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Improving yields of deuterated, methyl labeled protein by growing in H₂O

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Abstract

Solution NMR continues to make strides in addressing protein systems of significant size and complexity. A fundamental requirement to fully exploit the ¹⁵N-¹H TROSY and ¹³C-¹H₃ methyl TROSY effects is highly deuterated protein. Unfortunately, traditional overexpression *E. coli* during growth on media prepared in D₂O leads to many difficulties and limitations, such as cell toxicity, decreased yield, and the need to unfold or destabilize proteins for back exchange of amide protons. These issues are exacerbated for non-ideal systems such as membrane proteins. Expression of protein during growth in H₂O, with the addition of ²H-labeled amino acids derived from algal extract, can potentially avoid these issues. We demonstrate a novel fermentation methodology for high-density bacterial growth in H₂O M9 medium that allows for appropriate isotopic labeling and deuteration. Yields are significantly higher than those achieved in D₂O M9 for a variety of protein targets while still achieving 75–80% deuteration. Because the procedure does not require bulk D₂O or deuterated glucose, the cost per liter of growth medium is significantly decreased; taking into account improvements in yield, these savings can be quite dramatic. Triple-labeled protein is also efficiently produced including specific ¹³CH₃ labeling of isoleucine, leucine, and valine using the traditional ILV precursors in combination with an ILV-depleted mix of ²H/¹⁵N amino acids. These results are demonstrated for the membrane protein sensory rhodopsin II and the soluble proteins human aldoketoreductase AKR1c3, human ubiquitin, and bacterial flavodoxin. Limitations of the approach in the context of very large molecular weight proteins are illustrated using the bacterial Lac repressor transcription factor.

Keywords

protein deuteration; nuclear magnetic resonance; amino acid chromatography; ILVM methyl labeling; algal amino acids

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Contributor Roles

E.S.O., D.W.L. & A.J.W. designed the research. E.S.O., D.W.L., M.A.S., B.F., & T.G. prepared samples and carried out NMR experiments. C.T. assisted in method implementation. E.S.O. and A.J.W. wrote the manuscript.

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Introduction

Structural and dynamic characterizations of large biological macromolecules using solution NMR techniques (Fiaux et al. 2002; Religa et al. 2010; Sprangers and Kay 2007; Sprangers et al. 2008) inherently rely on various implementations of the transverse relaxation optimized spectroscopy (TROSY) effect (Ollerenshaw et al. 2003; Pervushin et al. 1997). To take full advantage of the TROSY effect, it is necessary to reduce external dipolar relaxation as much as possible; protein deuteration has become the standard approach (Ruschak and Kay 2010). In addition to its usefulness in NMR studies of large proteins, deuteration has extensive utility in small angle neutron scattering (SANS) studies (Neylon 2008) and electron paramagnetic resonance (EPR) spectroscopy such as the double electron-electron resonance (DEER) experiments (Jeschke 2012). Deuteration is typically achieved through recombinant expression in *Escherichia coli* (*E. coli*) during growth on highly deuterated media and in bulk D₂O. There are several limitations to this procedure. *E. coli* generally grows much slower in deuterated medium and often do not reach the same cell density as in hydrogenated media and solvent (Giovanni 1960), while the success of expression is often protein specific. Complicating matters further, purified protein must often be destabilized or refolded in H₂O to allow for backexchange of amide protons incorporated into fully folded structural elements (Englander 2006). Back-exchange is especially problematic for membrane proteins (Zhang et al. 2010).

Many expression strategies have attempted to improve protein yields, several of which incorporate a deuterated background. A common method for overexpression of unlabeled protein in a fermentation system involves growth to high cell density at 37°C, followed by a decrease in temperature and subsequent introduction of additional nutrients for induction of protein expression (Fike 2009). Cai et al. (1998) developed a very similar strategy that was designed specifically to incorporate ¹⁵N and ¹³C labeling uniformly throughout proteins by addition of ¹⁵NH₄Cl and ¹³C-glucose concurrent with protein expression. By performing an initial growth to high cell density, they are then able to induce expression in conjunction with addition of labeled compounds when the first round of *unlabeled* nutrients are exhausted, thus conserving isotopically labeled material for use when it is most needed – during protein expression (Cai et al. 1998). More recently, Cai et al. (2016) introduced a protocol using a modified M9 medium (M9+) that allowed for growth in deuterated medium to a much higher cell density. Total protein expression increased linearly with the cell density at induction up to an OD₆₀₀ of 10, which provides a ~10 fold increase in expressed protein for a given volume of M9 medium. This strategy results in a significant cost savings with respect to D₂O use, but only marginal savings on deuterated glucose. The protocol is applicable to proteins that express fairly well in D₂O medium using standard methods. Fiaux et al. (2004) described a method for expression of deuterated proteins in a protonated growth medium by adding deuterated amino acids derived from an algal extract during *E. coli* growth. Using a standard minimal medium prepared in H₂O and supplemented with deuterated amino acids, they were able to achieve a deuteration level ranging from 60–92% (Fiaux et al. 2004). Deuterated amino acids and relatively low levels of unlabeled glucose led to labeling efficiencies of ~85% while growth in D₂O medium leads to perdeuterated protein (95–97%) (Fiaux et al. 2004). Finally, cell-free protein expression strategies can also

produce ^2H protein during expression in bulk H_2O though it is currently largely limited to uniform labeling schemes incorporating ^2H algal amino acids (Linser et al. 2014). We sought an alternative method for generating specific methyl group labeling from H_2O media in a highly deuterated background, which is now a common labeling technique for large proteins. Here we take advantage of the more favorable aspects of these approaches. When implemented in the highly reproducible context of controlled “fermentation” we have devised a method of using ILV-depleted $^{15}\text{N}/^2\text{H}$ amino acids, in conjunction with standard $^{13}\text{C}^1\text{H}_3$ -ILV precursors, to produce highly deuterated, ^{15}N , $^{13}\text{C}^1\text{H}_3$ -methyl labeled protein samples grown in H_2O .

Materials and Methods

Uniform $^2\text{H}/^{15}\text{N}$ or $^2\text{H}/^{15}\text{N}/^{13}\text{C}$ labeling

A 25 mL overnight culture made from a fresh transformation of BL21(DE3) cells harboring the expression vector was used to inoculate 1 L M9 media. Growth was carried out at 37°C and 40% dissolved oxygen (DO) with 0.5 L/min air flow in a New Brunswick BioFlo 115 cell culture system in a 3 L vessel. The liter of M9 is composed of $1.5\times$ M9 salts (Table 1), which is supplemented prior to inoculation with the first master mix (MM #1) (Table 1). Upon saturation at an optical density (OD) at 600 nm (OD_{600}) of ~ 2.6 , the temperature is set to the optimal protein expression temperature (18°C for pSR11, 37°C for ubiquitin, 30°C for Lac repressor and AKR1c3, and 22°C for flavodoxin). Optimal induction temperatures were determined in trials conducted in shaker flasks. The pH is corrected to 7.4 with NaOH during temperature equilibration to avoid precipitous drops at elevated cell densities. Immediately upon depletion of the first round of unlabeled nutrients, unlabeled glucose (200 mg/L) is added during the temperature adjustment to ensure cell viability. As the temperature approaches the desired value for induction, the second master mix (MM #2) is added. The isotopic content of MM #2 is dependent on the desired labeling pattern (see Table 1). After equilibration for 10 minutes, protein expression is induced with previously optimized concentrations of IPTG. The culture is maintained at the same DO level until the second round of nutrients are exhausted, as characterized by a second spike in DO. It is useful to avoid complete exhaustion of nutrients after expression, and for this reason the controlled conditions of a regulated fermentor are highly recommended. The protein expression phase is typically 3–3.5 hours at 18°C , but is significantly shorter if more glucose is used in the first phase (higher induction OD), and significantly longer if more glucose is used in the second phase (higher maximum OD). Inductions at 37°C or 30°C under these conditions last approximately 45 minutes or 1.5 hours (respectively).

ILV removal from $^2\text{H}/^{15}\text{N}$ amino acids

Using a mixture of the 20 amino acids in pure dH_2O with 0.1% (v/v) TFA (final pH = 3.0, final concentration = 10 mg/mL amino acid), a C18 reverse phase (RP) high-performance liquid chromatography (HPLC) system was tested for its ability to isolate ILV(M) from the rest of the amino acids. A Discovery BIO Wide Pore HPLC column (300 Å pore size, 10 µm particle size, 25 cm length, 1.0 cm I.D.) stored in 95% acetonitrile (ACN) was progressively equilibrated with lower quantities of organic phase and washed with 5 column volumes of pure dH_2O with 0.1% TFA (v/v) at 4 mL/min. Amino acids were then loaded onto the

column, which continued to flow at 4 mL/min water equilibrant. 100 mL after injection of amino acids, a 100 mL 25% organic phase (ACN with 0.1% v/v TFA) gradient was started to efficiently elute the aromatic amino acids (see Fig. 5). Each peak was collected as a fraction, frozen, and lyophilized for 48 hours until completely dry. Fractions were then resuspended in 500 μ L D₂O and analyzed by 1D proton NMR. Loading more than 65 mg of amino acids resulted in a lack of resolution between ILV(MY) and the polar amino acids. Elution profiles for Martek algal amino acid mixtures (chosen for chromatography due to their purity relative to ISOGRO) are qualitatively similar to the amino acid standard mixture, with no major additional peaks and only minor residual peaks surrounding the phenylalanine elution peak.

After demonstrating successful separation of both amino acid standard as well as algal amino acid mixture, we attempted removal of I, L, and V from ¹⁵N/²H Martek algal amino acids. 1.0 g of ¹⁵N/²H Martek amino acids was dissolved in 100 mL (also 10 mg/mL) dH₂O. TFA was added to maintain the same pH (3.0) as for the standard amino acid mixture (final 0.16% v/v). After thorough mixing and heating in a 42°C heat bath, the solution was centrifuged at 4,000 rpm and the precipitate removed. The solution was added to the column 60 mg (6 mL) at a time. Five fractions were collected during each run of preparative separations; polar amino acids [fraction 1 in Fig. 5], IVM [fractions 2–4 in Fig. 5], YL [fraction 5 in Fig. 5], F [fraction 6 in Fig. 5], and W [fraction 7 in Fig. 5]. After the 17 runs needed to separate the whole mixture, the polar residues were mixed with F and W, while IVMYL were discarded. Most of the residual ~1 L of liquid (water/acetonitrile mixture) was removed through rotary evaporation until only ~40 mL remained. This ILV(MY) depleted amino acid mixture was then subsequently modified to pH 7.4 with NaOH and used for growth of deuterated pSRII with ¹³C¹H₃ methyl labeled ILV(M) residues [growth conditions described below]. As we determined from the initial studies, efficient ILV removal also requires removal of methionine (as it elutes in between valine and isoleucine and becomes inseparable at higher loads) and tyrosine (which co-elutes with leucine). Substituting back ¹³C¹H₃ labeled methionine to the growth allows for methionine methyl group labeling. Phenyl-deuterated tyrosine can also be added (if desired – if not, cells can produce tyrosine from the deuterated D-glucose used for induction or from the deuterated phenylalanine through a chorismate intermediate, though final tyrosine labeling using this method is unclear).

Preparation of deuterated ¹³C¹H₃ ILVM labeled pSRII

Methyl-labeled ILV(M) protein was grown according to Fig. 1 with the following modifications. While the temperature was dropped to 18°C (for pSRII) following the spike in dissolved oxygen concentration, 0.2 g of ²H-D-glucose was added to stabilize cells while the temperature equilibrated. With the temperature nearly equilibrated, standard amounts (60 mg alpha-ketobutyrate [Sigma-Aldrich 589276] and 100 mg alpha-ketoisovalerate [Cambridge Isotope Laboratories CDLM-7317-PK] with ¹³C¹H₃ methyl labeling of one isotopomer and ¹²C/²H labeled remaining atoms) of ILV precursors (Goto et al. 1999) were added and equilibrated with cells for 5 minutes. MM #2 was then added – it is composed (in this case) of 1 g ILVMY-depleted ¹⁵N/²H Martek amino acids in ~40 mL dH₂O, 1 g ²H D-glucose, 2 mM MgSO₄, 100 μ M CaCl₂, 50 mg phenyl-deuterated L-tyrosine [Sigma Aldrich 489808] and 50 mg ¹³C¹H₃/¹²CD L-methionine [Cambridge Isotope Laboratories

CDLM-8885-PK]. We use deuterated glucose when growing $^{13}\text{C}^1\text{H}_3$ ILV labeled protein with precursors because in order to convert the ketobutyrate and ketoisovalerate to leucine, valine, and isoleucine, carbons and protons are added from glucose – in this case, using deuterated glucose should improve the resulting deuteration of ILV residues (Goto et al. 1999). Cells were then induced with 1 mM IPTG following another brief 5 minute delay (and the addition of 1 mL 10 mM all-trans retinal dissolved in ethanol [10 μM final concentration] for expression of pSRII and another 10 μM aliquot of retinal every hour of the growth for a final concentration of 30 μM). Cells were induced for 3 hours at 18°C until the start of the second spike in dissolved oxygen concentration and were immediately spun down at 5,000 rpm for 35 minutes and stored at -80°C until purification.

Protein purification

pSRII was purified roughly as previously described (Gautier et al. 2008; Gautier et al. 2010; Gautier and Nietlispach 2012) with some modifications. Briefly, thawed cell pellets with overexpressed pSRII were resuspended in 20 mL 100 mM Tris-HCl pH 7.4, 400 mM NaCl and 20% glycerol. 20 mg lysozyme, 4 mg DNase, 20 mM final concentration MgCl_2 , 0.6 g dodecylmaltopyranoside (DDM), and 2 EDTA-free protease inhibitor tablets [COEDTAF-RO Roche] were then added. The mixture was then topped to 40 mL with deionized H_2O and mixed in the dark overnight. The crude lysate was then spun down at 15,000 rpm for 45 minutes at 4°C . The insoluble pellet was discarded and imidazole was added to the supernatant to a final concentration of 20 mM. The supernatant was then added at 0.7 mL/min to a nickel column preequilibrated with 50 mM phosphate buffer (pH 7.4), 150 mM NaCl, 10% glycerol, 20 mM imidazole, and 0.1% DDM detergent. The column was then washed with 5 column volumes (CV) of the same buffer, then with 5 CV of the same buffer with 750 mM NaCl and 50 mM imidazole. For the present studies, DDM detergent was exchanged for diheptanoylphosphocholine (DHPC) detergent as previously described (Gautier et al. 2008; Gautier et al. 2010), with deuterated d_{26} -DHPC [FB Reagents], then eluted with 0.28% d_{26} -DHPC in pH 6.0 50 mM phosphate, 300 mM imidazole with 50 mM NaCl and 0.02% sodium azide. Protein was then buffer exchanged for the same buffer without imidazole, and concentrated to 450 μL for NMR.

Ubiquitin (Wand et al. 1996), Lac repressor (Stetz et al. 2016), and flavodoxin (Liu et al. 2001) were purified as previously described.

1 L of cells expressing AKR1c3 were resuspended in 12.5 mL of 50 mM Tris/HCl at pH 8.6. 1 EDTA protease inhibitor tablet [COEDTAF-RO Roche], 100 μL 1 M DTT, 5 mg lysozyme, 612.5 μL 10% Triton X-100, 0.25 mg DNase and 125 μL 1 M MgCl_2 were then added to the suspension. Cells were sonicated and shaken at room temperature for 2 hours. The lysate was then centrifuged and the insoluble pellet discarded. Crude lysate was then dialyzed overnight at 4°C in 4 L of 10 mM Tris/HCl, 1 mM EDTA, 2 mM DTT at pH 8.6. Lysate was then added to a DEAE column equilibrated with 10 mM Tris/HCl and 1 mM DTT at pH 8.6. A five column volume 0–200 mM NaCl gradient was then performed to elute AKR1c3. Fractions containing AKR1c3 were pooled and separated further on size exclusion with an S75 column in 20 mM sodium citrate and 50 mM NaCl with 5 mM TCEP at pH 6.0. Purified AKR1c3 was then stored in the same buffer with 0.2% sodium azide.

Calculation of deuterium incorporation percentages

¹D proton 1 with full protonation as well as with unknown levels of deuteration (for flavodoxin and ubiquitin). Isolated methyl proton peaks in both proteins were integrated for each sample grown with a different amount of ¹⁵N/²H ISOGRO and compared to the integral for fully protonated protein (after normalization to amide protons). The maximum deuteration achieved here (~75–80%) is also in accordance with the ¹⁵N-TROSY spectral quality for intermediate (pSRII) and high (LacI) MW protein complexes (i.e. good quality for intermediate MW and poor spectral quality for large MW).

NMR spectroscopy

NMR data were collected at 600 or 750 MHz (¹H) on Bruker Avance III spectrometers equipped with TXI cryoprobes. For uniformly double (¹⁵N/²H) labeled proteins, ¹⁵N-TROSY (Rance et al. 1999) experiments were collected. For uniformly triple (¹⁵N/²H/¹³C) labeled proteins (AKR1c3 and Lac repressor), non-uniformly sampled 3-dimensional TROSY-HNCO (Grzesiek and Bax 1992) and TROSY-HNCA (Kay et al. 1990) experiments were collected with a 15% sampling density and a Poisson gap distribution (Hyberts et al. 2012). Data were reconstructed using iterative soft thresholding with istHMS (Hyberts et al. 2012). For ILVM methyl labeled pSRII samples, ¹³C-HMQC (Ollerenshaw et al. 2003) experiments were collected in the conditions described above for protein purification, as well as in Fig. 3. All experiments were processed in nmrPipe (Delaglio et al. 1995) and analyzed in Sparky (Goddard 2008).

Results

Strategy.

This study aims to design a protocol that takes advantage of useful aspects of several previously described methods (Cai et al. 1998; Cai et al. 2016; Fiaux et al. 2004). The first attempt began with a modified version of an industrial production method, where cells were grown to saturation in H₂O M9 medium with 2.0 g/L unlabeled glucose, followed by a change in temperature and addition of another 2.0 g of glucose concurrent with protein expression. This preliminary unlabeled growth, as a proof of principle, resulted in 15 mg/L of culture of pSRII. This represents the highest yield to date by a fairly wide margin, in contrast to ~5 mg/L of unlabeled protein produced in our hands and ~1–4 mg/L from other studies (Hohenfeld et al. 1999; Mironova et al. 2005). 5 mg/L of pure deuterated pSRII was obtained by Gautier et. al., but these growths depended on 5 g/L (rather than 2 g/L) deuterated glucose to achieve high cell densities and expression levels (Gautier et al. 2010; Gautier and Nietlispach 2012).

Next, isotopically labeled reagents were introduced to growths after the initial unlabeled phase. Upon consumption of the first 2.0 g of unlabeled glucose, a sharp spike in the dissolved oxygen (DO) concentration occurs in solution as cells enter lag phase and switch from glycolytic conditions (Fig. 1) (Fike 2009). This is very easy to observe on a fermenter with continual DO monitoring, but can potentially be approximated as the beginning of the lag phase by monitoring the OD₆₀₀ in a shaker flask. This is useful from a labeling perspective as cells can be grown to a high density before addition of isotopically labeled

reagents concurrent with protein expression. Rather than adding ^2H or $^2\text{H}/^{13}\text{C}$ glucose, the traditional source of these isotopes, the use of $^2\text{H}/^{15}\text{N}$ or $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ amino acids derived from algal extracts allows for direct incorporation into the protein of interest with minimal processing or scrambling. A schematic of the final growth protocol is shown in Fig. 1, where unlabeled M9 medium is grown to saturation, at which point pH is returned to 7.4 and temperature is modulated appropriately, followed by introduction of various amounts of $^2\text{H}/^{15}\text{N}$ algal amino acids (as well as additional unlabeled glucose) concurrent with protein overexpression.

Uniform double ($^{15}\text{N}/^2\text{H}$) labeling.

In order to test for how efficiently deuterons from algal amino acid mixtures can be incorporated into proteins grown in H_2O , various amounts (0.5 – 3.0 g/L) of $^2\text{H}/^{15}\text{N}$ amino acids were introduced in the second phase of growth along with a constant 1.0 g/L unlabeled glucose (to maintain various cell functions), along with supplementation of $^{15}\text{NH}_4\text{Cl}$ (to ensure uniform ^{15}N labeling). As one might anticipate, final deuterium incorporation has a clear relationship with the amount of deuterated amino acids introduced, as demonstrated in Fig. 2. Low levels (~30–40%) of deuteration are seen at 0.5 g/L amino acids, with an increase to ~65% deuteration at 1.0 g/L. Further improvements are seen at 2.0 and 3.0 g/L, where nearly 80% deuteration is achieved. Labeling efficiency appears to plateau at 2.0 g/L. Closer inspection of the 1D proton spectrum of double labeled ubiquitin grown reveals that leucines and isoleucines are nearly fully deuterated, while the majority of the residual protons arise from valine and alanine side chain protons (data not shown). This may result from catabolism of valine resulting in scrambling of the deuterons with solution. This does not happen in leucine due to the feedback inhibition in this pathway as discussed below. Using this protocol, 1 L of highly deuterated protein can be grown for less than \$300, representing a significant cost savings relative to traditional D_2O growths (>\$800) even before factoring in increases in protein yield due to growing in H_2O , inducing at much higher cell densities, and an avoidance of back-exchange (all discussed further below). High-quality 2D ^{15}N -TROSY NMR spectra can be collected for both pSRII (~60 kDa micellar system) and AKR1c3 (38 kDa) grown with 1.0 and 2.0 g/L (respectively) labeled amino acids (Fig. 3a & 3b). The maximum level of deuteration achieved using our growth method reaches a limitation with the ~70 kDa Lac repressor protein grown with 2.5 g/L $^2\text{H}/^{15}\text{N}/^{13}\text{C}$ algal amino acids (Fig. 3c). Nearly acceptable (but highly heterogeneous) ^{15}N -TROSY spectra can be achieved with significant signal averaging, but more complex triple resonance experiments proved largely unsatisfactory.

Uniform triple ($^{15}\text{N}/^2\text{H}/^{13}\text{C}$) labeling

of proteins is necessary for obtaining backbone resonance assignments for medium- to large-sized proteins or protein complexes. However, growth and purification of triple labeled protein presents the same difficulties as discussed above for uniform $^{15}\text{N}/^2\text{H}$ labeling. Therefore, a more cost-effective method of producing large quantities of triple labeled protein becomes necessary. Triple-labeled algal amino acids are only slightly more expensive than their double-labeled counterparts. Aldo-ketoreductase isoform 1c3 (AKR1c3) (Desmond et al. 2003; Penning 2017) and the Lac repressor (LacI) (Stetz et al. 2016) were expressed with triple labeled amino acids as described in Methods and the

resulting samples were tested with standard triple resonance NMR assignment experiments. TROSYHNCO and TROSY-HNCA experiments could be collected for AKR1c3 grown with 2.0 g/L $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ algal amino acids with reasonable signal to noise (16 scans for HNCO, 32 scans for HNCA) with non-uniform sampling with a Poisson gap distribution and reconstruction with istHMS (Hyberts et al. 2012). Representative strips of both experiments are shown in Fig. 4a. In total, 287 of 304 (94.4%) expected peaks in the HNCO and 555 of 608 (91.3%) expected peaks in the HNCA are readily apparent.

For comparison with a very large molecular weight system, LacI was grown with 2.5 g/L $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ algal amino acids. As discussed previously, the level of deuteration achieved with our growth method results in experimental challenges at the very large molecular weight of LacI and results in more heterogeneous TROSY-HNCO and TROSY-HNCA spectra (Fig. 4b). Indeed, 137 out of 254 (54%) peaks were apparent in the HNCO along with 217 of 392 (55%) peaks noted in fully deuterated reference spectra of LacI. We note that in order to achieve inter-residue connectivity in triple resonance experiments, it is best to increase labeling as much as possible because efficient transfer depends on full ^{13}C -labeling of *both* residues involved. For AKR1c3 grown with only 0.5 g/L $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ algal amino acids, the inter-residue transfer efficiency diminishes, with only ~75% of the predicted total number of peaks apparent. This is confirmed for LacI, where 64% of intra-residue CA peaks are apparent, as opposed to 42% of inter-residue CA peaks. Further, in the much smaller, separately tumbling headpiece domain of LacI, the current triple-labeling methodology is able to replicate results from a fully deuterated sample, with 98% of inter-residue CO and 95% of intra-residue CA peaks appearing the spectrum (along with 80% of inter-residue CA peaks).

Improvements in yield of deuterated proteins.

Final protein yields were compared to their traditional D_2O -growth counterparts. The yield of pSRII using the current methodology is more than 3-fold improved over traditional D_2O growths (7.1 mg/L versus 2.3 mg/L). The yield of ubiquitin also improves nearly 3-fold (28 mg/L versus 9.4 mg/L), while the yield of LacI improves more than 2-fold (27.2 mg/L versus 12.2 mg/L). Inconsistencies in the AKR1c3 purification procedure prohibited accurate comparisons between batches of protein, though no more than 3 mg/L of purified protein has been observed from D_2O medium as compared to the 11 mg/L achieved here. The nearly constant 3-fold improvement in protein yield corresponds nicely with the increase in cell density at which protein expression is induced (typically $\text{OD}_{600} \sim 0.7 - 1.0$ for D_2O shaker flask growths versus $\text{OD}_{600} \sim 2.6$ for the current methodology). This suggests the possibility of even further improvements if more unlabeled glucose is incorporated into the first growth phase, resulting in an even high OD_{600} at which protein is expressed.

ILV methyl $^{13}\text{CH}_3$ labeling.

In order to incorporate specific ILV methyl labels of interest, several modifications to the protocol are necessary. In typical M9 D_2O growths, α -ketobutyrate and α -ketoisovalerate precursors are added ~1 hour before induction of protein to allow for processing to the final amino acids (Goto et al. 1999). However, timing of precursor addition in the current method

must be adjusted to account for the greatly increased cell density at induction to prevent over-processing. We have found that addition of ILV precursors during temperature equilibration at 18°C (for pSRII growths), followed by a ~5 minute pause for processing that is followed by another 5 minute wait after addition of $^{15}\text{N}/^{2}\text{H}$ algal amino acids (10 minutes total before induction) allows for sufficient time for conversion to the final amino acids. The length of protein expression is comparable to growths without ILV precursors, as is the final protein yield.

Use of higher quantities of deuterated amino acids is preferable for improving the deuteration in the final protein sample. However, when adding specific ^{13}C methyl labels of interest, one must also consider the fact that the uniformly $^{2}\text{H}/^{15}\text{N}$ -labeled ILV already present in the amino acid mixture will compete for labeling with the ^{13}C -methyl-labeled precursors, leading to drastically diminished methyl labeling at higher deuteration levels. For example, at 1.0 g/L algal amino acids per growth there is about an equal mass of I, L, and V $^{15}\text{N}/^{2}\text{H}$ amino acids in the mixture as the $^{13}\text{CH}_3$ ILV precursors used per L (~160 mg), which would lead us to predict at ~50% labeling efficiency. Ubiquitin was expressed using the above scheme with 1.0 g/L $^{15}\text{N}/^{2}\text{H}$ amino acids to test the ILV labeling efficiency. Analysis of ^1H spectra indicated the incorporation to be 53%, which is near the mass-predicted value.

However, it became apparent that a much more significant problem occurs when combining ILV precursors with $^{15}\text{N}/^{2}\text{H}$ amino acids; nearly all leucine methyl groups were eliminated or greatly diminished (<10% the signal of valine methyl peaks). Leucine resonances are also significantly diminished when using 0.5 g/L $^{15}\text{N}/^{2}\text{H}$ amino acids (~25% intensity, data not shown). Upon transitioning to 1.0 g/L $^{15}\text{N}/^{2}\text{H}$ amino acids in order to improve deuteration, all leucines were uniformly depleted (illustrated for pSRII in Fig. 6b, blue spectrum). This phenomenon has been noted previously (Tzeng et al. 2012) though a mechanism was not proposed. Initial studies of the biosynthesis of the branched chain amino acids indicate that leucine serves as a product inhibitor of a decarboxylase that converts the leucine precursor (α -ketoisovalerate) to the full amino acid (Freundlich et al. 1962). Valine synthesis is presumably also repressed by excess product, but the repression must be weak enough so that no significant labeling inhibition is observed with excesses of free amino acid.

Rather than attempting to balance the benefits of improved deuteration with detriments of decreased methyl labeling efficiency, we sought a more beneficial solution that would allow complete methyl labeling *and* high deuteration. Since diminished ^{13}C -methyl ILV labeling and complete inhibition of leucine labeling are both caused by the *presence* of these amino acids in the algal mixture, the obvious solution is to remove them. The 20 amino acids have been separated previously by reverse phase (RP) high-performance liquid chromatography (HPLC) (Tripp and McCullagh 2012). In order to improve retention times of the hydrophobic amino acids and more carefully control the final pH of the amino acid mixture (pH 3.0), trifluoroacetic acid (TFA) was added at 0.1% v/v to the amino acid mixtures as well as running buffers. An example chromatogram demonstrating separation of a standard amino acid mixture is shown in Fig. 5, as observed by absorbance at 205 nm (all amino acids) and at 280 nm (aromatic amino acids, particularly tryptophan and tyrosine). Fractions were collected and analyzed by one- and two-dimensional ^1H NMR (see Methods for more

details) to determine retention times of each amino acid. The first fraction (1) that elutes at the effective void volume of the column is composed of the more polar amino acids. Valine (2) and methionine (3) follow after the polar amino acids with ~10 mL separation, followed immediately by isoleucine (4). Tyrosine and leucine then co-elute (5). An acetonitrile gradient is then used to efficiently elute phenylalanine (6) and tryptophan (7) (Fig. 5). Conveniently, the middle fraction(s) containing ILV (plus methionine and tyrosine) can be combined and set aside. The polar amino acids are then combined with phenylalanine and tryptophan to produce ILV(MY)-depleted amino acid mixtures. Because methionine and tyrosine are also removed in the process, they are added back to the mixture separately (50 mg each). Using $^{13}\text{CH}_3$ -labeled methionine provides more methyl probes in the final protein carbon spectrum, while phenyl-deuterated tyrosine can be supplemented in a cost-effective manner (~\$35/50 mg). If desired, further separation of the coeluting leucine/tyrosine fraction should be possible using mixed-mode chromatography or by modifying the pH of the amino acid solution (Tripp and McCullagh 2012). Supplementing the uniform double labeled growth described above with the addition of $^{13}\text{CH}_3$ ILV precursors and the ILV(MY)-depleted $^{15}\text{N}/^2\text{H}$ amino acids (with added $^{13}\text{CH}_3$ methionine and deuterated tyrosine) restores full leucine labeling (Fig. 6b, red spectrum) with 1.0 g/L amino acids as compared to the previous growth without removal of ILV (Fig. 6b, blue spectrum). The resulting ILVM methyl spectrum (Fig. 6a) is of excellent quality with high signal to noise sufficient for lower sensitivity experiments such as those employing multiple quantum relaxation (Sun et al. 2011).

Discussion

One of the main goals of this protocol was to diminish deuterium-related costs incurred while producing large quantities of deuterated product. Isotopically enriched reagents for traditional M9 growths (D_2O , deuterated glucose, $^{15}\text{NH}_4\text{Cl}$) cost approximately \$750/L. Using $^{15}\text{N}/^2\text{H}$ algal amino acids as described in the current protocol costs \$146 (1 g/L; ~65% deuteration) or \$276 (2 g/L; ~75–80% deuteration) per L of expression. Using 2 g/L labeled amino acids as the minimum required to approach 80% deuteration, this results in a roughly 3-fold decrease in reagent costs. A similar cost analysis for triple labeled $^{15}\text{N}/^2\text{H}/^{13}\text{C}$ amino acid growths shows the current protocol reduces costs by a factor of 2 (relative to M9 with $^{13}\text{C}/^2\text{H}$ glucose in D_2O). If the targets are difficult to express or refold (as in the current study), it may become necessary to grow large volumes of cells to achieve reasonable yields; this protocol can make such studies significantly more feasible.

Bacterial overexpression of proteins in D_2O is notoriously difficult and unpredictable (Etezady-Esfarjani et al. 2007). Upon transitioning from protonated to deuterated medium, nearly every protein displays a decrease in yield – for many common, stably expressed proteins this is a consistent and predictable decrease, ranging from minimal differences to factors of two or more. In the case of pSR11, this problem is exacerbated due to the low and unpredictable yield of deuterated protein, ranging from 1 to 3 mg/L (with an average of 2.3 mg/L), even with extensive D_2O adaptation of *E. coli* cells. Using this protocol with growth in bulk H_2O , the yield improved more than three-fold to 7.1 mg/L on average, and importantly produced no less than 6.6 mg/L, allowing for consistent sample preparation. Combining the three-fold decrease in costs with the three-fold improvement in final yield

results in a ~9 fold more cost-effective growth on a permilligram of purified protein basis, along with much more consistent and improved yields, albeit with a final deuteration level closer to 75–80%. This improvement in yield appears to be consistent (>2–3 fold improvement demonstrated for pSRII, ubiquitin and LacI). In situations where protein expression is weak in unlabeled medium (e.g. complex soluble human proteins or membrane proteins) and further decreases due to expression in D₂O medium are unacceptable, the present protocol can serve as a convenient way to maintain and likely improve yield while still obtaining extensive protein deuteration. We have also found this to be useful in cases where proteins must be back-exchanged by unfolding before ¹H-¹⁵N experiments can be collected; in the case of AKR1c3, nearly half the total protein yield typically irreversibly precipitates upon unfolding/refolding.

Uniform triple labeling for assignment experiments of intermediate to large molecular weight proteins or protein complexes is also facile using this technique, as demonstrated for the human protein AKR1c3, (Fig. 4b) with reasonable yields and high labeling efficiency at greatly diminished cost. However, deuterium incorporation percentages using this methodology start to be insufficient for NMR experiments conducted on very large molecular weight systems (>70 kDa), as demonstrated by the quality of the ¹⁵N-TROSY and TROSY triple resonance experiments for LacI.

Rounding out the list of the most popular labeling schemes for large proteins (after uniformly double [¹⁵N/²H] and triple [¹⁵N/²H/¹³C] labeling for obtaining protein backbone assignments) is methyl labeling, particularly ILV labeling from keto-acid precursors (Goto et al. 1999). Methyl probes are exquisitely useful for large protein NMR for several reasons; their three protons have strong spectral properties (Goto et al. 1999; Ollerenshaw et al. 2003; Ruschak and Kay 2010), they are abundant and fairly evenly distributed across protein structures, and they can be used to determine protein thermodynamic quantities such as changes in conformational entropy (Caro et al. 2017) and heat capacity. In order to fully exploit the advantages that methyl groups offer, the uniform labeling strategies outlined previously were modified to suit this purpose. Reverse phase HPLC sufficiently separates polar amino acids from branched chain hydrophobics from aromatics, but due to overlaps in elution, methionine and tyrosine also must be removed from the mixture to sufficiently remove ILV. Properly labeled methionine can then be supplemented in order to provide additional methyl probes, while deuterated tyrosine is also added to maintain high levels of protein deuteration [see Methods for more detail]. Combining the appropriate keto-acid ILV precursors with ILV(MY)-depleted ²H/¹⁵N amino acids for introduction to cells slightly before induction results in highly deuterated, fully methyl ILVlabeled protein as shown in Fig. 6. Comparison of proteins produced with and without removal of isoleucine, leucine, and valine from the amino acid mixture (i.e. with and without leucine labeling) can be very useful for determining resonance-type assignments in the crowded leucine/valine region. Separation of amino acid mixtures by HPLC can be a fairly labor intensive process. As described using a semi-preparative column, separation of 1 g of material takes approximately 21 hours of chromatography time due to limits on injection quantity. The use of preparative HPLC columns should dramatically decrease the amount of time required (~7 hours) making this a feasible separation procedure for most labs.

The potential for modification to the basic scaffold of the protocol presented here is extensive. Additional glucose in the initial phase of growth can lead to induction at even higher cell densities, possibly providing a further source of improvement to protein yield. For labs without a fermentation apparatus, this method can be performed in standard 2 L shaker flasks, though with less direct control over timing for nutrient introduction and cell harvest. Average levels of deuteration can also be tailored for the purpose, if desired, and maximum levels of deuteration occur at 2 g/L $^{15}\text{N}/^2\text{H}$ algal amino acids. This deuteration level should be sufficient for studies of all but the largest proteins and complexes, demonstrated successfully here for pSR_{II} (50–70 kDa micelle particle (Gautier et al. 2010)), AKR1c3 (37 kDa), with limitations encountered above 70 kDa (LacI). In summary, a methodology is described that is consistently capable of producing highly deuterated, ILV-methyl labeled protein in H_2O , at greatly diminished cost compared to traditional (Cai et al. 2016) D_2O -based growths.

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Fig. 1.

Idealized growth curve showing the cell density as measured by absorbance at 600 nm (OD_{600} , red) and the dissolved oxygen concentration (DO, black). The culture is inoculated (1) at an $OD_{600} \sim 0.1$ with the first master mix (MM #1) and grown as described in the Methods until saturation (2), at which point temperature and pH (7.4) are modified for induction. The second master mix containing either unlabeled reagents or labeled algal amino acids are then added when temperature stabilizes (3), and cells are induced to express protein 5–10 minutes after addition of additional nutrients. Cells grow and express protein until the second round of glucose is consumed, at which point cells are harvested (4) (ideally before the second DO spike).

Fig. 2.

Relationship between labeling percent for ubiquitin (blue squares) or flavodoxin (red triangles) and the amount of $^2\text{H}/^{15}\text{N}$ amino acids used in the growth. At 0.5 g/L, 30–40% deuteration is possible. This increases greatly at 1.0 g/L, where ~65% deuteration can be achieved, and further improvements are seen at 2.0 and 3.0 g/L, where >75% deuteration is possible. All growths use 1.0 g/L unlabeled D-glucose for the second induction phase; improvements are likely possible when deuterated D-glucose is used in the second master mix during protein induction.

Fig. 3.

^{15}N -TROSY spectra for pSRII at 750 MHz (^1H) (a), AKR1c3 at 600 MHz (^1H) (b), and LacI at 750 MHz (^1H) (c). pSRII was grown with 1.0 g/L $^{15}\text{N}/^2\text{H}$ amino acids, AKR1c3 was grown with 2.0 g/L $^{15}\text{N}/^2\text{H}/^{13}\text{C}$ amino acids, and LacI was grown with 2.5 g/L $^{15}\text{N}/^2\text{H}/^{13}\text{C}$ amino acids. pSRII is 350 μM in 4.5% d_{26} -DHPC micelles at 50°C, AKR1c3 is 300 μM with 1.2 mM NADP and 2.8 mM indomethacin at 25°C, and LacI is 100 μM in the apo state at 37°C. Buffer conditions are described in the Methods. Spectra of pSRII (64 scans, 6 hours) and AKR1c3 (16 scans, 1.5 hours) were collected with 128 and for LacI (64 scans, 11 hours) with 200 complex increments in the ^{15}N dimension. Moderate levels of deuteration are sufficient for intermediate molecular weight proteins (AKR1c3 and pSRII), while significant line broadening is observed for the highest deuteration possible in the current growth method for the large (~70 kD) LacI protein complex (c).

Fig. 4.

Example slices of TROSY-HNCO and TROSY-HNCA spectra at 600 and 750 MHz (^1H) of AKR1c3 (a) and LacI (b) (respectively). Both proteins were triple labeled with 2 g/L $^{15}\text{N}/^{2}\text{H}/^{13}\text{C}$ algal amino acids. AKR1c3 is at 300 μM concentration with 1 mM NADP and 1 mM indomethacin at 37°C with pH 6.0 citrate buffer, 50 mM NaCl, 50 mM TCEP, and 0.02% sodium azide. LacI was 100 μM in the apo state at 37°C.

Fig. 5. Separation of amino acids using C18 reverse phase high-performance liquid chromatography. The elution profile of the standard 20 amino acids as observed by absorbance at 205 nm (black trace) and at 280 nm (red trace). The acetonitrile gradient (blue dotted trace) used to elute the aromatic amino acids is shown. The major elution peaks are denoted by the numbers above the chromatogram.

Fig. 6. ILVM labeled pSRII. Panel (a) is the methyl region of ^{13}C -HMQC spectrum of ILVM labeled pSRII grown with 1.0 g/L ILV(MY)-depleted amino acids. Panel (b) is an expansion of the leucine/valine region to illustrate labeling differences with (red) and without (blue) removal of ILV from the deuterated amino acid mixture used in the growth (1.0 g for each). Leucine labeling is nearly completely eliminated without removal of ILV from the mixture; removal of these residues restores full labeling.

Table 1.

Composition of M9 growth medium and variable components needed for uniform or ILV labeling. This table describes the materials per liter needed in each stage of the expression protocol. All growths use a common M9 salts buffer and first master mix (MM #1) in the left hand column. Unlabeled glucose may be used in the first master mix because it will be nearly completely depleted prior to expression of protein. The second master mix (MM #2) contains several variable components depending on the labeling needed; these variations are specified in the second column for uniform double or triple labeling and in the third column for ILV methyl labeling.

Universal Components	Uniform Labeling	ILV Methyl Labeling
<i>First Phase</i>	<i>Second Phase</i>	<i>Second Phase</i>
<i>M9 salts</i>	<i>MM #2</i>	<i>MM #2</i>
72 mM Na ₂ HPO ₄	1.0 g D-glucose ^d	1.0 g ² H D-glucose ^g
33 mM KH ₂ PO ₄	N g algal aa's ^e	N g ILV(MY)-depleted aa's ^e
13.5 mM NaCl	100 μM CaCl ₂	100 μM CaCl ₂
pH 7.4	2 mM MgSO ₄	2 mM MgSO ₄
to 980 mL in dH ₂ O	to 50 mL in dH ₂ O ^f	50 mg methionine ^h 50 mg ² H-tyrosine ⁱ
<i>Master Mix #1 (MM #1)</i>	<i>Additional Glucose</i>	to 50 mL in dH ₂ O
2.0 g unlabeled D-glucose	200 mg D-glucose ^d	<i>ILV Precursors</i>
1.0 g NH ₄ Cl ^a	to 1.5 mL in dH ₂ O	
2 mM MgSO ₄		60 mg α-ketobutyrate ^j
100 μM CaCl ₂		100 mg α-ketoisovalerate ^j
200 mg yeast extract		to 10 mL in dH ₂ O
100 mg antibiotic ^b to 20 mL in dH ₂ O		<i>Additional Glucose</i>
15 mg FeSO ₄ ^c		200 mg ² H D-glucose ^g to 1.5 mL in dH ₂ O
15 mg ZnCl ₂ ^c		

Isotopic reagents:

¹⁵N/²H ISOGRO – 608300 Sigma-Aldrich

¹⁵N/²H/¹³C ISOGRO – 608297 Sigma-Aldrich

¹⁵N/²H mixed free amino acid powder – Martek Isotopes LLC

²H D-glucose – 552003 Sigma-Aldrich

¹⁵NH₄Cl – 299251 Sigma-Aldrich

2-ketobutyric acid-4-¹³C, 3,3-d₂ sodium salt hydrate – 589276 Sigma-Aldrich

α-ketoisovaleric acid (3-methyl-¹³C, 3,4,4,4-D₄) sodium salt – CDLM-7317-PK Cambridge Isotope Laboratories

phenyl-deuterated L-tyrosine – Sigma Aldrich 489808

¹³CH₃/²H methionine – CDLM-8885-PK Cambridge Isotope Laboratories

1,2-diheptanoyl-d26-sn-glycero-3-phosphocholine (d26-D7PC) – CD5011P CortecNet

^a¹⁵N or unlabeled as needed

^b or as appropriate for the plasmid of interest

^c added directly to the medium before inoculation, not to the master mix

^d unlabeled is sufficient, ²H or ²H/¹³C D-glucose can be used instead to (possibly) improve labeling efficiency

^e 0.0–3.0 g per liter for the desired labeling efficiency (see Fig. 2), ¹⁵N/²H amino acids for double labeled protein and ¹⁵N/²H/¹³C amino acids for triple labeled protein. We advise using at least 2.0 g/L triple labeled amino acids for efficient triple-resonance experiments

^f or until fully dissolved

^g²H D-glucose is needed for ILV labeling to improve deuteration in the final amino acid product, as some protons from glucose are transferred in the process

^h¹³CH₃, ¹²C/²H labeled for additional methyl probes, ²H if not

ⁱ phenyl-deuterated tyrosine should be sufficient in most situations

^j careful selection of ILV precursors is necessary. In this case, we opted for a ¹³CH₃ labeled methyl group with other heteroatoms ²H/¹²C labeled. ¹³CH₃ labeled methyl groups with other atoms ²H/¹³C can also be used for side chain methyl assignment experiments.