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Enhanced, simplified expression of perdeuterated hemoglobin for NMR structure and dynamics

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ABSTRACT

An advanced protocol is provided to adapt cells for enhanced proliferation in and expression from deuterated minimal media. For large proteins (>20–30 kDa), deuteration levels >90% are essential for NMR characterization of structure and dynamics. In addition, the low sensitivity of NMR demands can be achieved without major sacrifice to yield. We applied the approach to human adult hemoglobin (Hb A), a 64 kDa, tetrameric protein that requires significant post-expression processing. This aspect accentuates the need for high yield. Using specially adapted JM109(DE3) *Escherichia coli*, we developed a shake-flask approach to express >90% deuterated NMR samples. Typical yields were 2.5-fold higher than obtained from cells adapted by more-traditional methods, while deuteration levels were increased by 17%. Ultimately, a 200 mL culture was sufficient to obtain (^{2}H, ^{15}N)-labeled Hb A sufficient for a 200 μM, 400 μL NMR sample. This avoids need for additional equipment for fermentation, which was used in previous protocols to express Hb A. It also allows a much smaller culture volume than often required by such equipment, for corresponding linear reductions in the cost of labeled starting materials. We tested the adaptation protocol with both JM109 and JM109(DE3) *E. coli*, and with pre- and post-transformation with the Hb A expression plasmid (pHE7). The (DE3) strain consistently outperformed its parent strain in response to adaptation, with the latter failing to survive adaptation in multiple trials. In addition, pre-transformed cells were consistently more receptive to adaptation. Finally, we also detail updated protocols to isolate Hb A in its functional form.

Introduction

Modern nuclear magnetic resonance (NMR)[1] techniques are powerful probes of protein structure [1,2] and dynamics [3–6]. NMR has provided approximately 25% of atomic-resolution structures in the protein data bank. Meanwhile, it is also the premier probe of biomolecular dynamics, capable of elucidating motions over 14 orders of magnitude in time, from picoseconds to hours. The most common frustration to NMR studies is the production of samples of sufficient quality and concentration. For example, the low sensitivity of solution-state NMR typically demands purified isolation of ~500 μM protein in 500 μL volume (e.g., 5 mg of a 20 kDa protein). Of course, isotopic enrichment is also necessary. NMR-active nuclei, such as ^{15}N and/or ^{13}C, are required near 99%, while natural-abundance levels are only 0.4% and 1.1%, respectively. Proteins larger than about 20 kDa also require 80–90% deuteration. This provides enhanced resolution and improved signal-to-noise (S/N) at essential reporter nuclei like ^{15}N, ^{13}C and amide protons, which are back-exchanged from H_2O after expression. The enhancements stem from removal of background of spin–spin interactions with otherwise unused protons. The significance of this background is heightened in larger, slowly tumbling proteins, making deuteration an absolute necessity for detailed structural or dynamic characterization at >20-30 kDa.

Techniques to meet these labeling needs are widespread in many NMR-focused labs. Yet, as NMR is applied to increasingly challenging systems, improved protocols are needed to boost yield without compromising NMR constraints quality. Furthermore, retaining simplicity of both expression technique and equipment needs is critical. This will allow broad access to be maintained among researchers whose focus is spectroscopy, rather than cell or molecular biology.

We demonstrate a simple and effective approach to high-yield, perdeuterated expression, here applied to human adult hemoglobin (Hb A). The method improved Hb A yield and deuteration levels of protein expressed from shake-flask cultures by 150% and 17%, respectively, over results from more traditional shake-flask

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[1] Abbreviations used: Hb A, human adult hemoglobin; MAP, methionine amino peptidase; NMR, nuclear magnetic resonance; S/N, signal-to-noise-ratio; MALDI-TOF MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; ESI, electrospray ionization.

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techniques. Similar deuteration gains are also apparent in comparison to earlier, fermentation-based expression of Hb A [7]. The key here, inspired by Paliy et al. [8], is expression from cells selected by an advanced adaptation protocol to thrive in a deuterated, minimal environment. Incorporation of other essential isotopes (^{15}N, ^{13}C) is a trivial extension that we also demonstrate. Our results with Hb A culminate in high-resolution (^{1}H, ^{15}N) NMR spectra. The enabling adaptation procedure is general to any expression system.

We also detail several additional aspects required by the particular complexity of expression, processing and assembly of Hb A. Thus, we treat an excellent example of a challenging system demanding improved methods for labeled expression. Particular complexities to consider for Hb A include: (1) co-expression of α and β globin subunits with heme incorporation, (2) concurrent processing by co-expressed methionine amino peptidase (MAP), which serves to cleave N-termini of α and β globins to provide Hb A in its natural human form, (3) redox treatment of the hemes to ensure proper insertion [9–11] and (4) separation and reconstitution as a chain-selectively labeled tetramer [7], which enables simplified interpretation of NMR spectra. Shen et al. [9,10] developed the pHE-*x* family of *Escherichia coli* expression plasmids to accomplish the first two tasks, while Simplaceanu et al. [7] employed the plasmid pHE2 for fermentation-based expression of 80% deuterated ^{15}N- or ^{13}C, ^{15}N-labeled Hb A. This group also demonstrated chain-selective labeling based on earlier protocols for chain-separation and tetramer reconstitution [12]. Finally, several groups have noted heterogeneous, non-native heme insertion in recombinant Hb A [9–11], and reported that oxidation–reduction treatment is capable of restoration to fully functional Hb A. We explored a variety of approaches for heme reinsertion, and detail the most successful following Khalife et al. [13], to finally obtain homogeneous Hb A in its native form.

Functionally, Hb A is a 64 kDa tetramer of two α and two β globin subunits. It transports O_2 via a complicated process of cooperative binding by its four corresponding heme prosthetics. The complexity is extended by allosteric response of O_2-binding affinity to pH and the concentrations of Cl^- and small-molecule effectors. Recent solution NMR studies of Hb A structure [14–16] and dynamics [17–19] have been particularly valuable in opening a window to intramolecular and inter-subunit dynamics in Hb A. However, much additional study is warranted to elucidate the value of these dynamics to O_2 binding, transport and release. For example, extension to exploring mechanisms of glycation-induced disruption of O_2-transport capabilities [20–23] is of vital interest due to the excessive glucose addition that occurs in diabetics [24].

To date, multidimensional NMR studies of Hb A [14–19] have used the fermentation-based expression protocol of Shen et al. [9,10] and Simplaceanu et al. [7]. Reliance on fermentation allows automated, active pH control to maintain expression and deuteration levels and provides high cell mass for corresponding high protein yield. However, fermentation incurs significant cost and complexity as well as equipment needs not met in many NMR-focused labs. On costs, fermentation equipment often sets the minimum expression volume at 3–10 L, which can be daunting for expression from deuterated minimal media [e.g., >$1500/L mostly accounted in the costs of 99% D_2O (>$300/L) and 98% d_7-glucose (~$125/g at 10–20 g/L)]. Meanwhile, smaller fermentation units (down to ~300 mL culture volume) may avoid that expense, but are not inexpensive to own (e.g., ca. $20k) and thus can be similarly prohibitive. Methods instead based on expression from shake-flask cultures, in principle, set no restrictions on expression volume, operate without additional equipment needs and may be simpler to perform. Thus, shake-flask methods are an attractive alternative provided they do not unduly sacrifice expression quality.

Regardless of the chosen venue for cell growth, yield and deuteration levels are critical for NMR. Thus, cell adaptation to a fully deuterated minimal medium has become standard practice. However, improved approaches are needed (1) so that the compromises of shake-flask growth (lower cell densities, poorer pH control) do not force replacement with fermentation and (2) to overcome additional challenges inherent to complex systems such as Hb A. Previous deuteration protocols for NMR [25–29] typically adapt cells to a deuterated environment with successive steps from rich, protiated starting culture, to minimal media with progressively higher deuterium content. Another approach was suggested by Paliy et al. [8], where additional plate-based selection steps were included along with iterative application of steps in both liquid and solid media. Altogether, this can provide cultures with a uniform phenotype for proliferation in deuterated minimal media. We have updated this approach and proven its value (1) with a less specialized medium of more general use in NMR sample production, (2) with a distinct approach to combining adaptation with cell transformation, (3) with new comparative analysis of success vs *E. coli* strain. In addition, our direct comparison of protein deuteration levels from traditionally vs advance-adapted cells shows that the latter critically increases protein deuteration, in addition to improving the yield. Deuteration levels obtained are similar-to-higher than those previously obtained by fermentation-based expression of Hb A [7]. Ultimately, our protocol enabled production of a sample for multidimensional NMR from a culture of only 200 mL.

Materials and methods

Reagents

All isotopes were purchased from Cambridge Isotope Laboratories (Andover, MA) or Isotec, Inc. (Miamisburg, OH), including 99% D_2O, 98% $^{15}NH_4Cl$, 98% $^{15}NH_4OH$ (purchased as a 3 N solution in H_2O), and both 88% and 98% ^{2}H d_7-glucose (1, 2, 3, 4, 5, 6, 6-D7). A stock of IPTG (isopropyl β-thiogalactopyranoside) purchased from Fisher Scientific (Suwanee, GA), was prepared in 99% D_2O at final concentration of 40 mM. Hemin from Sigma–Aldrich (St. Louis MO) was dissolved at 10 mg/mL in the $^{15}NH_4OH$ solution. Post-expression reagents did not require deuterated solvent unless otherwise specified. These include PMSF (phenylmethysulfonyl fluoride) protease inhibitor from Fisher, prepared in pure ethanol and stored at −20 °C. The oxidizing agent, TQ (thymoquinone) from Fisher was dissolved in pure ethanol at 0.3 M. Reduced glutathione (GSH) from Fisher was dissolved in H_2O at 0.48 M. The matrix for MALDI-TOF (matrix-assisted laser-desorption ionization time-of-flight) mass spectrometry (MS) was prepared with sinapinic acid from Sigma–Aldrich, with 10 mg dissolved in 1 mL of 60% H_2O (or D_2O), 40% acetonitrile and 0.1% trifluoroacetic acid (TFA). When not specified, all other chemicals were from Sigma–Aldrich or Fisher Scientific.

Bacterial strain, plasmid and growth media

E. coli strains JM109 and JM109(DE3) from Promega (Madison, WI) were used for cell adaptation and protein expression. The JM109 strain has been used in many examples of Hb A expression [7,9–11] with the plasmid pHE7 [10] that was used here. The JM109(DE3) strain is distinguished by its incorporation of an IPTG-inducible chromosomal copy of the gene for T7 RNA polymerase. We also note that JM109 and the (DE3) variant both contain the *lacI*^{q} mutation on the F′ episome. This is noteworthy as a potentially unstable aspect of these strains, although F′ retention is encouraged in minimal media (see Results and Discussion). We tested both *E. coli* strains for Hb A expression and for successful response to adaptation. The plasmid pHE7 was the generous gift of Prof. Chien Ho of Carnegie Melon University (Pittsburgh, PA). It