



HHS Public Access

Author manuscript

Curr Opin Struct Biol. Author manuscript; available in PMC 2022 October 01.

Published in final edited form as:

Curr Opin Struct Biol. 2021 October ; 70: 44–52. doi:10.1016/j.sbi.2021.03.015.

NMR illuminates intrinsic disorder

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Abstract

NMR has long been instrumental in the characterization of intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs). This method continues to offer rich insights into the nature of IDPs in solution, especially in combination with other biophysical methods such as small-angle scattering, single-molecule fluorescence, EPR and mass spectrometry. Substantial advances have been made in recent years in studies of proteins containing both ordered and disordered domains and in characterization of problematic sequences containing repeated tracts of a single or a few amino acids. These sequences are relevant to disease states such as Alzheimer's, Parkinson's and Huntington's diseases, where disordered proteins misfold into harmful amyloid. Innovative applications of NMR are providing novel insights into mechanisms of protein aggregation and the complexity of IDP interactions with their targets. As a basis for understanding the solution structural ensembles, dynamic behavior, and functional mechanisms of IDPs and IDRs, NMR continues to prove invaluable.

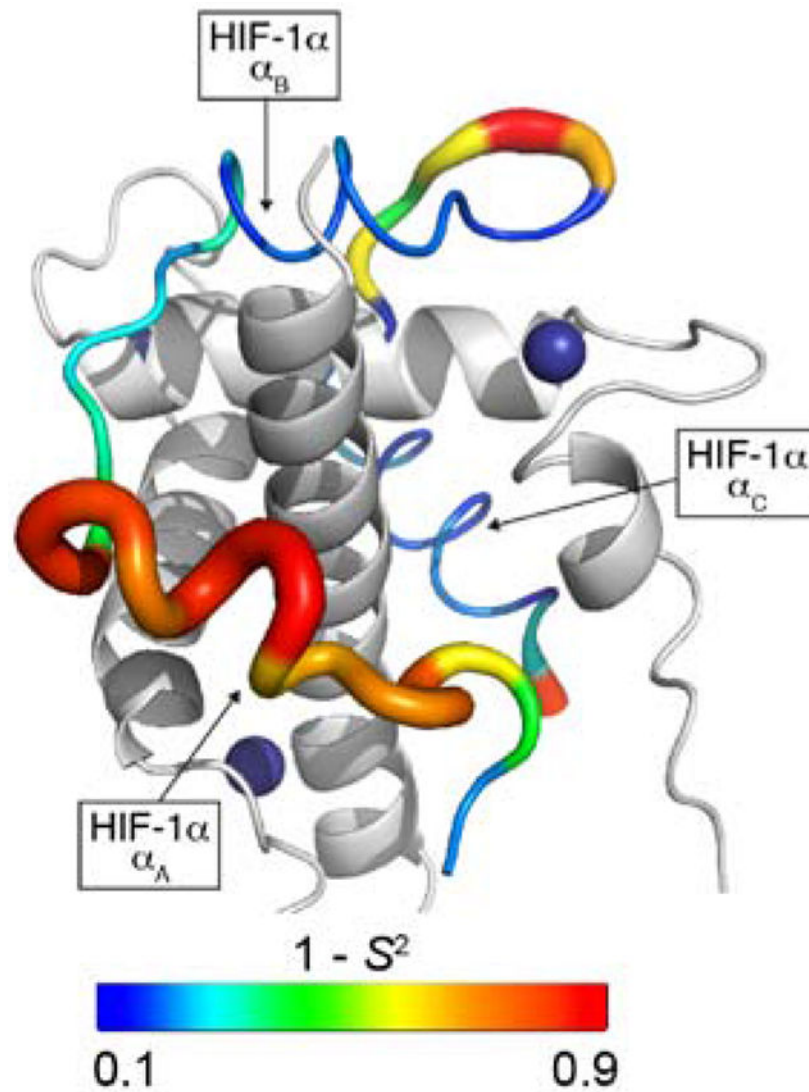
Graphical Abstract

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Declaration of Interest

The authors declare no conflict of interest.



Keywords

intrinsically disordered proteins; intrinsically disordered regions; amyloid; protein dynamics; isotopic labeling

Introduction

More than half of the proteins in the human proteome are entirely disordered (intrinsically disordered proteins; IDPs) or contain both structured and long disordered regions (IDRs). Molecular level characterization of proteins that contain both structured and disordered domains represents an enormous challenge to the traditional methods of structural biology. Most studies to date have relied upon a reductionist approach, in which the ordered and disordered regions are investigated in isolation. However, within the cell, the ordered and disordered domains act synergistically to allow a protein to perform its biological function;

a full understanding of the underlying molecular mechanism can only be achieved through a holistic, rather than reductionist, approach.

The original experimental identification of disordered regions of functional importance in proteins was accomplished primarily by NMR spectroscopy [1]. The sequence signatures of disordered protein regions have been identified in numerous bioinformatic studies and disorder predictions are available in databases [2]. The past few years have seen extensive progress in the biophysical characterization of IDPs and IDRs; NMR continues to be a primary tool and continuing innovations have greatly advanced fundamental studies aimed at characterizing IDPs, IDRs and their functional interactions, and in exploring particular disordered systems of biomedical interest. Despite intense current interest in the role of IDPs in liquid-liquid phase separation and the direct application of NMR in many studies, this review will make only brief mention of the field as recent reviews have covered the subject thoroughly [3].

NMR Technical Advances

IDPs are inherently dynamic and sample an ensemble of conformations in solution. Characterization and validation of these ensembles is challenging and requires a hybrid approach in which experimental restraints from NMR, small angle X-ray scattering, and biophysical methods such as single molecule FRET are combined with computer modeling to generate structural models of the overall ensemble [4]. Although local elements of transient helical structure can be readily identified and the population of helix quantitated from secondary chemical shifts, characterization of transient β -sheet has proved elusive. Recently, an approach designated TSD-NMR (Transient Structure from chemical Denaturation by NMR) has been developed which, in combination with chemical shift-based structure determination, revealed 10% population of a small β -sheet near the C-terminus of the Alzheimer's $A\beta^{1-42}$ peptide [5*]. Chemical denaturant titrations have also been used to overcome a problem that is often encountered in NMR studies of larger IDPs – broadening of resonances due to formation of collapsed, molten globule-like states. For the oncoprotein Myc, this problem was resolved by titration with guanidinium hydrochloride to allow assignment of resonances and calculation of structural ensembles using restraints from chemical shifts and paramagnetic relaxation rates, extrapolated to zero denaturant concentration [6*].

One of the major hurdles to tackling the NMR characterization of full-length proteins containing both ordered and disordered domains is their structural heterogeneity. This poses major challenges, especially for higher molecular weight proteins where resonances arising from residues in the structured domain(s) experience substantial broadening while those associated with the disordered domain(s) have narrow linewidths but may be poorly resolved due to limited spectral dispersion and extensive resonance overlap. Methods used previously to elucidate the structure and dynamics of isolated fragments comprising the disordered or structured regions do not provide the vital insights into the synergistic interactions between the various domains of the full-length molecules. This problem has been central to a number of the studies described in the past few years.

Protein ligation and isotopic labeling.

Deconvoluting the NMR resonances of large disordered and mixed ordered-disordered proteins is a challenge that can be addressed in the first instance by clever applications of stable isotopic labeling. Uniform isotopic labeling with ^{15}N and ^{13}C is the standard method, but complications due to resonance overlap persist even with the amelioration of the relaxation problems of large molecular systems by transverse relaxation-optimized spectroscopy (TROSY) and deuteration. Protein ligation technology has come into its own in the last few years, as a means of producing proteins that are differentially labeled at the level of individual domains. Recent innovations have included traceless salt-inducible intein ligation[7]., and flexible ligation using variants of a sortase A enzyme from *Staphylococcus aureus* that can be employed to produce circularized proteins or lipidated proteins, as well as mixed ordered-disordered proteins, or to provide anchoring to nanodiscs [8]. Cell-free expression has proved to be a powerful method to obtain NMR-labeled samples of low-complexity proteins, particularly homorepeat proteins containing long stretches of single amino acids such as proline or glutamine: a compendium of useful cell-free expression methods was recently published [9]. By combining cell-free protein synthesis with nonsense suppression, site-specific labeling of residues within polyglutamine, polyproline or other homorepeat tracts can be accomplished, opening the way to structural and dynamic characterization of proteins containing these regions [10].

^{15}N and ^{13}C detection for IDPs

Traditionally, heteronuclear NMR experiments have focused on the detection of proton magnetization since the sensitivity of the proton is so much greater than those of other spin $\frac{1}{2}$ nuclei (except for ^{19}F). However, for the specific case of disordered proteins, direct detection of ^{15}N and ^{13}C offers the advantages of narrower linewidths, enhanced resolution, and the ability to acquire spectra at physiological pH, where exchange of amide protons with water typically results in broadening or disappearance of resonances in conventional amide $^1\text{H}^{\text{N}}$ -detected spectra [11,12]. Recent advances include development of a sensitivity enhanced ^{13}C -detect experiment for monitoring phosphorylation of IDPs at physiological temperature and pH where kinases are active [13]. A suite of triple resonance experiments utilizing $^1\text{H}^{\alpha}$ -detection provides a 3-5-fold increase in sensitivity compared to their ^{13}CO -detect analogs for samples at physiological or higher pH [14].

New ways to use NMR for IDPs

The community has produced a number of innovative new experimental techniques designed to give information beyond simple chemical shift assignments and relaxation measurements. These include NMR exchange spectroscopy for elucidating fuzzy interactions [15]., use of solvent paramagnetic relaxation enhancement (PRE)[16]., to identify low populations of locally structured states and to characterize the conformational ensemble [17]., the use of ^{15}N chemical exchange saturation transfer (CEST) to obtain amide proton exchange rates [18]., and a combination of CEST and pulsed double electron-electron resonance (DEER) EPR to give information on interactions of huntingtin peptides with micellar nanoparticles [19]. In an innovative approach to overcome the problem of extreme IDP resonance broadening due to fast exchange of amide protons with solvent at physiological

pH values, a weak ^1H B_1 field is swept through a three-dimensional $^1\text{H}^a$ -detect spectrum to determine amide proton resonance frequencies [20*]. A novel method that combines transferred NOEs, ^{13}C -methyl labeling, and isotope-edited/isotope-filtered NOESY has been reported for mapping interactions of intrinsically disordered short linear motifs (SLiMs) with target proteins [21]. This method should find general utility for mapping weak, fast-exchange interactions between IDPs and targets of up to ~80 kDa molecular weight. Weakly interacting, slowly-exchanging systems can be characterized by off-resonance $R_{1\rho}$ in cases such as liquid-liquid phase separation where there are large differences in relaxation rates but no differences in chemical shift between free and bound states [22].

Dynamics measurements

Since IDPs consist of ensembles of distinct structures rather than the single or closely-similar structures characteristic of globular proteins, the issue of the dynamics of the ensemble, and the kinetics of interconversion between members of the ensemble is a central one for disordered proteins. Considerable emphasis has been placed in the past few years on the characterization of IDP dynamics and its relevance to biological function. Extensive ^{15}N NMR relaxation measurements have been reported for many IDPs in their unbound states. Distinct dynamic modes associated with fast (< 50 ps) librational motions, transitions between Ramachandran substates on an ~1 ns time scale, and slower (> 5 ns) segmental motions of the polypeptide chain have been identified [23,24]. Measurements of IDP relaxation data under conditions of molecular crowding show that the intermediate and slow time scale motions are strongly coupled to the friction of the solvent [25*]. These studies led to a unified model of IDP dynamics in complex environments such as phase separated states and the cellular milieu.

NMR relaxation measurements have been used to characterize fast (ps – ns) time scale dynamics in IDP complexes to provide insights into binding energetics, binding mechanisms, and allostery. Many IDPs bind their targets through multiple linear motifs separated by weakly interacting linkers; the overall affinity is determined by the binding free energy of each motif and the strength of thermodynamic coupling between them. NMR relaxation measurements provide a direct method for probing the heterogeneous energy landscape of an IDP complex. Several studies have focused on complexes formed by the TAZ1 domain of the transcriptional coactivators CBP and p300, which binds the disordered activation domains of several cellular transcription factors. TAZ1 exhibits structural plasticity and both the backbone and side chain dynamics are differentially modulated by binding of different IDP ligands [26*,27]. The IDP ligands themselves exhibit heterogeneous dynamics; regions that do not interact with TAZ1 or make only fuzzy interactions undergo large amplitude motions while sequence motifs that dominate binding are motionally restricted, underscoring the complexity of IDP-target interactions [26*]. (Figure 1) Dynamics measurements provided important insights into the molecular mechanism by which the negative feedback regulator CITED2 promotes dissociation of the hypoxia inducible factor HIF-1 α from TAZ1 to very efficiently down-regulate the hypoxic response. By binding to a region of TAZ1 where HIF-1 α interactions are highly dynamic, CITED2 forms a transient ternary complex that drives a conformational change in TAZ1 and

dissociation of HIF-1 α [26*,28]. The plasticity of TAZ1 and the dynamic heterogeneity of the HIF-1 α and CITED2 complexes are central to the mechanism of this molecular switch.

A particularly fruitful approach appears to be the combination of NMR dynamics measurements with molecular dynamics calculations, which have been used, for example, in the delineation of solvent-dependent segmental dynamics [29]. A new strategy has been reported for generation of conformational ensembles for flexible multidomain proteins using residual dipolar coupling restraints and Langevin dynamics [30]. Solvent deuterium isotope effects were shown to have deleterious effects on the accuracy of ^{15}N transverse relaxation measurements but this problem can be overcome by using a very low fraction of D_2O in the buffer [31].

An IDP Problem: Repetitive low complexity tracts

Varying lengths of repeating sequences, sometimes of short peptide motifs and sometimes of just a single amino acid, are characteristic features of IDPs and IDRs. Such repetition is problematic for the residue-level characterization of IDPs by NMR, and several approaches have been successfully implemented to assign resonances in these regions. Proline-rich sequences are common in disordered regions, where they frequently participate in molecular recognition in signaling networks or may perform diverse roles such as a disorder promoter or compaction facilitator [32]. Proline is a problematic amino acid in the commonly-used $^1\text{H}^{\text{N}}$ -detected NMR experiments, as it lacks an amide proton. Proline-rich sequences can be assigned using ^{13}C -detected CON-based strategies. Rather than requiring 4- and higher dimensional spectra, a simplified approach, of particular utility in systems with a very high proportion of prolines, uses a single 2D CON spectrum, with two 3D triple-resonance spectra to directly correlate adjacent CON units [33]. The CON spectrum provides a useful fingerprint for proline-rich IDPs, analogous to the ^1H - ^{15}N HSQC spectrum [34]. A comprehensive characterization of the behavior of prolines in disordered proteins is essential for the description of a number of systems, such as a proline-rich domain (containing 30% proline residues) of the adaptor protein ALIX [35]. The CON strategy has also been used to identify the interactions between arginine-rich and aromatic-rich regions that drive phosphorylation-dependent, liquid-liquid phase separation in the FMRP-CAPRIN1 system at physiological pH (7.4) [36**].

Glutamine is another problematic amino acid that is frequently enriched in disordered regions of disease-related proteins. The problem here is not the absence of the amide proton, but the extreme resonance overlap caused by long tracts of polyglutamine. For huntingtin, where disease pathology in Huntington's Disease is directly related to expansion of polyglutamine repeat sequences, characterization of these regions by NMR has required extensive design of specific labeling strategies [10], which have led to the insight that the structural properties of the polyglutamine repeat regions are strongly influenced by the nature of the flanking regions [37**].

Fuzzy Complexes

Although early studies of the complexes formed by IDPs frequently showed the disordered domains in a folded state in the complex, we and others increasingly began to observe that in even quite tight complexes ($K_d \sim \text{nM}$), the bound IDP or regions of it can remain disordered. For example, the adenoviral E1A oncoprotein binds to the TAZ2 domain of the transcriptional activator CBP with two binding modes – part of the ligand folds to form a structured helix on the surface of TAZ2 [38]. but the binding affinity is increased 4-fold by the presence of an N-terminal region of E1A that makes only transient interactions with TAZ2 [39]. Complexes that display structural polymorphism and structural disorder have been termed fuzzy complexes [40].

The functional advantages of fuzziness have been described elsewhere [40]. and there are now numerous published examples in a number of biomedically important systems. Recent examples include a comprehensive study that identified an extremely high-affinity interaction between two disordered proteins, prothymosin- α and histone H1, that is mediated through electrostatics and shows all of the hallmarks of a fuzzy complex [41,42]. Other examples include the C-terminal domain of the splicing factor FBP21, which binds with high specificity to a large site on the Brr2 helicase but remains highly dynamic in the complex [43]. and binding of the transcription factor c-Jun to the ubiquitin ligase SCF^{Fbw7} and the proline isomerase Pin1 through dynamic interactions with multiple phospho-degron motifs located in intrinsically disordered regions of c-Jun [44]. A particularly interesting and complex case involved an order-disorder transformation of a redox switch in the chloroplast protein CP12 upon interaction with glyceraldehyde-3-phosphate dehydrogenase [45]. which is interpreted as contributing to an entropy increase that amplifies the affinity of the complex.

Increasingly, NMR is combined with molecular dynamics simulations to provide a molecular level description of fuzzy complexes. By combining solution and solid-state NMR with computer simulations, a recent study provided novel insights into the highly dynamic interactions of a disordered protein, corresponding to the cytoplasmic region of the divisosome protein ChiZ, with an acidic membrane. Interactions occurred predominantly through arginine side chains and the membrane lipids were observed to redistribute to adopt arginine-proximal positions [46*]. In a very elegant study, methyl TROSY NMR, paramagnetic relaxation enhancement, small angle neutron scattering (SANS), and molecular modeling were used to determine the solution structure of the complex between the acetyltransferase Rtt109, the histone chaperones Asf1 and Vps75, and the histone H3:H4 dimer [47**]. Electrostatically-mediated, fuzzy interactions between the intrinsically disordered Vps75 C-terminal region and the disordered histone H3 tail promote binding of H3 lysine residues in the Rtt109 catalytic site and thereby enhance acetylation specificity with minimum entropic cost.

Chaperone interactions

Among the paradigmatic interactions of folded proteins with unfolded domains are those between molecular chaperones and nascent or unfolded proteins. One of the most prominent

functions of chaperones is to sequester nascent protein chains as they emerge from ribosomes. NMR has been used with great success in the past to elucidate the disordered conformations of nascent chains [48]. More recently, an NMR and ESI-mass spectrometry study showed that the nascent polypeptide-associated complex, a ribosome-associated chaperone, exhibits conformational flexibility that allows it to bind weakly to nascent chains and IDPs with widely varying polypeptide sequences [49]. The chaperone HdeA becomes disordered when activated, exposing hydrophobic surfaces for binding clients [50]. CEST experiments were used to identify local acid-sensitive regions that regulate the structural change and exposure of the client binding sites [51]. NMR experiments also show that another small chaperone, HspB1, responds to phosphorylation of IDR clients by local unfolding and increased exposure of hydrophobic sites [52]. and that the same type of hydrophobic exposure mediates interactions of HspB1 with the disordered protein tau [53].

Besides their function as “holdases” in the stabilization of nascent transcripts emerging from ribosomes, and as “foldases” facilitating the correct folding of globular proteins in the cytoplasm, chaperones are also heavily utilized in secretion pathways. The requirement for secreted proteins to be unfolded before passing through the membrane in complex with chaperones has long been known. Differences in the mechanisms of these processes in the case of the *Yersinia* virulence factors YopE and YopH have recently been elucidated with methods combining NMR, SAXS and molecular modeling [54]. Bacterial virulence factors are secreted in complex with a specific chaperone. For YopE, the secretion complex was shown to be ordered, while YopH employed an entirely different mechanism with part of the C-terminus extruded from the surface of the complex.

Recent NMR studies give a definitive mechanistic picture of the interaction between the chaperone Hsp70 and client proteins, in this case a destabilized mutant domain of chicken brain α -spectrin termed R17* and an SH3 domain [55]. Both proteins exist in equilibrium between folded and unfolded states in solution. ^1H - ^{13}C CEST and zz -exchange experiments were used to define the fluxes through induced-fit (IF) and conformational selection (CS) pathways of binding of R17* to Hsp70. The Hsp70 binds both clients by CS mechanism, preferentially binding to the unfolded state rather than the natively folded form. In an alternative approach, DEER EPR was recently used to characterize the interaction of a disordered protein, Tau, with the chaperone Hsp90 [56].

Progress in the Study of Aggregation Diseases

Protein aggregation diseases such as Alzheimer’s, Huntington’s and Parkinson’s diseases are among the most frustratingly resistant to treatment. Considerable effort has been expended in characterizing the mechanisms of aggregation and toxicity of the proteins involved. In-cell NMR gave extensive insights into the molecular state of the Parkinson’s disease protein α -synuclein, confirming that it remains disordered even in the crowded environment of the cell [57]. A more recent study employing in-cell NMR showed that a specific motif in α -synuclein interacts with Hsp70 and Hsp90 chaperones both *in vitro* and in the cell; inhibition of this interaction in the cell causes α -synuclein to re-localize to mitochondria and aggregate [58*].

New NMR methods have been introduced to probe transient oligomerization processes of intrinsically disordered proteins that lead to aggregation and amyloid formation. A novel pressure-jump NMR method has been applied to study Alzheimer's A β oligomers at site-specific resolution [59*]. By cycling between low and high pressure, the structure and dynamics of the oligomeric state can be probed by monitoring NMR spectra of the monomer to provide new insights into the earliest aggregation events. A set of NMR relaxation experiments, including relaxation dispersion, rotating frame relaxation, and exchange-induced chemical shifts, recorded over a range of protein concentrations, has been used to monitor early oligomerization processes in a truncated region of the huntingtin protein [60**]. A branched kinetic scheme for oligomerization, involving on- and off-pathway processes, was derived and structural models of the productive intermediates were determined from PRE data and validated by DEER EPR measurements. The approach was later extended to the full huntingtin exon 1 region containing a tract of 7 glutamines and two polyproline tracts [61]. Human profilin I inhibits huntingtin aggregation by binding to one of the polyproline tracts and abrogating the productive oligomerization pathway.

NMR Approaches to Full-Length Proteins Containing IDRs

The experimental and computational methods outlined above (and other techniques that are beyond the scope of this review) form part of an arsenal that can be used to investigate full-length proteins that contain fully-folded and disordered domains. These efforts are clearly ongoing, but good progress has been made in recent years for a number of widely different systems.

Viral proteins

Protein disorder is particularly prevalent in transcriptional and cell cycle control [62], and these processes are targeted by viruses that use virally encoded IDPs to hijack cellular pathways [63]. Disordered viral proteins target, for example, the retinoblastoma protein pRb and the transcriptional coactivator CBP/p300, through sequence-specific and high-affinity interactions that compete with disordered cellular factors such as the tumor suppressor p53. Recent NMR studies include characterization of the interaction of the disordered activation domain of the human T cell leukemia virus (HTLV-1) protein HBZ, which maintains chronic infection and promotes leukemogenesis, with the KIX domain of CBP [64]. The TAZ2 and NCBD domains of CBP/p300 interact with the adenoviral E1A protein [38,65], as well as a previously-unrecognized sequence motif in the the N-terminal IDR of CBP [66].

Multi-domain viral proteins have been a major focus of several labs over a number of years. Early studies of the Measles virus capsid protein employed the reductionist approaches that were standard at the time, but recent work has captured many of the features of the full-length proteins, including liquid-liquid phase separation upon mixing the phosphoprotein and capsid protein to form droplets that facilitate encapsidation of RNA [67]. Two recent studies have focused on the Nipah virus phosphoprotein that contains both disordered and structured regions. Using ^{13}C -detected and conventional $^1\text{H}^{\text{N}}$ detected experiments, ~90% of the residues in a disordered, 406 amino acid N-terminal domain were successfully assigned [68]. . Characterization of the structural ensemble of the full-length Nipah virus

phosphoprotein tetramer exemplifies the challenges of characterizing the structure of large proteins and illustrates the power of a combined approach that applies NMR, X-ray crystallography, SAXS and other biophysical methods [69].

Tumor Suppressor p53

The voluminous literature on p53 has received a number of additions in the past two years. It contains both structured (the DNA-binding domain (DBD) and the tetramerization domain) and disordered domains (the N-terminal activation domain (NTAD), the proline-rich domain (PRD) and the C-terminal regulatory domain). p53 has been extensively studied for many years, but recent advances in NMR and isotopic labeling methods have allowed new, atomic-level insights into synergistic interactions between domains that modulate the behavior of the full-length protein. Two recent NMR studies using either full-length p53 or an NTAD-DBD fragment showed the presence of intramolecular interactions between the DBD and the NTAD that function to enhance discrimination between cognate and non-cognate DNA sequences [70,71]. Studies of the full-length protein were facilitated by the use of intein methods to segmentally ¹⁵N label the p53 tetramer so that only the NTAD resonances are observed in the NMR spectra [70]. These studies were subsequently extended to investigate the role of Thr55, located within the NTAD, which functions as a phosphorylation-dependent switch that regulates DNA binding [72].

Conclusion

NMR remains one of the most powerful techniques for characterization of the structure, dynamics, interactions, and mechanism of action of proteins, particularly those that contain regions of intrinsic disorder. Our current survey of the most recent literature identifies a few recurring themes. New advances in NMR methodology are constantly stretching the limits on the types of biological systems that can be studied. Innovative and elegant isotope labeling strategies are opening the way to NMR studies of large proteins containing both ordered and disordered domains and are beginning to provide unprecedented insights into complex systems. Combined approaches using multiple biophysical methods, including NMR, small angle scattering, single molecule FRET, DEER EPR, and X-ray crystallography, coupled with molecular dynamics simulations, promise to greatly advance our understanding of disordered proteins. We now have tools available to tackle some of the most challenging multi-domain proteins and can anticipate the emergence of a new, non-reductionist era of structural biology in which the functional synergies between ordered and disordered domains is fully revealed.

Acknowledgements

This work was supported by grants GM131693, CA214054, CA229652, and GM075995 from the National Institutes of Health.

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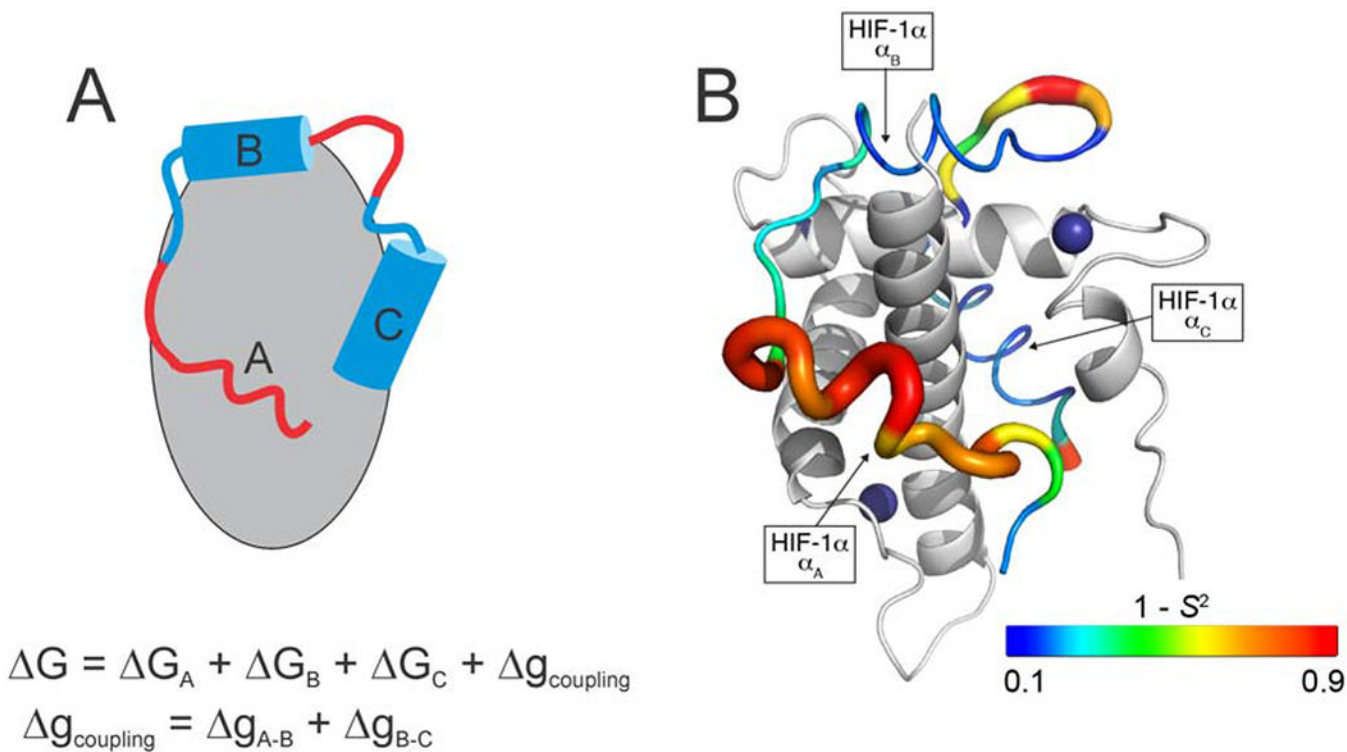
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**Figure 1.**

A. Schematic representation of heterogeneous interactions between an IDP and its target. Interactions with motif A are fuzzy and highly dynamic while motifs B and C interact strongly and specifically with the target such that their motions are restricted. The overall binding free energy is the sum of the free energies of interaction of each motif and the free energy associated with thermodynamic coupling (Δg) between the individual binding motifs.

B. Backbone dynamics for the IDP HIF-1 α in complex with TAZ1. The ribbon width of the HIF-1 α transactivation domain peptide is scaled by $1 - S^2$, where S^2 is the order parameter describing the amplitude of N-H bond motions. The backbone color gradient ranges from blue ($1 - S^2 = 0.1$) in regions where motions are restricted by strong interactions with TAZ1 to red ($1 - S^2 = 0.9$) where intermolecular interactions are weak and there are large amplitude motions of the HIF-1 α backbone. TAZ1 is shown in the cartoon representation in grey. Zinc atoms are shown as dark blue spheres. The secondary structural elements of HIF-1 α are labeled. (Adapted from [26*] with permission).

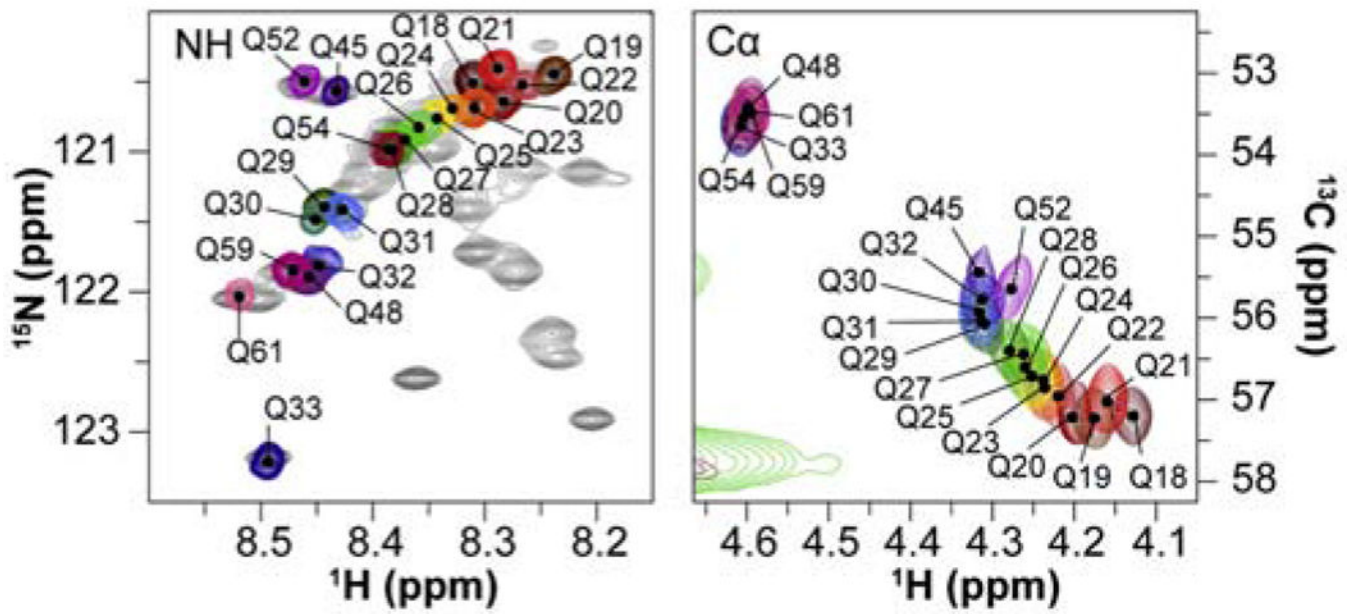


Figure 2. Portions of ^1H - ^{15}N HSQC (left) and ^1H - ^{13}C HSQC (right) spectra showing cross peaks belonging to members of a polyglutamine tract in huntingtin. The color-coded peaks were assigned by site-specific isotopic labeling [10,37**]. (adapted from reference [37**], with permission).