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Methyl-specific isotopic labeling: a molecular tool box for solution NMR studies of large proteins

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Nuclear magnetic resonance (NMR) spectroscopy is a uniquely powerful tool for studying the structure, dynamics and interactions of biomolecules at atomic resolution. In the past 15 years, the development of new isotopic labeling strategies has opened the possibility of exploiting NMR spectroscopy in the study of supra-molecular complexes with molecular weights of up to 1 MDa. At the core of these isotopic labeling developments is the specific introduction of [¹H,¹³C]-labeled methyl probes into perdeuterated proteins. Here, we describe the evolution of these approaches and discuss their impact on structural and biological studies. The relevant protocols are succinctly reviewed for single and combinatorial isotopic-labeling of methyl-containing residues, and examples of applications on challenging biological systems, including high molecular weight and membrane proteins, are presented.

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Nuclear magnetic resonance (NMR) spectroscopy is an established and powerful tool for studying the structure, dynamics and interactions of biomolecules at atomic resolution. The contribution of NMR spectroscopy to the study of high molecular weight proteins has evolved considerably since the mid-1990s. Advances in NMR hardware (e.g., cryogenic probes and higher field NMR spectrometers), the introduction of optimized NMR

experiments (e.g., TROSY [1]) and the use of deuterated [¹³C,¹⁵N]-labeled samples [2–6] have enabled NMR studies of proteins up to ca. 50 kDa. Strategies for the specific introduction of [¹H,¹³C]-labeled methyl groups in perdeuterated proteins have substantially extended that upper molecular weight limit. Indeed, these advances have permitted solution NMR studies of supra-molecular complexes up to 1 MDa, protein targets that were previously inaccessible to the technique.

NMR-driven studies of high molecular weight systems can provide site-resolved information on local structure, thermodynamics, kinetics, molecular dynamics, post-translational modifications and function. Much of the groundbreaking work in the NMR analysis of supramolecular assemblies has been conducted on the proteasome. The 20S core proteasome particle consists of two subunits, α and β . Each subunit forms a heptameric ring, which assemble to form a $\alpha_7\beta_7\beta_7\alpha_7$ oligomer. Methyl-specific labeling and methyl-TROSY NMR [7•] approaches developed and applied by Kay and colleagues have been used to characterize oligomerization, substrate binding and protein dynamics as well as screening for small molecule inhibitors [8••,9–12]. One elegant example, which also highlights the enormous experimental flexibility that comes from analyzing these protein complexes in solution, concerns the measurement of pK_a values for catalytic groups in the 20S core proteasome particle [13•]. Despite a number of high-resolution structural studies, there had been ongoing debate about the identity of the base that deprotonates the catalytic hydroxyl group of Thr-1 of the β -subunit. NMR analysis of ionization equilibria in smaller proteins is routine. Application of this approach to the 20S core particle required a sample with threonine-specific methyl labeling [13•]. The pH-dependent changes in the chemical shift of the γ_2 -methyl group of Thr-1 were fitted to different models describing the ionization of neighboring functional groups. These analyses were consistent with a reaction mechanism in which the N-terminal α -amino group deprotonates the γ -OH group of the catalytic threonine.

NMR spectroscopy can report information on molecular dynamics across a range of time scales, from picoseconds to hours. Typically, these data are accessed by measuring how an NMR signal relaxes or changes with time. Methyl-specific labeling and methyl-TROSY experiments permit NMR relaxation studies of larger proteins and supramolecular assemblies. A further strength of NMR spectroscopy is

Figure 1

Examples of specific methyl-labeling of the 468 kDa amino peptidase TET2 from *Pyrococcus horikoshii*. Proton densities presented on the 3D structure of the homododecameric TET2 protein (PDB code: 1Y0R) for (a) U-[^1H , ^{13}C] TET2 sample and (b) U-[D] TET2 with specific [^1H , ^{13}C]-labeling of the β -methyl group of alanine. 2D (^1H , ^{13}C)-HMQC spectra of samples of (c) U-[^1H , ^{13}C] TET2 compared to U-[D] TET2 with specific [^1H , ^{13}C]-labeling of (d) the β -methyl group of alanine using alanine as precursor [37], (e) the δ_1 -methyl group of isoleucine using 2-ketobutyrate [20**], and (f) the proS methyl group of valine using 2-acetolactate [30]. The 1D traces extracted at the ^{13}C frequency of Ala-344 are shown in (c) and (d). In the U-[^1H , ^{13}C] TET2 sample, the intense dipolar interactions with nearby protons broaden the lines beyond the threshold of detection (c); whereas in the U-[D] TET2 sample specifically protonated on the β -methyl group of alanine, the

its ability to provide site-resolved information for intrinsically disordered proteins or regions of proteins. These two strengths were combined in a study of the gating mechanism of the 20S proteasome [14^{*}]. The degradation of protein substrates occurs in a central cavity formed by the two 7-mer β rings. The catalytic cavity is accessed via pores formed by the two 7-mer α rings. Access through the pore is regulated by the N-termini of the α -subunits, which are flexible and which had not been resolved in crystallographic studies. Methionine ϵ -methyl-specific labeling was used to probe the dynamics of the N-terminal gating regions. Resonance assignment was performed through site-by-site Met-to-Ala mutation. Methyl-TROSY spectra revealed that certain methionine residues yielded multiple NMR signals that corresponded to whether the N-terminus was inside or outside the proteasome pore. Analysis of the stoichiometry revealed that, on average, two of the seven termini were in the pore, while five were not. NMR EXchange Spectroscopy (EXSY) demonstrated that these states interconvert on the seconds time-scale as well as revealing longer-distance allosteric affects, which were detected over 80 Å from the proteasome pore entrance.

The study of longer time-scale protein dynamics can also be probed by NMR spectroscopy. Real-time analysis of enzyme turnover [15] or protein folding [16] can be performed at site-resolved resolution through the collection of a series of 2D NMR experiments. The upper molecular weight limit of this approach was enhanced greatly by the optimization of the SOFAST HMQC experiment [17] for sparsely-protonated, methyl-labeled high molecular weight proteins. Consequently, 2D (^1H , ^{13}C) correlation spectra of very large proteins can be acquired on a second time-scale [18]. Rizo and colleagues reported a nice example of the use of real-time NMR studies of protein function [19]. Using 2D methyl TROSY NMR experiments, they showed that the rate at which the Syntaxin-1 protein restructures to form an active SNARE complex is affected by other protein components of the synaptosome.

Below, we will review the methods for selective protonation and isotopic labeling of methyl groups that have permitted such studies. We will also highlight how methyl-labeling can be applied to study the structure of larger proteins.

Development of protocols for isotopic labeling of methyl groups in proteins

NMR spectroscopy is a well-established technique for characterizing the structure, dynamics and function of proteins of less than 30 kDa. Spectroscopists have access

to an enormous array of multidimensional heteronuclear NMR experiments designed for [^{13}C , ^{15}N]-labeled proteins. Above 30 kDa, the reduction in molecular tumbling rate increases the rate at which NMR signal relaxes, causing signal broadening and compromising experimental sensitivity. In higher molecular weight proteins, the major mechanism that drives ^1H NMR relaxation is dipolar interactions between the large number of neighboring protons (Figure 1a). For proteins of 30–80 kDa, detrimental dipolar interactions can be reduced by expressing the protein in a perdeuterated culture medium. A basal level of protons, typically around 20%, is then reintroduced at labile sites (e.g., NH, OH, NH_3^+ , among others), by purifying or, if necessary, refolding the protein in H_2O -based buffers. The resulting protein has protonated amide groups, which ensures that backbone-directed NMR experiments can still be applied to proteins in this size range.

To apply solution NMR spectroscopy to proteins above 100 kDa requires an even greater reduction in overall level of protonation (Figure 1a–d). However, to benefit from the high intrinsic sensitivity of the ^1H nucleus it is necessary to retain some residual protonation at key sites. Methyl groups are ideal candidates for NMR studies of high molecular weight proteins, as they yield highly intense and well-resolved NMR signals due to the multiplicity of protons and the rapid rotation about the three-fold methyl symmetry axis. Furthermore, methyl groups are enriched in the hydrophobic cores of proteins and at protein–protein interfaces. To reduce ^1H signal relaxation rates requires a protein sample in which only selected methyl groups are protonated while the remainder of the protein is completely deuterated. Such a labeling scheme can be achieved by supplementing a fully deuterated *Escherichia coli* minimal culture medium with one or more specifically [$^{13}\text{CH}_3$]-labeled amino acids or biosynthetic precursors (Figure 2). The approach used to produce methyl labeled proteins depends on the biosynthetic pathway of the target methyl group, with the simplest cases involving amino acids that are the products of irreversible metabolic pathways. The earliest methyl-specific labeling protocol reported in the literature concerned the δ_1 -methyl group of isoleucine [20^{**}]. The precursor 2-keto-3-[D₂],4-[^{13}C]-butyrate was used as the sole source of protons in a perdeuterated culture media, to produce a [U-D], Ile- δ_1 -[$^{13}\text{CH}_3$]-labeled protein (Figure 1e). A similar strategy was subsequently developed to isotopically label the prochiral methyl groups of leucine and valine. This protocol used 2-keto-3-[D]-[$^{13}\text{CH}_3$, $^{13}\text{CH}_3$]-isovalerate, a precursor of both leucine and valine, which labels both methyl groups in both amino

(Figure 1 Legend Continued) reduction of local proton density makes it possible to recover a narrow NMR signal detectable by solution NMR (d). In (c), the two observable signals annotated by * correspond to the methyl resonances of valine-4 located on the flexible N-terminal end of the TET2 sequence. The insets of (d) to (f) show the chemical structure of the precursor or amino acid used for specific labeling. NMR spectra were acquired at 50 °C on a NMR spectrometer operating at a proton resonance frequency of 800 MHz.

Figure 2

Simplified biosynthetic pathway for methyl-containing amino acids in *E. coli* (adapted from KEGG <http://www.genome.jp/kegg/kegg2.html>). The different precursors cited in the text are indicated. Carbon atoms derived directly from pyruvate or alanine (via deamination of alanine catalyzed by *E. coli* transaminases) are indicated in green, while carbon atoms derived from aspartate are shown in red. Enzymes: (1): α -hydroxy acid synthase (AHAS); (2): ketol-acid reductoisomerase (KARI) and dihydroxy-acid dehydratase (DHAD); (3): branched-chain amino acid aminotransferase (BCAT); (4): *E. coli* alanine transaminases (AlaA, AlaC and AvtA); (5): biosynthetic threonine deaminase (IlvA).

acids [21–23]. Later, 2-keto-3-[D]-[$^{13}\text{CH}_3$, $^{12}\text{CD}_3$]-isovalerate was used to label only one of the prochiral methyl groups in Leu/Val, which greatly reduced the local proton concentration and thereby methyl relaxation rates [24, 25, 26]. However, this precursor is produced as a racemic mixture and consequently each prochiral methyl group in the protein is labeled at 50%, which means that both sites are detectable by NMR but with reduced sensitivity. Stereospecific labeling of leucine and valine was achieved using 2-acetolactate (2-hydroxy-2-methyl-3-ketobutyrate), a more upstream precursor in the Leu/Val biosynthetic pathway (Figure 2) that could be chemically synthesized to give 100% pro*S* or pro*R* labeling in the final protein product [27]. Protocols for the labeling of leucine but not valine, and vice versa, have also been proposed. [$^{13}\text{CH}_3$]-labeled leucine [28] or 2-ketoisocaproate [29] have been introduced for either stereo-specific or non-stereospecific labeling of leucine. Alternatively, 2-acetolactate [30] or valine [28] can be supplemented in conjunction with deuterated leucine to label solely valine methyl groups (Figure 1f). The Ile- γ_2 methyl group can be labeled using 2-hydroxy-2-ethyl-3-keto-butyrate [31, 32], while the ϵ -methyl group of methionine can be labeled by supplementing the medium with the residue itself [33, 34, 35].

Specific protonation of either alanine (Figure 1d) or threonine is more complicated, as both of these amino acids are either synthesized in reversible reactions or are precursors of other methyl-containing amino acids (Figure 2). Typically, alanine [36, 37] and threonine [38] can be supplemented directly in the M9/D $_2$ O medium with an appropriate cocktail of deuterated metabolic intermediates (Table 1) to suppress scrambling (i.e., leak of ^{13}C or/and ^1H into undesired atomic positions).

Combinatorial labeling of methyl-containing residues increases the number of NMR probes in large proteins

The high level of deuteration that is needed to detect signals of protonated methyl groups leads to a substantial reduction in the number of NMR-visible probes and consequently to a significant loss of structural information. The six naturally occurring methyl-containing amino acids represent up to 30–40% of the amino acids in proteins, including up to 50% in hydrophobic cores [39] and up to 24% at protein–protein interfaces [40]. Simultaneous labeling of several types of methyl group-containing amino acids is an obvious way to increase the number of NMR-visible probes from which useful information

Table 1**Incorporation of $^{13}\text{CH}_3$ groups in perdeuterated proteins**

Methyl groups	Name of precursors	Quantity (mg/L) ^c	Scrambling/incompatibilities	Ref.
Ile- δ_1	2-Ketobutyrate or 2-(S)-hydroxy-2-ethyl-3-ketobutyrate	60 60	Both precursors have co-incorporation incompatibilities with 2-acetolactate ^d	[20**], [44*]
Ile- γ_2	2-Hydroxy-2-ethyl-3-ketobutyrate (racemic)	100	Scrambling (7%) in L/V ^{pro-R} group ^e . Co-incorporation incompatibility with 2-acetolactate ^d	[31], [32]
Met- ϵ	L-Methionine or 4-Methyl-thio-2-ketobutyrate	100–250	–	[33**], [34]
Leu/Val	2-Ketoisovalerate ^a	80–120	–	[24*], [26]
Leu/Val ^{pro-S}	2-Acetolactate ^b	300	Co-incorporation incompatibility with isoleucine precursors ^d	[27**]
Leu	2-Ketoisocaproate ^a	150	–	[29]
Leu ^{pro-S}	Stereospecifically labeled L-Leucine ^b	20	–	[28]
Val ^{pro-S}	2-Acetolactate ^b + U-[D] L-Leucine	300 20	Scrambling in L ^{pro-S} groups (<1%). Co-incorporation incompatibility with isoleucine precursors ^d	[30]
Val ^{pro-S}	Stereospecifically labeled Valine ^b + U-[D] Leucine	100 20	–	[28]
Ala- β	L-Alanine	600	Scrambling in Ile- γ_2 , L/V* (up to 25%), minor scrambling in other CH and CH ₂ sites ^f	[37*], [43], [44*]
Thr- γ_2	L-Threonine	50–100	Scrambling in Ile- δ_1 (ca. 50%), minor scrambling in glycine ^g	[38*]

^a This precursor is produced as a racemic mixture, and consequently overall labeling of each prochiral methyl is 50%, which means that both pro-S and pro-R sites are detectable by NMR but with reduced sensitivity.

^b This precursor permits stereospecific labeling of pro-S (or pro-R) methyl groups. Selected methyl groups are labeled at ca. 100%, enhancing sensitivity for the detection of structural meaningful NOEs between remote methyl groups by up to a factor of 4.

^c Quantity of precursors to add 1 hour prior to induction of protein expression in M9/D₂O media containing 2 g/L of deuterated glucose. Variation of carbon sources may interfere with incorporation of specifically labeled precursors into overexpressed protein.

^d Co-incorporation incompatibilities can be suppressed by addition of 2-acetolactate 40 minutes before isoleucine precursor (added 20 minutes before induction) [44*].

^e Scrambling suppressed by co-addition of 200 mg/L U-[D] 2-ketoisovalerate [32].

^f Scrambling suppressed by co-addition of 200 mg/L U-[D] 2-ketoisovalerate, 60 mg/L U-[D]-isoleucine or 2-(S)-hydroxy-2-ethyl-3-ketobutanoic acid, 2.5 g/L U-[D]-succinate and 2.5 g/L U-[D]-glycerol [37*,44*].

^g Scrambling suppressed by co-addition of 60 mg/L of U-[D]-isoleucine + 100 mg/L U-[D] glycine [38*].

can be obtained. A number of combinatorial [$^{13}\text{CH}_3$]-methyl-labeling schemes have been reported, including ILV (Ile- δ_1 /Leu- δ /Val- γ) [22,26,41,42], MILV (Met- ϵ /Ile- δ_1 /Leu- δ /Val- γ) [33**], AILV (Ala- β /Ile- δ_1 /Leu- δ /Val- γ) without [43] or with stereospecific labeling of leucine and valine [44*], and MILVT (Met- ϵ /Ile- δ_1 /Leu- δ /Val- γ /Thr- γ_2) [45**].

Methyl groups resonate in a narrow region of 2D (^1H , ^{13}C) NMR spectra. As a consequence, the motivations for combining different methyl groups must be considered:

- *The residue type-dependence of [$^{13}\text{CH}_3$] resonance dispersion.* Signal overlap can be alleviated by choosing

suitable amino acids to label simultaneously [46]. Figure 3a illustrates an example of a well-resolved 2D methyl-TROSY spectrum of malate synthase G (MSG; 82 kDa) in which Met- ϵ , Ile- δ_1 and Thr- γ_2 methyl groups have been labeled simultaneously. These methyl groups typically resonate in non-overlapping regions of the spectrum.

- *Specific labeling of one methyl group per residue.* The regio-specific or stereo-specific labeling of a single methyl group in Ile, Val or Leu can greatly increase sensitivity and resolution [20**,27**,28,30–32,44*] (Figure 3b). Optimal resonance dispersion is achieved by labeling the pro-S methyl groups of leucine and valine at the same time as the Ile- δ_1 methyl group, as these groups

Figure 3

Scrambling-free combinatorial isotopic labeling to optimize sensitivity and resolution. 2D (^1H , ^{13}C) HMQC spectra of (a) U-[D], (Ile- δ_1 , Met- ϵ , Thr- γ_2)-[$^{13}\text{CH}_3$] or (b) U-[D], (Ala- β , Ile- δ_1 , Leu/Val $^{\text{proS}}$)-[$^{13}\text{CH}_3$]-labeled MSG [44 $^{\bullet}$]. Spectra were acquired at 37 $^{\circ}\text{C}$ on a NMR spectrometer operating at a proton resonance frequency of 800 MHz. The colored ellipses represent the location of methyl resonances for each type of methyl group. The x and y boundaries of the semi-transparent ellipses demonstrate the second standard deviations associated with each chemical shift. (^1H , ^{13}C) chemical shifts were taken from the BioMagResBank (BMRB, <http://www.bmrb.wisc.edu>).

tend to have wider chemical shift dispersion [47] and limited overlap.

- *Prevention of co-incorporation incompatibilities of precursors.* If the metabolic precursors used for combinatorial labeling share the same metabolic pathway, the enzymatic machinery can exhibit preference towards one precursor over others. Combinatorial labeling of Ile/Val/Leu with 2-ketobutyrate and 2-acetolactate is affected by this phenomenon. Incompatibility of the two precursors (Table 1) leads to a 2-fold reduction in enrichment at the Leu/Val $^{\text{proS}}$ site unless 2-acetolactate is added to the media before 2-ketobutyrate [44 $^{\bullet}$].
- *Prevention or exploitation of isotope scrambling.* Amino acids that are intermediates or involved in reversible metabolic pathways generate isotope scrambling. This isotopic leak can be suppressed, or exploited if advantageous (Table 1). For example, isotopic scrambling to Ile- γ_2 methyl groups occurs when 3-[^{13}C]-Ala and 2-ketobutyrate are used simultaneously [37 $^{\bullet}$]. 3-[^{13}C]-Ala is converted into 3-[^{13}C]-pyruvate, which causes 2–5% scrambling in Isoleucine γ_2 position [44 $^{\bullet}$]. The replacement of 2-keto-3-[D $_2$]-4-[^{13}C]butyrate with 2-hydroxy-2-[2'-(^{13}C)-1'-(D $_2$)]ethyl-3-keto-4-[D $_3$]-butyrate resolves this problem. Likewise, the activity of threonine deaminase causes enrichment of Ile- δ_1 methyl groups when the medium is supplemented with 2-[D]-3-[D]-4-[^{13}C]-Thr [38 $^{\bullet}$]. This scrambling can be suppressed by adding deuterated isoleucine to retro-inhibit threonine deaminase or exploited by labeling both Ile- δ_1 and Thr- γ_2 sites simultaneously.

NMR structural analysis of methyl labeled protein samples

The Nuclear Overhauser Effect (NOE) is a key observable for 3D structure determination by solution NMR

spectroscopy. In smaller proteins, NOEs can be detected between ^1H – ^1H pairs separated by up to 6–7 Å. However, the sparse density of protons in selectively methyl-protonated proteins has allowed the detection of accurate long-range methyl/methyl distances of up to 12 Å in 10–20 kDa protein [48]. Although NOE magnetization transfer is more efficient for large proteins, the detection of methyl/methyl NOEs in such systems is limited by the signal broadening that results from faster relaxation (Figure 4). Nonetheless, NOE cross-peaks can be detected for methyl groups separated by up to 10 Å, in MSG (82 kDa, 37 $^{\circ}\text{C}$; $\tau_c \approx 50$ ns; [44 $^{\bullet}$]), while in a 0.5 MDa protein complex, it is possible to detect NOEs between methyl groups separated by 7–8 Å (50 $^{\circ}\text{C}$; $\tau_c \approx 220$ ns [30]).

Combinatorial selective protonation of methyl groups has underpinned numerous structural studies of high molecular weight proteins. The first large protein whose structure was determined using restraints from ($^{13}\text{CH}_3$)-NMR spectroscopy was the 82 kDa monomeric protein MSG [49 $^{\bullet\bullet}$]. Methyl-TROSY NMR studies have also contributed significantly to structural studies of challenging membrane proteins. Examples include the structure of the voltage-dependent anion channel (VDAC), an integral membrane protein that allows the diffusion of small molecules across the eukaryotic outer mitochondrial membrane [50], and the hexameric p7 cation channel from hepatitis C virus [51]. Methyl NMR approaches were also used to elucidate the 3D structure of the phototaxis receptor sensory rhodopsin II, a seven trans-membrane-helix protein that exhibits many structural similarities to G-protein coupled receptors [52]. In larger protein assemblies, specific labeling of methyl probes has been used to model the interaction between functional

Figure 4

Detection of long-range methyl-methyl NOEs in high molecular weight proteins. **(a)** Variation of the *maximal distance* for which methyl/methyl NOEs are detectable as a function of the correlation time of the protein. Methyl probes separated by the *maximum distance* have a 50% probability of yielding observable NOEs. **(b)** Distribution of experimentally detected inter-methyl NOEs in U-[D], (Ala- β , Ile- δ_1 , Leu/Val^{proS})-[¹³CH₃]-MSG [44[•]] and U-[D], (Ile- δ_1 , Val^{proS})-[¹³CH₃]-TET2 systems [30]. 3D HMQC-NOESY data sets were recorded on a Varian (Agilent) DirectDrive spectrometer operating at a proton frequency of 800 MHz equipped with a cryogenic triple resonance probehead. The experiments were recorded at 37 °C for 96 hours with a 1 mM U-[D], (Ala- β , Ile- δ_1 , Leu/Val^{proS})-[¹³CH₃]-MSG sample and a NOE mixing time of 500 ms, (50 °C, 64 hours and 400 ms for U-[D], (Ile- δ_1 , Val^{proS})-[¹³CH₃]-TET2 sample). Each point represents the percentage of experimentally detected NOEs between pairs of methyl groups for a given distance (± 0.5 Å). The total number of methyl/methyl pairs for a given distance was predicted using the 3D structures of MSG (82 kDa; red curve; PDB code: 1D8C) or TET2 (468 kDa; blue curve; PDB code: 1Y0R). Examples of methyl/methyl pairs for which long-range NOEs were experimentally detected in MSG **(c)** and TET2 **(d)**.

signal-peptides and the 204 kDa translocase SecA [33^{••}], to characterize the interaction between the trigger factor chaperone and substrates at the atomic level [45^{••}], and to model the structure of the 390 kDa box C/D RNP enzyme bound to substrate RNA [53^{••}].

Extending methyl-specific labeling strategies

Specific isotopic labeling and protonation of methyl groups in very large perdeuterated proteins is essential for their investigation by solution NMR spectroscopy. However, the possible drawbacks of this approach need to be considered. The first concerns the restricted number of observable NMR probes compared to standard uniform

labeling. As discussed, it is possible to reduce this effect through combinatorial labeling schemes. In addition, non-native methyl groups can be introduced at strategic sites through mutation [14[•],35] or by chemical modification of lysine [54] or cysteine [55] residues.

Perhaps the largest obstacle is the requirement to produce milligram quantities of protein in deuterated culture medium. D₂O negatively affects bacterial growth and can severely impact recombinant protein expression and solubility to such an extent that methyl labeling ceases to be economically viable. Protocols for the methyl labeling of perdeuterated proteins in the yeast *Kluyveromyces fragilis*

have been proposed [56] as well as approaches for methyl-specific labeling via the insect cell/baculovirus expression system [57**]. Cell-free protein expression alleviates the requirement that the expression organism must be viable in perdeuterated expression medium while also offering tremendous control over the isotopic composition of the reaction mixture and therefore the final protein. In addition, cell-free protein synthesis can be tuned to maximize the production of challenging systems, for example, improving membrane protein production through the addition of lipids and detergents to the reaction mixture [58–60].

Residue-selective protonation of methyl groups in perdeuterated proteins opens the possibility of NMR investigations of the structure, dynamics and function of challenging biological systems at atomic resolution. A growing array of isotopically-enriched metabolic precursors is now available for isotopic-labeling of different types of methyl groups in protein targets in *E. coli*. In the next decade, advances in emerging approaches for methyl labeling, such as cell-free protein synthesis or eukaryotic expression systems, will be key to ensuring that greater numbers of biologically important protein targets can be investigated by solution NMR spectroscopy.

Conflict of interest

None declared.

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