Computational, biochemical, and NMR-driven structural studies on histone variant H2A.B

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Chapter 2. Isotope-labeling strategies for solution NMR studies of macromolecular assemblies

This chapter is based on:
Zhang, H. & van Ingen, H. Isotope-labeling strategies for solution NMR studies of macromolecular assemblies. Current Opinion in Structural Biology. 2016 June; 38:75-82. Doi: 10.1016/j.sbi.2016.05.008.

Abstract

Proteins come together in macromolecular assemblies, recognizing and binding to each other through their structures, and operating on their substrates through their motions. Detailed characterization of these processes is particularly suited to NMR, a high-resolution technique sensitive to structure, dynamics, and interactions. Advances in isotope-labeling have enabled such studies to an ever-increasing range of systems. Here we highlight recent applications and bring to the fore the range of options to produce labeled proteins and to control the specific placement of isotopes. The increased labeling control and affordability, together with the possibility to combine strategies will further deepen and extend the range of protein assembly investigations.

Introduction

Proper cellular functioning depends critically on networks of biomolecular interactions. Proteins at the nodes of these networks interact with and operate on other proteins, nucleic acids, and small-molecule ligands. Thus, understanding protein function at the molecular level is a key goal in life sciences research. Structural biologists and biochemists pursue this goal by investigating the structures, dynamics, and interactions of proteins. The key technologies used include crystallography, nuclear magnetic resonance spectroscopy (NMR), electron paramagnetic resonance, cryo-electron microscopy, and small-angle scattering. NMR has the unique advantages that it allows to study proteins and protein interactions at atomic resolution, in solution, and that it is exquisitely sensitive to a wide range of protein motions. Such studies require the incorporation of NMR-active isotopes of nitrogen (^{15}N) and carbon (^{13}C), sometimes in combination with deuterium (^{2}H), to allow residue and atom-specific interpretation of the NMR spectrum.

Here, we review recent developments in isotopic labeling strategies in solution-state NMR, focusing on the study of macromolecular assemblies (Figure 2.1). The size of such complexes and/or the complexity of the protein of interest generally require different approaches from the conventional uniform [^{2}H,^{13}C,^{15}N]-labeling, briefly reviewed below. These strategies require restricted placement of isotopes in order to reduce the number of signals, usually in combination with deuteration of unwanted signals to enhance sensitivity. Here, we review recent progress in and highlight examples of selective labeling of methyl-groups, defined protein segments, or specific subunits. These strategies are applied separately or in combination to achieve high-quality spectra for demanding systems. Finally, we review ^{19}F fluorine labeling and isotopic labeling in cell-free systems, yeasts and insect cells that enable NMR studies of challenging eukaryotic proteins.

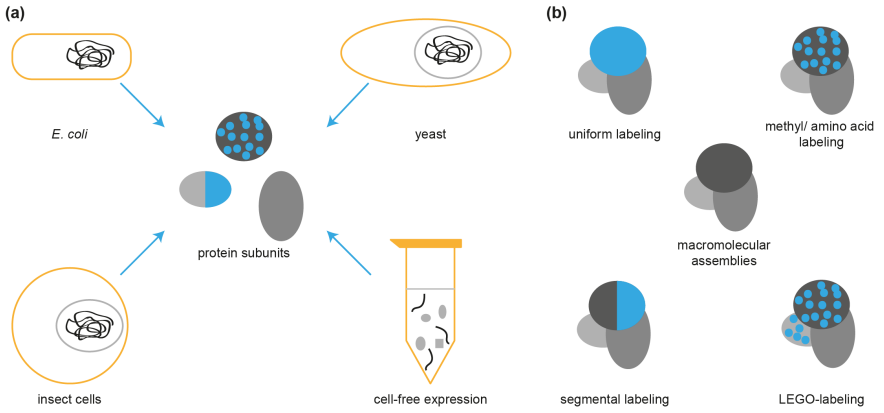

Figure 2.1. Overview of labeling strategies available for the study of macromolecular protein assemblies. Schematic overview of **(a)** different expression hosts available to produce isotope-label proteins with ^{2}H, ^{13}C and ^{15}N, and **(b)** different labeling schemes that can be applied. Blue proteins are NMR-active, isotope-labeled, gray proteins are unlabeled (or deuterated) and NMR-inactive. Expression in *E. coli* is compatible with all labeling methods, cell-free expression with uniform, methyl-selective, amino-acid selective and segmental labeling, yeast-based expression with uniform and methyl-selective labeling, insect-cell-based expression with uniform or amino-acid selective and methyl-selective labeling. Notably, reconstitution of the complex takes place *in vivo* after expression of subunits in LEGO-NMR labeling, whereas the other cases depicted in **(b)** require reconstitution *in vitro*.

Conventional uniform labelling

In the typical uniform labeling strategy, proteins are overexpressed by manual induction of a suitable T7-based *E. coli* strain [1], grown in M9 minimal medium supplemented with ^{13}C-labeled glucose and $^{15}NH_4Cl$ as the sole carbon and nitrogen sources, respectively. Proteins larger than 20-25 kDa are typically deuterated by using $^{2}H_2O$ (D_2O) in the cell growth medium instead of $^{1}H_2O$, optionally combined with the use of fully deuterated and ^{13}C-labeled glucose as the carbon source. Combining [^{2}H,^{13}C,^{15}N]-labeling and transverse relaxation optimized spectroscopy (TROSY [2]), allows structural and dynamical characterization of proteins in complexes in the 100 kDa size range, for recent examples see [3-4], up to 1 MDa [5-6]. Additionally, uniform labeling is valuable to study individual subunits in the ‘divide-and-

conquer' strategy. For single-chain proteins beyond 50 kDa, however, the sheer amount of signals complicates the spectra, and assignment becomes increasingly difficult.

In case of large assemblies, simple uniform labeling can be exploited to selectively observe highly flexible regions, as is nicely illustrated in a recent study on the nucleosome [7]. Its histone H3 subunit has a highly flexible N-terminal tail that is effectively decoupled from the slow overall molecular tumbling of the nucleosome (~220 kDa). Due to the large overall size, signals of the rigid part of uniformly [^{15}N,^{13}C]-labeled H3 are effectively broadened beyond detection, leaving a simplified spectrum of N-terminal tail. Using this approach, Stützer *et al*. were able to show that the H3 tail interacts with linker DNA and that this reduces the modifiability of the histone tail.

As an alternative to manual induction, auto-induction media have been developed offering overexpression in an unattended manner, better reproducibility, and higher levels of soluble protein expression [8]. Auto-induction media are composed of glucose, lactose and glycerol as carbon sources, triggering T7-based expression strains to be automatically induced by lactose after consuming all glucose present. For uniform ^{13}C or ^{2}H-labeling, such media are prohibitively expensive due to the need for labeled lactose. Recently, Guthertz and his colleagues showed that only the glucose moiety of lactose needs to be isotope-labeled, taking advantage of the inability of *E. coli* BL21 to metabolize the galactose moiety [9]. Specifically labeled lactose was synthesized from unlabeled galactose and ^{13}C or ^{2}H-labeled glucose, and used to produce uniformly ^{13}C or ^{2}H-labeled proteins.

Interestingly, O'Brien *et al*. proposed a novel method to produce deuterated proteins in H_2O medium [10]. The uniform ^{2}H, ^{15}N, ^{13}C-labeling is achieved by adding ^{2}H, ^{15}N, ^{13}C labeled nutrients prior to IPTG induction in the H_2O M9 medium where the unlabeled nutrients are exhausted. This approach was optimized to achieve 80% deuteration for ^{2}H, ^{15}N uniform labeling, however is less sufficient for triple ^{2}H, ^{15}N, ^{13}C labeling, Nevertheless, this approach provides a more cost-effective and feasible uniform as well as methyl-specific isotope labeling approach for NMR studies.

Methyl-TROSY labelling

The method of choice for the quantitative study of high molecular weight systems (> 100 kDa) is the specific labeling of methyl groups in a highly deuterated background [11-12]. Methyl groups are ideal candidates to be specifically isotopic labeled because they are abundant, found both in the core and on the surface of protein structures [13]; they carry three protons, and their symmetry and rapid rotation can be exploited to yield intense and well-resolved NMR signals [14-15]. Originally developed in the Kay lab for Ile-δ1, Leu, Val methyl groups, this labeling strategy requires perdeuterated proteins, into which specific [^{1}H,^{13}C]-labeled methyl groups are introduced using deuterated amino acids precursors that only [^{1}H,^{13}C]-labeled on the methyl group of interest [16-17]. Methyl-labeling has since been extended to Ile-γ2, Ala, Met, Thr methyl groups [18-22] and is thoroughly reviewed in [23].

Developments during the last 5-6 years have focused on reducing overlap and increasing sensitivity of methyl-TROSY spectra by independently labeling Leu and Val methyl groups, and extending this capability to the stereo-specific labeling of these prochiral methyl groups [24]. In the original protocol, these methyl groups cannot be separated as they originate from a common precursor. Lichtenecker *et al*. developed protocols to selectively label Val or Leu methyl groups using custom synthesized Leu precursors [25-26]. Selective and stereo-specific labeling of Val methyl groups was achieved by Mas *et al*. using specifically labeled 2-acetolactate as Leu/Val precursor together with addition of perdeuterated Leu in the culture medium to prevent conversion of the precursor to labeled Leu [27]. In a third approach, the culture medium is supplemented with custom synthesized stereo-specifically labeled Leu and Val amino acids rather than their precursors [28]. With this approach fully independent labeling of either pro-*R* or pro-*S* methyl group of either Leu or Val, e.g. pro-*R* Leu-δ1 with pro-*S* Val-γ2, is possible by proper choice of amino acid supplement. The Boisbouvier lab recently developed a protocol where Ala-β, Ile-δ1, Leu-pro*S* and Val-pro*S* are simultaneously methyl labeled, relying on a custom synthesized Ile-precursor to avoid co-incorporation incompatibility and isotopic scrambling [29]. They demonstrate the suitability of this scheme to measure methyl-methyl

distances for structural studies of high molecular weight systems. A different approach was developed by Miyanoiri et al. using auxotrophic *E.coli* strain of which biosynthesis pathways of Ile, Leu and Val are blocked to achieve stereo-specific labeling of $^{13}CH_3$-Ile, -Val, and -Leu without any amino acid scrambling [30].
Survey of recent literature shows many great examples of how this labeling strategy can generate exciting insights in the structure-dynamics-function relationship of protein-protein, protein-DNA, and protein-small molecule complexes involved in protein folding [31-33], regulation of protein expression [34-38], protein signal-transduction [39-40] and protein secretion [41-43]. We highlight here the work from the Kalodimos lab on the interaction of the 50 kDa trigger factor (TF) chaperone with a 48 kDa unfolded substrate, alkaline phosphatase (PhoA) [44]. Taking advantage of the modular nature of the PhoA-TF complex, Saio *et al.* were able to show that three TF molecules are required to interact with the entire length of PhoA, resulting in a ~200 kDa complex in solution. Using methyl-group labeled samples as the cornerstone in their NMR data collection and analysis, high-resolution NOE-based structures were determined for each TF bound to a PhoA segment. The resulting structures show how the same substrate-binding region in the chaperone engages different hydrophobic stretches of the unfolded PhoA.

Segmental labelling

Isotope-labeling of selected segments of a protein can greatly reduce the complexity of NMR spectra. Labeled and unlabeled protein segments are produced separately, and then fused via a thioester-intermediate to ultimately form a native peptide bond (Figure 2.2a,b). Rooted in native chemical ligation where both parts are produced synthetically [45], recombinant protein segments are fused using either inteins [46-49] or sortase [50]. Both methods require a judicious choice of the ligation point, typically in a domain-connecting loop.
Inspired by protein splicing, the intein-based approaches rely on the use of internal protein domains (inteins) that can excise themselves from a protein in a traceless manner. In expressed protein ligation (EPL [51-52]), the required thioester intermediate is formed after expression of the N-terminal protein fused to an intein, allowing subsequent ligation

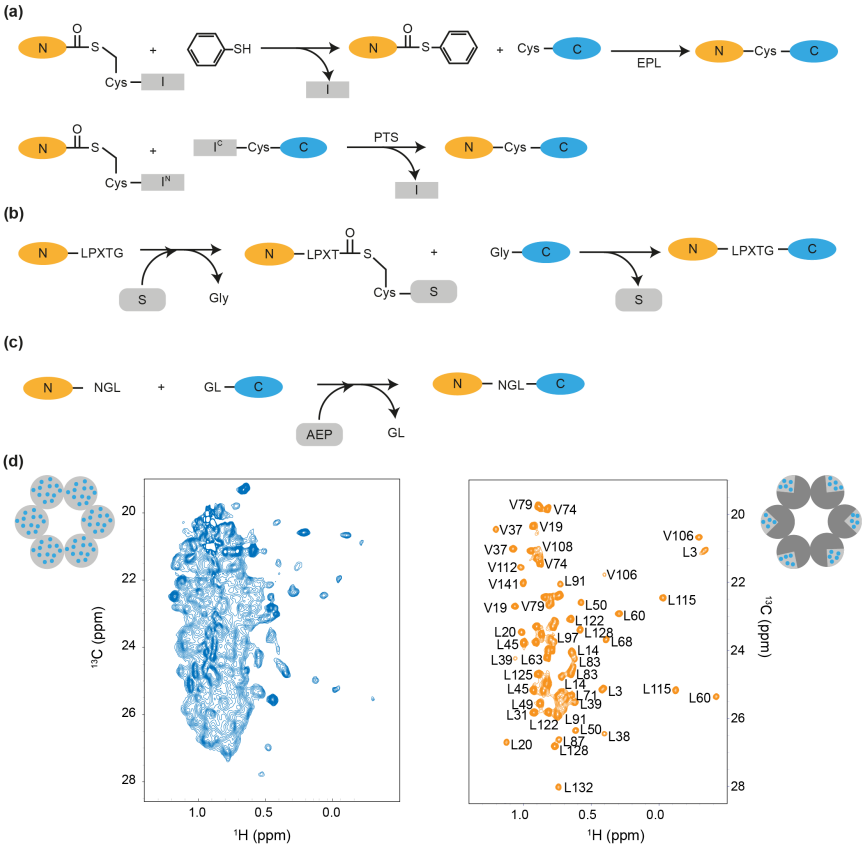

Figure 2.2. General scheme and example of segmental isotope-labeling. Schematic overview of (a) intein-based and (b) sortase based protein ligation. (a) In expressed protein ligation (EPL, top), expression of the N-terminal protein (*N*) fused to an intein (*I*), typically Mxe GyrA, results in formation of a thioester due to a N to S-acyl shift. Via an *in vitro* transthioesterification reaction, a highly reactive thioester is formed that is attacked by the N-terminal nucleophile, typically the thiol of a cysteine, to result in a thioester of N and C-terminal fragments (*C*). After another N to S-acyl shift, a native peptide bond between the two segments is formed. In protein trans-splicing (PTS, bottom), the intein, typically based on Ssp or Npu, is split in two halves, each fused to either N- or C-terminal protein segment. The affinity between the split inteins drives reassembly of the full, active intein, which subsequently excises itself, ligating the two external sequences. (b) Sortase (*S*) cleaves the C-terminal Gly of the LPXTG-motif, forming a thioester with the N-terminal protein fragment. Here, the nucleophile is the N-terminal Gly of the C-terminal protein. Attack of this Gly on the thioester results in ligation of the two protein fragments, restoring the LPXTG motif. (c) asparaginyl endopeptidases (AEP) catalyzed protein ligation with the reported recognition sequence. (d) Recent work of Rosenzweig *et al.* [64] on the 580

kDa hexameric ClpB chaperone illustrates the dramatic improvement in spectral quality in a segmental methyl-selective labeled complex (right) over the uniformly methyl-selective labeled complex (left). Color coding of the assembly cartoon as in Figure 2.1. Figure adapted from [64] with permission from the authors.

with the C-terminal part (Figure 2.2a). In protein trans-splicing (PTS), both parts of the protein are fused to a split intein, and expressed either separately, or sequentially from different promoters, to allow differential labeling [48, 53]. The split intein-fusions are reassembled *in vitro* or *in vivo* to an active intein that excises itself, resulting in a native, fused target protein [53] (Figure 2.2a). Notably, intein activity in PTS may depend critically on the protein context and unwanted "cross-labeling" may occur when splicing is carried out *in vivo* [54].

Development in intein-based segmental protein production has focused mainly on the identification of better split inteins for PTS [55-57]. Recently, a highly active and extremely stable split intein was designed promising higher yields and increased robustness in PTS [58]. In addition, generic gene insert was designed containing a split intein, termed PTS cassette, to screen split intein insertion sites for any target proteins under the control of T7 promoter [59].

An attractive alternative to intein-based segmental labeling is the *in vitro* ligation approach based on the transpeptidase Sortase A (SrtA) [50], in which protein segments are produced with or without isotopic labeling, purified separately and ligated *in vitro*, without risking cross-labeling contamination. The sortase enzyme recognizes an LPXTG motif on the N-terminal segment and catalyzes the formation of a new peptide bond with the C-terminal part (Figure 2.2b). To highlight, Bobby lab used this powerful method to study ligand-bromodomain interaction at high resolution by strategically labelling on the C-terminal bromodomain whereas the N-terminal bromodomain remained unlabeled [60]. Recently, the Sattler lab developed a modified ligation protocol, addressing the reversibility of sortase reaction [61]. Using a centrifugal concentrator to continuously remove the cleaved glycine and a clever combination of cleavable and non-cleavable purification-tags, ligation efficiency for tested proteins (a 32 kDa dual RRM-domain protein and the 57 kDa Hsp90 chaperone) was improved up to two-fold.

While EPL, PTS, and SrtA methods have been successfully applied for segmental isotope labeling of multidomain proteins, it is more

challenging to apply to single domain globular proteins. This is because the split fragments of globular proteins are usually insoluble, which requires extra refolding steps. To solve this, a new approach using asparaginyl endopeptidases (AEP) was proposed [62-63] (Figure 2.2c). Compared to sortase, AEP recognizes a shorter motif of NGL on the N-terminal segment of the protein and leaves a shorter ligation tag in the catalyzed protein ligation, which is less likely to disturb the solubility of the split fragments. In the demonstrated case of MAP, the two fragments were folded and purified before ligated by AEP *in vitro*. This new strategy, in combination with PTS, provides new possibilities for production of more complex protein conjugates with various biophysical probes.

Recent work from Rosenzweig *et al*. on the substrate recognition of the 580-kDa hexameric ClpB chaperone demonstrates the dramatic spectral improvement segmental labeling can offer [64] (Figure 2.2d). The N-terminal domain (NTD, 16 kDa) of the ClpB monomer (97 kDa) was expressed as an intein-fusion, with methyl-group specific isotope-labeling, whereas the remainder of ClpB was fully deuterated. The ligated, segmentally labeled ClpB monomer was subsequently reassembled into its functional hexameric form. The resulting high-quality methyl-TROSY spectra were used to determine microscopic binding affinities of a client protein to two separate sites on ClpB. Together with biochemical assays, these results established the NTD as a protein aggregate sensor that binds client protein before they are shuttled though the ClpB active channel for unfolding.

LEGO-NMR subunit labelling

Protein complexes are typically reconstituted *in vitro*, permitting the selective labeling of one or more subunits. This approach may fail for complexes for which the individual subunits have poor solubility. The LEGO-NMR strategy was recently introduced to overcome this problem [65]. In a method akin to *in vivo* PTS, all subunits are co-expressed in a single *E. coli* cell from two plasmids, one inducible by arabinose with glycerol as carbon source, and the other by IPTG with glucose as carbon source. This setup permits the selective labeling of a subset of subunits and the *in vivo* assembly of labeled and unlabeled subunits into a functional complex. Mund *et al.* demonstrated this

technique to label, express and generate oligomers (LEGO) on a ~75 kDa complex, comprised of 7 subunits, which was selectively [^{2}H,^{15}N]-labeled on three or single subunits, allowing precise mapping of an RNA binding site. Furthermore, compatibility with selective methyl-labeling was neatly demonstrated by preparation of a complex with selective methyl-labeling of Met in 3 subunits and of Ile-δ1 in the remaining 4 subunits.

Fluorine-labelling

As an alternative to ^{1}H/^{15}N/^{13}C isotope-labeling, incorporation of ^{19}F isotopes can offer a highly sensitive probe of conformational changes, dynamics and interactions because of its high abundance, gyromagnetic ratio and chemical shift range (for a recent review see [66]). Uniform labeling with fluorinated amino acids analogs is achieved using bacterial strains auxotrophic for the substituted amino acid, or using the amber-codon approach to achieve site-specific labeling. Alternatively, fluorinated tags, such as 3-bromo-1,1,1-trifluoroacetone, are attached to cysteine-thiol groups or other labile groups. Recently, chemical shift sensitivity of CF_3 tags has been compared to optimize resolution [67]. CF_3 tags with distinct chemical shifts were also used for differential ^{19}F labeling of proteins to study individual behavior of each protein in their mixtures [68]. Combination of paramagnetic and ^{19}F labeling was recently demonstrated to obtain precise long-range distance measurements [69]. Furthermore, enzymatic ^{19}F labeling of glutamine side chain carboxamide group by transglutaminase was developed to study the drug-protein and protein-protein interactions, as demonstrated on the complexes of about 100 kDa [70]. The advantages of ^{19}F labeling are nicely illustrated in recent studies where the chemical shift sensitivity of ^{19}F was exploited to identify different conformational states of GPCRs [71-72] and substrate-arrestin complexes [73].

Isotope-labelling in yeast and insect cells

Expression in *E. coli* is widely used due to its high-level of protein production and cheap growth media. It may fail, however, to produce functional recombinant proteins, especially in case they require

eukaryotic folding machineries, glycosylation or other post-translational modifications. Cells from higher organisms, most commonly yeasts and baculovirus infected insect cells [74] are necessarily used as expression systems to isotope-label these proteins, permitting NMR studies of otherwise intractable protein assemblies.

Expression in yeast is attractive because of the low-cost minimal growth medium and relatively high protein expression yields. Recently, selective [^{1}H,^{13}C]-labeling of Ile-δ1 methyl groups in perdeuterated proteins has been described in glucose-controlled *Kluyveromyces lactis* [75] and methanol-controlled *Pichia pastoris* [75-76]. The 42 kDa maltose-binding protein was perdeuterated to high levels (≥90%) with Ile-δ1 labeling efficiency of 45% and 67% for *P. pastoris* and *K. lactis*, respectively. For both systems, methyl-selective Leu/Val labeling was <5%, although significant improvement is possible through co-expression of metabolic enzymes or labeled Leu/Val supplementation [75].

Isotope-labeling in insect cells requires the use of labeled amino acids as medium-supplement. The associated high costs are raised even further for large proteins requiring deuterated amino acids. Recently, protocols for cheaper media have been proposed based on custom-made isotope-labeled yeast extracts, demonstrating the feasibility of uniform ^{15}N-labeling [77], and uniform [^{2}H,^{13}C,^{15}N]-labeling [78]. Opitz *et al.* achieved >80% ^{13}C/^{15}N incorporation and ~60% deuteration, producing samples suitable for triple resonance experiments and detailed structural analysis [78]. Sitarska and colleagues optimized a protocol based on commercially available isotope-labeled algae extracts, resulting in triple-labeled proteins with similar efficiency and costs compared to the yeast-based method [79].

Here, we highlight recent studies of solubilized membrane proteins that are expressed and isotope-labeled on specific amino acids in insect cells [80-85]. Nygaard *et al.* used specific ^{13}C-labeling of Met methyl groups to study the conformational heterogeneity of a detergent-GPCR complex in diverse ligand-bound states [80]. In a subsequent study, the GPCR was embedded in lipid bilayer nanodiscs and deuterated up to 90% using a combination of ^{2}H-labeled algae extracts and ^{2}H-amino acids [81]. Recently, the Grzsiek lab studied the β1-adrenergic receptor GPCR as a 100 kDa detergent-GPCR complex using specific ^{15}N-labeling of Val residues, resulting in highly quality TROSY spectra

where 21 out of 28 possible Val probes could be resolved and assigned [82]. Ligand binding caused chemical shift changes at the opposite end of the GPCR, which correlated linearly to the G-protein activation efficiency of each ligand, demonstrating an allosteric coupling between the extracellular ligand binding site and the intracellular G-protein binding site.

Cell-free isotope-labelling

The exemplification of cell-free based isotope-labeling is stereo-array isotope-labeling (SAIL) where a cocktail of specifically [^{2}H,^{13}C,^{15}N]-labeled amino acids is used to produce proteins with optimal NMR properties [86]. The SAIL method takes full advantage of the lack of isotope scrambling in cell-free protein synthesis and the smaller amounts of amino acid supplementation required, compared to *in vivo* expression. Other advantages of cell-free expression are that it offers possibility to express toxic proteins, to improve protein production by adjusting the cell-extract with various factors [87], and to produce solubilized membrane-proteins without co-purification of endogenous lipids [88].

Recently, three new strategies have been put forward to optimize labeling of large proteins in cell-free expression. First, combination of cell-free expression with segmental labeling was proposed to generate multi-domain proteins with a specific pattern of amino acid labeling restricted to each domain [89]. This was demonstrated on a two-domain protein, where a ^{15}N-Lys labeled intein-fusion was ligated using EPL to a [^{13}C,^{15}N]-Lys labeled domain. Second, the high cost of selective methyl-group labeling has been reduced greatly, making use of hydrolyzed methyl-labeled inclusion bodies derived from *E. coli* to replace commercial labeled amino acids [90]. This approach was illustrated on an Ile-δ1, Val/Leu-pro*S* methyl-labeled eukaryotic membrane protein, toxic to *E. coli*. While this second method still relies partially on the cellular expression, the most recent strategy uses additional branched chain aminotransferase IlveE to directly convert precursors into L-Val and L-Leu (and potentially L-Ile as well) for the synthesis of the target protein in cell-free system [91].

Conclusion

Here, we highlighted the increasing range of options regarding expression system and labeling strategy that is available for solution NMR studies of protein complexes. The availability of affordable deuteration and methyl-labeling protocols for non-*E. coli* based expression, as well as the LEGO-NMR approach, widen the application window to otherwise intractable systems. Control over the restricted placement of isotopes offers an extremely valuable degree of flexibility, in particular when both backbone and methyl-TROSY spectra are of good quality. The 'best' labeling strategy remains case-dependent: the size and behavior of complex and its subunits, the question at hand, and the spectral quality required versus costs and time affordable will dictate the strategy chosen. We anticipate that especially the combination of labeling strategies, such as segmental methyl-labeling, will prove extraordinarily powerful in the dissection of the inner workings of Nature's molecular machines.

Acknowledgements

We thank all members of the MacBio group for stimulating discussions. This work was supported by a VIDI grant from the Dutch Science Foundation NWO to HvI (NWO-CW VIDI 723.013.010).

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