Current Topics

Protein Nuclear Magnetic Resonance under Physiological Conditions[†]

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ABSTRACT: Almost everything we know about protein biophysics comes from studies on purified proteins in dilute solution. Most proteins, however, operate inside cells where the concentration of macromolecules can be >300 mg/mL. Although reductionism-based approaches have served protein science well for more than a century, biochemists now have the tools to study proteins under these more physiologically relevant conditions. We review a part of this burgeoning postreductionist landscape by focusing on high-resolution protein nuclear magnetic resonance (NMR) spectroscopy, the only method that provides atomic-level information over an entire protein under the crowded conditions found in cells.

The inside of a living cell is one of the ultimate physiologically relevant environments, but this environment is difficult to define. The cytoplasm comprises all the material in the cell, excluding the plasma membrane and the nucleus (if present). The cytosol is the cytoplasm without organelles and other subcellular structures, such as ribosomes and the fibrous proteins that determine cell shape, motility, and material transport. However, the extent to which these structures need to be considered in physiologically relevant nuclear magnetic resonance (NMR) studies remains unclear. Furthermore, it is difficult to state precisely the conditions in the cytosol. The temperature for optimal growth is often considered the physiologically relevant temperature, 37 °C for *Escherichia coli* and animal cells and 30 °C for yeast, although cells can survive at least for limited periods above or below these temperatures. As a general rule, the pH of the cytosol is 7.2, but it depends on extracellular conditions. The pH in *E. coli* cells has been monitored with NMR and covers a range from pH 6 to 8 (*1*). This range of values raises a key point: conditions inside the cell depend on the conditions outside the cell to a certain extent. Studies in *E. coli* show that the composition of its cytosol can be manipulated over a wide range of ionic strengths, water concentrations, and solute concentrations (*2*). Cells can compensate for changes in conditions, including protein overexpression, through a variety of means, from simply adjusting the concentration of small hydrophilic solutes called osmolytes (*3*) to spatially organizing entire biosynthetic pathways (*4*). Finally, even the exact state of the protein under study may be difficult to define in time and space because of trafficking and posttranslational modification.

Independent of these details, there is so much yet to learn that even semireductionist approaches involving in vitro studies of "artificial cytosol" will be important for understanding proteins under physiological conditions.

If the composition and condition of the cytosol are moving targets, the stationary target that captures the biggest difference between the conditions used in most in vitro studies and physiologically relevant conditions is volume exclusion. That is, much of the volume of a cell is inaccessible (i.e., excluded) to the protein being studied. Water comprises only 50−70% of a cell's volume, depending on extracellular conditions (*2*). Macromolecules account for at least 30% of the volume, and their total concentration can reach more than 300 g/L (*5*). To put these numbers in perspective, an egg white is only 100 g/L protein.

Fluorescence spectroscopy and NMR spectroscopy are two of the few biophysical techniques that allow the observation of a particular protein in the sea of proteins and other macromolecules in cells. Some fluorescence-based approaches involve attaching green fluorescent protein (GFP)[1] to the protein of interest (*6*). Other approaches use fluorescent dyes that target amino acid motifs engineered into the protein of interest (*6*). Unfortunately, the former approach adds 27 kDa of bulk to the target protein, and both provide information only about the environment of the fluorophore, which occurs only once in the target protein. Here, we focus on

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[1] Abbreviations: BSA, bovine serum albumin; CI2, chymotrypsin inhibitor 2; CK2, casein kinase; GB1, B1 domain of streptococcal protein G; HSQC, heteronuclear single-quantum coherence; GFP, green fluorescent protein; PVP, poly(vinylpyrrolidone).

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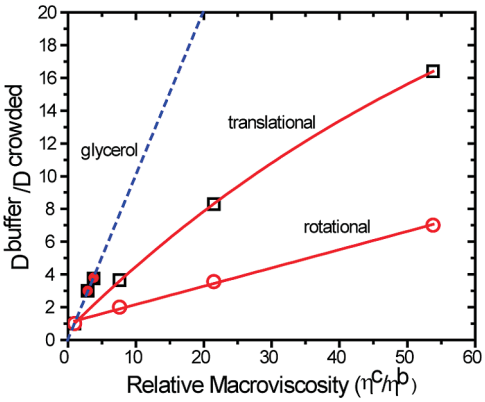

FIGURE 1: Effects of large- and small-molecule crowders on the diffusion of CI2. The ratio of the diffusion coefficient in buffer to that in the cosolute is plotted for rotational and translational diffusion as a function of the relative macroscopic viscosity. The translational diffusion was assessed by using the X-STE (*92*) pulse sequence, and rotational diffusion was assessed from the ^{15}N T_1/T_2 ratio (*93*). The experiments were performed on a 600 MHz spectrometer at 298 K in acetate buffer (pH 5.4).

NMR spectroscopy, which has the potential to give-atomic level information across the entire protein molecule.

NMR Parameters and Crowding. For the basic concepts of protein NMR, we refer the reader to the recent text by Rule and Hitchens (*7*). Briefly, the ability to detect a protein by high-resolution NMR depends on its rotational dynamics. These dynamics are reflected in the relaxation times, T_1 (longitudinal) and T_2 (transverse), for each observed nucleus. If T_1 is too long, the nuclei do not relax between pulses, lowering the sensitivity of the experiment. If T_2 is too short, the resonances are too broad to observe. The time scale of rotational diffusion is captured in a parameter called the rotational correlation time.

Increasing the molecular mass of a protein or the viscosity of the sample increases a protein's correlation time. Larger correlation times are reflected in longer T_1 values and shorter T_2 values, both of which are detrimental to data acquisition. Consequently, NMR studies of globular proteins under physiological conditions are currently limited to small proteins that tumble freely. There are, however, notes of optimism. The detectable size range is larger for intrinsically disordered proteins (*8*); larger protein complexes can be studied by enriching just one of the partners (*9*), and the development of TROSY (*10*) has expanded the usable range of molecular masses in dilute solution to include proteins up to 100 kDa.

Diffusion. The cellular contents impede protein diffusion (*11*), which affects attempts to obtain high-resolution NMR data. The theory relating crowding to NMR studies of protein diffusion has been reviewed by Bernado et al. (*12*). To illustrate how crowding alters diffusion, we used NMR to quantify the diffusion of chymotrypsin inhibitor 2 (CI2, a 7.4 kDa globular protein) in two cosolutes: a macromolecular crowder, 40 kDa poly(vinylpyrrolidone) (PVP), and a small molecule, glycerol. The results are shown in Figure 1. In glycerol, both rotational and translational diffusion follow the Stokes−Einstein relationship, which predicts a linear response. Macromolecular crowding leads to a different result. In PVP, both translational and rotational diffusion are less affected than they are in glycerol. The change in behavior occurs because the size of crowding molecules approaches the size of the test protein. Most importantly for NMR studies under physiological conditions, rotational diffusion is less affected.

There are few NMR-based studies of protein diffusion in cells. Using ^{19}F NMR to quantify rotational diffusion, Williams et al. (*13*) showed that the cytoplasm of yeast halves the diffusion coefficient of two glycolytic enzymes compared to dilute solution (a third glycolytic enzyme could not be observed, probably because it is immobile). All other in-cell diffusion data from eukaryotic cells (fibroblasts, epithelial cells, and erythrocytes) are based on fluorescence. These studies used a variety of probes, including small molecules (*14, 15*), latex beads (*16*), dextrans (*17*), Ficolls (*17, 18*), and GFP (*19*). Most of this work focused on translational motion, but one study examined rotational dynamics (*19*). All these data are consistent with the results from the ^{19}F study; the eukaryotic cytoplasm decreases diffusion coefficients 1.5−3.8-fold, compared to dilute solution.

Studies of protein diffusion in the bacterium *E. coli* give a remarkably different picture. These data are all based on using fluorescence to measure the translational diffusion of GFP, either by itself or fused to other proteins. Whereas the eukaryotic cytoplasm slows diffusion no more than 4-fold, the cytoplasm of *E. coli* slows diffusion 10−30-fold (*20*−*22*). As suggested by the data in Figure 1, even a 30-fold decrease in translational diffusion should have only a small effect on rotational diffusion, the most important property for NMR. Nevertheless, these data suggest that the cytoplasm of *E. coli* is much more viscous than that of eukaryotes. All the *E. coli* data, however, have a common element, GFP. Without information on other proteins, it remains unclear whether the cytoplasm of *E. coli* is more viscous or GFP interacts strongly with more immobile components.

Equilibrium. Macromolecular crowding agents excludes otherwise accessible volume from the protein being crowded. As a result, proteins should favor compact states. Crowding-induced compaction has repercussions for a number of equilibrium properties, including stability, ligand binding, and protein−protein interactions. The mathematical basis of crowding effects has been examined extensively (*23*). Here we focus on predictions arising from these examinations.

The native state of a globular protein is structurally defined and highly compact (*24*). The denatured state, on the other hand, is a thermodynamic state, defined by a broad ensemble of mostly expanded structures (*25, 26*). For a stable protein, the native state is favored over the denatured state. Due to the compact nature of the native state, crowding agents should further destabilize the denatured state, leading to an increase in stability for the native state. Predictions of the magnitude vary (*27, 28*), but the crowding effect could increase globular protein stability by several kilocalories per mole.

For aggregation and binding, additional complications arise. There are three principal types of protein aggregates: folded, misfolded, and unfolded. In all instances, the final aggregated state is highly compact and should be favored by crowding. For folded aggregates, the protein goes directly from the native state to the aggregated state, without intermediates. For misfolded or unfolded aggregates, the protein must unfold prior to aggregation. Crowding is expected to decrease the stability of these less compact forms

relative to the native state, which might attenuate aggregation (*29, 30*).

Crowding can alter not only structure-based properties like stability and aggregation but also function-based properties like ligand binding and catalysis. There are two major equilibrium states in protein binding: the free state and the bound state. In general, the ligand−protein complex will be more compact than the sum of its individual components. This compaction is mainly due to the binding-induced decrease in the ligand's solvent accessibility. Thus, the addition of crowding agents may not affect the ligand when it is bound to the protein but can bias the system toward bound states (*23*). Macromolecular crowding has also been shown to affect catalytic efficiency (*31, 32*).

IN VITRO NMR

Here, we examine the most reductionist of experiments: studies performed with purified proteins in the presence of macromolecular crowding agents.

Artificial Cytosol. Even though it is now possible to obtain high-resolution NMR data from proteins in living cells (vide infra), there remains room for reductionism. These experiments typically employ a system comprising three components: a buffer, a macromolecular crowding agent, and the protein being tested. Compared to in-cell experiments, this type of system allows tight, independent control of pH, concentration, viscosity, crowding agent size, and types of interaction.

The physical properties of the crowding agent are critical. To obtain relevant data, a crowding agent must fulfill a number of requirements. It must be water-soluble up to several hundred grams per liter. From a practical point of view, price and purity may also be constraints because a large amount of the agent will be required. The presence of the crowder must not interfere with protein detection. For synthetic polymers, the polydispersity of the crowder will need to be considered when comparing theory to experiment.

Most importantly, the crowding agent should be chemically inert with respect to the test protein. Without this property, the effects attributed to excluded volume may really arise from interactions between the crowding molecule and the protein or interactions of the protein with itself. These interactions, even if they are weak, will affect T_1 and T_2. As mentioned above, the increased viscosity of crowded samples also affects T_1 and T_2. The problem is separating the effects of increased viscosity from those of binding. One idea is to focus on the product of $1/T_1$ and $1/T_2$ because this product becomes insensitive to viscosity but remains sensitive to intermolecular interactions (*33*).

Several macromolecular crowding agents, including Ficoll, poly(ethylene glycol) (PEG), poly(vinylpyrrolidone) (PVP), and proteins themselves, have been used and provide a pool of potential candidates for NMR studies (*34*). Here, we concentrate on crowders that have been used in NMR experiments.

PEG is a highly water-soluble polymer available in multiple molecular weights. In NMR studies, PEG has been shown to broaden certain ^{1}H and ^{15}N resonances of proteins such as cytochrome *c*, but the proteins are still detectable (*35*). Unfortunately, these experiments also suggest that PEG interacts with cytochrome *c*. These site-specific changes may make it difficult to isolate macromolecular crowding effects from other types of interactions. PEG has also been used in an NMR study to assess changes in the rates of folding and unfolding for apocytochrome b_{562} (*36*). As predicted, PEG-induced crowding increases the folding rate.

An alternative, PVP, is also highly water-soluble and available in multiple molecular weights. PVP has been used to determine the stability of the globular protein CI2 under crowded conditions (*37*). The results from NMR-detected hydrogen exchange experiments (*38*) showed that residues in regions of the protein that exchange only during complete unfolding were stabilized by more than 3 kcal/mol in the presence of 300 g/L 40 kDa PVP. The value agrees with predictions that macromolecular crowding should have a significant effect on globular protein stability (*27*).

A slightly less reductionist approach involves the use of natural proteins as crowding agents. Globular proteins such as bovine serum albumin (BSA) and ovalbumin have been used (*39*−*41*). The results from these studies are discussed in the section on intrinsically disordered proteins.

Lysates and Extracts. The next logical step is to use mixtures of proteins obtained from cells. Cell disruption yields a mixture of soluble and insoluble macromolecules, and these ex vivo conditions are used to mimic the cytoplasm of intact cells (*42*). The term lysate usually refers to ruptured cells, while the term extract usually refers to centrifuged (cleared) lysates, although the two terms are sometimes used interchangeably. Lysates and extracts approximate the cell cytoplasm and cytosol, respectively.

Lysates and extracts offer some advantages compared to in vitro and in-cell NMR preparations. As discussed below, in-cell protein spectra can be difficult to obtain due to resonance broadening. The cell membrane and cytoskeletal components, however, are disrupted in lysates and extracts. Thus, these mixtures may enable the observation of proteins by NMR that are otherwise not detected in cells because they bind the intact structures (*13, 43*). Additionally, the homogeneity of lysates and extracts improves the quality of NMR spectra (*41*).

Lysates and extracts prepared from cells expressing the isotopically enriched protein of interest offer a convenient way to acquire NMR spectra without needing to purify the protein (*44*−*46*). Proteins can be screened to assess their suitability for more detailed NMR studies (*47*). Lysates and extracts allow the selective addition and removal of proteins and small molecules to alter biological processes and can be used to study cellular processes because many biological reactions in cells also occur in these mixtures (e.g., ref *48*).

Protein NMR spectra of cell lysates and extracts from bacteria (*43*−*47, 49*), yeast (*13*), and *Xenopus laevis* oocytes and eggs (*41, 48, 50*) have been published. The results mainly agree with NMR spectra obtained in living cells, indicating the potential of these mixtures to complement in-cell NMR.

Lysates and extracts, however, have limitations. Isotopic enrichment of small background molecules and other components can make conclusive assignment of resonances difficult (*46*). Thus, for high-resolution structure determination, in vitro NMR of purified protein may be preferred. Furthermore, proteins that function in enzyme complexes may be disrupted in lysates or extracts. Cell lysis also disrupts the proteolytic machinery in cells, which may lead to proteolysis of the test protein.

IN-CELL NMR

The ultimate postreductionist approach is to use NMR to examine proteins inside living cells. First, we discuss the history and generalities of in-cell NMR, including some caveats. Next, we focus on studies in *E. coli* and then on nucleated higher eukaryotic cells.

Isotopic Enrichment and Labeling. Living cells have been used for protein NMR experiments for several decades. One of the earliest examples involved assigning the peaks corresponding to the protein chromogranin A in a proton spectrum of adrenal medulla (*51*). Most experiments, however, focused on small metabolites rather than proteins. To differentiate between in-cell studies of protein and metabolites, protein detection is commonly termed in-cell NMR, while small molecule detection is often called in vivo NMR.

The first generation of experiments focused on proteins that are naturally present at high levels. For example, the proton resonances from the surface histidines of hemoglobin were used to monitor the internal pH in human erythrocytes (*52*), which lack nuclei and contain large amounts of hemoglobin. NMR detection of a protein in more typical cells is difficult for two reasons; the protein of interest must be selectively enriched or labeled with NMR active nuclei to distinguish it from other proteins, and it must be expressed at sufficiently high levels to be detected.

In one of the earliest examples of labeling, Brindle et al. used fluorinated amino acids to incorporate ^{19}F into 3-phosphoglycerate kinase in the yeast *Saccharomyces cerevisiae* (*53*). In a series of seminal publications, Brindle and colleagues went on to investigate ligand binding, protein mobility, and protein−protein interactions (*13, 54, 55*). Although the large chemical shift anisotropy of ^{19}F makes this nucleus sensitive to changes in chemical environment, it also leads to efficient relaxation, which broadens resonances.

A more complete technique for in-cell NMR was pioneered by the Dötsch group (*56*). *E. coli* cells are grown in rich media and then transferred to media containing ^{15}N as the sole nitrogen source just before induction, so that backbone and side chain amide nitrogens are uniformly enriched in ^{15}N. The protein of interest is expressed at high levels relative to other cellular components and therefore is enriched with this NMR-active isotope. Using this technique, they reported the first high-resolution heteronuclear single-quantum coherence (HSQC) protein spectrum in *E. coli* cells overexpressing the N-terminal domain of bacterial mercury-detoxification protein (*57*).

Uniform ^{13}C enrichment can provide advantages over uniform ^{15}N enrichment. Most importantly, ^{13}C is 2.5 times more detectable than ^{15}N (*7*). Due to their fast internal rotation, methyl groups have longer T_2 values. Furthermore, methyl groups contain three protons compared to the single proton in an amide. Unlike amide protons, the protons in methyl groups do not exchange with water. The fast internal motion of methyl groups also allows detection of proteins or protein complexes that may be too large to observe by ^{15}N in-cell NMR (*58*). One of the biggest disadvantages of ^{13}C is its narrower chemical shift range compared to that of ^{15}N. Another potential disadvantage is the high abundance of carbon in small molecules (i.e., metabolites). The spectra often exhibit high backgrounds, which complicate assignment. Selective labeling can overcome these obstacles.

Developments have been made to improve ^{19}F protocols as well (*59*). Fluorine NMR has several advantages over NMR with other labels. First, the sensitivity of ^{19}F in NMR experiments is greater than that of ^{15}N and ^{13}C. Second, the protein spectra are simple since only one, or a few, labeled amino acids are used.

Mehl and co-workers (*60*) have developed a site-specific technique in which a codon in the gene of interest is mutated to an amber stop codon, TAG. The *E. coli* host is then cotransformed with another plasmid, which encodes the orthogonal RNA synthetase needed to incorporate a fluorinated unnatural amino acid at the inserted amber stop codon. Also, since fluorine is not native to the cell, only the protein of interest will contain ^{19}F, minimizing background metabolite signals.

Caveats. Even in-cell protein NMR lacks absolute physiological relevance, at least as currently practiced. Here, we discuss some shortcomings. Until they are overcome, we suggest that it is best to use the term "in-cell" rather than "in vivo" protein NMR.

NMR is insensitive. Collecting data for longer times and increasing the density of cells in the sample both increase the sensitivity. Both options, however, will most likely decrease cell survival by depriving cells of necessary oxygen and nutrients. Cell death and shifts in intracellular pH have been observed in longer NMR experiments (*8*).

Sensitivity can also be increased by using a cold probe (*61*). Even though high ionic strengths can degrade cold probe performance (*62*), and the ionic strength inside cells is hundreds of millimolar (*3*), we and others find that in-cell experiments often benefit from the application of this technology.

Even with a cold probe, the acquisition of high-quality spectra on the time scale of minutes to tens of hours requires protein concentrations of at least tens of micromolar and, more generally, hundreds of micromolar. This concentration range should be compared to the concentration of the most abundant natural protein in *E. coli*, EfTu, approximately 1 μM (*63*), and the concentration of ubiquitin in HeLa cells, approximately 10 μM (*64*). Others suggest that the protein of interest must represent at least 1−2% of the total soluble protein or be present at an intracellular concentration of ~150 μM for ^{15}N enrichment or ~50 μM for ^{13}C enrichment (*58*).

Thus, the protein to be studied by NMR must be overexpressed, or an unnaturally large amount of the isotopically enriched or labeled protein must be introduced by other means. Even though overexpression does not appear to have a large effect on the total protein concentration in *E. coli* (*39*), problems may arise because of the high "monolithic" concentration of the protein under study. For instance, when the goal is to study protein−protein interactions but only one component is overexpressed, only the free protein will be detected. Coordinating the expression of the components can avert this difficulty (*9*).

Another problem arises when the overexpressed protein binds (specifically or nonspecifically) to large macromolecular components and the number of binding sites is greater than the concentration of the isotopically enriched protein; the protein becomes invisible. We noted this problem with CI2 (*8*); the Gierasch laboratory has encountered this problem with several additional small globular proteins (personal communication), and it has been discussed by others

(*13, 54, 65*). Simulation experiments show that even millimolar dissociation constants for a complex comprising a protein the size of CI2 (7.4 kDa) and bovine serum albumin (68 kDa) can lead to a 4-fold increase in line width (C. Li, unpublished observation). A quick way to test for these invisible proteins is to lyse the cells (*65*). Unless the protein is tightly associated with larger species, lysis and dilution should cause the resonances to appear.

Some proteins leak from cells. We observed this problem for two proteins, CI2 and apocytochrome b_5. Since the solution bathing the cells is less viscous than the intracellular environment and there are fewer chances for protein−protein interactions, isotopically enriched extracellular proteins are easier to detect because they give sharper resonances. Misattribution of the resonance source for apocytochrome b_5 resulted in one of us (G. J. Pielak) retracting (*66*) two papers (*67, 68*), even though SDS−PAGE showed that ~90% of the protein remained in the cell at the end of the experiment. Although glycerol has been used to stabilize cells, the definitive control is to subject the sample used for the in-cell NMR experiments to gentle centrifugation and then subject the resulting cell-free supernatant to the same NMR experiment used for the in-cell sample. The observation of protein resonances in the supernatant suggests leakage.

The last caveat we will discuss is sample inhomogeneity. That is, the mere presence of cells in the sample leads to inhomogeneity in the magnetic susceptibility, which makes shimming difficult and causes resonance broadening. This problem can be minimized by using solid-state NMR techniques, specifically magic angle spinning. Using this technique, Lippens and co-workers collected a ^{13}C HSQC spectrum of glucans in the periplasm of *Ralstonia solanacearm* (*69*). Even insoluble proteins in *E. coli* can be observed by solid-state NMR (*70*). As compared to liquid state-NMR, this approach allows much higher cell densities, which improves signal detection, but has been shown to lead to cell death and protein leakage (*50*).

IN-CELL NMR IN *E. coli*

Protein−Protein Interactions and Signal Transduction. Understanding protein−protein interactions is one of the main objectives of the postgenomic era (*71*). Conventional in-cell NMR spectroscopy characterizes one protein at a time. An obvious method for studying protein−protein complexes is to enrich all the proteins in the complex with an NMR-sensitive isotope, but this approach will give more complicated spectra. Shekhtman and co-workers have overcome this difficulty (*9*).

Their method, called STINT-NMR (structural interactions using NMR), works by enriching only one protein in a complex. Expression from orthogonal induction systems is the key. In their seminal paper, Shekhtman and co-workers induced ubiquitin in ^{15}N-enriched media with L-arabinose by using the pBAD promoter. The cells were then pelleted, washed, and resuspended in unenriched media. The partner protein was then expressed by using the PT7/lac system induced with IPTG. The interactions between the target and its partner are associated with changes in the width or chemical shift of the resonances of the enriched protein. In addition to the spectral simplification, STINT-NMR also benefits from the facile nature of many protein−protein complexes; if the stability of the complex is high and the off-rate is low, the decreased rotational motion of the complex will broaden the cross-peaks beyond detection. STINT-NMR can also be used to study phosphorylation.

Phosphorylation, the posttranslational addition of a phosphate group to serine, threonine, histidine, tyrosine, aspartic acid, or lysine side chains by kinases, can alter protein properties and thereby affect cellular signaling, regulation, and division. Furthermore, alterations in normal phosphorylation patterns are linked to disease (*72*). NMR allows noninvasive detection of protein phosphorylation events inside living cells. Fluorescence-based experiments involving GFP fusion proteins (*73, 74*) and fluorescently labeled antibodies against the test protein (*75*) have also been used to measure protein phosphorylation and the location of the protein in living cells.

NMR is well suited to the study of protein phosphorylation because protein resonances are sensitive to changes in the chemical environment induced by phosphorylation. These phosphorylation events are observed regardless of the number of sites or their positions in the protein. Additionally, the degree of a protein's phosphorylation can be determined from the size of the resonance.

Shekhtman's laboratory has applied STINT-NMR to the study of signal transduction by simultaneously expressing three proteins: the target protein (ubiquitin), a large (>100 kDa) heterodimeric affector complex, and a specific kinase (*76*). First, they assessed interactions between ubiquitin and the complex. Next, they induced expression of the kinase, which phosphorylates the affector complex. This phosphorylation was detected by examining changes in the spectrum of ^{15}N-enriched ubiquitin. The ubiquitin was expressed under three conditions: alone, with coexpression of the affector complex, and with coexpression of the complex and kinase. A key point is that changes induced by phosphorylation can be sensed by ubiquitin even though the complex is quite large.

Intrinsically Disordered Proteins. Intrinsically disordered proteins lack stable tertiary structure in dilute solution. Many members of this recently defined class play important roles in cell signaling, regulation, and control. Disordered proteins are associated with disease states, including amyloidoses and neurodegenerative disorders (*77*). Although the properties of globular proteins do not change significantly over a wide range of solution conditions, the properties of intrinsically disordered proteins can vary (*78*). This sensitivity to solution conditions should make them attractive targets for studies of physiologically relevant conditions. Despite this sensitivity, and the known relationships between protein disorder and disease, little is known about the intracellular structure of this protein class.

Disordered proteins are easier to detect by in-cell NMR than are globular proteins of the same size. This increased sensitivity arises from differences in global and local motions for globular and disordered proteins (*8*). Because of their rigidity, the relaxation rates for globular proteins are most sensitive to global motion, which is described by a single rotational correlation time. Disordered proteins, on the other hand, are flexible. Their motions involve an ensemble of interconverting conformers where different residues have different effective correlation times. That is, the flexible nature of disordered proteins mitigates the deleterious effect of viscosity on their spectra.

The first in-cell NMR study of an intrinsically disordered protein was reported by Dedmon et al. (*39*). The protein, FlgM, regulates flagellar synthesis upon binding a transcription factor. The intracellular environment in *E. coli* causes the C-terminal half of FlgM to gain structure while the N-terminal half remains unstructured. Similar characteristics were noted in vitro in solutions containing high levels of glucose, BSA, and ovalbumin. These data show that it is important to study disordered proteins under physiologically relevant conditions.

α-Synuclein, a 140-residue cytosolic eukaryotic protein, is the primary component of the intracellular protein aggregates called Lewy bodies (*79*). These aggregates are present in substantia nigra neurons of patients with Parkinson's disease. Studying α-synuclein under crowded conditions may provide information related to its role in the disease. McNulty et al. (*40*) used in-cell NMR to investigate the structure of α-synuclein in *E. coli*. These authors first noticed a difference between $^{1}H-^{15}N$ HSQC spectra in dilute solution acquired at 10 and 35 °C (*80*). Spectra collected at the higher temperature presented fewer cross-peaks (∼35) compared to spectra collected at 10 °C (∼70). Experiments using an α-synuclein comprising only the first 100 residues showed that cross-peaks in the spectrum acquired at 35 °C are from the C-terminal third of the protein. This temperature-dependent behavior was associated with an increase in hydrodynamic radius and a gain in the level of secondary structure at 35 °C. These changes were reversible when the temperature was decreased to 10 °C. The authors concluded that the N-terminal two-thirds of the protein exchanges between a more structured extended state and more disordered but more compact state. Heating the protein increases the rate of exchange between these states, causing the cross-peaks to disappear.

The in-cell spectrum of α-synuclein at 35 °C looks like the spectrum recorded at 10 °C in dilute solution. In vitro experiments in 300 g/L BSA yield spectra similar to those acquired from *E. coli* at 35 °C. These observations give rise to the idea that crowding in these cells or in vitro keeps α-synuclein in a disordered but more compact state.

These conclusions, however, have been questioned. Croke et al. suggest the difference between the in-cell and dilute solution results reflects a mismatch in pH between the samples, which results in higher amide proton exchange rates and a concomitant loss of resonances at 35 °C (*81*).

IN-CELL NMR IN HIGHER EUKARYOTIC CELLS

In-cell studies in *E. coli* will continue to provide fundamental information about crowding effects, but the medical relevance of higher eukaryotic cells makes them attractive targets for in-cell NMR. In addition, in-cell NMR in higher eukaryotic cells may be easier because the cytosol of eukaryotic cells seems to have a lower apparent viscosity (*5*).

The first in-cell NMR study of isotopically enriched proteins in nucleated higher eukaryotic cells was conducted in *X. laevis* oocytes (*41*). Their large size (∼1 mm diameter) allows microinjection of isotopically enriched protein into the cytosol. The B1 domain of streptococcal protein G (GB1) was expressed and ^{15}N-enriched in *E. coli*, purified, and injected into 200 oocytes. The authors studied a range of intracellular GB1 concentrations between 50 and 500 μM. The positions of the GB1 resonances in the HSQC spectra remained the same in dilute solution, in oocytes, and in solutions containing 250−300 mg/mL BSA, showing that neither crowding nor the environment in the cell changes the structure of GB1. Different behavior was observed, however, in terms of cross-peak intensity. Resonances of amides involved in intramolecular hydrogen bonds showed diminished intensity in cells and in BSA compared to dilute solution. This observation is consistent with the idea that more dynamic parts of the protein are less affected by the increased viscosity in cells (*8*).

Sakai et al. studied protein behavior after microinjection of ^{15}N-enriched ubiquitin and calmodulin into *Xenopus* oocytes (*82*). The in-cell spectrum of wild-type ubiquitin presented fewer resonances compared to its in vitro spectrum. Mutations in the hydrophobic patch on the β-sheet of ubiquitin confirmed that the loss of the resonances is associated with interactions between ubiquitin and other proteins. Calmodulin spectra in oocytes presented two patterns correlated with the presence or absence of extracellular calcium ions. When calcium ions were coinjected with the calcium-free protein, the in-cell spectrum was characteristic of calcium-bound calmodulin, while the spectrum was characteristic of the calcium-free form if calcium was not coinjected. These observations show that the physiological intracellular calcium concentration (∼0.1 μM) does not affect calcium-free calmodulin.

We end this section with a note of caution. Oocytes are fragile, so conditions that preserve them will facilitate longer acquisition times, thereby decreasing the amount of protein required for detection by NMR. Bodart et al. (*50*) showed that oocytes can be preserved by embedding them in a 20% Ficoll solution. The embedded cells remained intact for 20 h, allowing the detection of 5 μM intracellular protein. Nevertheless, care must be taken to monitor protein leakage. Sakai et al. (*83*), working with enriched calmodulin and ubiquitin, have developed an oocyte coinjection method involving GFP that is useful for detecting leaks.

Phosphorylation in Higher Eukaryotic Cells. Selenko et al. (*48*) used NMR to detect phosphorylation of the viral SV40 T antigen in intact *X. laevis* oocytes by endogenous casein kinase 2 (CK2). CK2 phosphorylates two serine residues in the regulatory region of the T antigen, altering the nuclear-import properties of the full-length protein. These investigators provide the first NMR observation of an in vivo protein substrate phosphorylation event inside living cells by an endogenous protein kinase. Time-resolved NMR spectra in oocytes show sequential phosphorylation of the substrate. The investigators conclude that CK2 phosphorylation occurs in a two-step reaction with intermediate release of the substrate and preference of the kinase for the unphosphorylated substrate. The results obtained in oocytes agree with NMR spectra acquired in dilute solution and in egg extracts, implying that the kinetics of phosphorylation are not affected by macromolecular crowding.

Phosphorylation of the intrinsically disordered protein tau in *X. laevis* oocytes has also been studied with NMR (*50*). Bodart et al. detected novel signals in the in vivo spectrum that they assigned to phosphorylated residues of tau. Comparison of the in-cell spectrum to the in vitro spectrum containing two known oocyte kinases revealed shifts in resonance positions. These shifts suggest that unidentified

kinases in oocytes may alter the positions of resonances observed in vitro. This study provides the first analysis of an intrinsically disordered protein in live eukaryotic cells by in-cell NMR.

CHALLENGES AND FUTURE DIRECTIONS

Internal Dynamics. In the past few years, NMR has led the way in pinpointing atomic-level relationships between enzyme function and protein motion in dilute solution (*84*). There is tantalizing evidence that macromolecular crowding affects enzyme-mediated catalysis (*31*). To the best of our knowledge, however, there are no valid (*66*−*68*) NMR-based studies that track the effects of macromolecular crowding on internal protein motion, although a study of encapsulated ubiquitin showed only small changes in picosecond-to-microsecond dynamics (*85*).

For both in vitro and in-cell studies, one challenge is the requirement that the protein remain monomeric. Crowding-induced interactions affect the protein's rotational correlation time, a key parameter for analyzing NMR-based dynamical data (*86*). Unfortunately, independent determination of protein correlation times is difficult. Another challenge is the long time required for data acquisition. For in-cell experiments, the challenges are to keep the cells alive and the test protein in the cell.

Studies inside Higher Eukaryotic Cells. Studies in oocytes were important first steps, but in terms of translational research, a key goal is to apply in-cell NMR to more conventional higher eukaryotic cells. Such endeavors will open the way for additional studies of protein modifications and protein−protein interactions and will provide a platform for the study of protein−drug interactions in cells.

One challenge is expressing proteins in higher eukaryotic cells at sufficient levels to overcome the low sensitivity of NMR spectroscopy. Besides injection, one approach is to use an orthogonal tRNA−aminoacyl tRNA synthetase pair to introduce an enriched, unnatural amino acid into the protein (*87*). Another approach is to insert isotopically enriched proteins expressed in *E. coli* into higher eukaryotic cells using cell-penetrating peptides, a new class of biomolecules that translocate proteins across the plasma membrane of eukaryotic cells. These peptides can be covalently attached to the protein of interest (*88*) or be used to form a complex with the protein through electrostatic and/or hydrophobic interactions (*89*). Such peptides may be capable of delivering enough intracellular protein to overcome the problems related to the insensitivity of NMR techniques. Another difficulty associated with the low sensitivity of NMR spectroscopy in living systems is the long times required to collect reasonable data. Encapsulation and immobilization of cultured cells at high densities (*90*) for NMR studies can prolong the viability of these living systems.

The insensitivity of NMR, the delicate nature of most cells, and the fact that nearly all higher eukaryotic cells are most at home when anchored to something make it certain that the future depends on cell encapsulation and on the development of bioreactors (*91*) that supply cells with nutrients and gases while removing waste products.

In summary, there are at least three challenges to using NMR for studying proteins under physiological conditions. The first challenge is the crowded environment itself. High macromolecular concentrations favor weak intermolecular interactions. These interactions, whether called "aggregation" or "nonspecific binding", present a challenge to both acquiring high-resolution NMR data and parsing any observed effects between volume exclusion and chemical interactions. Fortunately, NMR is quite sensitive to weak interactions. The second challenge is sensitivity. The low sensitivity of NMR necessitates high protein concentrations. Investigators must not only ensure the presence of high concentrations of the isotopically enriched test proteins inside cells but also ensure that the test protein neither changes the biology nor leaks from the cell. The third challenge is location. Currently, investigators assume the test protein is located in a single environment in a single compartment (e.g., the cytosol or the periplasm). Proteins, however, are dynamic entities. It is common for a protein to traverse several compartments and be modified several times during the course of its duty. Can in-cell NMR track these locations and modifications?

Despite these challenges, the data reviewed here show that NMR is, and will continue to be, a powerful tool for understanding protein properties under the conditions where they fulfill their biological role.

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