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Biomolecular NMR

Isotope Labeling Methods for Protein Dynamics Studies

Eric D. Watt and J. Patrick Loria, Ph.D.

Department of Chemistry, Yale University, Kline Chemistry Laboratory, 225 Prospect Street, New Haven, CT 06520

Protein structure determination by solution NMR spectroscopy has long relied on the uniform stable isotopic enrichment with ^{13}C and ^{15}N to alleviate resonance overlap and to allow multiple distance and angular restraints, at as many atomic sites as possible, to facilitate computing the optimal three-dimensional structural model.(1) Recently, the optimization of these labeling techniques has increased the range of protein sizes amendable to study, enhanced the quality of three-dimensional structures, and simplified the analysis of experimental data.(2) Similarly, the field of protein dynamics has benefited from advances in isotopic labeling techniques that have allowed researchers to study the motional properties of ever larger proteins over a broad range of timescales while more accurately describing the protein motions. In many ways, improvements in isotopic labeling for dynamics have mirrored those used in structural studies. However, spin-relaxation experiments designed for studying protein dynamics have their own unique requirements for residue labeling, requiring special developments in isotope enrichment techniques better suited for these demanding studies.

The Need for Isolated Spin Systems in Dynamics

Solution NMR is a powerful method for characterizing protein motions over a wide range of time scales by the measurement of relaxation rates of the desired nuclei. The design of these relaxation experiments as well as the analysis and interpretation of data are significantly simplified if the protein position of interest can be treated as an isolated spin pair. In cases such as this, pulse sequence design is not overburdened by the necessity of accounting for and manipulating multiple undesirable coherence pathways and the relaxation rates obtained are straightforwardly measured from monoexponential decay profiles of the peak intensities. However, the presence of multiple large one-bond couplings can needlessly complicate experimental results through multiexponential relaxation pathways and signal-to-noise degradation. Because of this, much of the work on labeling schemes has been to provide a means to isotopically label different isolated spin pairs within proteins such that one-bond scalar (J) couplings do not pose an experimental roadblock.

^{15}N-labeling

To date, most dynamics studies have been performed using uniform ^{15}N enrichment. ^{15}N is a good target to study for a variety of reasons. The necessary nitrogen needed for cell growth can be controlled by use of ^{15}N-enriched minimal or nutrient-rich growth media that are readily available, allowing for easy sample preparation. Uniform ^{15}N labeling results in an isolated spin system (^{1}H-^{15}N) that lends itself well to relaxation experiments. Every ^{15}N position whether in the protein backbone or sidechain is separated from another ^{15}N atom by at least two bonds. Therefore there are no $^{1}J_{NN}$ couplings that could lead to complicated multiexponential relaxation behavior, which would be difficult to accurately measure and would cloud the interpretation of the associated motions.

However, ^{15}N enrichment by itself does not provide a complete picture of the motions a protein undergoes. Nitrogen makes up only 1/3 of the protein backbone and is only sparsely populated in the sidechains in 6 of the 20 amino acids. Therefore, except for a few select positions (Asn, Gln, His, Trp, Lys, and Arg) ^{15}N relaxation experiments do not allow substantial protein-wide dynamical coverage to provide a full picture of sidechain and backbone motions. In particular, amide relaxation may be relatively insensitive to motions in the hydrophobic core of the protein. It has also been noted that certain motional modes of the protein backbone will not be detected by monitoring relaxation at the amide positions. Nonetheless, the simplicity of ^{15}N protein labeling, the powerful experiments that exist and the sensitivity of nitrogen to structural, electrostatic, and hydrogen bonding effects make ^{15}N an essential part of any dynamics study.

^{13}C-labeling

On the surface, carbon relaxation experiments provide many additional opportunities for molecular dynamics studies by NMR spectroscopy. The abundance of carbon in each amino acid provides still more probes of enzyme dynamics. These carbons are contained both in the backbone and the side-chains, providing information on dynamics throughout the entire protein. Methyl residues, typically buried in the hydrophobic core, are particularly suited to provide information on the dynamic contribution to protein folding and stability. The dependence of ^{13}C chemical shifts, particularly C^{α}, on the protein secondary structure is more clearly understood than that for the amide nitrogen, allowing for easier interpretation of chemical shift changes retrieved from certain spin-relaxation experiments.

Unfortunately, ideal carbon labeling methods are not as straightforward as those for nitrogen. The great coverage of protein dynamics provided by the significant portion of carbon in the protein is the same feature that poses the biggest problem to its study; the large

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number of carbon atoms means that almost all carbons are adjacent to another carbon atom. Therefore, uniformly labeled ^{13}C protein samples result in large one bond ^{13}C-^{13}C couplings for many residues, making straightforward interpretation of relaxation data difficult or intractable in many cases. The only position that does not suffer from the aforementioned $^{1}J_{CC}$ couplings is the methionine C^{ϵ} methyl group, which is isolated from the rest of the protein by the sulfur atom. Thus, in a uniform ^{13}C-labeled protein there are limited opportunities for dynamics studies. While methionine relaxation data may be extremely useful, it does not provide the level of dynamic coverage that could be obtained from a more complete carbon labeling scheme. Because of this, many isotope labeling methods that take advantage of the known bacterial metabolic pathways have been developed.

One method that was used to isolate ^{13}C labels focused on partial labeling using 15% ^{13}C-acetate with the remainder being ^{12}C.[3] This method results in dilution of the ^{13}C labels to sufficient levels to make relaxation experiments feasible, though with a concomitant reduction in signal-to-noise due to the reduced fraction of labeled protein.

By using [3-^{13}C] pyruvate as the sole carbon source, ^{13}C labeling of Leu^{δ}, Val^{γ}, and Ile^{γ}, methyl groups can be achieved at >90% incorporation levels.[4] More importantly, the isotope labeling is not scrambled to directly bonded carbons to any great extent, allowing relaxation measurements for these residues. Thus, good single-to-noise and monoexponential relaxation behavior is observed for these methyl positions. Like methionine, these residues allow insight into the dynamics of protein motion in the hydrophobic core.

The use of α-keto acids also provides a cost-effective way to produce ^{13}C-methyl labeled amino acids that is also compatible with high levels of deuteration at other carbon sites.[5, 6] Deuteration allows dynamic studies to be performed on much larger protein systems than otherwise possible. Another benefit of this approach is that there is minimal scrambling of the ^{13}C labels thereby minimizing the aforementioned problems.

The use of 1- or 2- positionally labeled glycerol with an auxotrophic cell line allowed alternating labels to be incorporated for the majority of carbons throughout the protein sidechains. Typically, two protein samples are needed to obtain as complete coverage of atomic positions as possible.[7] Aromatic residues can complement the data obtained for methyl groups as they are typically found in the hydrophobic core as well. Specific labeling in these residues is especially important given the strong J-couplings as well as the small range of chemical shifts. Early studies showed that growth on [2-^{13}C]-glycerol will result in isotope enrichment at alternating carbons in most amino acids, including isolated aromatic carbons in Phe, Tyr, and Trp. Growth on [1,3-^{13}C]-glycerol will give the opposite labeling pattern. An alternative to this is using [1-^{13}C]-glucose as the sole carbon source. Aromatic rings are labeled at Phe^{δ}, Tyr^{δ}, $His^{\delta2/\epsilon1}$ and $Trp^{\delta1/\epsilon3}$.[8] [1-^{13}C]-glucose is beneficial because it is not only more affordable, but produces higher protein yields.

More recently, it has been shown that [1-^{13}C]-glucose will also lead to ~45% enrichment of the methyl residues of Ala, Val, Leu, Met and Ile separated by two or more bonds from other ^{13}C labeled atoms.[9] In the same study, expression with [2-^{13}C]-glucose as the only carbon source was shown to lead to 20-45% enrichment at C^{α} positions with no C′ sites labeled, and only Leu, Val and Ile C^{β} sites labeled. This labeling allows CPMG relaxation experiments to be run on the C^{α} positions, providing a wealth of data that complements that obtained for the more common ^{15}N CPMG experiment.

Finally, isotopic labels can be incorporated quite specifically via introduction of the desired labeled amino acid directly into the growth media.[10, 11] Typically, to avoid scrambling and dilution of the label the desired amino acid is included in a mixture containing all the other amino acids in unlabeled form. Thus cell growth is quite robust due to the nutrient rich nature of the growth medium. Obviously, depending on the position of the desired label synthesis may not be straightforward and it may be quite time consuming. This technique has proved useful in dynamical and structural studies and has recently been used in NMR structure determination of large proteins.[2]

Future Prospects

As more and more bacterial metabolic pathways are employed to provide specific isotopic labeling, solution NMR relaxation experiments that can take advantage of these advancements will be developed, allowing for an extremely detailed description of protein dynamics across a wide range of residue types.

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Complex Growth Media

Typical Procedure for Growing E. coli *Using ISOGRO® Powder*

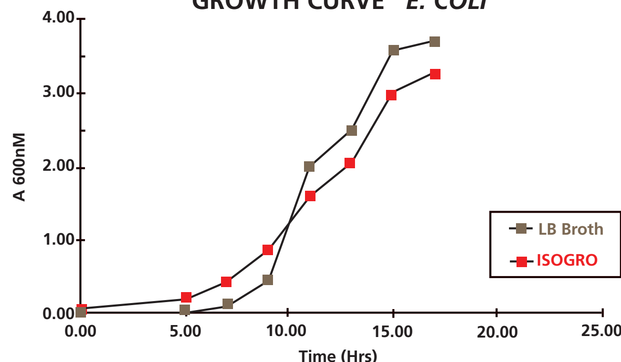
To prepare 100 mL ISOGRO medium:

1. Dissolve 1.0 g of ISOGRO powder in about 90 mL of Millipore® water.
2. Make stock solutions of the following salts and use the quantities indicated in the medium preparation:

Salt	Conc. of Stock Soln.	Qty/100 mL medium
K_2HPO_4	100 g/L	1.8 mL
KH_2PO_4	50 g/L	2.8 mL
$MgSO_4$	50 g/L	2.0 mL
$CaCl_2 \cdot H_2O$	37 g/L	30μL

3. Adjust pH to 7.0 with NaOH and bring solution up to 100 mL with Millipore water.
4. Pass the solution through a 0.22 μm filter and transfer the filtrate to an autoclaved shaker flask (for example: 50 mL medium in a 500 mL flask).
5. The culture is inoculated with a loop of *E. coli* which has been maintained on a nutrient agar slant.
6. Shake the culture flask in a 37 °C water bath.
7. The absorbance of the culture is measured at 600 nm with a 1:3 dilution into water.

Note: Researcher's specific expression applications do vary, so our preparation should serve as a guideline.



The suitability of ISOGRO as a culture medium has been demonstrated in our labs by growing *E. coli* strain W3110, ATCC 27325, in comparison with ATCC LB broth under identical conditions, with no significant differences in the two curves. The **LB Broth**, used as a comparison medium, is made up as described in the ATCC Catalog.

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ISOGRO-^{13}C,^{15}N,D Powder -Growth Medium	99 atom % ^{13}C 98 atom % ^{15}N 97-99 atom % D	**608297-1G**
ISOGRO-^{15}N Powder -Growth Medium	98 atom % ^{15}N	**606871-1G**
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