HHS Public Access

Author manuscript

J Magn Reson. Author manuscript; available in PMC 2020 September 01.

Published in final edited form as:

J Magn Reson. 2019 September ; 306: 187–191. doi:10.1016/j.jmr.2019.07.037.

Protein NMR: Boundless Opportunities

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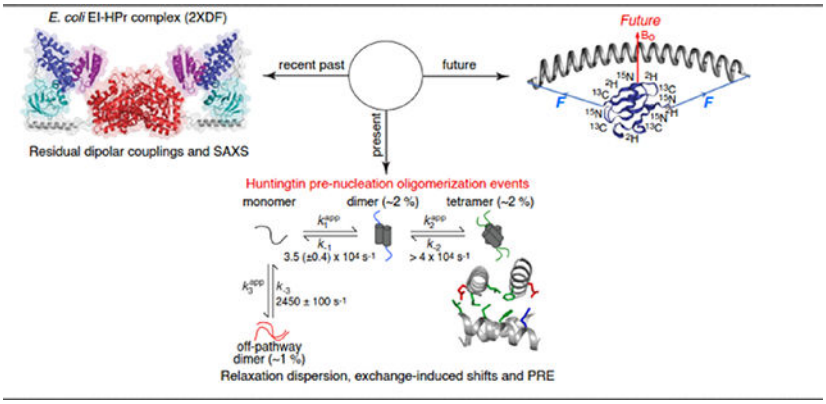
Abstract

Over the past approximately three decades, isotope-directed NMR spectroscopy has become a powerful method for determining 3D structures of biological macromolecules and their complexes in solution. From a structural perspective NMR provides an invaluable tool for studying systems that are not amenable to crystallization, including intrinsically disordered proteins and weak complexes. In contrast to both X-ray crystallography and cryo-electron microscopy which afford a largely static view of the systems under consideration, the great power of NMR lies in its ability to quantitatively probe exchange dynamics between interconverting states, and to reveal and characterize at atomic resolution the existence of transient states that may be populated at levels as low as 1%. Such “excited” states play a key role in macromolecular recognition, allostery, signal transduction and macromolecular assembly, including the initial events involved in aggregation and amyloid formation. Optimal application of NMR to such systems of fundamental biological interest requires a sound footing of the physical underpinnings of today’s and tomorrow’s sophisticated NMR experiments.

Graphical Abstract



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Keywords

heteronuclear NMR; structure; dynamics; conformational exchange; sparsely-populated excited states; supramolecular machines; megadalton assemblies

The enormous advances in solution biomolecular NMR over the last approximately 30 years hinged on six separate developments: uniform isotope slabeling (^{13}C and/or ^{15}N with or without ^{2}H) that enabled the extension from 2D ^{1}H-^{1}H NMR to heteronuclear multidimensional (3D and 4D) NMR, thereby overcoming spectral degeneracy and chemical shift overlap, and largely solving the assignment problem; improvements in magnet technology leading to higher magnetic fields which afford an increase in sensitivity and resolution which scale as $B_o^{3/2}$ and B_o^{N}, respectively, where N is the spectral dimensionality; the introduction of cryogenic probes that reduce thermal noise three- to four-fold, leading to substantial increases in sensitivity; improvements in spectrometer hardware, including pulsed field gradients and the availability of four separately controllable radiofrequency channels; the introduction of non-uniform sampling and associated processing algorithms that permit one to take full advantage of the increases in resolution as data collection in multidimensions is no longer restricted by limitations in data acquisition times; and last but not least the development of a vast array of sophisticated NMR experiments that permit the rich information contained in nuclear spins to be extracted and exploited in unambiguous ways.

Initially, solution biomolecular NMR primarily focused on 3D structure determination of proteins, nucleic acids and their complexes. In the mid to late 1980s, the first structures ranging from 5–10 kDa were determined by 2D ^{1}H-^{1}H NMR. The introduction of multidimensional heteronuclear NMR in the late 80s and early 90s led to considerable increases in the size of systems that could be investigated. Further NMR and computational advances led to *de novo* structures in the 30–40 kDa range by the late 1990s and an 82 kDa structure of malate synthase in 2005 [1]. The study of such larger systems was made possible by two independent technical developments: the TROSY technique coupled with perdeuteration to reduce the negative impact of large transverse relaxation rates in high molecular weight systems for both backbone amide as well as methyl resonances [2, 3], and residual dipolar couplings (RDCs) that provide long-range orientational information relative to an external alignment tensor [4].

However, even today, solving *de novo* NMR structures at an accuracy comparable to 2-Å resolution crystal structures still entails a large amount of work, including the acquisition of many observables in addition to NOEs, including J couplings and an extensive set of RDCs. Thus, with the routine introduction of synchrotron sources, X-ray crystallography commonly remains the high-resolution structural method of choice when a protein or protein complex can be crystallized. That being said, and as discussed below, there are many examples where NMR in the context of structure determination remains invaluable.

An area where the potential of protein NMR was exploited early on relates to ligand binding [5, 6]. It often is straightforward to find ligand binding sites by monitoring the perturbations in the ^{1}H-^{15}N HSQC spectrum upon titrating ligand into a protein sample. Here, protein

NMR serves as much more than a “screening tool” as it doesn’t just provide unambiguous information regarding whether there is an interaction with the ligand, but also yields the site of binding, the affinity, and the kinetics, while also revealing any allosteric effects that may be induced by the ligand. In terms of drug-design, chemical shift mapping has been used with great success to initially screen and map the binding sites of weak ligands (e.g. millimolar affinity) and subsequently covalently linking two non-competing ligands (that bind to proximate, non-overlapping sites) to generate much tighter binding lead compounds [7]. The importance of such studies is widely recognized, and the merits of this type of protein NMR cannot be overstated. However, from an NMR-technology perspective, this area of application appears fairly mature and we therefore expect it will simply continue to thrive while benefitting from mostly modest further refinements in future years.

The real question then remains: what new roles will protein NMR play in biology in years to come? Do the rapid technological advances in cryo-electron microscopy (cryo-EM) pose a threat or an opportunity for protein NMR? Which outstanding fundamental questions in molecular biophysics will become tractable by NMR? As outlined below, we believe that with its technological basis now firmly established, protein NMR is ripe to address a plethora of fundamental questions.

The role of motion in biological function

Motion and motional control are at the heart of much of biology. X-ray crystallography and cryo-EM provide snapshot images of the macromolecular machinery from which, in favorable cases, the mechanism by which it functions can be deduced. However, much of the path followed often remains elusive and more detailed analysis then hinges on insights gained from coarse-grained molecular dynamics simulations. The unique power of NMR to quantify the time scales and amplitudes of motions at the atomic level from NMR relaxation rates has long been appreciated [8]. However, the potential of NMR to characterize motions stretches far beyond the classical relaxation measurements [9, 10].

Interpretation of RDCs in motional terms is particularly powerful as it not only provides the amplitude but also the directions of such motions [11]. When alignment is enacted by a paramagnetic metal, interpretation of the differential degrees of alignment for multi-domain structural assemblies in terms of motion becomes unambiguous [12]. Such studies are particularly insightful once high-resolution structures of its domains are available. The enormous advances made by the classical structural methods have therefore opened the door to quantitative analysis of interdomain movements.

The introduction of methyl TROSY experiments now permits the study of very large systems, up to 1 MDa, by chemical shift mapping and *zz*-exchange spectroscopy [13]. Such experiments have led to considerable insights into conformational transitions and switching within numerous systems, including, for example, the 20S proteasome, the RNA exosome, the Hsp70 chaperone, and the KcsA potassium channel reconstituted in micelles [14]. Assignment of the methyl resonances is a prerequisite for such studies and has been tackled by methyl-specific isotopic labeling [15], but innovative computational solutions to this problem also appear powerful [16, 17].

Intrinsically disordered systems

Many, often highly conserved eukaryotic proteins are intrinsically disordered (IDPs) or contain large segments that remain dynamically disordered and therefore defy the traditional structure determination methods [18]. Such disordered proteins often fold upon binding to their biological targets (coupled folding and binding) and play important functional roles. Although locally disordered, these systems often display dynamic structural preferences that include a degree of long-range order that can be key to their biological function. Ensemble descriptions of these proteins commonly are generated from a wide range of NMR input parameters, including paramagnetic relaxation enhancement (PRE), J couplings, chemical shifts, and RDCs [19, 20]. IDPs also can be key to liquid-liquid phase separation, an emerging area in biology where NMR can provide unique structural insights [21].

The importance of transient states

Biology requires chemical reactions, signaling and control, transport and directional movement. Almost invariably, this involves the requirement for conformational states that differ from the aesthetically pleasing atomic resolution structures obtained by X-ray crystallography. As an example of an obvious case, an enzyme with its substrate in place often cannot release its product without transiently opening a “flap” that covers its active site. Whether the prevalent, substrate-free state is open or closed often cannot be established with certainty from static images, but the fact that such a transition must occur is unambiguous, and the equilibrium can be established experimentally by NMR [22].

More interesting cases are where the presence of the transient state is unexpected, but key to function. As an example, we highlight a case where static structures have dictated our understanding of nucleic acid biology but a transient state may define function. As demonstrated by Al-Hashimi and co-workers [23], low-populated and short-lived A.T and G.C Hoogsteen base pairs at CA and TA steps are transiently present inside canonical duplex DNA. The observation that such Hoogsteen base pairs are also found in DNA duplexes specifically bound to transcription factors implies that the DNA double helix intrinsically codes for such Hoogsteen base pairing, thereby expanding the structural complexity of DNA recognition.

Many critical events in biology rely on the presence of such transient states but commonly escape detection by traditional, static methods but are amenable to solution NMR. A minimal population of the transient state, typically on the order of one percent is needed, although lower populations can sometimes be accessed by PRE [24]. Much structural and dynamic information on the transient species often can be extracted from relaxation dispersion experiments, which relies on significant chemical shift differences between resonances in major and minor species and can probe events on time scales ranging from ~ 50 μs to ~10 ms [25–27]. Such measurements not only reveal populations and time scales of interconversion between the major and transient species, but also can yield the chemical shifts of the minor species which can then be interpreted in structural terms [28]. For cases where the exchange process is too slow for relaxation dispersion, chemical-exchange saturation transfer (CEST) is a particularly powerful alternative [29]. Newer versions of this

experiment, which “multiplex” the selective CEST irradiation scheme can substantially alleviate the time needed to record such spectra [30].

Folding, misfolding, and amyloid disease.

Some of today’s most challenging and acute health problems relate to the increased prevalence of amyloid diseases, such as Alzheimer’s and Parkinson’s disease, associated with today’s aging population. Misfolding of a protein or polypeptide into β-sheet rich structures that form microscopically visible inclusion bodies is believed to be at the heart of such diseases. Remarkably, many of these amyloidogenic proteins are intrinsically disordered or contain large disordered regions. The dearth of structural information on the physical processes underlying the conversion to the misfolded state challenges the traditional, rational design of inhibitors to prevent the cascade of events leading to the diseased state. Whereas solid-state NMR spectroscopy has proven particularly powerful at developing atomic resolution structural models of various amyloid fibrils [31–33], little remains known about the oligomeric species that are believed to be on-pathway to this pathologic state and are often held responsible for the toxic effects associated with the amyloid disease [34]. The conversion from disordered monomeric protein to oligomer to amyloid is a highly dynamic process where structural techniques other than solution NMR typically fall short of providing atomic resolution insights.

Dark state Exchange Saturation Transfer (DEST) combined with lifetime line broadening provides an avenue to characterize the exchange dynamics between amyloid β monomer and megadalton protofibrils, thereby revealing at atomic resolution the dynamics of the earliest stages involved in amyloid β aggregation [35]. DEST relies upon very large differences in transverse relaxation rates (and hence molecular weight) between major and minor species and can probe transitions on time scales ranging from 20 μs to 100 ms [36].

Combining DEST, lifetime line-broadening, small exchange-induced shifts and relaxation dispersion can characterize substrate interactions, as exemplified for the chaperonin GroEL [37], an 800 kDa supramolecular machine that plays a critical role in protein homeostatis and protein folding within the cell. This work established the passive effect exerted by apo GroEL on protein substrates in the absence of ATP and the co-chaperone GroES, and showed that apo GroEL stabilizes a SH3 folding intermediate relative to the folded state, while increasing the rate of interconversion between folded and intermediate states 500-fold.

Whereas acquisition of NMR signals is typically much slower than folding, unfolding, or misfolding, NMR nevertheless provides a powerful method to probe such processes if the structural equilibrium can be perturbed repeatedly. Optically triggered protein folding studies provide just one example [38]. Pressure-jump NMR, which synchronizes data acquisition with a pressure-induced change in the structural equilibrium, provides another effective approach to study protein folding [39]. The observation that amyloid β fibrils can be converted to their monomeric, soluble peptide state by the application of high pressure [40] opens new opportunities to access the early stages of misfolding.

Transmembrane signal transduction

Many key questions in biology focus on the communication between the inside and the outside of a cell, with the signal transmitted by integral membrane proteins. These include G-protein coupled receptors (GPCRs), integrins, T-cell receptors (TCRs), and many others. Considerable structural details on each of these systems has been derived from X-ray crystallographic and, more recently, cryo-EM studies. Although these represent magnificent breakthroughs in the structural biology of these complex systems, they typically present a single static snapshot of either a ligated or unligated state, shedding limited light on the actual mode the protein uses to carry the signal from the exterior to the cellular interior. Simple, one-dimensional ^{19}F NMR experiments pointed to a switch in the dynamic equilibrium of the conformational states of helices VI and VII upon ligand binding by the β2-adrenergic receptor [41]. However, 2D NMR studies of the dimethyllysine modified receptor foreshadowed a more complex behavior with a dynamic equilibrium between multiple substates, not seen in crystallographic studies [42]. Subsequent studies by Prosser and Kobilka utilizing $^{13}C^{\varepsilon}H_3$-Met labeled material provided site-specific resolution of multiple Met reporter groups, confirming the complex, allosterically driven mode of the signaling pathway [43]. Recent ^{19}F NMR studies, using advanced deconvolution analysis of the 1D NMR spectra, now appear in agreement with these conclusions [44, 45].

Traditional triple resonance NMR studies, which potentially could reveal a far more complete picture of such a complex signaling apparatus, have remained challenging for two reasons: (1) bacterial expression, a prerequisite for isotopic labeling at a reasonable cost, has proven difficult, and (2) such studies generally require perdeuteration by growing the protein in D2O, followed by back-exchange of the amide protons. This back-exchange is not yet a solved problem for the water-inaccessible amide groups of the transmembrane helices. However, the potential value of such studies is overwhelmingly clear after Grzesiek and co-workers succeeded in ^{15}N enrichment of all Val residues in the pi-adrenergic receptor [46], providing a first extensive view of the response of the transmembrane helices to ligand binding. This study, relying on baculovirus-infected insect cells and extensive mutagenesis [47]), represents a tour-de-force. Recent breakthroughs in the bacterial expression of eukaryotic integral membrane proteins hold promise to open this field to the larger biological NMR community [48]. Challenges with back-exchange of perdeuterated proteins potentially can be addressed with direct ^{15}N detection, an approach made feasible by the development of highly sensitive cryogenic probe technology [49].

Importantly, methyl groups are abundant in such systems and can serve as highly sensitive probes of structure and dynamics, in particular in a perdeuterated background [50]. Multiple approaches to solve the methyl assignment problem for such systems have been put forward [51, 52]. If the protein’s structure is known from X-ray crystallography or cryo-EM, as will frequently be the case when carrying out mechanistic studies, assignment protocols that take advantage of this information appear particularly powerful [16].

The effect of force and its direction on molecular interaction

The above areas are already well recognized as fertile ground for further development. We envision, however, that the scope of protein NMR can be extended to new areas that were largely intractable prior to the tremendous technological advances of the past decade. As just a single example, we point to the importance of the directionality of intermolecular forces, which are key in muscle contraction and all inter- and intracellular transports. In fact, without directionality of such forces higher level organisms could never have developed. Catch-bonds are key in establishing the directional properties of such intermolecular forces [53, 54]. Whereas monitoring the effect of directional forces has long remained the domain of single-molecule optical trapping experiments, it should become possible to study this all-important biophysical property by NMR spectroscopy. Specifically, it is possible to connect two sites on a protein, or protein complex, by a molecular spring, thereby adding a directional force between the two sites of attachment [55]. Together with segmental isotope labeling strategies, the above-mentioned advances in TROSY technology directly draw this class of studies into the NMR-feasible arena.

Concluding remarks

Isotope-directed NMR of proteins has been an area where explosive growth in the technology now enables the study of complex, meaningful problems in biology. Initially much of its focus was aimed at determining three-dimensional structures, analogous to, but in competition with the generally more powerful X-ray crystallographic methods. However, its future hinges upon exploiting this structural information to gain insights into function, involving signaling, transport, and a wide array of other critical elements of biology. A potential hurdle lies in the complexity and diversity of many of the NMR experimental schemes. In particular, with increased focus on biological applications, training in the physical aspects of the actual spectroscopic techniques appears to be in decline, jeopardizing the opportunity to take full advantage of NMR's unparalleled power. We therefore urge the community to retain sufficient emphasis on in-depth training of the next generation of researchers, such that the full potential of NMR can be brought to bear on solving today's, and tomorrow's, critical questions in biology and biomedicine.

Acknowledgment

Work in the authors' laboratories is supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Disease

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Highlights

- NMR remains pivotal to structural studies of dynamic and partially disordered systems
- Complementary strategies provide access to biologically important transient states
- Both DEST and pressure-jump NMR provide access to the study of amyloid formation
- Advances in protein expression and labeling will reveal key transmembrane signaling
- Even unexplored areas such as force spectroscopy may become accessible to NMR