

Use of biosynthetic fractional ^{13}C -labeling for backbone NMR assignment of proteins

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Abstract We describe a simple approach to classify amino acid residue types in NMR spectra of proteins for supporting the backbone resonance assignments. It makes use of the differences in biosynthetic pathways of the 20 amino acids in *Escherichia coli*. Therefore, it is distinct from the parameters routinely exploited in the backbone resonance assignment such as chemical shifts and spin topology information. The combination of biosynthetically directed fractional ^{13}C -labeling and uniform ^{15}N -labeling enables us to obtain both residue-type specific information and sequential connectivities from a single protein sample. The residue-type classification exploiting biosynthetic pathways can be used for accelerating the conventional backbone assignment procedure.

Keywords Isotope labeling · Backbone resonance assignment · Amino acid metabolism

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Introduction

Sequence-specific assignment of the resonances in the spectra is a prerequisite for any NMR study of proteins. It is usually based on (i) classifying the amino acid spin systems or amino acid residue types, and (ii) establishing sequential connectivities between these spin systems using through-space coupling in NOESY experiments or heteronuclear scalar coupling in triple resonance experiments such as HNCA, CBCA(CO)NH, HNCACB, etc. (Wüthrich 1986; Clore and Gronenborn 1994). The characteristic $C\alpha$ or $C\beta$ chemical shifts of residues such as Ala, Thr/Ser, and Gly play a pivotal role for the residue-type identification in the sequential resonance assignment. However, the residue-type identification usually has to be supported by information on the side-chains topology, for example, from ^{13}C - ^{13}C or ^1H - ^1H TOCSY-type experiments, or from the pulse schemes designed for exploiting the spin topology of specific side-chains and chemical shifts (Dötsch et al. 1996a, b; Schubert et al. 1999). Both approaches cannot provide unambiguous classification when the magnetization transfers are insufficient. Alternatively, selective amino acid labeling is a very powerful method to unambiguously distinguish amino acid types in the spectra, as it does not require any additional magnetization transfer to the side-chain atoms in order to obtain residue-type information (Muchmore et al. 1989; McIntosh and Dahlquist 1990). Its drawbacks are the required sample preparation and its inherent loss of the sequential connectivities.

In this article, we present a simple and cost-effective approach to obtain amino acid specific information as well as sequential connectivities from a single sample,

exploiting differences in the biosynthetic pathways of amino acids with minimal magnetization transfer steps. It uses biosynthetically directed fractional ^{13}C -labeling of proteins to classify residue types in the protein NMR spectra.

Materials and methods

Sample preparation

100% ^{15}N - and 10 or 20% fractionally ^{13}C -labeled samples were prepared using M9 medium containing 10% or 20% $^{13}\text{C}_6$ D-glucose (ISOTEC)/90% or 80% natural isotope abundance D-glucose (Fluka) (w/w) and 100% $^{15}\text{NH}_4\text{Cl}$ as the sole carbon and nitrogen sources. The plasmid for the expression of the DNA binding domain of phage 434 repressor, 434 (1–63) or bacteriophage lambda capsid stabilizing protein, gpD (12 kDa) was transformed into *Escherichia coli* ER2566 (New England Biolab). The bacterial culture (0.75 l) was grown in a 2 l Erlenmeyer flask without baffle or baffled flask (NALGENE) at 37°C with shaking at 180 rpm. Expression of the proteins was induced with 1 mM isopropyl-thio- β -D-galactoside at an OD_{600} of 0.5–0.6, followed by further incubation for 4.5 h. The cells were harvested and frozen at -80°C for further purification. The proteins were purified as described previously (Luginbühl et al. 1997; Iwai et al. 2004). The samples were concentrated to ca. 0.7–1.0 mM in 200 μl volume.

Data acquisition and analysis

NMR measurements were performed at 25°C at the ^1H frequency of 600 MHz on a spectrometer equipped with a triple resonance cryogenic probehead. For the HNCA experiment with 100% ^{15}N - and 10% fractionally ^{13}C -labeled 434 (1–63), a total of 32 (ω_1) \times 64 (ω_2) \times 512 (ω_3) complex points were collected, with $t_{1,\text{max}} = 11.0$ ms, $t_{2,\text{max}} = 15.1$ ms, $t_{3,\text{max}} = 66.4$ ms. The total measurement time was 21 h. For the $\text{C}\alpha$ -coupled HNCO experiment in which the 180° pulse for $\text{C}\alpha$ during the C' evolution time is omitted (Grzesiek and Bax 1992), a total of 30 (ω_1) \times 60 (ω_2) \times 768 (ω_3) complex points were collected, with $t_{1,\text{max}} = 10.2$ ms, $t_{2,\text{max}} = 30.6$ ms, $t_{3,\text{max}} = 106.9$ ms. The total measurement time was 21 h. Similar parameters were used for recording spectra with the 20% fractionally ^{13}C -labeled samples of 434(1–63) and of gpD. The fractions of singlet I_s in the multiplet fine structures were determined by least square fitting using inverse Fourier transformation of the cross-sections from the

HNCO spectra using the program INFIT that was modified to fit the multiplet components (Szyperski et al. 1992a).

Results and discussion

Biosynthetic pathways can be different even for amino acids having similar spin topologies and chemical shifts (Fig. 1). We exploited these biosynthetic pathways to classify residue types for the backbone resonance assignments. In order to extract this information from the NMR spectra, we applied biosynthetically directed fractional ^{13}C -labeling of proteins, a method in which the sole carbon source available during bacterial growth and protein expression is a mixture of 10–20% $^{13}\text{C}_6$ D-glucose and 80%–90% natural isotope abundance glucose. This 10% fractional ^{13}C -labeling has been routinely used for stereo-specific assignment of diastereotopic methyl groups in valine and leucine (Neri et al. 1989, 1990; Senn et al. 1989; Szyperski et al. 1992b; Szyperski 1995). When combined with uniform ^{15}N -labeling, this method also becomes adequate for triple resonance experiments with a 1 mM protein sample. As shown in Fig. 2, sensitive NMR experiments such as HNCA and HNCO can yield spectra with a good S/N using a cryogenic probehead. The HNCA spectrum can be used for establishing the sequential connectivities based on chemical shifts (Fig. 2a). As $\text{C}\alpha$ – $\text{C}\beta$ passive coupling and intra-/inter-residual N – $\text{C}\alpha$ couplings are not simultaneously active in all the molecules in a fractionally ^{13}C -labeled sample, one could also exploit a longer evolution period in order to improve the resolution of a HNCA experiment.

At the same time, the use of biosynthetically directed fractional isotope labeling gives rise to non-random ^{13}C distribution patterns, as intact 2-carbon and 3-carbon fragments originating from the $^{13}\text{C}_6$ -glucose

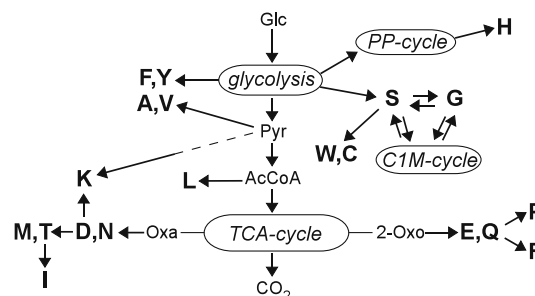
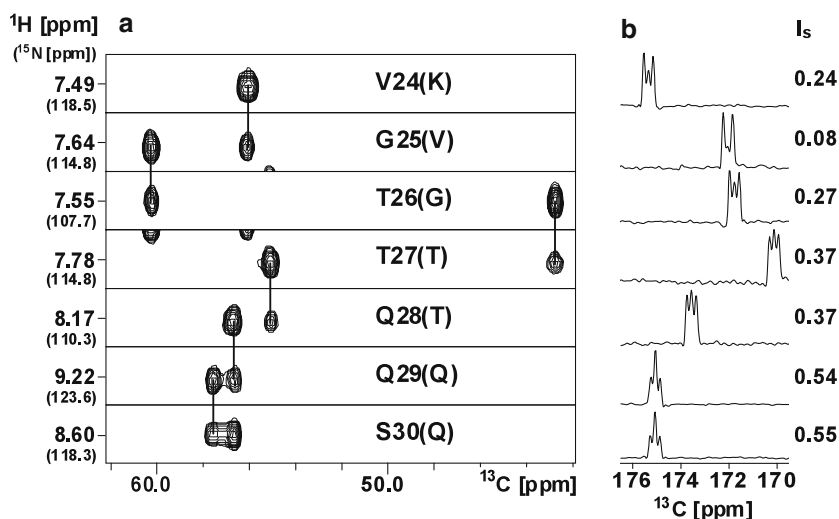


Fig. 1 A simplified scheme of the amino acid biosynthetic pathways in *E. coli*. The origins of the $\text{C}\alpha$ – C' fragment in the 20 amino acids are depicted by arrows

Fig. 2 (a) Strips from the HNCA spectrum for residues 24–30 of the 100% ^{15}N - and 10% fractionally ^{13}C -labeled 434(1–63) prepared from *E. coli* cell cultures in standard Erlenmeyer flasks. The amino acid type of the preceding residue, i.e., the residue determining the ^{13}C fine structure, is indicated in parenthesis. (b) Cross-sections of the corresponding resonances in the $C\alpha$ -coupled HNCO spectrum with their I_s values



molecules are incorporated into the amino acids during biosynthesis (Fig. 1). This amino acid specific information remains encoded in the expressed proteins and can be observed as ^{13}C fine structures in the NMR spectra. A similar strategy has been previously proposed for ^1H NMR assignments (Szyperski et al. 1992b), but has not been widely used even for small proteins because of the complexity of the fine structures and of the limited resolution. On the other hand, carbonyl carbons show a simple ^{13}C fine structure (Fig. 3a) arising only from the relatively large $C\alpha$ – C' coupling (~ 55 Hz), which can be observed in a HNCO experiment when combined with 100% ^{15}N -labeling. They are also free from ^1H – ^{13}C dipolar relaxation, making HNCO one of the most sensitive triple resonance experiments at the ^1H -frequency of 600 MHz.

The C' fine structure can readily be obtained from a $C\alpha$ -coupled HNCO experiment with a 100% ^{15}N - and 10% fractionally ^{13}C -labeled sample, in which $C\alpha$ -decoupling is omitted during the C' evolution period (Grzesiek and Bax 1992). The C' fine structure observed for residue i in this spectrum reflects the metabolic information of the preceding residue ($i - 1$) (Fig. 2), and is composed of a singlet and a doublet component (Fig. 3a). The relative fractions of these components are characteristic signatures of the 20 amino acid residues in proteins, which can be used for the residue-type classification.

Classification of residue types based on known biosynthetic pathways in *E. coli*

Based on current knowledge of the amino acid metabolic pathways in *E. coli*, we have divided the 20 amino acids into six groups according to the expected singlet/

doublet ratios in the C' fine structures of a protein produced in exponentially grown *E. coli* cells with 10% fractional ^{13}C -labeling (Szyperski 1995; Neidhardt et al. 1996). The first group contains the amino acids with the highest expected fraction of singlet component, namely Pro, Glu, Gln, and Arg (group **A**). Their carbon skeletons are mainly derived from 2-oxoglutarate. The isotope incorporated in 2-oxoglutarate is scrambled *via* the various reactions of the TCA cycle. Concomitantly, the probability of having both C' and $C\alpha$ positions originating from a single glucose molecule of the residues in group **A** decreases. The fraction of singlet component in the ^{13}C fine structure at the C' position (I_s) is expected to be 0.45–0.60 for these residues (Fig. 3c). The second group (group **B**) concerns the amino acids derived from another TCA cycle intermediate, oxaloacetate, and includes Asp, Asn, Ile, Met, and Thr. The I_s values for group **B** are expected to be in the range of 0.30–0.45 due to the contribution from the TCA cycle (Szyperski 1995). Gly is derived from Ser, which is synthesized from a glycolysis metabolite. In addition, Gly might undergo the reversible conversion to C1 unit and CO_2 . Thus, Gly is expected to have a higher I_s value than Ser. Biosynthesis of Lys occurs *via* the diaminopimelate pathway in *E. coli*. The resulting C' fine structure of Lys is expected to represent an intermediate situation between those observed for amino acids derived from glycolysis (group **D**, see below), and from oxaloacetate (group **B**) (Szyperski 1995; Neidhardt et al. 1996). Both Gly and Lys can be placed into the same group (group **C**), as their I_s values are clearly distinct from groups **B** and **D**.

The $C\alpha$ and C' carbons of the remaining amino acids (Cys, Ser, Trp, Phe, Tyr, Ala, Val, Leu, and His) are mainly synthesized from the breakdown of a single

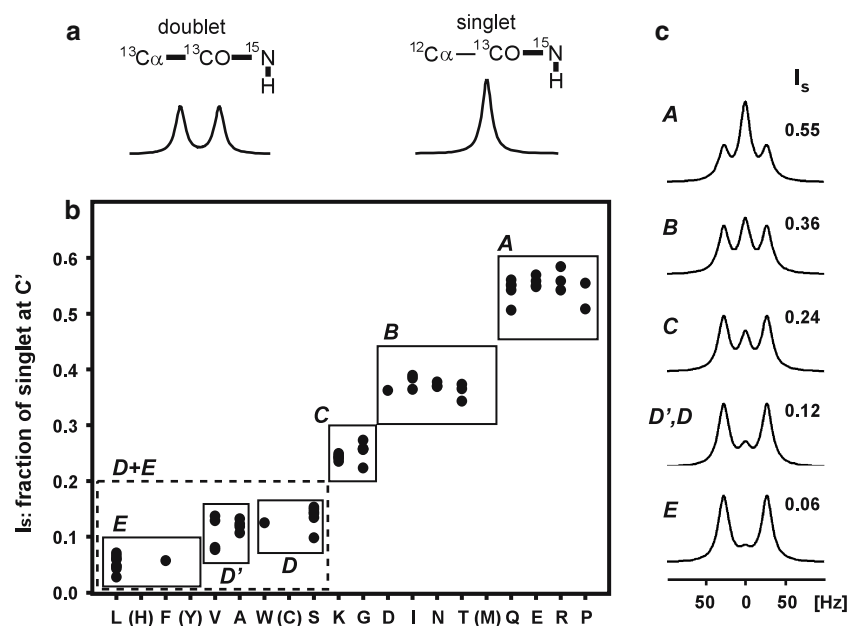


Fig. 3 (a) Possible ^{13}C -labeling patterns of the backbone in a fractionally ^{13}C -labeled protein. Thick bars represent the active scalar coupling used in the $C\alpha$ -coupled HNCOC experiment. In this experiment, a singlet for the C' signal is expected when the neighboring $C\alpha$ is a ^{12}C atom (right). A doublet is generated when this position is occupied by a ^{13}C atom (left). The observed C' fine structure is a superposition of the singlet and doublet components (see c). (b) Fraction of singlet I_s in the C' fine structure versus residue type as obtained from the 100% ^{15}N - and 10% fractionally ^{13}C -labeled 434(1–63) prepared from

E. coli cultivated in standard Erlenmeyer flasks. Thick boxes indicate the residue groups based on the expected and observed results for aerobic and exponentially growing *E. coli*. Missing residue types in 434(1–63) are shown in parentheses. (c) Simulated C' fine structures in a biosynthetically directed fractionally ^{13}C -labeled protein for various fractions of singlet I_s , representing the residue-type groups **A–E**. Parameters for the simulation were 55 Hz for $C\alpha$ – C' coupling and 8 Hz for the linewidth

glucose molecule, resulting in a high fraction of doublet ($I_s < 0.20$). However, Cys, Ser, and Trp are also affected by the C1 unit metabolism, which increases their I_s values. They therefore constitute a separate group **D**. Likewise, one expects slight increases of the I_s values for Ala and Val compared to Phe and Tyr (Ala, Val; $I_s = 0.10$ – 0.20). This effect is due to pyruvate precursor molecules shuffled through the pentose phosphate pathway (Fig. 1). These residues are then classified into another group **D'**. On the other hand, Phe and Tyr are synthesized directly from glycolysis intermediates with little shuffling of the carbon fragments. The $C\alpha$ – C' fragment of Leu originates from Acetyl-CoA that is produced exclusively from the breakdown of a single glucose molecule. Finally, the backbone fragment of His is also synthesized directly from ribose-5-P molecules generated from glucose in the non-oxidative branch of the pentose phosphate pathway (Szyperski 1995; Neidhardt et al. 1996). Therefore, the C' fine structures for Phe, Tyr, Leu, and His should be composed almost exclusively of the doublet component ($I_s < 0.10$). These residues can be classified into group **E** (Fig. 3).

Observed C' fine structures in the 10% fractionally ^{13}C -labeled proteins

In order to investigate the actual C' fine structures in proteins for residue-type classification, a model protein, the DNA binding domain of phage 434 repressor, 434(1–63) was prepared with 100% ^{15}N - and 10% fractional ^{13}C -labeling. Figure 2b shows typical C' fine structures obtained from the 100% ^{15}N - and 10% fractionally ^{13}C -labeled 434(1–63) in the $C\alpha$ -coupled HNCOC spectrum. Analysis of the C' fine structures indicates that the fraction of singlet I_s decreases according to (**A**) Arg, Pro, Gln, Glu > (**B**) Asp, Asn, Ile, Thr > (**C**) Lys, Gly > (**D, D'**) Ser, Trp, Ala, Val > (**E**) Phe, Leu. Overall, this trend is consistent with the expected values described above. However, when I_s values become smaller ($I_s < 0.20$), the quantification of the singlet component also becomes more prone to errors, making the further classification of the amino acids groups less reliable. Indeed, the differences between groups **D** and **E** seem too small to distinguish them reliably even for this small protein. We therefore clustered the residues into four groups

corresponding to the groups **A**, **B**, **C**, and **D + E**, which is depicted by a dotted box in Fig. 3b. The information for Tyr, Met, His, and Cys is missing in the 434(1–63) data set, as the protein does not contain these residues. However, the observed I_s values for Tyr, His, and Met in another protein gpD are in a good agreement with the expected values (Fig. 4). Thus, these missing residues are likely to be consistent with the expected values.

Effect of the $^{13}\text{C}/^{12}\text{C}$ -glucose ratio in the culture medium

The disadvantage of 10% fractional ^{13}C -labeling is the reduced sensitivity due to the dilution of ^{13}C atoms. While increasing the fraction of $^{13}\text{C}_6$ -glucose could improve the S/N in the spectra, it yields C' fine structures with a higher proportion of doublet and thereby attenuates the differences between the residue groups (Fig. 4). To test the effect of the fraction of $^{13}\text{C}_6$ -glucose in the medium, we analyzed the ^{13}C fine structures of 20% fractionally ^{13}C -labeled sample of 434(1–63) (Fig. 4). Overall, I_s in the fine structures follows the same trend: **(I)** Arg, Pro, Gln, Glu > **(II)** Asp, Asn, Ile, Met, Thr > **(III)** Lys, Gly > Ala, Val, Ser, Trp > Phe, Tyr > Leu, His. With this 20% ^{13}C -labeled sample, it has become impossible to draw a clear cut between the group **C** and **D + E**. However, three main groups **I–III** can be distinguished (Fig. 4, dotted boxes). In practice, the fraction of $^{13}\text{C}_6$ -glucose can thus be chosen in the range of 10%–20% depending on the required sensitivity. We applied this method to another 12 kDa protein, gpD with 20%

fractional ^{13}C -labeling and obtained virtually identical results (Fig. 4).

Influence of the sample preparation conditions

Commonly used *E. coli* strains for the protein production are usually either B-strains such as BL21(DE3), ER2566, or K-12 strains such as W3110, which do not differ significantly in their amino acid metabolism, unless they are specially modified by knocking out the genes involved in the metabolic pathways (Neidhardt et al. 1996; Sauer et al. 1999; Fischer and Sauer 2003). Therefore, the proposed method should be directly applicable to commonly used *E. coli* strains. When expressing proteins in different organisms such as *Bacillus subtilis*, *Saccharomyces cerevisiae* or *Pichia pastoris*, the classification of the 20 amino acids will have to be adjusted to the particularities of their metabolic pathways (Sauer et al. 1997; Maaheimo et al. 2001; Fiaux et al. 2003; Sola et al. 2004).

While the amino acids metabolism is usually insensitive to small variation of pH or mineral additives in the M9 medium, significant metabolic switches occur upon oxygen limitation or accumulation of metabolic byproducts. Assuming that exponentially growing cells are in a physiological pseudo steady-state, aeration is only the key parameter that might influence our result significantly. Therefore, we tested protein expression either in a standard Erlenmeyer flask or a baffled Erlenmeyer flask to see how this small change in aeration would affect the C' fine structures. In general, we found that the use of baffled flasks had only a minor effect on the fine structures (Supplemental Table). In particular, there is little influence on groups **D** and **E**.

Anaerobic growth conditions lead to interruption of the TCA cycle and are expected to decrease the fraction of singlet component for the residues in groups **A** and **B**, as well as to shift Ala and Val to group **C** due to the reversible action of pyruvate formate-lyase (Szyperski 1995; Fiaux et al. 1999). In theory, this metabolic switch under anaerobic conditions might be utilized to classify an additional group.

Possible application to larger proteins

Rapid backbone resonance assignment of larger proteins is of importance particularly for studying protein–ligand interactions. Biosynthetically directed fractional ^{13}C -labeling could accelerate such backbone resonance assignments by providing additional information about the residue types, which can be used as anchoring points during the sequential assignment. The lineshape

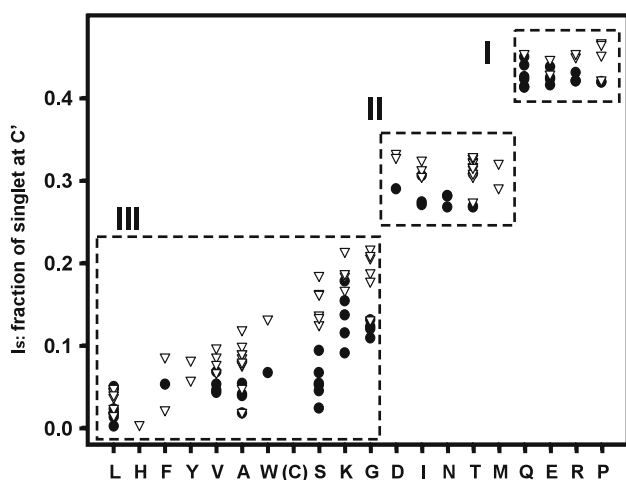
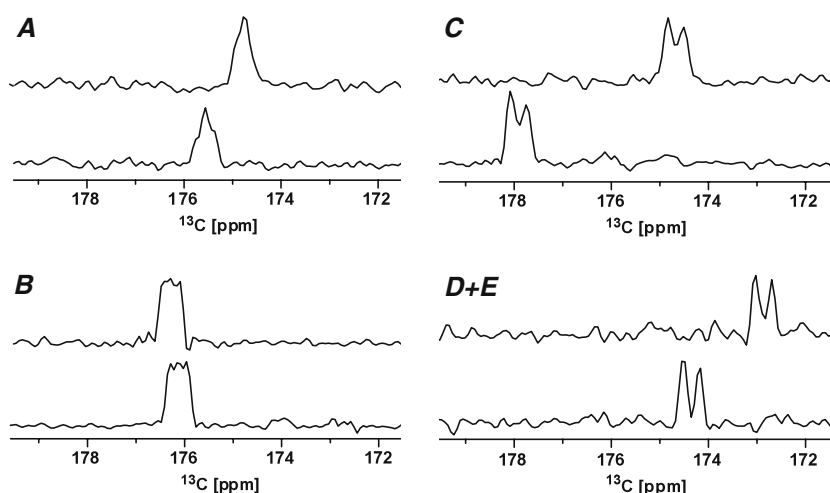


Fig. 4 Fraction of singlet I_s in the C' fine structure versus residue type obtained from the 20% fractionally ^{13}C -labeled 434(1–63) (filled circles) and gpD (open triangles) prepared from *E. coli* cultivated in Erlenmeyer Flasks with baffles

Fig. 5 Typical cross-sections of the $C\alpha$ -coupled HNCO spectrum measured with 0.6 mM 100% ^{15}N - and 10% fractionally ^{13}C -labeled sample of a 29 kDa protein, mRFP. The cross-sections can be categorized into four different groups **A**, **B**, **C**, and **D + E**, based on their lineshapes. A total of 40 (ω_1) \times 80 (ω_2) \times 512 (ω_3) complex points were collected for this spectrum, with $t_{1,\text{max}} = 18.2$ ms, $t_{2,\text{max}} = 32.0$ ms, $t_{3,\text{max}} = 68.2$ ms. The total measurement time was 33 h



analysis of the C' fine structure from the $C\alpha$ -coupled HNCO spectra of larger proteins may become difficult because of the line broadening of C' signals. However, the $C\alpha$ - C' coupling (~ 55 Hz) is usually large enough to distinguish the singlet and doublet, as shown in the spectra of the 29 kDa monomeric red fluorescent protein (mRFP) (Fig. 5). Alternatively, the ratio of singlet/doublet can be quantified without any lineshape analysis by using a HN(CO)CA-type experiment, at the expense of the sensitivity because of the additional magnetization transfer (Iwai and Permi, manuscript in preparation). The line broadening of C' signals will be more profound at higher fields since the linewidth of C' is proportional to the square of the magnetic field strength. To overcome the signal overlap problem arising with larger proteins at the lower field and the required concentration (ca. 1 mM) due to the dilution of ^{13}C , the low-cost fractional ^{13}C -labeling can be combined with a more sophisticated labeling method such as segmental isotope-labeling (Yamazaki et al. 1998; Züger and Iwai 2005).

Conclusion

The combination of 100% ^{15}N - and fractional ^{13}C -labeling can yield both sequential connections and residue-type information from a single sample in a cost-effective manner. This information obtained from biosynthetic pathways is distinct from the chemical shift and spin-system topology parameters routinely used in backbone resonance assignments of proteins. Since 10% fractional ^{13}C -labeling is routinely applied for stereo-specific assignment of diastereotopic methyl groups, our approach can easily be implemented without additional preparation. Moreover, the combination with any other information obtained with

conventional heteronuclear experiments should facilitate the backbone resonance assignment in general, and could also be included in automatic assignment procedures. It might be particularly useful for larger proteins as their backbone resonance assignment based on chemical shifts becomes difficult.

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References

- Clore GM, Gronenborn AM (1994) Multidimensional heteronuclear nuclear-magnetic-resonance of proteins. *Methods Enzymol* 239:349–363
- Dötsch V, Oswald RE, Wagner G (1996a) Amino-acid-type-selective triple-resonance experiments. *J Magn Reson B* 110:107–111
- Dötsch V, Oswald RE, Wagner G (1996b) Selective identification of threonine, valine, and isoleucine sequential connectivities with a TVI-CBCACONH experiment. *J Magn Reson B* 110:304–308
- Fiaux J, Andersson CIJ, Holmberg N, Bülow L, Kallio PT, Bailey JE, Szyperski T, Wüthrich K (1999) C-13 NMR flux ratio analysis of *Escherichia coli* central carbon metabolism in microaerobic bioprocesses. *J Am Chem Soc* 121:1407–1408
- Fiaux J, Cakar ZP, Sonderegger M, Wüthrich K, Szyperski T, Sauer U (2003) Metabolic-flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. *Eukaryot Cell* 2:170–180
- Fischer E, Sauer U (2003) Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS. *Eur J Biochem* 270:880–891
- Grzesiek S, Bax A (1992) Improved 3D triple-resonance NMR techniques applied to a 31-Kda protein. *J Magn Reson* 96:432–440
- Iwai H, Forrer P, Plückthun A, Güntert P (2004) Letter to the editor: assignments of H-1 and N-15 resonances of the

- bacteriophage lambda capsid stabilizing protein gpD. *J Biomol NMR* 28:89–90
- Luginbühl P, Pervushin KV, Iwai H, Wüthrich K (1997) Anisotropic molecular rotational diffusion in N-15 spin relaxation studies of protein mobility. *Biochemistry* 36:7305–7312
- Maaheimo H, Fiaux J, Cakar ZP, Bailey JE, Sauer U, Szyperski T (2001) Central carbon metabolism of *Saccharomyces cerevisiae* explored by biosynthetic fractional C-13 labeling of common amino acids. *Eur J Biochem* 268:2464–2479
- McIntosh LP, Dahlquist FW (1990) Biosynthetic incorporation of N-15 and C-13 for assignment and interpretation of nuclear-magnetic-resonance spectra of proteins. *Q Rev Biophys* 23:1–38
- Muchmore DC, McIntosh LP, Russell CB, Anderson DE, Dahlquist FW (1989) Expression and N-15 labeling of proteins for proton and N-15 nuclear-magnetic-resonance. *Methods Enzymol* 177:44–73
- Neidhardt FC, Curtiss R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznokoff WS, Riley M, Schaechter M, Umberger HE (eds) (1996) *Escherichia coli* and *Salmonella typhimurium*, vol. 1, American Society for Microbiology Press, Washington
- Neri D, Szyperski T, Otting G, Senn H, Wüthrich K (1989) Stereospecific nuclear magnetic-resonance assignments of the methyl-groups of valine and leucine in the DNA-binding domain of the 434-repressor by biosynthetically directed fractional C-13 labeling. *Biochemistry* 28:7510–7516
- Neri D, Otting G, Wüthrich K (1990) H-1 and C-13 NMR chemical-shifts of the diastereotopic methyl-groups of valyl and leucyl residues in peptides and proteins. *Tetrahedron* 46:3287–3296
- Sauer U, Hatzimanikatis V, Bailey JE, Hochuli M, Szyperski T, Wüthrich K (1997) Metabolic fluxes in riboflavin-producing *Bacillus subtilis*. *Nature Biotechnol* 15:448–452
- Sauer U, Lasko DR, Fiaux J, Hochuli M, Glaser RW, Szyperski T, Wüthrich K, Bailey JE (1999) Metabolic flux ratio analysis of genetic and environmental modulations of *Escherichia coli* central carbon metabolism. *J Bacteriol* 181:6679–6688
- Schubert M, Smalla M, Schmiede P, Oschkinat H (1999) Music in triple-resonance experiments: amino acid type-selective H-1-N-15 correlations. *J Magn Reson* 141:34–43
- Senn H, Werner B, Messerle BA, Weber C, Traber R, Wüthrich K (1989) Stereospecific assignment of the methyl H-1-NMR lines of valine and leucine in polypeptides by nonrandom C-13 labeling. *FEBS Lett* 249:113–118
- Sola A, Maaheimo H, Ylonen K, Ferrer P, Szyperski T (2004) Amino acid biosynthesis and metabolic flux profiling of *Pichia pastoris*. *Eur J Biochem* 271:2462–2470
- Szyperski T, Güntert P, Otting G, Wüthrich K (1992a) Determination of scalar coupling-constants by inverse fourier transformation of in-phase multiplets. *J Magn Reson* 99:552–560
- Szyperski T, Neri D, Leiting B, Otting G, Wüthrich K (1992b) Support of H-1-NMR assignments in proteins by biosynthetically directed fractional C-13-labeling. *J Biomol NMR* 2:323–334
- Szyperski T (1995) Biosynthetically directed fractional C-13-labeling of proteinogenic amino-acids – an efficient analytical tool to investigate intermediary metabolism. *Eur J Biochem* 232:433–448
- Yamazaki T, Otomo T, Oda N, Kyogoku Y, Uegaki K, Ito N, Ishino Y, Nakamura H (1998) Segmental isotope labeling for protein NMR using peptide splicing. *J Am Chem Soc* 120:5591–5592
- Wüthrich K (1986) *NMR of Proteins and Nucleic Acids*. Wiley, New York, NY, USA
- Züger S, Iwai H (2005) Intein-based biosynthetic incorporation of unlabeled protein tags into isotopically labeled proteins for NMR studies. *Nat Biotechnol* 23:736–740