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Efficient Condensed-Phase Production of Perdeuterated Soluble and Membrane Proteins

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Abstract

Protein perdeuteration approaches have tremendous value in protein NMR studies, but are limited by the high cost of perdeuterated media. Here, we demonstrate that *E. coli* cultures expressing proteins using either the condensed single protein production method (cSPP), or conventional pET expression plasmids, can be condensed prior to protein expression, thereby providing high-quality ²H,¹³C,¹⁵N-enriched protein samples at 2.5 - 10% the cost of traditional methods. As an example of the value of such inexpensively-produced perdeuterated proteins, we produced ²H,¹³C,¹⁵N-enriched *E. coli* cold shock protein A (CspA) and EnvZb in 40X condensed phase media, and obtained NMR spectra suitable for 3D structure determination. The cSPP system was also used to produce ²H,¹³C,¹⁵N-enriched *E. coli* plasma membrane protein YaiZ and outer membrane protein X (OmpX) in condensed phase. NMR spectra can be obtained for these membrane proteins produced in the cSPP system following simple detergent extraction, without extensive purification or reconstitution. This allows a membrane protein's structural and functional properties to be characterized prior to reconstitution, or as a probe of the effects of subsequent purification steps on the structural integrity of membrane proteins. We also provide a standardized protocol for production of perdeuterated proteins using the cSPP system. The 10 - 40 fold reduction in costs of fermentation media provided by using a condensed culture system opens the door to many new applications for perdeuterated proteins in spectroscopic and crystallographic studies.

Introduction

The production of high quality isotope-enriched protein samples is a prerequisite for applying modern NMR methods for protein structure determination. Although NMR spectroscopy is well suited for rapid semi-automated structure determination of small proteins (MW < 15 kDa), solving structures of larger proteins and multimeric complexes is

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considerably more challenging. As the size of the molecule increases, so does the molecule's rotational correlation time and, consequently, the efficiency of ^1H - ^1H relaxation mechanisms. One way to suppress these effects is to incorporate deuterium into the protein sample, diluting the ^1H - ^1H relaxation networks and increasing ^{13}C and ^{15}N relaxation times, resulting in sharper line widths for ^{13}C , ^{15}N and remaining ^1H nuclei, and dramatically improved signal-to-noise ratios (Grzesiek et al., 1993; LeMaster and Richards, 1988; Markley et al., 1968). Perdeuteration is generally required for studies of larger proteins (Gardner and Kay, 1997; Gardner and Kay, 1998; Rosen et al., 1996; Venters et al., 1995b), particularly membrane proteins (Fernandez et al., 2004; Hiller et al., 2008). ^2H , ^{13}C , ^{15}N -enriched protein samples are also central to certain strategies for fully automated analysis of small protein structures (Zheng et al., 2003), and for protein neutron diffraction studies (e.g., Kossiakoff and Spencer, 1981).

While deuterium incorporation into protein samples can greatly improve the quality of data collected, the sample preparation itself can be challenging. Cell growth is affected by increasing $^2\text{H}_2\text{O}$ concentration, and cells must be gradually acclimated to high $^2\text{H}_2\text{O}$ concentration in incremental steps (Venters et al., 1995a). Once acclimated, additional isotopically-enriched reagents are introduced, and protein expression can proceed. As fermentation media costs for production of uniformly ^2H , ^{13}C , ^{15}N -enriched samples range from \$1500 - \$3,000 per liter, or higher, perdeuteration methods are generally employed only when absolutely required; for this reason, many potential applications of perdeuterated samples in routine protein NMR applications have not been pursued.

Here we describe a method for efficient production of perdeuterated, ^{13}C -, ^{15}N -enriched protein samples at a fraction of the cost of standard techniques. By optimizing the previously described condensed single protein production (cSPP) method of protein production in *E. coli* (Suzuki et al., 2007; Suzuki et al., 2006; Suzuki et al., 2005), we can produce high levels of specific proteins in deuterium-based, isotope-enriched minimal medium. Alternatively, cultures harboring pET expression cassettes can also be considerably condensed prior to protein induction, as has been shown previously for condensations of up to 4-fold without a reduction in per-cell protein expression levels (Marley et al., 2001).

Briefly, the cSPP system utilizes MazF, a cellular endoribonuclease, to induce a quasi-dormant state within the cell (Suzuki et al., 2006). MazF specifically cleaves single-stranded RNA at ACA sequences, thereby destroying cellular mRNAs, and shutting down protein synthesis. Engineering a target gene devoid of ACA triplets therefore renders transcripts resistant to MazF cleavage. Upon expression of MazF and induction of a quasi-dormant state, it is possible to not only selectively produce a single protein in a living cell, but to express targeted proteins in highly-condensed culture conditions.

The selective isotope-enrichment of the target protein in the absence of host protein expression, unique to the cSPP system, is a considerable advantage over a pET condensed culture. Two versions of the cSPP method are currently in use, cSPP(IPTG) (Suzuki et al., 2007) and cSPP(tet) (Schneider et al., 2009). The two methods differ in that while the cSPP(IPTG) system relies on IPTG for induction of both the MazF toxin and the target gene, the induction of the target gene can be temporally regulated with use of the cSPP(tet)

method (Schneider et al., 2009). Where required, the ability to temporally regulate target gene induction can lead to a significant reduction in accumulation of unlabeled protein expression, and improved homogeneity of enrichment in the resulting protein sample.

In this work, we demonstrate that both the pET and cSPP methods can be optimized to provide as much as 40-fold condensation (cSPP) of the culture prior to induction of target protein expression with no significant reduction in protein yield per cell. Using such perdeuterated samples of the *E. coli* cold shock protein A (CspA) and EnvZb, we have generated high quality NMR spectra. As a second application, we demonstrate the use of the cSPP system for cost-effective production of ^2H , ^{13}C , ^{15}N -enriched membrane proteins, including *E. coli* plasma membrane protein YaiZ and outer membrane protein X (OmpX).

Methods and Materials

General Protocol for Production of [^1H - ^{13}C]-l(+)LV, ^{13}C , ^{15}N , ^2H -enriched Proteins Using Anhydrotetracycline-Inducible SPP Vectors

Anhydrotetracycline-inducible pCold vectors, containing target protein under the control of the *tet* operator (*tetO_I*), are described in (Schneider et al., 2009). The following protocol is used for production of ^2H -enriched proteins with these vectors.

- i. Pick a single colony (containing pMazF and the expression plasmid), using a toothpick, from freshly plated transformed cells grown overnight on MJ9-CAA medium based plates (MJ9 medium (Jansson et al., 1996) supplemented with 0.2% casamino acids) containing 25 $\mu\text{g/ml}$ chloramphenicol and 100 $\mu\text{g/ml}$ ampicillin at 37°C and inoculate MJ9-CAA medium for overnight growth on a shaker (at approximately 180 r.p.m.). Throughout this protocol, the MJ9 medium, which is vitamin and buffer supplemented (Jansson et al., 1996), can be replaced with standard M9 medium, though better protein expression yields are usually obtained with MJ9 medium.
- ii. Inoculate MJ9 medium containing 25 $\mu\text{g/ml}$ chloramphenicol and 100 $\mu\text{g/ml}$ ampicillin at 5 $\mu\text{L/ml}$ (CAA should NOT be added). Grow cultures overnight at 37°C on a shaker (at approximately 180 r.p.m.).
- iii. Inoculate 10% of desired culture volume from overnight culture into fresh MJ9 medium containing 25 $\mu\text{g/ml}$ chloramphenicol and 100 $\mu\text{g/ml}$ ampicillin, and incubate at 37°C on a shaker (at approximately 180 r.p.m.). Note: if protein is to be labeled with specific amino acids or precursors, the same **unlabeled** amino acids or precursors (see b. below) should be added at this time to suppress the corresponding biosynthesis pathways from glucose during cell culture growth. Specifically, for preparations labeling the isopropyl methyl groups of Leu and Val, add 100 mg/L α -ketoisovaleric acid (Goto et al., 1999); for preparations labeling Ile, add 50 mg/L α -ketobutyric acid (Ile) (Gardner and Kay, 1997; Goto et al., 1999); for preparations labeling Phe, Tyr, or Trp, add 75 mg/L shikimic acid (Rajesh et al., 2003) or 50 mg/L of the individual amino acids Phe, Tyr, and/or Trp (Crespi and Katz, 1969), to suppress the corresponding biosynthetic pathways.

- iv. Monitor the optical density of the culture at 600 nm. The initial OD₆₀₀ should be approximately 0.2 after inoculation. Make sure the culture is growing exponentially. The OD₆₀₀ of the culture should increase linearly in a graph of log OD₆₀₀ verses time.
- v. When the OD₆₀₀ reaches ~ 0.5, remove the flask from the shaker and chill the culture rapidly by shaking the flask in an ice water bath for 10 min to reach a target temperature of 15°C.
- vi. Add IPTG to a final concentration of 1 mM to induce expression of MazF. Harvest a sample of cells from 1.5 ml of the culture by centrifugation (12,000 × g, 5 min, 4°C) and store the resulting pellet at –20°C for subsequent analysis of protein expression levels by SDS-PAGE.
- vii. Continue the culture at 15°C with shaking for 2 more hours. This 2 hour pre-incubation step before culture condensation and isotope labeling is important to prevent background isotope incorporation and prepare the cells for subsequent condensation.
- viii. Centrifuge (5000 × g, 10 min, 4°C) the culture to collect the cells and resuspend the cell pellet at 2.5% the initial culture volume (40X) in chilled 100 mM phosphate buffer, pH 7.5, in ²H₂O to wash the cells. Repeat centrifugation step to re-pellet the cells.
- ix. Resuspend the cell pellet in the desired culture volume of MJ9 medium prepared in ²H₂O, and containing specifically labeled amino acids or precursors together with 25 µg/ml chloramphenicol, 100 µg/ml ampicillin, 1 mM IPTG and anhydrotetracycline (see b. below for anhydrotetracycline concentration). For production of [¹H-¹³C]-I(+)LV, ¹³C, ¹⁵N, ²H-enriched proteins, the condensed fermentation contains 1 g / L ¹⁵N-NH₄Cl; 4 g / L ¹³C, ²H-glucose; 50 mg/L α-¹³C-ketobutyric acid; 100 mg/L α-¹³C-ketoisovaleric acid. For labeling of Phe, Tyr, and/or Trp [¹H, ¹³C]-enriched sites, add 75 mg/L of ¹H, ¹³C-enriched shikimic acid or 50 mg/L each of isotopically-enriched Phe, Tyr, and/or Trp amino acids.
 - a. The degree of condensation allowing for optimal expression varies among the different proteins expressed (Fig. 1-3). 20-fold condensed (5% of initial volume) is a good starting point; however, optimal fold condensation can be worked out in small scale pilot experiments if it is desired. Dilute a portion of the condensed culture to have approximately 5 ml at 1X and induce separately for an uncondensed control.
 - b. Uncondensed cultures should be induced with 0.2 µg/ml of anhydrotetracycline. Culture condensation requires that the concentration of anhydrotetracycline is increased linearly up to 20× condensation. To calculate this, take the fold condensation of the culture and multiply by 0.15 µg/ml of anhydrotetracycline. For example, a 20× condensed culture would require (20 * 0.15 µg/ml = 3.0 µg/ml) of anhydrotetracycline for induction.

- x. Incubate the condensed culture at 15°C with shaking overnight. Harvest the cell pellet by centrifuging ($12,000 \times g$, 5 min, 4°C) and store them at -80°C.

General Protocol for Production of [^1H - ^{13}C]-I(†)LV, ^{13}C , ^{15}N , ^2H -enriched Proteins Using IPTG-Inducible SPP Vectors

Using IPTG inducible pCold vectors (Suzuki et al., 2007), available from Takara Bioscience, the protocol for production of ^2H -enriched proteins is essentially identical to that used for the anhydrotetracycline-induced vectors, except that no anhydrotetracycline is added in step (ix).

Preparation of [^1H - ^{13}C]-I(†)LV, ^{13}C , ^{15}N , ^2H - CspA and EnvZb for Mass Spectrometry and NMR Studies

Isotope-enriched samples of CspA and EnvZb were prepared as described in the protocol above, with the following details. Competent *E. coli* BL21(DE3) cells containing the pACYCmazF (Suzuki et al., 2005) plasmid were transformed with pColdI(SP-4) (Suzuki et al., 2007) plasmid (Takara Bioscience, Inc) containing ACA-less *cspA* or *envZb* cloned into the *Nde* I- *Bam*HI sites. The resulting constructs include a 16-residue N-terminal tag, consisting of a translation enhancing element (TEE), a His₆ tag, and a Factor Xa cleavage site. Protein expression was performed essentially as described above, with the following details: single colonies were selected and used to inoculate 2.5 ml LB medium at 37°C for 6 hrs. 2 ml of the LB culture was inoculated into 100 ml of MJ9 minimal medium at 37°C overnight. When OD₆₀₀ reached 1.8 - 2.0 units, the culture was centrifuged at $3000 \times g$ for 15 min at 4°C. The cell pellet was resuspended in 1 L of fresh MJ9 medium and cells were grown at 37°C until OD₆₀₀ reached 0.5. At this point the culture was chilled on ice for 5 min and shifted to 15°C for 45 min to acclimate the cells to cold shock conditions. Target protein (CspA) was then expressed along with MazF for 1.5 hrs by addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) prior to expression in isotope enriched medium. Cultures were then centrifuged at $3000 \times g$ for 15 min at 4°C, resuspended in 2.5% volume (40×) in deuterated ($^2\text{H}_2\text{O}$) wash solution (7.0 g/L Na₂HPO₄; 3.0 g/L KH₂PO₄; 0.5 g/L NaCl; pH 7.4)], centrifuged again, and resuspended in 25 ml of deuterated MJ9 minimal medium containing 1 g / L $^{15}\text{NH}_4\text{Cl}$; 4 g / L ^{13}C , ^2H -glucose; 50 mg/L α- ^{13}C -ketobutyric acid; 100 mg/L α- ^{13}C -ketoisovaleric acid; and 1 mM IPTG. Protein expression continued at 15°C for 24 hrs. Cells were harvested by centrifugation as described above and cell pellets were stored at -80°C. For SDS-PAGE analysis, 100 μL of uncondensed cell culture (1×) or 2.5 μL of 40-fold condensed cultures (40×) were analyzed by Coomassie Blue stain. All isotopes were purchased from Cambridge Isotope Laboratories.

CspA and EnvZb Purification and Concentration

Cell pellets were resuspended in 40 ml of lysis buffer [50 mM Na₂HPO₄-NaH₂PO₄; 300 mM NaCl; 5 mM imidazole; 5 mM 2-mercaptoethanol (βME); with 1 EDTA-free protease inhibitor tablet (Roche Cat. # 11 873 580 001) per 50 ml at pH 8.0] and sonicated to lyse the cells. Lysed cells were then centrifuged at 4°C for 1 hr at 16,000 rpm in a Sorvall SS-34 rotor. Proteins were further purified by binding to NiNTA agarose at 40 ml of soluble extract per 1 ml of bed resin at 4°C overnight. Resin was washed twice with 25 ml of wash

buffer [50 mM Na₂HPO₄-NaH₂PO₄; 300 mM NaCl; 25 mM imidazole; 5 mM 2-mercaptoethanol (βME), pH 8.0], and protein was eluted in 8 ml of elution buffer [50 mM Na₂HPO₄-NaH₂PO₄; 300 mM NaCl; 300 mM imidazole; 5 mM 2-mercaptoethanol (βME), pH 8.0]. The protein solution was then dialyzed overnight at 4°C into NMR Buffer (20 mM Na₂HPO₄-NaH₂PO₄, 50 mM KCl, 0.02% NaN₃, 5 mM MgCl₂, pH 7.0 for EnvZB, and 50 mM KH₂PO₄, 1 mM NaN₃, adjusted to pH 6.0 with 5 M KOH for CspA). Protein solutions were then concentrated with Amicon Ultra 4 centrifugal filter devices (5000 MWCO) by centrifugation (2500 × g) to a volume of 1 ml. 3 ml of NMR Buffer containing and 5% ²H₂O was added and samples were again centrifuged to 1 ml. This process was repeated and samples were further concentrated to a volume of 500 μL at a final concentration of 2 - 3 mg/ml (~ 0.2 mM) for NMR studies.

Preparation of Uniformly Labeled ²H, ¹³C, ¹⁵N-HR3461D Protein For Mass Spectrometry Studies

NESG Target HR3461D is the SH2 domain (residues 453-557) of the human proto-oncogene tyrosine-protein kinase FER (Uniprot ID P16591; FER_HUMAN), which has been selected for study as part of the NESG Cancer Protein-Protein Interaction Network project (Huang et al., 2008). Uniformly ²H, ¹³C, ¹⁵N-HR3461D protein was prepared according to the protocol describe above, however, no amino acid precursors were present in the expression medium.

Expression of *E. coli* Plasma Membrane Protein YaiZ in the cSPP System

Uniformly ²H, ¹³C, ¹⁵N-enriched YaiZ was prepared according to the protocol describe above, with the following details. *E. coli* BL21 (DE3) cells, transformed with pACYCmazF (Suzuki et al., 2005) and pColdI(SP-4) (Suzuki et al., 2007) plasmids harboring the ACA-less yaiZ target gene, were grown in M9-glucose medium at 37° C. When OD₆₀₀ reached 0.5 - 0.6 units, the culture was chilled on ice for 5 min and shifted to 15°C for 45 min in order to acclimate the cells to cold temperature. After the cold-shock treatment, the expression of both MazF and the target gene were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cell pellet was washed twice with 10 ml of M9 salt buffer (no NH₄Cl) in ²H₂O, and finally suspended in 50 ml of deuterated M9 minimal medium (20× condensed phase) containing 1 g/L ¹⁵NH₄Cl; 4 g/L ¹³C, ²H-glucose, and 1 mM IPTG. Protein production was induced at 15°C for ~ 36 hr and the cells were then harvested by centrifugation.

Expression of *E. coli* Outer Membrane Protein OmpX in the cSPP System

Uniformly ²H, ¹³C, ¹⁵N-enriched OmpX was prepared according to the protocol describe above, with the following details. An ACA-less gene coding for *E. coli* outer membrane protein OmpX was cloned into pColdIV(SP-4) (Suzuki et al., 2007). *E. coli* strain BL21(DE3) was engineered to be ompA⁻ and ompF⁻ for two major outer membrane proteins, a strategy aimed at enhancing target outer membrane protein expression. These *E. coli* BL21(DE3) cells were transformed with pACYCmazF (Suzuki et al., 2005) and pColdI(SP-4) (Suzuki et al., 2007) plasmids harboring the target gene and grown in 2 L M9-glucose medium at 30°C. When OD₆₀₀ reached 0.5 - 0.6 units, the culture was chilled on ice

for 5 min and shifted to 15°C for 45 min in order to acclimate the cells to cold temperature. After the cold-shock treatment, the expression of both MazF and the target gene were induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 45 min. The cells were then harvested by centrifugation at $3000 \times \text{rpm}$ for 30 min at 4°C. The cell pellet was washed twice with 10 ml of M9 salt buffer (no NH_4Cl) in $^2\text{H}_2\text{O}$, and finally suspended in 50 ml of deuterated M9 minimal medium (40 $\times$ condensed phase) containing 1 g/L $^{15}\text{NH}_4\text{Cl}$; 4 g/L ^{13}C , ^2H -glucose, and 1 mM IPTG. Protein production was induced at 15°C for ~ 36 hr and the cells were then harvested by centrifugation.

Expression of Proteins in pET Vectors

Single colonies of BL21(DE3) cells containing pET expression vectors harboring the various protein constructs were inoculated into MJ9-CAA containing 100 $\mu\text{g/ml}$ ampicillin and grown overnight at 37°C while shaking. Overnight growth was used to inoculate fresh MJ9 containing 100 $\mu\text{g/ml}$ ampicillin at $\text{OD}_{600} = 0.2$, grown to $\text{OD}_{600} = 0.5 - 0.6$ in unlabeled media. Cells were subsequently condensed to various degrees by resuspension in deuterated MJ9 media containing ^2H , ^{13}C , ^{15}N in the form of [^2H , ^{13}C]-glucose and ^{15}N - NH_4Cl , and induced with 1 mM IPTG at 37°C while shaking for 2 hr (condensation experiments). For conventional expression of HR3461D, overnight cultures were resuspended directly in deuterated MJ9 containing ^2H , ^{13}C , ^{15}N in the form of [^2H , ^{13}C]-glucose and ^{15}N - NH_4Cl , grown to $\text{OD}_{600} = 0.5 - 0.6$ and then induced with 1 mM IPTG at 37°C while shaking for 2 hr. Aliquots of cell cultures were used for SDS-PAGE analysis to compare protein expression and the remaining culture was harvested and stored at -70°C for purification. Sonicated cells were centrifuged and soluble fractions were purified on NiNTA resin. Elution fractions containing protein were subsequently analyzed by mass spectrometry.

NMR Sample Preparation of YaiZ

The cell pellet provided by a 20 $\times$ condensed culture originating as a 1 L fermentation was suspended in 10 ml of 50 mM Tris buffer (pH 7.4). Cells were then lysed by a French press at 15,000 psi. The membrane fraction was collected by centrifugation at $100,000 \times g$ for 1 hr at 4°C. The membrane pellet was resuspended in 1 ml of 50 mM Tris buffer (pH 7.4) by sonication, centrifuged at $100,000 \times g$ for 1 hr at 4°C and the membrane pellets were stored at -80°C. The NMR sample of [^2H , ^{13}C , ^{15}N]-YaiZ was prepared by simple detergent solubilization of the plasma membrane fraction by 25 mM MES buffer, pH 6.0, containing 10% LOPG in 95% H_2O / 5% $^2\text{H}_2\text{O}$. The final concentration of YaiZ in the NMR sample was ~ 0.2 mM.

NMR Sample Preparation of OmpX

The cell pellet provided by a 40X condensed culture originating as a 1 L fermentation was suspended in 4 ml of 20 mM potassium phosphate buffer, pH 6.4, containing 0.5% sodium lauryl sarcosinate (Sarkosyl) and incubated at room temperature for 20 min, followed by centrifugation at $135,000 \times g$ for 30 min at 4°C. The outer membrane fraction was then isolated as a pellet (Filip et al., 1973). The NMR sample of [^2H , ^{13}C , ^{15}N]-OmpX was then prepared by simple detergent extraction from the outer membrane fraction using 20 mM

potassium phosphate buffer, pH 6.4, containing 5% DPC and 5% $^2\text{H}_2\text{O}$. The sample was briefly treated at about 80°C to allow extensive back-exchange of amide protons. The final concentration of OmpX in the NMR sample was ~ 0.2 mM.

Mass Spectrometry for Analysis of Isotope Incorporation

Purified protein samples were run on a 15% SDS-PAGE followed by Coomassie Blue staining. In-gel digest was performed in 50 mM NH_4HCO_3 , pH 7.9 overnight. Peptides were extracted from the gel with 60% acetonitrile, 5% formic acid, and lyophilized. Samples were then solubilized in 0.1% trifluoroacetic acid (TFA), pH 2.5-3.0, prior to liquid chromatography mass spectrometry / mass spectrometry (LC-MS/MS) analysis. Chromatography was performed using an ultimate nano-LC system (Dionex/LC Packings) and a fritless nanoscale column (75 $\mu\text{m} \times 15\text{ cm}$) packed in-house with 3 μm , 200 Å pore size Magic C18 stationary phase (Michrom Bioresource, Auburn, CA). The column was equilibrated in 0.1% formic acid (Solvent A), and samples were eluted using a linear gradient from 2% to 45% solvent B (0.1% formic acid in acetonitrile) over 30 min at a flow rate of 250 nL/min and analyzed by an LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nanospray source (Proxeon Biosystems).

To select peptides suitable for monitoring isotopic enrichment, the tryptic peptides were analyzed by MS/Zoom scan / MSMS scan. For HR3461D, peptide IEAQELLKK was selected. Quantitation of isotope incorporation was performed with MS in profile mode, by integration of peak areas. The percent of isotope incorporation was determined by comparing the mass estimated from the isotope-enriched peptide mass distribution to the expected mass computed assuming 100% isotope incorporation. It is expected that all of the carbon and nitrogen atoms present in a fully-enriched samples are ^{13}C and ^{15}N isotopic forms and all of the rapidly-exchanging atoms will back-exchange to ^1H when purified in H_2O buffers. Therefore, all NH, SH, and OH groups are assumed to be ^1H isotopes in the fully isotope-enriched peptide fragments. To compare isotope incorporation (^2H , ^{13}C , ^{15}N) for protein expressed in the cSPP system as compared to condensed and uncondensed conventional methods (pET), the protein HR3461D was subject to MS analysis following the methods of sample preparation described in the Materials and Methods section.

NMR Spectroscopy

NMR measurements of uniformly ^2H , ^{13}C , ^{15}N -enriched YaiZ in 10% LOPG were obtained using a 800 MHz Bruker AVANCE spectrometer with a cryoprobe at 40°C. TROSY [$^1\text{H}^{\text{N}}$ - ^{15}N] HSQC NMR data were acquired using spectral widths of 14 ppm in ^1H dimension and 32 ppm in ^{15}N dimension. The matrix size of collected spectra was 1024 $\times$ 256 total data points. NMR measurements of uniformly ^2H , ^{13}C , ^{15}N -enriched OmpX in 5% DPC were obtained using a 600 MHz Bruker AVANCE spectrometer with a cryoprobe at 50°C. TROSY [$^1\text{H}^{\text{N}}$ - ^{15}N] HSQC NMR data were acquired using spectral widths of 14 ppm in ^1H dimension and 34 ppm in ^{15}N dimension. The matrix size of collected spectra was 1024 $\times$ 256 total data points. NMR spectra of CspA and EnvZB were recorded at 20 °C using an 800 MHz Bruker AVANCE spectrometer with cryogenic probe, except where noted otherwise. Non-constant time [^{13}C - ^1H]-HSQC spectra were acquired for both CspA and EnvZB with [^1H - ^{13}C]-I(†)LV, ^{13}C , ^{15}N , ^2H -enrichment. [$^1\text{H}^{\text{N}}$ - ^{15}N] HSQC spectrum of

CspA was collected as 1024×256 total data points with spectral widths of 14 ppm in ^1H dimension and 34 ppm in ^{15}N dimension. In order to make backbone and side-chain assignments, deuterium-decoupled triple resonance pulse sequences as well as ^{13}C - and ^{15}N -edited NOESY spectra were acquired for $[^1\text{H}-^{13}\text{C}]\text{-I}(\dagger)\text{LV}$, ^{13}C , ^{15}N , ^2H -enriched CspA. All NMR spectra were processed and examined with NMRPipe and NMRDraw software package (Delaglio et al., 1995). The program SPARKY (Goddard and Kneller, 2008) was used for data visualization and analysis. Chemical shifts of proton were referenced to external DSS. ^{13}C and ^{15}N chemical shifts were referenced indirectly based on the proton referencing.

Analysis of Resonance Assignments

AutoAssign (Moseley et al., 2001) software was used for analysis of backbone and side chain $^{13}\text{C}^\beta$ resonance assignments for CspA. Sidechain ^{13}C and ^1H methyl resonances of Leu, Val and Ile ($\dagger$) were determined by manual analysis of $[^{13}\text{C}-^1\text{H}]\text{-HSQC}$ and 3D ^{15}N -edited NOESY spectra.

Results

Culture Condensation Using pET and cSPP Methods

In order to compare protein expression levels in deuterium-based minimal medium under condensed culture conditions using either a pET vector or the cSPP system, a panel of proteins were chosen for expression. Figure 1 presents the results of protein expression using a pET vector in deuterated and non-deuterated minimal medium at various levels of culture condensation. We find the maximal level of culture condensation without loss of expression is protein dependant, but generally ranges from 10-20 fold for proteins produced with a pET vector. Figure 2 presents examples of two ACA-less *E. coli* proteins expressed in the cSPP system under conditions of growth arrest and cessation of cellular protein production. The levels of CspA and EnvZB produced *per cell* from a 40-fold (40 $\times$) condensed H_2O -based culture is comparable to that of the uncondensed (1X) culture, where cells were treated identically differing only in the volume of media used for resuspension during the protein expression induction phase (Fig 2 a and b, lanes 2 and 3). CspA protein production is also unaffected by expression in 40X condensed $^2\text{H}_2\text{O}$ -based medium, while EnvZB displays only a modest reduction in expression (Fig. 2 a and b, lane 4). A second independent analysis of EnvZB at 20 $\times$ and 40 $\times$ condensation showed no observable decrease in expression in $^2\text{H}_2\text{O}$ -based medium (Fig 3, panel f).

To further demonstrate the generality of these condensation methods, Figure 3 displays nine additional proteins encompassing an eclectic collection of target proteins produced using the cSPP system. In addition to the *E. coli* proteins (Figure 2 and panel f of Figure 3), expression for four membrane proteins (panels a-d), two eukaryotic proteins (panels e and g), and three viral proteins expressed in the cSPP system are presented in Figure 3. The figure includes expression data from both the cSPP(IPTG) as well as the cSPP(tet) condensed-phase expression methods. In combination, these data of Figs 1 - 3 demonstrate that no acclimation of *E. coli* cells is required for preparing perdeuterated proteins at high yield in 10 to 40 -fold condensed phase using either pET and cSPP expression systems.

Isotope Incorporation

Proteins were purified by a single step Ni-NTA affinity purification (see Materials and Methods) and further analyzed by mass spectrometry (MS). The resulting isotope-enriched samples are extensively perdeuterated. Figure 4 presents LTQ linear ion trap mass spectrometry data for representative trypsin fragments from protein samples generated using 20-fold condensed cSPP(tet) (panel a), 20-fold condensed pET (panel b), and uncondensed conventional pET expression methods (panel c) with uniform ^2H , ^{13}C , ^{15}N -enrichment. The peak at 358.1 mass units present in each MS spectrum, <1% (panel a), 25% (panel b), and 8% (panel c) represents a portion of the total protein with no label incorporation. This unlabeled population likely results from leaky expression in unenriched medium, prior to introduction of the isotope enriched medium. Condensed cultures exhibit more heterogeneity in the isotope enriched protein population relative to protein produced by non-condensed conventional methods (cf. Figs. 4a and b with 4c).

Protein produced with the cSPP(IPTG) method yields a greater percentage of unlabeled protein as compared to the cSPP(tet) method (Schneider et al., 2009), while the heterogeneity of the isotope enriched fraction is essentially the same (Suppl. Fig. S1). While the unlabeled fraction results in a reduced effective yield of isotope-enriched protein, the unlabeled protein is essentially invisible in standard ^{15}N and/or ^{13}C -edited NMR spectra, and its presence would therefore not be problematic for routine triple-resonance NMR studies for protein structure determination, except to the degree that it lowers the effective sample concentration. In condensed-phase fermentations, the percent of overall isotope incorporated into the CspA and EnvZB proteins (i.e. in the isotope-enriched fractions indicated in Suppl. Fig. S1 and Suppl. Fig S2.) is ~85% using either the IPTG or dual IPTG/Tet inducible systems. In the absence of protonated precursors for isoleucine, leucine, and valine, ^2H incorporation also ranges from 85-100% (Suppl. Fig. S3). To determine whether this heterogeneity unveiled by MS analysis would affect subsequent NMR experiments, 40-fold condensed cultures expressing *E. coli* proteins CspA and EnvZb with uniform ^2H , ^{13}C , ^{15}N -enrichment and ^1H - ^{13}C labeling of Ile($\dagger$), Leu, and Val methyl groups [referred to here as ILV-perdeuterated samples (Gardner and Kay, 1997; Rosen et al., 1996)] were produced with the cSPP(IPTG) method and subsequently analyzed by NMR.

NMR Analysis of 40-fold Condensed cSPP(IPTG) Protein Samples

The quality of the [^1H - ^{13}C]-I($\dagger$)LV, ^{13}C , ^{15}N , ^2H -enriched proteins produced with the IPTG system were assessed by 2D [^1H - ^{13}C] HSQC NMR analysis (Fig. 5). These data demonstrate the expected ^{13}C - ^1H peaks of the Ile($\dagger$), Leu, and Val methyl groups in the upfield region of the spectrum, and little or no resonance peak intensity in other regions of the spectrum, demonstrating that these highly-perdeuterated protein samples are suitable for triple-resonance NMR studies. Similar results are obtained using CspA produced with the dual control IPTG/tet induced system (data not shown).

As an example of the utility of inexpensive production of perdeuterated proteins using the cSPP system, we evaluated the use of these samples for rapid determination of the 3D structure of an 86-residue construct of *E. coli* CspA. A 0.2 mM sample of ILV-perdeuterated CspA (produced with the IPTG system) was used for collection of deuterium-decoupled

triple resonance experiments, including HNCO, HN(ca)CO, HNCA, HN(co)CA, HNCACB, and HN(co)CACB experiments collected over a period of 3.5 days at 800 MHz, which were then used to determine backbone resonance assignments. Figure 6 shows the excellent quality of the resulting 2D [$^1\text{H}^{\text{N}}$ - ^{15}N] -HSQC spectrum. Perdeuterated protein samples produced with the cSPP system thus provide high-quality NMR spectra suitable for subsequent structural studies.

Membrane Proteins

Perdeuteration is essential for solution NMR studies of membrane proteins, and can be achieved at a fraction of the cost of conventional methods by using the cSPP system. As a second example of the utility of the cSPP system for cost-effective production of larger perdeuterated proteins, we also have demonstrated the production of perdeuterated membrane proteins, including the 8 kDa, 70-residue *E. coli* plasma membrane protein YaiZ, and the 16 kDa, 148-residue *E. coli* protein OmpX outer membrane protein. The NMR sample of YaiZ was expressed using 20 $\times$ condensation and OmpX was expressed using 40 $\times$ condensation in perdeuterated media. Both OmpX and YaiZ were expressed at high levels (Fig. 3 panels b and c). YaiZ was found exclusively in the inner membrane fraction, but not in the outer membrane fraction and inclusion bodies. An NMR sample of YaiZ was prepared by detergent extraction directly from membrane fractions using 10% 1-oleoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LOPG). OmpX assembles into the outer membrane (Suppl. Fig. S4), rather than as inclusion bodies as typically observed for outer membrane proteins (Arora et al., 2001; Fernandez et al., 2001). An NMR sample of OmpX sample was prepared by solubilizing the plasma membrane fraction with 0.5% sodium lauryl sarcosinate (Sarkosyl) (Filip et al., 1973), separating the insoluble outer membrane fraction, and then extracting OmpX into 5% dodecylphosphocholine (DPC) detergent.

These ^2H , ^{13}C , ^{15}N -enriched YaiZ and OmpX samples in simple detergent extracts were studied using [$^1\text{H}^{\text{N}}$, ^{15}N] TROSY NMR (Fernandez et al., 2001; Pervushin et al., 1997) at 800 and 600 MHz, respectively (Figs. 7a and 7b). The YaiZ and OmpX spectra exhibit essentially all of the amide peaks expected. The distribution of amide peaks in the OmpX spectrum is similar, but not identical, to that reported for reconstituted OmpX samples (Fernandez et al., 2001). While further purification does improve the spectra, these samples of YaiZ in a plasma membrane detergent extract and OmpX in a outer membrane detergent extract are suitable for some structural and functional studies by NMR without any further purification; however further purification of these ^2H -enriched samples would be recommended for extensive NMR structural studies or crystallization. These results demonstrate the utility of the cSPP system for production of perdeuterated membrane proteins.

Discussion

These results demonstrate an important new technology for producing perdeuterated proteins for NMR and other biophysical studies. The cSPP and pET expression systems can be used to produce perdeuterated, ^{13}C , ^{15}N -enriched protein samples, with methyl, aromatic, or other ^1H - ^{13}C labeling patterns, as required, at a fraction of the cost of conventional

systems with similar protein production yields. NMR data acquired from these samples can be used to generate data suitable structural studies. These methods have additional applications for studies of larger proteins, protein-protein complexes, and membrane proteins.

The most important feature of the cSPP method is that cells are no longer growing following MazF induction. Therefore, the only protein being produced in the “semi-dormant state” is the target protein – i.e. all of the ^{13}C , ^{15}N isotope is incorporated into the target protein, and none into other proteins, providing the ability to selectively detect the target protein using ^{13}C and/or ^{15}N -edited NMR experiments. Using pET expression and other conventional methods, these isotopes are incorporated into many different proteins – i.e. the labeling would not be selective to the targeted protein. The cSPP method can also be used in some cases to condense cells to very high densities (e.g. 40X or higher) in isotope-enriched media with no significant reduction in protein production yields per cell.

Condensed cell cultures, or high density cell cultures, harboring a pET expression cassette can also yield high levels of isotopically labeled protein (Marley et al., 2001; Sivashanmugam et al., 2009). The maximal level of culture condensation in the pET system has not been extensively explored, however, very high levels condensation (40X) are routinely obtained with the cSPP method. The optimal level of condensation and the method applied (cSPP or pET) varies among proteins (i.e. compare calmodulin (CaM) expression/condensation in pET vs. cSPP methods Figure 1 panel (a) and Figure 3 panel (e)) and therefore should be empirically determined on a small scale prior to large scale production. Using the SPP(tet) system, essentially all of the protein produced is isotopically-enriched.

Mass spectrometry analysis of HR3461D (Fig. 4) reveals a more heterogeneous profile of isotope incorporation with condensed samples as compared to samples produced in a conventional fashion. This heterogeneity likely results from the remaining unlabeled metabolites present in the cell at the time of induction (Marley et al., 2001). Providing a lag period following condensation prior to protein induction should significantly decrease this observed heterogeneity (Marley et al., 2001). Nevertheless, this low degree of heterogeneity in deuterium enrichment is not problematic (see Fig. 5), and samples produced in condensed conditions have proven highly suitable for structural studies.

Routine access to inexpensive perdeuteration methods provides a route to fully automated analysis of backbone assignments and 3D structures of smaller (< 12 kDa) proteins (Zheng et al., 2003). Our study demonstrates cost-effective production of ^2H , ^{13}C , ^{15}N -enriched proteins in the cSPP and pET systems. The resulting sample of cSPP induced CspA, purified with a single-step Ni-NTA affinity chromatography, allowed data collection and automated analysis of backbone $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}^{\beta}$, $^{13}\text{C}^{\alpha}$, as well as sidechain $^{13}\text{C}^{\beta}$, assignments in only a few days. This approach has tremendous value in generating assignments and structural information for small molecule screening studies, as well as in high-throughput structural and functional genomics studies.

In the case of membrane proteins, the selective labeling provided by the single protein production system allows NMR studies of a targeted membrane protein following simple

detergent extraction. NMR spectra can be obtained without extensive purification or reconstitution, allowing a membrane protein's structural and functional properties to be characterized prior to reconstitution, or as a probe of the effects of subsequent purification steps on the structural integrity of membrane proteins.

Conclusion

In conclusion, the condensed-phase SPP and pET technology for protein production allows cost-effective production of milligram quantities of perdeuterated proteins, facilitating many NMR, neutron diffraction, and other biophysical applications. The current technology typically provides samples that are ~ 85% enriched in ^2H , and 100% enriched in ^{15}N and ^{13}C . As we demonstrate here for CspA, this labeling is sufficient for obtaining high quality NMR spectra suitable for subsequent structure studies of small soluble proteins and for production of valuable perdeuterated samples of larger soluble proteins and membrane proteins. The 10 – 40 fold reduction in costs of perdeuterated fermentation media provided by using the pET or cSPP system opens the door to many new applications for perdeuterated proteins in spectroscopic and crystallographic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. SDS-PAGE analysis of various proteins expressed in condensed-phase pET system
Comparison of expression in H₂O and ²H₂O (a-d). The names of each protein, degree of condensation, and solvent (H₂O or ²H₂O) are indicated in each panel. Panel a, calmodulin (CaM), (eukaryotic); panel b, adenovirus E1B19K; panel c, domain of human proto-oncogene tyrosine protein kinase FER, residues 453-557 (HR3461D), (eukaryotic); panel d, N-terminal domain of murine leukemia virus integrase, residues 9 - 105 (IN NTD).

Fig. 2. SDS-PAGE analysis of protein expression from cSPP produced protein

CspA (a) and EnvZB (b) in *E. coli* in minimal medium. Lanes 2-5 contain the whole cell extract equivalent to 100 μ L of uncondensed culture. Lane 1, molecular weight marker (M); lane 2, uncondensed expression in H₂O; lane 3, 40-fold condensed in H₂O; lane 4, 40-fold condensed in ²H₂O; lane 5, 40-fold condensed control cells (without pCold I expression vector). Arrows mark CspA and EnvZB protein band accordingly. These data demonstrate cost effective production of ²H-enriched proteins in condensed cultures, without acclimation of *E. coli* cells, using only 2.5% of media costs compared to a conventional fermentation to obtain similar protein yields.

Fig. 3. SDS-PAGE analysis of various proteins expressed in condensed-phase SPP system
 Comparison of expression in H₂O and ²H₂O (a-j). The names of each protein, degree of condensation, and solvent (H₂O or ²H₂O) are indicated in each panel. Panels a-d illustrate 4 *E. coli* membrane proteins expressed using the cSPP(IPTG) system. Panel a, OmpA; panel b, OmpX; panel c, YaiZ, panel d, YnaJ. Panels e-f illustrate two soluble proteins expressed using the cSPP(IPTG) system. Panel e, calmodulin (CaM), (eukaryotic); panel f, EnvZB (*E. coli*). Panels g-j illustrates four additional eukaryotic (HR 3461D) and viral (E1B19K, CTD, NTD) proteins expressed with the cSPP(tet) system. Panel g, domain of human proto-

oncogene tyrosine protein kinase FER, residues 453-557 (HR3461D); panel h, C-terminal domain of murine leukemia virus integrase, residues 287 - 381 (IN CTD); panel i, adenovirus E1B19K; panel j, N-terminal domain of murine leukemia virus integrase, residues 9 - 105 (IN NTD).

Fig. 4. Isotope incorporation resulting from cSPP and pET expression in various culture conditions

LTQ linear ion trap mass spectrometry data documenting extensive ^2H , ^{13}C , and ^{15}N incorporation. (a) 20× condensed $^2\text{H}_2\text{O}$ media using the dual IPTG/Tet-induced cSPP system. (b) 20× condensed and expressed from a pET vector. (c) Produced conventionally using a pET vector. The tryptic fragment of the HR3461D protein, IEAQELLKK ($z = +3$), displayed here demonstrates that the extent of isotope incorporation in the two systems is similar. Compare peaks of labeled protein at 388.6, 393.6, and 398.6 mass units (a), peaks 389.6, 394.6, and 399.6 mass units (b), and peaks 394.6, and 399.8 mass units (c). The dual

induction cSPP system (a) results in significantly less (<1%) unlabeled protein (peak at 358.1 mass units) as compared to the 25% of total unlabeled protein in (b) and 8% unlabeled in (c). Predicted mass for complete isotope incorporation is 400.4 mass units.

Fig. 5. [^{13}C - ^1H]-HSQC spectra of ^1H -IL(+) V , ^2H , ^{13}C , ^{15}N -enriched protein

CspA (a) and EnvZB (b) produced with the IPTG induced system. Peaks are present in the region of the spectrum where methyl signals appear, but little or no ^1H - ^{13}C signal is detected for the rest of the protein. This method does not detect the fraction of protein molecules (~10%) with ^1H - ^{12}C isotopic composition. Inset: Expanded methyl region of (a), with sequence-specific resonance assignments.

Spectrum collected at pH 6.0 and 20 °C of sample of [^1H - ^{13}C]-I(+)LV, ^{13}C , ^{15}N , ^2H - enriched CspA, produced with the IPTG-induced system, and recorded on an 800 MHz NMR spectrometer. Assigned backbone H^{N} resonances are labeled by one-letter amino acid code followed by their sequence numbers.

Fig. 7. NMR spectra of *E. coli* membrane proteins YaiZ and OmpX

(a) 800 MHz TROSY- $[\text{H}^{\text{N}}, ^{15}\text{N}]$ -HSQC NMR spectrum of uniformly ^2H , ^{13}C , ^{15}N -enriched YaiZ obtained at 40 °C following simple detergent extraction. (b) 600 MHz TROSY- $[\text{H}^{\text{N}}, ^{15}\text{N}]$ -HSQC NMR spectrum at 50 °C of uniformly ^2H , ^{13}C , ^{15}N -enriched OmpX following simple detergent extraction. Target proteins (YaiZ or OmpX) were selectively isotope-enriched and perdeuterated using the cSPP system. The NMR sample of YaiZ was prepared by simple detergent solubilization of the inner membrane fraction by 25 mM MES buffer, pH 6.0, containing 10% LOPG in 95% H_2O / 5% $^2\text{H}_2\text{O}$. The NMR sample of OmpX

by simple detergent extraction of the outer membrane fraction by 20 mM potassium phosphate buffer, pH 6.4, containing 5% DPC in 95% H₂O / 5% ²H₂O.