Protein Isotope Labelling | Hot Paper

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Local Deuteration Enables NMR Observation of Methyl Groups in Proteins from Eukaryotic and Cell-Free Expression Systems

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Abstract: *Therapeutically relevant proteins such as GPCRs, antibodies and kinases face clear limitations in NMR studies due to the challenges in site-specific isotope labeling and deuteration in eukaryotic expression systems. Here we describe an efficient and simple method to observe the methyl groups of leucine residues in proteins expressed in bacterial, eukaryotic or cell-free expression systems without modification of the expression protocol. The method relies on simple stereo-selective ^{13}C-labeling and deuteration of leucine that alleviates the need for additional deuteration of the protein. The spectroscopic benefits of "local" deuteration are examined in detail through Forbidden Coherence Transfer (FCT) experiments and simulations. The utility of this labeling method is demonstrated in the cell-free synthesis of bacteriorhodopsin and in the insect-cell expression of the RRM2 domain of human RBM39.*

NMR spectroscopy provides a rich source of information about the dynamics and interactions of proteins that are essential for function. The side-chain methyl groups of Ile, Leu and Val form critical hydrophobic contacts and stabilize the structural cores and interaction grooves of proteins, making them powerful probes in therapeutically relevant systems.[1] The symmetry of the methyl group ensures that all three hydrogens contribute to a single intense signal, further enhanced by the favourable relaxation properties due to rapid rotation around the C–C bond. In combination with the methyl-TROSY experiment,[2] which selects ^{13}C-^{1}H$_3$ coherences that relax much slower, methyl resonances can be observed even in megadalton-size proteins and used to characterize their interactions and dynamics.[3] However, for such large protein complexes, observation of the methyl signal requires replacement of surrounding protons with deuterons to reduce signal loss due to dipole-dipole relaxation.[4] The most common method for achieving specific labelling at methyl positions is to grow bacteria in perdeuterated medium supplemented with the appropriate ^{13}C^{1}H$_3$-methyl-labeled biosynthetic precursors of the amino acids targeted for observation.[5] However, using this method, significant dipole-induced relaxation can also arise from intra-residue proton-proton interactions, for example, between the geminal methyl groups in Leu or Val.[4,6] The introduction of pro-chiral amino acid precursors[1b] eliminated intra-residue dipole-dipole relaxation in bacterially expressed proteins.

Yet, biologically relevant targets, including most protein kinases, integral membrane proteins (GPCRs, ABC transporters and TCRs), and therapeutic antibodies often cannot be functionally expressed in bacteria. *In vitro* protein expression with ribosomal extracts represents an increasingly viable alternative for obtaining functional proteins; however the [^{13}C,^{1}H,^{2}H]-labeled amino acids required for production of methyl-labeled proteins by this method remain expensive (especially for the Leu precursor)[7] and/or require elaborate experimental protocols[7b,8] to achieve methyl labeling. Furthermore, expressing functional G-protein coupled receptors (GPCRs) *in vitro* remains a challenge.[9] Recently, yeast strains able to produce functional GPCRs, survive deuteration, and process the metabolic precursor from Ile have been developed.[10] Met,[11] Ala[12] and methylated lysines[13] and cysteines[14] have been used to probe GPCR dynamics, and the

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NMR signal was boosted by judicious "local inter-residue deuteration", that is, addition of deuterated amino acids of the types that occur in the spatial vicinity of the methyl group of interest. Alternatively, insect cell culture is frequently the method of choice for the production of proteins that have stringent requirements for proper folding and post-translational modifications. However, higher eukaryotic cells are not viable in ^2H_2O concentrations higher than 30%. Still up to 75% protein deuteration has been achieved by the addition of deuterated amino acids to the growth medium.[11c,15] Local deuteration surrounding the desired [^{13}C,^{1}H]-methyl signals was found to improve spectral quality significantly.[11c,15a] Despite progress, there remains an unmet need for simple and broadly applicable labeling of protein methyl groups, for example, through the use of affordable stereo-specifically labeled amino acids.[11a,12]

The stereo-selective isotope enrichment of branched amino acids is amply documented in the chemistry literature.[16] An impressive set of [^{13}C,^{15}N,^{2}H]-labeled amino acids optimally designed for automated NMR structure determination was developed[7a] and applied to obtain stereo-specific Leu labeled 150 kDa IgG2b glycoprotein produced in mouse hybridoma cells,[1d] which produced better spectra than its protonated counterpart in an otherwise protonated background. However, due to the complex chemistry and expensive starting materials, these amino acids remain prohibitively expensive. In light of this, we set out to produce a more affordable version of Leu containing the essential features needed for observing methyl resonances in large proteins.

Here we report a straightforward synthesis of Leu in which the desired methyl group is [^{13}C,^{1}H]-labeled while some of the proximal hydrogens are deuterium-enriched (Figure 1). The "locally" deuterated, methyl ^{13}C-labeled leucine (**5**, Figure 1) is hereafter referenced as *Leu-meth$_{LD}$*. We present an analysis of the benefits of this labeling configuration and report significantly increased spectral resolution and sensitivity even in an otherwise protonated background. We incorporate *Leu-meth$_{LD}$* into the 42-kDa maltose binding protein, the 12-kDa human RBM39$^{245-332}$ encapsulating RRM2 domain, and the 25-kDa membrane protein bacteriorhodopsin using *E. coli*, insect cell and cell-free protein expression, respectively, without modification of the original expression protocols. This method of labeling is flexible, simple and promises to considerably expand the range of isotopically labeled proteins available to NMR-based structural studies.

In our hands the most economical route to methyl-labeled leucine was a simplified version of a synthetic route by Siebum et al.[17] (Figure 1a). The isopropyl oxazolidinone (**1**) was acylated with deuterated propionyl chloride, and the product was stereo-selectively methylated with small excess of $^{13}CH_3$-methyl iodide, with an enantiomeric excess of 94%. Reductive cleavage with $LiAlH_4$ ($LiAlD_4$ can be used for deuteration of the β positions), followed by treatment with PPh_3/Br_2 yielded the stereoselective methyl-labeled isobutyl bromide (**4**). Chiral alkylation of activated glycine (**6**) yielded L-Leu, but the reaction proved sluggish unless stoichiometric quantities of expensive Maruoka catalyst[18] were used. To save cost we opted for the racemic DL-Leu which retained the

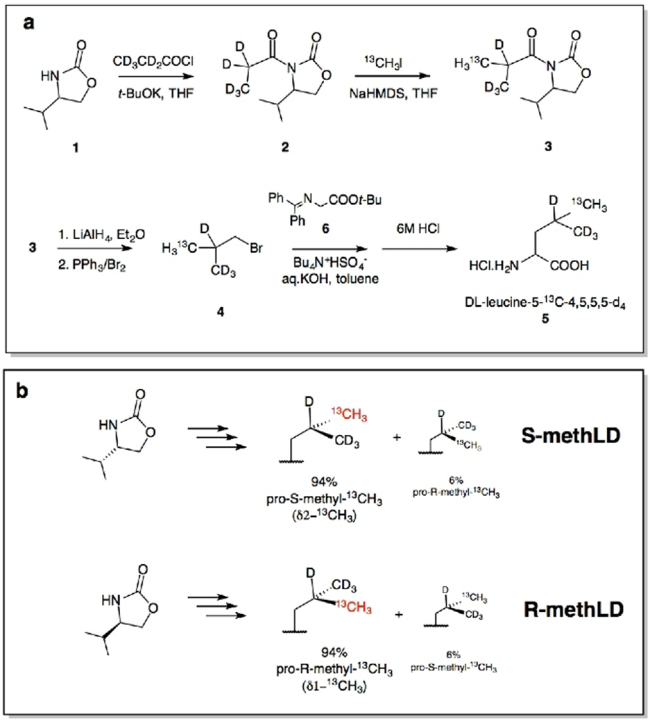

Figure 1. Simple and inexpensive synthesis of "locally" deuterated $^{13}CH_3$-methyl leucine. A) Synthesis route of Leu-meth$_{LD}$, (**5**); b) The pro-R-$^{13}CH_3$ or pro-S-$^{13}CH_3$ methyl configuration of **5** is obtained with 94% stereoselectivity starting from the corresponding Evans' chiral auxiliary **1**.

stereochemistry of the labeled methyl groups (Figure 1a). The final *Leu-meth$_{LD}$* (5-^{13}C-5,5,5,4-d$_4$-DL-Leu, **5**) contained 94% of the desired $^{13}C^1H_3$-methyl DL-leucine and 6% of the methyl-inverted configuration (Figure 1b). We synthesized and tested the pro-*R* isotopomer *(R-meth$_{LD}$)* in overall yield of 24% from d$_5$-propionic acid and 18% from $^{13}CH_3$-methyl iodide. The cost of reagents per milligram of *R-meth$_{LD}$* was less than 1 €. By comparison, a labeled ketoacid used to generate an analogous Leu labeling pattern in cell-free expression was reportedly obtained for 20 € mg^{-1}.[7a] A summary of the cost of *Leu-meth$_{LD}$* (**5**) used to label the proteins in this study is given in the supplementary information.

Using theoretical calculations, we compared the contribution of intra- and inter-residue ^{1}H to dipole-dipole relaxation of Leu-methyl protons in the 42-kDa protein MBP. We used the atom coordinates from a previously deposited NMR structure (PDB ID: 1EZO) and calculated all inter-proton distances between leucine methyl protons and the rest of the protons in MBP (averaged over all the conformations). The details of the computation are described in the supplementary information. Deuterating four of the closest intra-residue protons, namely Hδ21, Hδ22, Hδ23 and Hγ, translates to a 37% reduction in dipole-dipole interactions (Figure S1) leading to narrower linewidths. This is comparable to the dipole-dipole relaxation caused by all the remaining inter-residue protons (≈38%). This suggests that local deuteration, including stereo-specific methyl deuteration could sufficiently reduce the rate of transverse relaxation and

improve resolution and sensitivity in the absence of complete protein deuteration.

To experimentally validate these findings and quantify the effect of local deuteration, we used forbidden coherence transfer (FCT) experiments.[19] FCT experiments translate into a build-up curve where the slope reports on local dynamics (order parameter S^2) and the plateau on the local ^1H density that contributes to relaxation. Fits show that deviation from the control is higher for ^1H density than for S^2 parameters, as expected (Figure S4, supplementary information). As examples, we highlight Leu7 and Leu121 in MBP that was (i) δ1,2-[^{13}C,^1H]-methyl-labeled and fully protonated, and (ii) δ1-[^{13}C,^1H]-methyl, δ2-[^{12}C,^2H]-methyl, γ-[^2H]-labeled in an otherwise fully protonated background. The samples were produced in *E. coli* with the addition of (i) ^{13}C-α-ketoisovalerate[20] or (ii) *R-meth*$_{LD}$ (Figure 1b) to the aqueous M9 minimal medium 1 h before induction (Figure S4). The ratio of values to which the two FCT curves in Figure 2b plateaus gives us an estimate of the linewidth reduction (≈50%) due to the reduced dipole-dipole interaction from the geminal methyl protons. We present additional FCT curves for other Leu residues in MBP in supplementary Figures S2,S3.

These results show that "local deuteration" of Leu will reduce transverse relaxation sufficiently to produce usable NMR spectra from large, otherwise non-deuterated protein complexes. It represents a new avenue for proteins that cannot faithfully be expressed in bacteria. To validate this hypothesis, we expressed the seven-transmembrane-helix protein bacteriorhodopsin (bR) in an *E.-coli*-based cell-free system and the 12-kDa human RBM39$^{245–332}$ encapsulating RRM2 domain in *Sf*-9 cells. For bR, uniformly ^{13}C-labeled non-deuterated L-Leu (8.5 mg) or *R-meth*$_{LD}$ (Figure 1b) (17 mg) was used, and no difference in expression yield was observed between the samples (see supplementary methods for more details). bR was refolded and purified in *n*-dodecyl-β-D-maltoside (DDM) micelles, leading to a final protein-detergent complex of approximately 100 kDa.[21] Heteronuclear multiple quantum correlation (HMQC) spectra were acquired on both samples (see Figure 3a black vs. red). Similarly, for RBM39$^{245–332}$, medium prepared simply by replacing uniformly labeled Leu with 75 mg L^{-1} of *R-meth*$_{LD}$ was used and heteronuclear single quantum correlation (HSQC) spectra (Figure 3f) were measured. Notably, the quality of the RBM39$^{245–332}$ spectrum was sufficient to also detect the much weaker signals arising from the residual 6% pro-S (δ2) $^{13}C^1H_3$-methyl groups. In both cases (bR and RBM39), the $^1J_{CC}$ couplings in the [U-^{13}C,^1H]-Leu sample were absent in the *Leu-meth*$_{LD}$ sample, obviating the ^{13}C constant-time evolution component of the pulse sequence[22] and enabling applications of non-uniform sampling. A projection of the 2D spectra (along ^1H dimensions, Figure 3c,e,h,j) showed a 30%–50% increase in resolution and on average an approximately 6-fold increase in intensity. While an intensity gain of 2-fold is expected due to the absence of ^{13}C-^{13}C ^1J-couplings (Figure 3d,i), the reduced ^1H-^1H dipolar couplings in the *Leu-meth*$_{LD}$ sample contribute to the further enhanced spectral features. Notably, for bR in DDM micelles the residue-specific intensity gains can be well above 10-fold and depend on dynamic range and local protonation levels of the system favouring peaks that also require signal enhancements the most (see Figure S10 for more details). Overall, use of *Leu-meth*$_{LD}$ in the otherwise natural abundance expression system improved the quality of methyl spectra sufficiently to tip the scales in favour of NMR studies of binding interactions, dynamic and allosteric mechanisms in numerous challenging systems.[3a,13a]

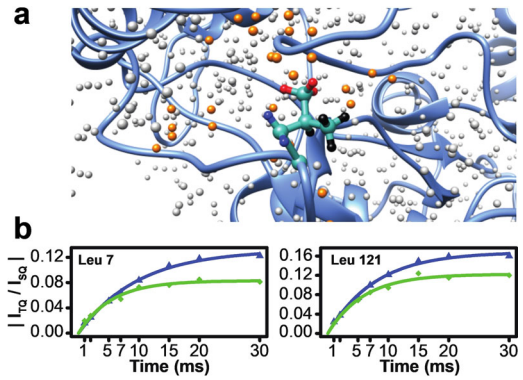

Figure 2. Local deuteration reduces the transverse relaxation rate of Leu methyl groups. (a) Enlarged view of the NMR structure of maltose binding protein (MBP, PDB:1EZO) around Leu121. Leu121 is shown as a ball and stick model with deuterated protons in black, Hβ2 and Hβ3 in blue, and the methyl protons being studied in red. The orange spheres are the inter-residue protons within 6 Å of the Cδ1 atom of Leu121, and the grey spheres are the rest of the protons in MBP. (b) Experimentally determined FCT curves of Leu7 δ1[$^{13}CH_3$] and Leu121 δ1[$^{13}CH_3$] in natural hydrogen abundance MBP (green curve) and MBP with δ1[$^{13}CH_3$] that is "locally" δ2 and γ-deuterated with Leu-meth$_{LD}$ (blue curve).

The method reported here provides a relatively inexpensive route to produce stereospecific methyl labeling of Leu residues, which is directly applicable for most commonly used protein production systems, including insect cells and *in vitro* expression without a need for modification of the existing expression protocols. Our simulations and experiments further clarify the effects of local deuteration on transverse relaxation rates. While the frequency of Leu in the eukaryotic proteome is around 9%, making it one of the most abundant amino acids, analogous site-selective [^{13}C,^1H/^2H] enrichment of Val and Ile should produce similar gains in the quality of protein NMR spectra. Further improvements in the protein spectrum are expected if surrounding residues and/or environment would be additionally deuterated or if *Leu-meth*$_{LD}$ is additionally deuterated at the beta protons, for example, by using $LiAlD_4$ (an additional 20–40% increase in the cost of reagents) in step 3 (Figure 1). However, the significant improvement in the absence of further deuteration makes this approach particularly appealing for the wide range of systems that so far eluted detection due to stringent requirements on the expression system. We therefore expect this methodology to enable further characterizations of challenging protein targets, including also the use of solid-state NMR.[23] The simplified protein spectra, the enhanced S/N, and the flattened dynamic range favor the use of non-uniform data sampling,[24] which will lead to further gains in sensitivity for protein complexes up to the megadalton regime.

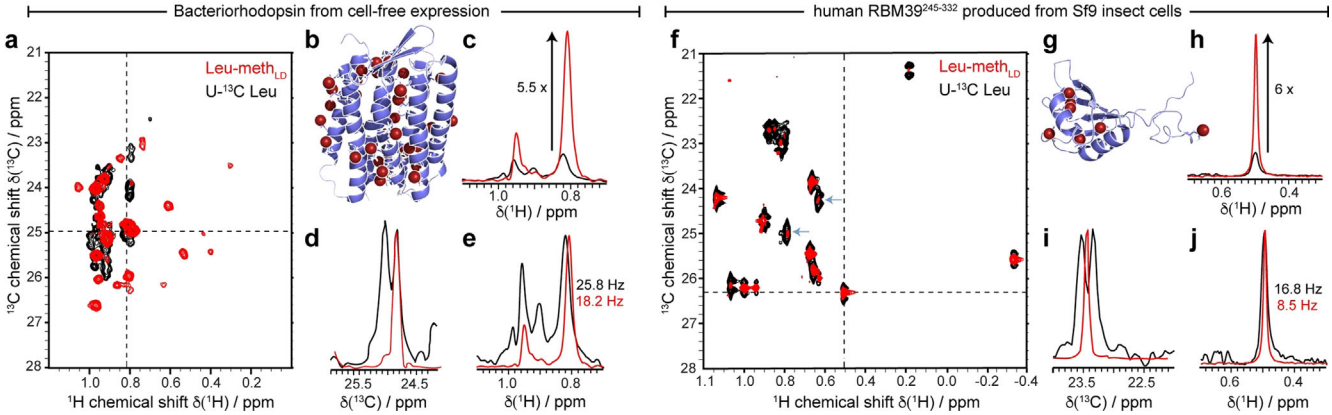

Figure 3. Stereo-specific $CD_3/^{13}CH_3$-labeled Leu with Leu-$meth_{LD}$ facilitates NMR characterization of proteins expressed *in vitro* and in insect cells. (a) [^{13}C,^{1}H]-HMQC spectra of cell-free expressed bR in DDM micelles with [U-^{13}C, ^{1}H] Leu (black) or Leu-$meth_{LD}$ (red). (b) Structure of bacteriorhodopsin (bR, PDB ID:1R84) with [^{13}C, ^{1}H]-labeled Leu methyl groups highlighted as red spheres. One-dimensional slices at indicated frequency in the ^{1}H/^{13}C dimension are shown in (c)–(e). (c) Signal intensity is increased by 5.5-fold. Comparison of normalized data shows that a singlet is obtained instead of doublet facilitating high resolution acquisition in the ^{13}C dimension (d) and that linewidth at half-height is reduced by 29% in the ^{1}H dimension (e). (f) [^{13}C,^{1}H]-HSQC spectra of the human RBM39$^{245–332}$ encapsulating RRM2 domain expressed in insect cells with [U-^{13}C, ^{1}H] Leu (black) or Leu-$meth_{LD}$ (red). (g) Structure of the CAPER-RRM2 domain (PDB ID: 2JRS) with [^{13}C,^{1}H] labeled Leu methyl groups highlighted as red spheres. Some of the residual 6% of inverted methyl labeled leucines are also observable (example marked by a blue arrow). Analog as for bR (c–e), one-dimensional slices at indicated frequency in the ^{1}H/^{13}C dimension are shown in (h)–(j). (h) Signal intensity is increased by 6-fold. (j) Linewidth at half height is reduced by 49% in the ^{1}H dimension.

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Conflict of interest

Vladimir Gelev is the founder or FB Reagents Ltd, a manufacturer of stable isotope labeled lipids, detergents, and amino acids.

Keywords: cell free protein expression · Forbidden Coherence Transfer · eucaryotic protein expression · methyl labeled leucine · methyl TROSY

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Communications

Protein Isotope Labelling

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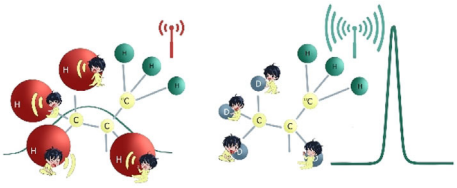
Local Deuteration Enables NMR Observation of Methyl Groups in Proteins from Eukaryotic and Cell-Free Expression Systems



Methyl-based magnetization usage can strongly benefit investigations of large protein systems via NMR spectroscopy. Here, a simple and cost-effective method to obtain locally deuterated leucine suitable for methyl NMR and to quantify its benefits is presented. The approach substantially expands the applicability of methyl labeling in emerging expression systems including cell-free and eucaryotic systems.

