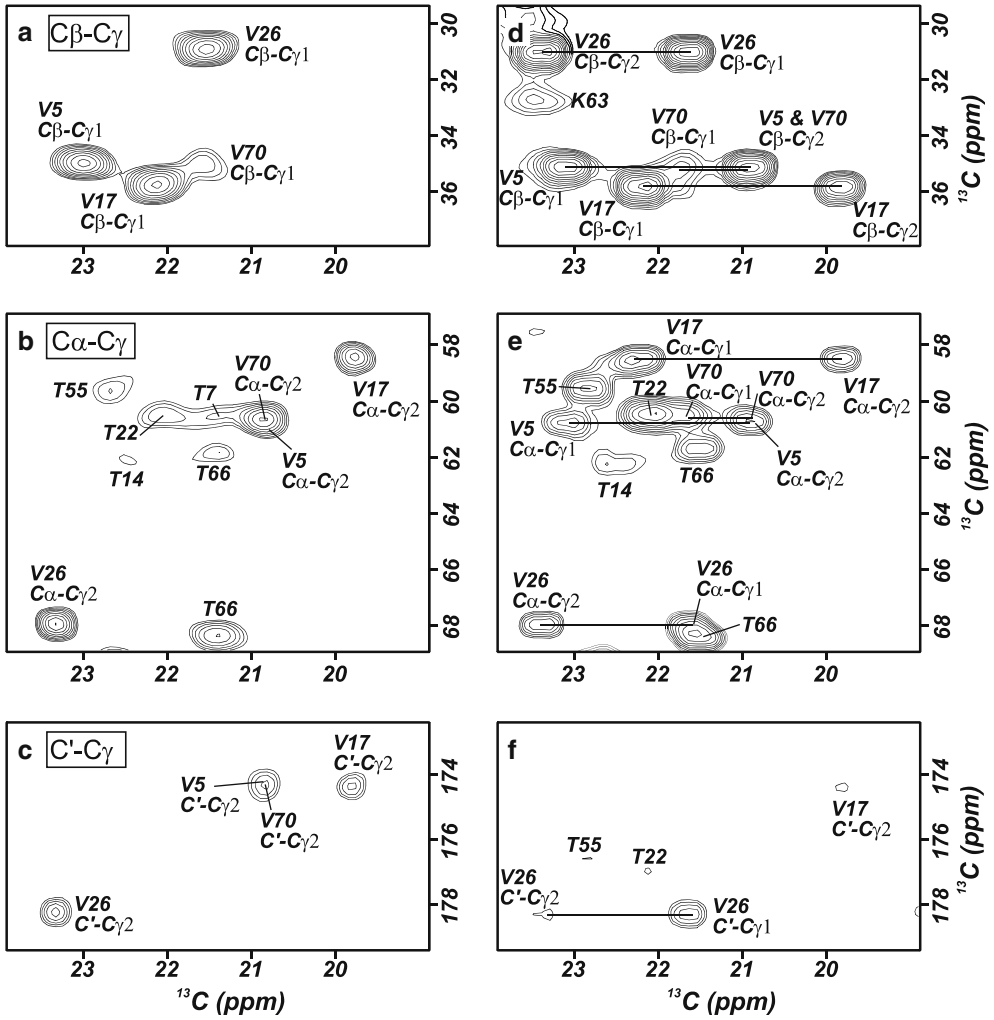


*Figure 3*. PDSD spectrum of 10% $^{13}C$/100% $^{15}N$ labeled (a–c) and uniformly $^{13}C/^{15}N$ labeled ubiquitin (d–f). Sections (a) and (d) show the region of Val C$\beta$–C$\gamma$ cross-peaks, (b) and (e) Val C$\alpha$–C$\gamma$, (c) and (f) Val C′–C$\gamma$ cross-peaks. Mixing times were 500 ms for the 10% $^{13}C$ and 30 ms for the 100% $^{13}C$ sample.

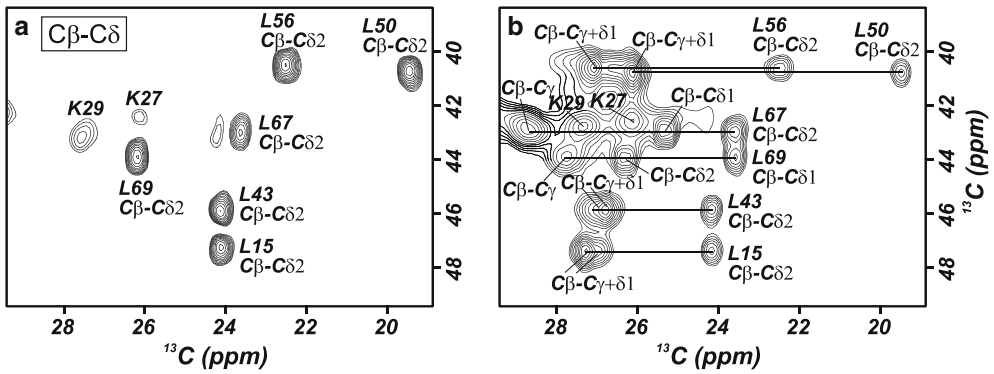


*Figure 4*. PDSD spectrum of 10% $^{13}C$/100% $^{15}N$ labeled (a) and uniformly $^{13}C/^{15}N$ labeled ubiquitin (b). Shown is a region with Leu C$\beta$–C$\delta$ cross-peaks. Mixing times were 500 ms for the 10% $^{13}C$ and 30 ms for the 100% $^{13}C$ sample.

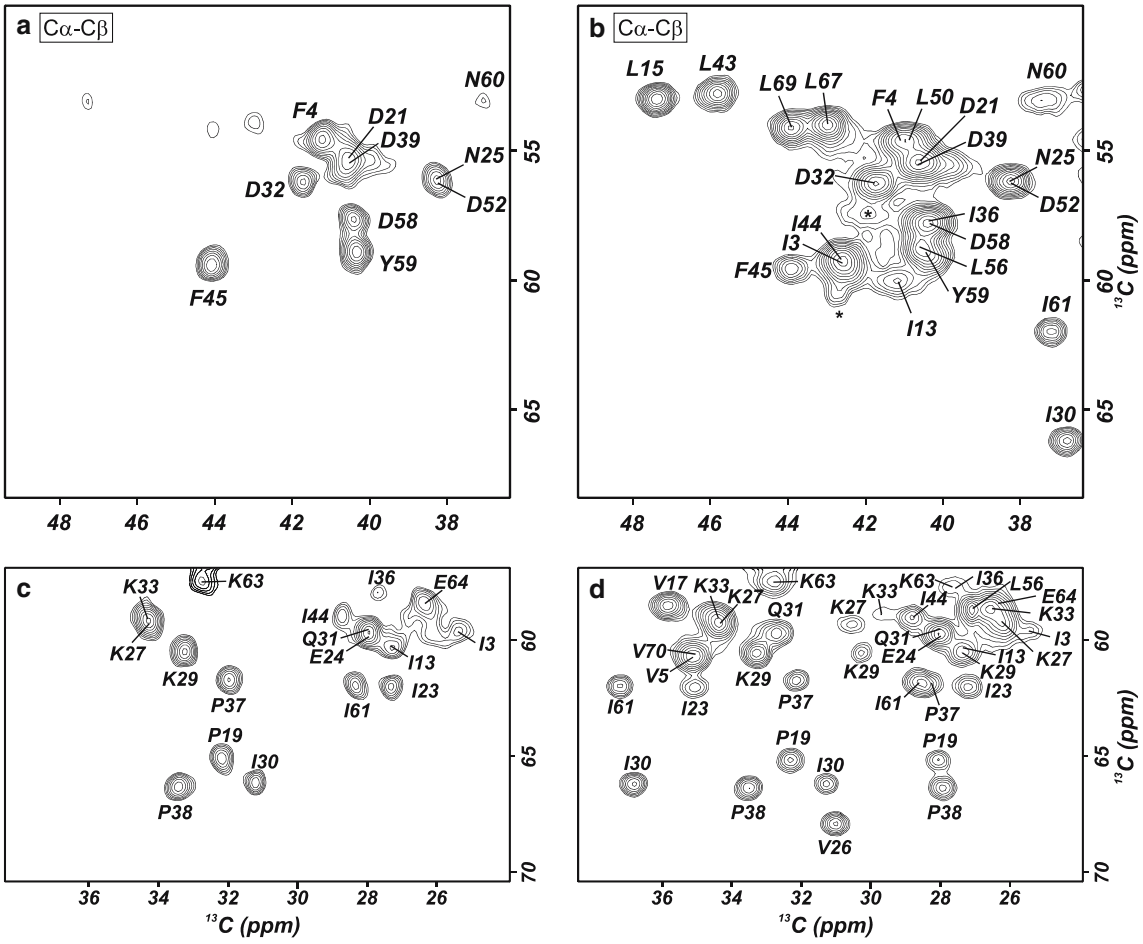


*Figure 5*. Regions of PDSD spectra of 10% $^{13}$C/100% $^{15}$N labeled (a) and (c) and uniformly $^{13}$C/$^{15}$N labeled ubiquitin (b) and (d). A region containing Cα–Cβ cross-peaks is shown in (a) and (b). In addition to Cα–Cβ cross-peaks, weak Lys Cα–Cε correlations are present in (b) and are indicated by an asterisk. The region in (c) and (d) contains Cα–Cβ, Cα–Cγ and Cα–Cδ cross-peaks of Lys, Pro, Glu, Gln, Val and Ile.

Figure 5a and b shows that the signal overlap is greatly reduced. In particular, the signals from Ile and Leu are missing in the 10% sample and the identification of cross-peaks from of Phe, Tyr, His, Asn, and Asp is facilitated. Since Cα–Cβ cross-peaks of those residues are the only signals in aliphatic $^{13}$C–$^{13}$C correlations, spectral overlap can be particularly problematic, e.g. the Cβ of F4 and Y59 have not been assigned previously. The spectra from the 10% $^{13}$C sample can be used to overcome these difficulties. Cα–Cβ correlations of F4 and Y59 are clearly visible in Figure 5a. In order to assign amino-acid types to the aromatic Cα–Cβ cross-peaks, Cγ/Cδ/Cε–Cα/Cβ correlations in a PDSD spectrum of uniformly $^{13}$C/$^{15}$N labeled ubiquitin were analyzed (Figure S2 of the supplementary material). Since there is only one His and one Tyr in ubiquitin, H68 and Y59 can be assigned based on the observed pattern and used as a starting point for backbone assignment. The assignments of F4, F45, Y59 and H68 were confirmed with NC correlations and a 3D NCOCX spectrum (supplementary material).

A further example of the spectral simplification is shown in Figure 5c and d: signals from Lys and Pro are more easily identified in the 10% labeled sample because Cα–Cβ cross-peaks resulting from Ile and Val are absent or weak.

We can amend the previously published $^{13}$C assignment for microcrystalline ubiquitin (Igumenova et al., 2004a) by the stereospecific assignments of Val/Leu isopropyl groups, the chemical

shifts of aromatic ring carbons and C$\beta$ resonances of F4 and Y59 (see Tables S1 and S2 of the supplementary material).

## Conclusion

The 10% non-random labeling scheme allows the simplification of $^{13}C$–$^{13}C$ correlation spectra and the unambiguous stereospecific assignment of Val C$\gamma$1, C$\gamma$2 and Leu C$\delta$2 $^{13}C$ chemical shifts. Such data can help to improve the quality of protein structures obtained by solid-state NMR spectroscopy. A 10% $^{13}C$ labeled protein sample also facilitates the assignment of backbone and side-chain resonances of Phe, Tyr, His, Asp, Asn, Pro, Lys and Ile. This may prove important for larger proteins with more spectral overlap.

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