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Enhanced biosynthetically directed fractional carbon-13 enrichment of proteins for backbone NMR assignments

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ABSTRACT

Routes to carbon-13 enrichment of bacterially expressed proteins include achieving uniform or positionally selective (e.g. ILV-Me, or $^{13}C'$, etc.) enrichment. We consider the potential for biosynthetically directed fractional enrichment (e.g. carbon-13 *incorporation* in the protein less than 100%) for performing routine n-(D)dimensional NMR spectroscopy of proteins. First, we demonstrate an approach to fractional isotope addition where the initial growth media containing natural abundance glucose is replenished at induction with a small amount (e.g. 10%$^{w/w}$ *u*-^{13}C-glucose) of enriched nutrient. The approach considered here is to add 10% (e.g. 200 mg for a 2 g/L culture) *u*-^{13}C-glucose at the induction time (OD_{600} = 0.8), resulting in a protein with enhanced ^{13}C incorporation that gives almost the same NMR signal levels as an exact 20% ^{13}C sample. Second, whereas fractional enrichment is used for obtaining stereospecific methyl assignments, we find that ^{13}C incorporation levels no greater than 20%$^{w/w}$ yield ^{13}C and ^{13}C–^{13}C spin pair incorporation sufficient to conduct typical 3D-bioNMR backbone experiments on moderate instrumentation (600 MHz, RT probe). Typical 3D-bioNMR experiments of a fractionally enriched protein yield expected backbone connectivities, and did not show amino acid biases in this work, with one exception. When adding 10% *u*-^{13}C glucose to expression media at induction, there is poor preservation of $^{13}C_\alpha$–$^{13}C_\beta$ spin pairs in the amino acids ILV, leading to the absence of C_β signals in HNCACB spectra for ILV, a potentially useful editing effect. Enhanced fractional carbon-13 enrichment provides lower-cost routes to high throughput protein NMR studies, and makes modern protein NMR more cost-accessible.

1. Introduction

An excess of signal still exists in routine 3D-NMR experiments of liquid phase protein samples, even when employing gradient coherence selection and non-traditional sampling (e.g. non-uniform sampling, NUS) to minimize experimental times [1]. Bacterial host expression protocols continue to improve, aiding researchers in achieving soluble 0.5–1 mM protein samples [2–6], while steady progress continues to be made in improving sensitivity through new probe designs (e.g. 77 K and 20 K cryogenic probes, reduced coil diameters), improvements in console electronics, and increasing static field strengths. It would be desirable to identify means to realize value from situations of excess NMR signal in liquid phase protein NMR. Cost savings could also be paired with automation of data analysis to aid high throughput structural studies by NMR [7–16]. In principle, ^{13}C incorporation into proteins at percentages less than 100%, conserving adjacent spin labels without amino acid bias, would reduce dependence on stable isotopes in many 3D NMR experiments on proteins, such as those for performing backbone assignments that normally require expensive isotopic enrichment. The practice of 10%$^{w/w}$ carbon-13 biosynthetically directed fractional (herein denoted 10%$^{w/w}$-BDF) enrichment has been well developed for obtaining stereospecific methyl side chain assignments [17–20]. Further, there is strong fundamental support that carbon-13 spin pairs and triples will be highly conserved in amino acid synthesis and thus in proteins produced by 10%$^{w/w}$-BDF [18,21,22]. The ^{13}C-BDF method should therefore have high utility for enabling broader 3D-NMR experiments on proteins, but does not appear to have been investigated for this use to date. This work investigates two questions relating to fractional ^{13}C incorporation into biosynthetically expressed proteins.

First, we considered if it would be possible to enhance the incorporation of the ^{13}C isotope into expressed proteins by adding the fractional amount of *u*-^{13}C-glucose at the time of induction. We find that the addition of 10% *u*-^{13}C-glucose by mass into cultures at the time of induction results in about 18% carbon-13 incorporation

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into the expressed protein, and have confirmed this effect under several conditions including different induction temperatures and durations, and also different glucose concentrations. We denote such an approach *opt*-10%$^{w/w}$-BDF. Second, we investigated whether fractional enrichment of proteins on the scale of 10–20% is suitable for producing proteins that are amenable to typical 3D-NMR backbone assignment experiments. We find that no more than 20% carbon-13 incorporation is needed for acquiring even challenging 3D-bioNMR experiments (e.g. 3D-HN(CA)CO) on moderate instrumentation (600 MHz, RT probe), while less incorporation easily provides enough signal for others (e.g. 3D-HNCO).

Uniform, fractional and specific isotopic enrichment for protein NMR structural studies have been well developed [2,17,19,23–28]. Protocols for specific amino acid enrichment include selective ILV methyl enrichment [29], selective alanine methyl enrichment [30,31], selective carbon enrichment of the protein backbone [32], and amino-acid selective enrichment at the carbonyl position [33]. These and other targeted labeling approaches [25,34–36] enable the study of very large proteins, but their efficacy is dependent on the complicated biosynthetic and catabolic pathways that they rely upon. Broadly, uniform enrichment approaches focus on the use of gram quantities of *u*-^{13}C-glucose, or other ^{13}C-enriched metabolites, in the growth media. Although costly, *u*-^{13}C-glucose is still among the least expensive sources of ^{13}C-enriched carbon suitable for *Escherichia coli* metabolism. Due to the low cost of $^{15}NH_4Cl$, protein samples are produced with 100% ^{15}N, which will be assumed for the remainder of this report.

As a result of these considerations, *u*-^{13}C-glucose and 100% $^{15}NH_4Cl$ enriched amino acids and their precursors, have gained widespread adoption in bacterial host protein expression of samples for NMR study. Although there are more methods of bacterial protein production than can be reviewed here, three general bacterial growth protocols for isotopic incorporation can be identified. The most common is to provide the specifically or uniformly enriched metabolites in the base media prior to growth [6,28,37–39]. A second strategy introduces the enriched metabolites into resuspended cell media during the expression phase once a certain optical density (OD_{600}) has been achieved; specifically, the culture is grown with natural abundance nutrients, centrifugally pelleted, and then resuspended into media containing enriched metabolites [40]. However, the weak centrifugation followed by resuspension has the potential to stress cellular membranes. Finally, pre-induction isotope addition, guided by monitoring dissolved oxygen as a function of glucose consumption [32,33], avoids the stresses of centrifugation and resuspension, but has been aimed at achieving ~100% incorporation instead of at identifying the minimum incorporation required for n-D NMR protein structural studies, and also relies on oxygen sensing [24]. In principle, cell-free expression also offers routes to employing lower quantities of enriched metabolites in the production of uniformly enriched biomolecules; the application of cell-free protein expression for obtaining large-scale and enriched samples [41,42] is promising but appears to still be in early phases of adoption and development.

A general feature of pre-induction isotope addition is the benefit of using somewhat reduced amounts of isotopically labeled metabolites, where the goal to date has been to achieve uniform carbon-13 incorporation (e.g. 100% ^{13}C). Alternately, the question may be posed, what level of incorporation of isotopes into proteins can still facilitate the acquisition of typical 3D-NMR experiments that require ^{13}C–^{13}C spin pairs? Prior detailed work by Szyperski demonstrated that 10%$^{w/w}$-BDF with *u*-^{13}C-glucose in the initial growth media will result in produced proteins that retain ^{13}C–^{13}C spin pairs and triples [18,43], which suggests that such samples could be suitable for broader NMR analysis. We show that the density of spin pairs in 10%$^{w/w}$-BDF enriched protein samples facilitates all typical 3D-NMR backbone assignment experiments on moderate instrumentation (600 MHz, RT probe), and introduce a simple method to enhance the carbon-13 BDF isotope incorporation into proteins. Specifically, introducing the 10%$^{w/w}$ *u*-^{13}C-glucose into the base media at the time of induction (OD_{600} = 0.7–0.8 for 2 g/L glucose, OD_{600} = 1.0–1.1 for 4 g/L glucose) produces samples that give spectra consistent with ca. 20%$^{w/w}$ incorporation, and which also does not depend on the use of internal glucose monitoring. This work suggests that BDF and *opt*-BDF enrichment strategies will significantly lower the cost per sample of protein NMR studies and enable employing NMR more broadly in high throughput structural studies. This methodology targets routine NMR structural investigations of well-behaved proteins and has implications for high throughput work. For NMR structural studies at the leading edge that target intrinsically difficult systems (high MW, low solubility, complexes, dark states, disordered proteins, etc…), uniform enrichment at the highest possible sample concentrations are normally required.

In other words, isotope incorporation is recast as a minimization problem to identify the least amount of *u*-^{13}C-glucose subject to the constraint of enabling all typical ^{13}C-dependent 3D-NMR backbone experiments of small to moderate size proteins.

2. Experimental

Yeast ubiquitin (8.6 kDa) was chosen as a model protein. A plasmid containing the yeast ubiquitin sequence under control of the *lac* operon (Courtesy Prof. J. Morgan, University of California San Francisco) was transformed into *E. coli* BL21 competent cells. All samples of yeast ubiquitin were prepared from 1 L cultures and induced at OD_{600} = 0.8 unless otherwise indicated. Identical cell growth conditions were enforced, including 100–105 rpm shaking in Fernbach flasks at 37 °C in M9 media (40 mM Na_2HPO_4, 20 mM KH_2PO_4, 8 mM NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 μg/ml thiamine, 1.0 g/L NH_4Cl) and containing 1.0 g $^{15}NH_4Cl$, followed by a 48–64 h induction at 18 °C or 5.5 h at 37 °C (see Table 1). The samples of yeast ubiquitin tested two possible parameters: fractional *u*-^{13}C-glucose in the initial media at inoculation, or timing the addition of *u*-^{13}C-glucose to the culture to occur at induction. All expression tests used 2.0 g/L or 4.0 g/L of glucose in M9 buffer (2.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 μg/mL thiamine, 100 μg/ml Kanamycin) supplemented with a commercial vitamin mix (BME, B6891 Sigma Aldrich) and a cocktail of supplemental metals (Fe, Ca, Mn, Co, Cu, Zn, Mo, Mg), whose primary component is iron. For cold induction, the incubation chamber was equilibrated to 18 °C for 20 min before adding IPTG.

This work reports results from over twenty independent preparations of yeast ubiquitin (Tables 1 and 2, Figs. 1–5, Fig. 9, S.3). Trials 1–9 comprise 18 of these samples. Each trial consists of a control sample that yields a known isotope incorporation (e.g. 10%$^{w/w}$-BDF, 20%$^{w/w}$-BDF) and an enhanced fractionally enriched sample (e.g. *opt*-10%$^{w/w}$-BDF, *opt*-20%$^{w/w}$-BDF) that were treated as a pair with respect to all experimental procedures (i.e. same incubator, same dialysis, sharing a centrifugal rotor, etc…). Each control sample was prepared with exactly 10%$^{w/w}$ or 20%$^{w/w}$ of *u*-^{13}C-glucose in the initial M9 growth media (e.g. 200 mg *u*-^{13}C glucose + 1.8 g *na*-glucose, or 400 mg *u*-^{13}C glucose + 3.6 g *na*-glucose). Each enhanced fractionally enriched sample was prepared by growing cultures with *na*-glucose initially, and then adding the fractional amount of *u*-^{13}C-glucose at induction. The induction period was followed by centrifugation (4 °C) of the media and freezing of the resulting cell pellet (−20 °C). The pellet was thawed and resuspended in a phosphate buffer (50 mM $NaPO_4$, 0.5 M NaCl, 10 mM Imidazole, and 2%[m/v] glycerol), and membranes were disrupted through sonication (5 × 30 s, 4 °C).