A simple biosynthetic method for stereospecific resonance assignment of prochiral methyl groups in proteins

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A simple biosynthetic method for stereospecific resonance assignment of prochiral methyl groups in proteins

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Abstract A new method for stereospecific assignment of prochiral methyl groups in proteins is presented in which protein samples are produced using U-[^{13}C]glucose and subsaturating amounts of 2-[^{13}C]methyl-acetolactate. The resulting non-uniform labeling pattern allows pro$R$ and pro$S$ methyl groups to be easily distinguished by their different phases in a constant-time two-dimensional ^{1}H-^{13}C correlation spectra. Protein samples are conveniently prepared using the same media composition as the main uniformly-labeled sample and contain higher levels of isotope-enrichment than fractional labeling approaches. This new strategy thus represents an economically-attractive, robust alternative for obtaining isotopically-encoded stereospecific NMR assignments of prochiral methyl groups.

Keywords Isotope labeling · Stereospecific assignments · Acetolactate · Prochiral methyl groups

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Introduction

NMR spectroscopy is a well-established technique for elucidating atomic resolution 3D structures of proteins. The final quality of an NMR-elucidated 3D structural ensemble is heavily dependent on a number of factors, including the accuracy and completeness of resonance assignments, and the number and type of structural constraints used. Inter-proton distances derived from NOE spectra remain the major source of information used in NMR structure calculations. The quality and precision of an NMR-elucidated structural ensemble can greatly benefit from correct assignment of resonances from prochiral groups (e.g. methylene protons, aromatic ring protons, isopropyl methyl groups, etc; Billeter et al. 1989; Driscoll et al. 1989; Güntert et al. 1989; Folmer et al. 1997; Kainosho et al. 2006). Perhaps the most important prochiral class in proteins comprises the methyl groups of Leu and Val residues which are often found in the hydrophobic core of proteins and form important long-range inter-residue contacts (Janin et al. 1988; Lo Conte et al. 1999; Tugarinov and Kay 2005). NOEs between methyl groups are therefore crucial for resolving the structure of the hydrophobic core of a protein. Stereospecific assignments can greatly improve the precision of NMR structures by allowing NOEs to be accurately assigned to specific methyl groups and thus avoiding the use of floating prochiral assignments or pseudo-atoms (Güntert et al. 1989).

The methyl groups in leucine (Leu) or valine (Val) side chains are useful sources of structural information. In general methyl groups have strong NMR signals due to proton multiplicity and rapid rotation around the 3-fold methyl symmetry axis, and consequently give rise to easily detected NOE crosspeaks (Tugarinov and Kay 2003). In the past 25 years several approaches have been developed

to allow stereospecific assignment of the prochiral methyl groups of Leu and Val. Such methods have tended to be based on either (a) isotope-labeling (Neri et al. 1989; Ostler et al. 1993; Tate et al. 1995; Atreya and Chary 2001; Kainosho et al. 2006); (b) measurement of NMR parameters (Sattler et al. 1992; Vuister et al. 1993; Tang et al. 2005); or (c) computational prediction of prochirality from NMR data or structures (Pristovsek and Franzoni 2006). The most widely used approach to date involves fractional isotope labeling of the target protein. In this protocol the protein is expressed from a minimal culture medium containing 10% [^{13}C]glucose and 90% [^{12}C]glucose (Neri et al. 1989). The signals of prochiral methyl groups in the resulting protein can then be differentiated by their phase in a constant-time (CT) 2D ^{1}H-^{13}C correlated spectrum. This elegant and simple method has found great use for determining stereospecific assignments in small and large proteins alike (Tugarinov and Kay 2004; Yee et al. 2006) and has spawned subsequent variations (Atreya and Chary 2001), and specifically devised NMR experiments (Hu and Zuidereg 1996), as well aiding the assignment of other nuclei (e.g. Jacob et al. 2002). One of the main practical drawbacks is that fractional labeling requires an additional and separate expression and purification of the target protein. Due to the low level of isotope enrichment, and therefore reduced sensitivity in subsequent experimental analysis, a fractional labeling strategy often necessitates preparing culture volumes comparable to those required for the primary [^{13}C,^{15}N]-labeled samples used for resonance assignment and NOE analysis.

Here, a method is presented that allows simple and efficient spectroscopic-differentiation of prochiral methyl groups in proteins. This new strategy utilizes the stereospecific enzymatic reactions that occur in the early stages of Leu and Val biosynthesis in *E. coli*. The final protein sample contains a mixture of isotope-labeling at prochiral sites with partial uniform [^{13}C]-labeling of Leu and Val side chains and partial [^{13}C^{1}H$_3$]^{proS}-specific labeling. This labeling pattern allows NMR signals of pro*R* and pro*S* methyl groups in the resulting protein to be distinguished by their sign in CT heteronuclear NMR spectra.

We recently reported the use of a metabolic intermediate in the biosynthesis pathway of Leu and Val, 2-hydroxy-2-methyl-butanoate (or acetolactate), for stereospecific labeling of pro*S* methyl groups in large proteins (Gans et al. 2010). The addition of 2-[^{13}C]methyl-4[^{2}H$_3$]-acetolactate to an *E. coli* expression culture produces a target protein with a Leu/Val-[^{13}C^{1}H$_3$]^{proS}, [^{12}C^{2}H$_3$]^{proR}-labeling pattern. This labeling strategy is ideal for high molecular weight proteins, which require a high-level of background deuteration. U-[^{2}H,^{13}C,^{15}N]-labeled proteins with specific Leu/Val-[^{13}C^{1}H$_3$]^{proS}-labeling can be conveniently prepared from a D$_2$O-based expression medium supplemented with 2-[^{13}C]methyl-4[^{2}H$_3$]-acetolactate and U-[^{2}H,^{13}C]glucose. Acetolactate is thus an attractive alternative to using α-ketoisovalerate (Goto et al. 1999; Lichtenecker et al. 2004; Tugarinov and Kay 2003). The main advantage in using acetolactate stems from the fact that α-ketoisovalerate is only available as a racemic mixture and thus results in equal labeling of pro*R* and pro*S* methyl groups across the sample, although with only a single [^{1}H,^{13}C]-labeled methyl group per residue (Tugarinov and Kay 2003).

In a 2D CT ^{1}H,^{13}C heteronuclear NMR spectra of a uniformly [^{13}C]-labeled protein all methyl group resonances have the same phase and consequently prochiral-specific assignments of Leu/Val methyl groups can not be determined (Fig. 1a). However, it is possible to manipulate the labeling patterns of the side chains of Leu and Val by supplementing the expression medium with suitable metabolic precursor. Using 2-[^{13}C]methyl-acetolactate produces a labeling pattern in which the pro*S* methyl group is [^{13}C]-labeled while the remainder of the side chain is [^{12}C]-labeled. As Leu-C$^\gamma$ and Val-C$^\beta$ sites are [^{12}C]-labeled the signal arising from a pro*S* methyl group is not affected by the evolution of a $^1J_{CC}$ coupling during the CT period and thus the corresponding signals appear 180° out of phase compared to the same signal in a uniformly [^{13}C]-labeled sample (Fig. 1b).

Adding a saturating amount of 2-[^{13}C]methyl-acetolactate removes crosspeaks originating from pro*R* methyl groups (ignoring the residual [^{13}C]-labeling derived from natural abundance). However, if a smaller, non-saturating amount of 2-[^{13}C]methyl-acetolactate is used in addition to U-[^{13}C]glucose then both pro*R* and pro*S* resonances will be detected as, over the whole NMR sample, certain prochiral sites would be derived from glucose and others from 2-[^{13}C]methyl-acetolactate (Fig. 1c). With this mixed-labeled sample the pro*S* crosspeak observed is the sum of the opposite-phased glucose-derived and acetolactate-derived pro*S* crosspeaks. The phase of the pro*S* peak can therefore be manipulated by changing the amount of 2-[^{13}C]methyl-acetolactate used. For example, if 66% of the metabolic pool of Leu and Val has been produced using 2-[^{13}C]methyl-acetolactate then the glucose-derived pro*S* signal would be completely cancelled out. This scenario would result in a spectrum in which the pro*R* and pro*S* crosspeaks appear 180° out of phase (Fig. 1c). Furthermore, the pro*R* and pro*S* crosspeaks would have equal intensity but at 33% of the signal intensity normally observed in spectra of a protein produced using U-[^{13}C]glucose alone.

Protein production for NMR assignment or NOE-based structural analysis commonly involves the expression of the target protein from several liters of U-[^{13}C,^{15}N]-enriched minimal expression medium. This strategy is outlined in Fig. 2a and is essentially routine for the characterization
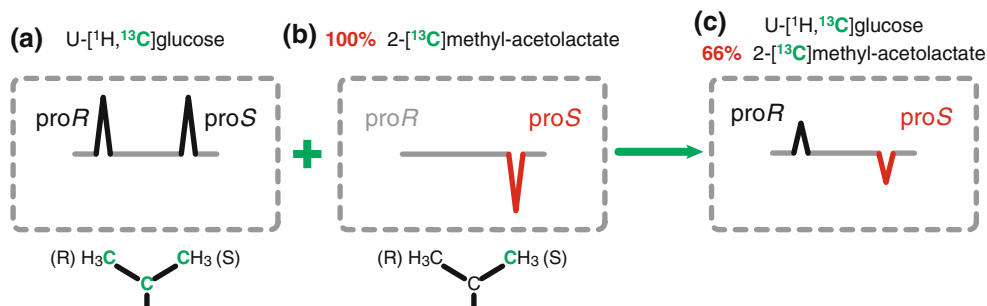
Fig. 1 Isotope-labeling patterns in prochiral methyl groups. **a** media containing U-[^{13}C]glucose as the sole carbon source produces proteins in which the side-chains of Leu and Val are uniformly [^{13}C]-labeled and consequently both methyl peaks have the same phase in a CT HSQC spectrum; **b** supplementing the expression medium with a saturating amount of 2-[^{13}C]methyl-acetolactate produces Leu and Val residues in which only the pro$S$ group is [^{13}C]- labeled, regardless of whether the glucose used was [^{13}C]-labeled or not. The pro$S$ crosspeak would appear 180° out of phase to those in (**a**). **c** using sub saturating amounts 2-[^{13}C]methyl-acetolactate in addition to U-[^{13}C]glucose results in a protein sample with a mixture of labeling patterns for Leu and Val side-chains. In a CT HSQC spectrum of such a sample the pro$R$ and pro$S$ crosspeaks appear 180° out of phase
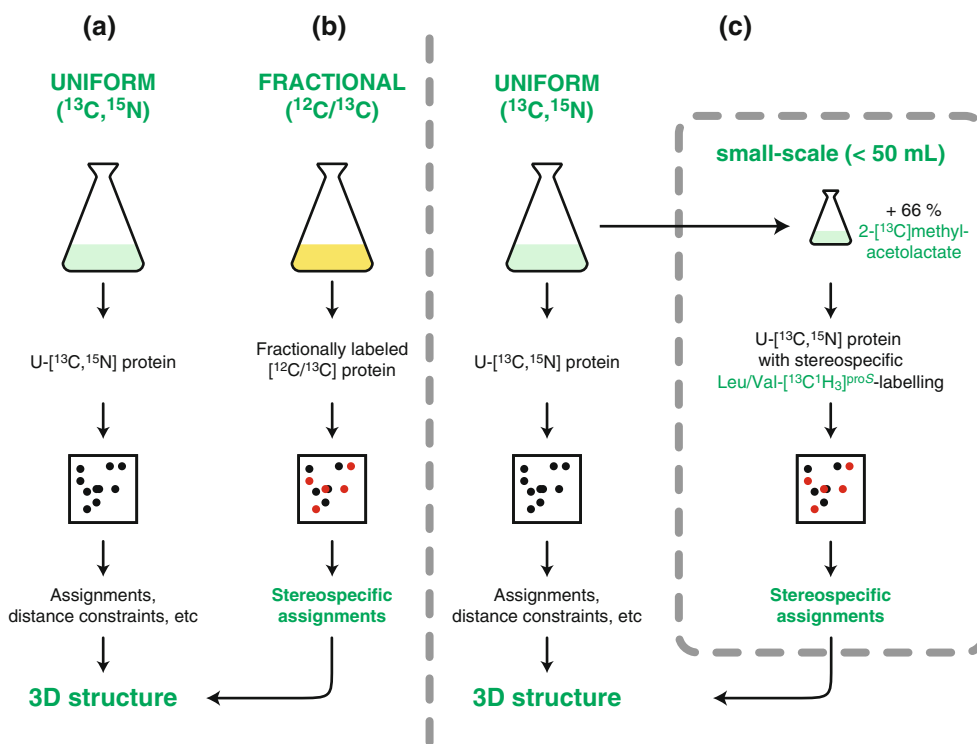

Fig. 2 Production of isotope-labeled samples for analysis by NMR spectroscopy. **a** an example of standard large-scale protocol for the production of U-[^{13}C,^{15}N]-labeled proteins. Proteins are expressed from a uniformly [^{13}C,^{15}N]-labeled expression medium. This sample is ideal for backbone and side chain assignment and the acquisition of structural restraints, but it cannot be easily used for determining stereospecific assignments of prochiral methyl groups. **b** A fractional labeling approach can be used to establish stereospecific resonance assignments of prochiral methyl groups. In this instance the protein is expressed from a large-scale culture medium containing only 10% [^{13}C]glucose and 90% [^{12}C]glucose. In a CT HSQC spectra recorded of this sample the crosspeaks of pro$R$ and pro$S$ methyl groups will be 180° out of phase. These stereospecific assignments can then be included in subsequent structural analyses. **c** Obtaining stereospecific assignments using 2-[^{13}C]methyl-acetolactate and a standard uniformly [^{13}C,^{15}N]-labeled expression medium. A small aliquot (4–50 mL) of the primary expression culture is taken at an optical density of 0.7. 2-[^{13}C]methyl-acetolactate is added at sub-saturating levels (the amount added is dependent on the amount of glucose used—see main text) and protein expression is induced 1 hour later (Gans et al. 2010). In a CT HSQC spectra recorded of this sample the crosspeaks of pro$R$ and pro$S$ methyl groups will be 180° out of phase. These stereospecific assignments can then be included in subsequent structural analyses

of smaller proteins (e.g. < 25 kD). To obtain stereospecific assignments using a fractionally-labeled sample (e.g. with 5 or 10% [^{13}C]-labeling) requires a separate round of protein expression using a specially prepared expression medium containing a different isotope composition (Fig. 2b; Neri et al. 1989).

The availability of acetolactate means that an alternative protein production strategy for isotope-encoded differentiation of prochiral methyl group crosspeaks can be proposed. As outlined above, it is possible to supplement acetolactate to a glucose-containing medium at level that suppresses the glucose-derived proS signal but retains the proR signal. This labeling pattern would give a final effective level of isotope labeling of approximately 33%, which is significantly higher than the 5-10% obtained using a fractional labeling protocol. As a sample produced using acetolactate has a higher effective concentration less sample is required for NMR analysis and consequently sufficient protein can be obtained from a much smaller volume of culture. Furthermore, acetolactate can be added to a uniformly-labeled culture medium. These two factors allow a new expression protocol to be proposed (Fig. 2c) in which a small aliquot (< 50 mL) is taken from the primary U-[^{13}C,^{15}N]-labeled expression culture to which a sufficient amount of 2-[^{13}C]methyl-acetolactate is added to ensure that approximately 66% the metabolic reservoir of Leu and Val is derived directly from the precursor. Protein expression is induced 1 hour later. As outlined in Fig. 1c, a CT HSQC spectrum of a protein produced in this way will still contain all the information that would be available using a U-[^{13}C,^{15}N]-labeled sample. However, the crosspeaks originating from proS methyl groups would be inverted with respect to the corresponding signal from a uniformly labeled sample.

The implementation of this approach in protonated expression media required a slight modification of the published synthesis scheme for acetolactate. The previously reported protocol produces 2-[^{13}C]methyl-4[^{2}H$_3$]-acetolactate (Gans et al. 2010). This molecule is optimal for studying larger molecular systems as deuteration of the proR methyl group reduces the inter-methyl group dipolar interaction (Tugarinov and Kay 2003; Gans et al. 2010). When using protonated glucose to produce a uniformly protonated sample (e.g. using the protocol outlined in Fig. 2) it is necessary that the proR methyl group and directly bonded carbon (i.e. Leu-C$^{\gamma}$ or Val-C$^{\beta}$) is protonated as this ensures that the signal of the proS group produced on adding 2-[^{13}C]methyl-acetolactate has an identical chemical shift and linewidth to that produced from uniform [^{13}C]-labeling with glucose. Thus, 2-[^{13}C]methyl acetolactate was prepared by deprotection of ethyl 2-hydroxy, 2[^{13}C]methyl-3-oxobutanoate in H$_2$O at pH 13.0 using the conditions previously described (Gans et al. 2010). Using fully-protonated 2-[^{13}C]methyl acetolactate instead of 2-[^{13}C]methyl-4[^{2}H$_3$]-acetolactate resulted in spectra in which the acetolactate-derived crosspeaks overlapped perfectly with their glucose-derived counterparts.

This new labeling approach for determining stereospecific assignments of Leu and Val methyl groups was tested on three proteins: ubiquitin (8 kD), a T50V mutation of the CAP-Glycine domain of the p150^{Glued} subunit of dynactin (8 kD; Plevin et al. 2008) and YajG, (20 kD; Boudet et al. 2007). Each target protein was prepared from a small volume culture of [^{13}C,^{15}N]-labeled minimal medium. The volume used was dependent on the expression level of the target protein and typically between 4 and 50 mL. Each culture was supplemented with 2-[^{13}C]methyl-acetolactate when the optical density at 600 nm (OD$_{600}$) reached 0.7. Protein expression was induced one hour later using IPTG (Gans et al. 2010). In each instance the protocol for cell growth and protein expression was identical to that employed for the preparation of a U-[^{13}C,^{15}N]-labeled sample, up to the addition of 2-[^{13}C]methyl-acetolactate. Following protein expression each sample was purified in a single step using immobilized-nickel affinity chromatography. Eluted samples were then concentrated to 0.5 mL before undergoing 3–4 rounds of buffer exchange by ultrafiltration. The final protein concentrations used for NMR analysis were between 50 and 200 μM.

CT ^{1}H-^{13}C HSQC spectra were recorded of each protein using an unmodified pulse sequence from the BioPack library (Varian) with the CT period set to 27 ms. Fig. 3 shows examples of 2D spectra recorded of U-[^{13}C,^{15}N]-labeled samples using U-[^{13}C]glucose alone and those prepared using U-[^{13}C]glucose and 2-[^{13}C]methyl-acetolactate. As predicted from Fig. 1C, a subsection of crosspeaks in spectra recorded of samples expressed in the presence of 2-[^{13}C]methyl-acetolactate change phase (Fig. 3). Plotting the negative and positive crosspeaks in different spectra unambiguously differentiates the two classes of prochiral methyl groups and easily allows stereospecific assignments to be made (Fig. 4).

The amount of acetolactate required to achieve perfect cancellation of glucose-derived proS crosspeaks is dependent on the amount of glucose used in the expression medium. 50-100 mg/L of 2-[^{13}C]methyl-acetolactate was found to be sufficient for media containing 2 g/L [^{13}C]-glucose (Figs. 3, 4 and Supplementary Figure 1) while the amount 2-[^{13}C]methyl-acetolactate required increased to 180-220 mg/L when using 3 g/L glucose (Supplementary Figure 1). There exists the possibility of minor culture-by-culture variations in the uptake and/or incorporation of the precursor, however the results presented here (Figs. 3, 4 and Supplementary Figure 1) demonstrate that our new approach is sufficiently robust to tolerate these differences.

The overall net level of isotope labeling in the final sample is 100% and approximately 33% for non-prochiral and prochiral methyl sites, respectively, which allows a 2D CT HSQC spectrum of sufficient intensity and resolution to be collected in less than one hour, depending on the sample concentration. To achieve the same level of sensitivity with a sample prepared using the fractional-labeling approach
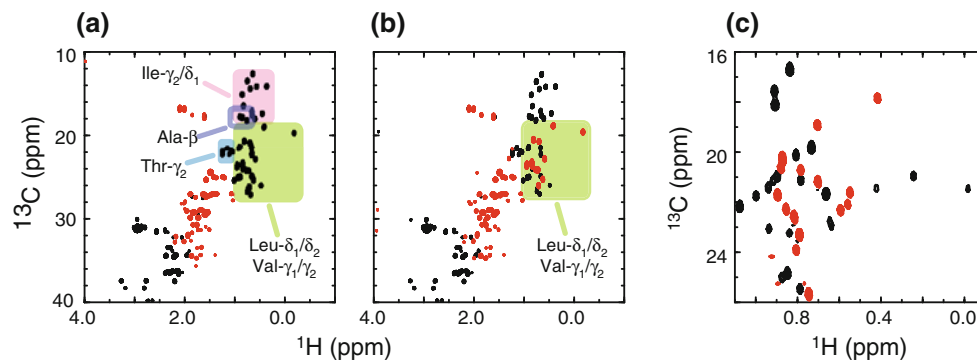
Fig. 3 Examples of 2D CT HSQC spectra of proteins prepared in the absence (**a**) and presence (**b**, **c**) of 2-[^{13}C]methyl-acetolactate (positive contours, black; negative contours, red). All protein samples were prepared using 2 g/L U-[^{13}C]glucose using small-scale (<50 mL) expression cultures. Spectra are presented for ubiquitin (**a**, **b**) and the CAP-Gly domain of the p150^{Glued} subunit of dynactin (**c**). The boxes in (**a**) indicate the resonance frequencies for each type of methyl group. The only crosspeaks to change sign in spectra of proteins prepared using 2-[^{13}C]methyl-acetolactate (supplemented at 50–100 mg/L) are those from the region containing the methyl groups of Leu and Val. NMR data were collected using an 800 MHz Varian Direct Drive spectrometer equipped with pulsed-field gradients and a cryogenically-cooled triple resonance probe head. CT HSQC spectra were recorded with (**a**, **b**) 256 (t_1) × 640 (t_2) complex points and acquisition times of 21 ms (t_1) and 64 ms (t_2); or (**c**) 256 (t_1) × 1,048 (t_2) complex points and acquisition times of 16 ms (t_1) and 78 ms (t_2). 16 scans were collected per indirect increment with the CT delay set to 27 ms
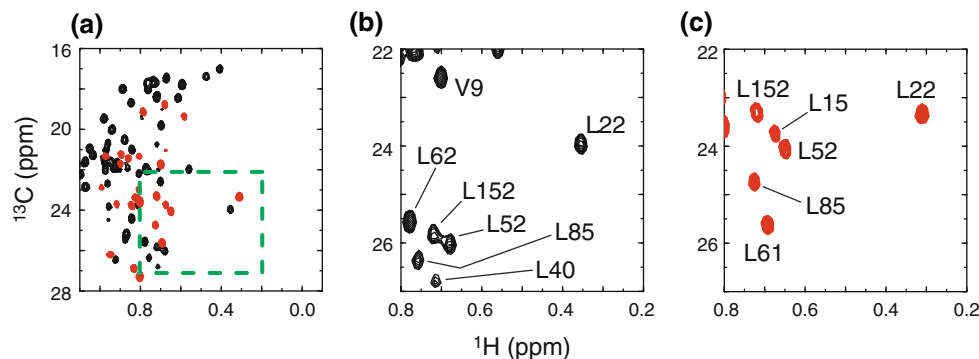

Fig. 4 Stereospecific assignment of Leu and Val methyl resonances. (**a**) region of the CT HSQC spectrum of YajG (positive contours, *black*; negative contours, *red*); Assignments for crosspeaks within the green box in (**a**) are provided in two sub spectra showing either the positive (**b**) or negative (**c**) contours of (**a**). pro*R* methyl groups yield positive signals (**b**) while pro *S* groups yield negative signals (**c**). NMR data were collected using an 800 MHz Varian Direct Drive spectrometer equipped with pulsed-field gradients and a cryogenically-cooled triple resonance probe head. CT HSQC spectra were recorded with 256 (t_1) × 1,048 (t_2) complex points, acquisition times of 16 ms (t_1) and 78 ms (t_2) and 16 scans per indirect increment. The CT delay set to 27 ms

would require either 3–4 times as much protein (Supplementary Figure 2) or 9–16 times more spectrometer time.

It was recently reported that acetolactate derivatives for isotope-labeling of protein samples are commercially available (Ruschak et al. 2010). The data presented in Figs. 3 and 4 was obtained from protein samples prepared using 2 g/L U-[^{13}C]glucose and <100 mg/L 2-[^{13}C]methyl-acetolactate. For a small scale culture (e.g. 50 mL) the total isotope consumption is only 0.1 g U-[^{13}C]glucose and ∼5 mg 2-[^{13}C]methyl-acetolactate. The additional cost of supplementing a 50 mL expression media with a subsaturating amount of 2-[^{13}C]methyl-acetolactate would be less than $10.

Most importantly, the background isotope labeling scheme of the expression medium (e.g. U-[^{13}C]glucose, U-[^{15}N]NH$_4$Cl) is identical to that used in the preparation of the main uniformly-labeled sample used for resonance assignment and NOESY data collection. This means that, using the protocol detailed here, it is not necessary to produce multiple large-scale cultures with different isotope enrichment schemes. As outlined in Fig. 2c, it is sufficient to take a small aliquot of the main culture and use this to prepare protein samples for prochiral methyl group assignment in parallel.

One of the advantages of expressing stereospecifically labeled samples from a standard uniformly-labeled expression media is that the changes to the resulting CT HSQC spectra are limited to the prochiral methyl groups (Figs. 2a, b). This is not the case for fractionally labeled samples (Supplementary Fig. 2c) where additional changes

in the spectrum are observed, such as the disappearance of Ile-δ_1 methyl resonances and the decreases in signal intensity of crosspeaks of Thr-γ_1 methyl groups which could potentially overlap with Leu or Val prochiral methyl peaks.

For larger proteins or for spectra with considerable overlap of methyl resonances, removing crosspeaks associated with proR methyl groups may greatly ease the process of resonance assignment. This can easily be achieved by increasing the amount of 2-[^{13}C]methyl-acetolactate used. Supplementing the expression media with saturating levels of acetolactate would cause complete suppression of proR peaks and thus decrease spectral crowding (Supplementary Figure 1).

In conclusion, the isotope-labeling strategy outlined here is a simple yet robust method for producing a protein in which the stereospecific assignment of prochiral resonances are isotopically pre-encoded. This new approach benefits from an improved level of isotope-enrichment and the fact that stereospecific assignments can be obtained from a 2D CT-HSQC. Both of these factors considerably decrease the amount of protein required and the time needed for protein production, as well as reducing spectrometer and analysis time. Consequently, the small extra isotope expense incurred using 2-[^{13}C]methyl-acetolactate is very easily offset by the reduction in spectrometer costs and more efficient use of human resources. This new protocol therefore represents a widely-applicable, straightforward, cost-effective and reliable method for obtaining stereospecific assignments of prochiral methyl groups.

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