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The Quiet Renaissance of Protein NMR

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Abstract

From roughly 1985 through the start of the new millennium, the cutting edge of solution protein nuclear magnetic resonance (NMR) spectroscopy was to a significant extent driven by the aspiration to determine structures. Here we survey recent advances in protein NMR that herald a renaissance in which a number of its most important applications reflect the broad problem-solving capability displayed by this method during its classical era during the 1970s and early 80s.

“Without receivers fitted and kept in order, the air may tingle and thrill with the message, but it will not reach my spirit and consciousness.”

Mary Slessor, Calabar, circa 1910

The first NMR spectrum of a protein, that of ribonuclease A, was reported in 1957 by the lab of Martin Saunders.¹ The introduction of radiofrequency-pulsed excitation, signal averaging, and Fourier transform data analysis into NMR spectroscopy in the mid-1960s and the production of the first superconducting magnets a few years after led to dramatic advances in both basic NMR spectroscopy and applicability to biological systems.² By the mid-1970s solution NMR had come to play a prominent role in biochemistry and molecular biophysics as a way of probing biomolecular structure and interactions, as well as providing read-outs for various assays. A superb account of this classical 1-D era of biomolecular NMR is found in a 1981 book by Jardetzky and Roberts “Nuclear Magnetic Resonance in Molecular Biology”.³ That the future is difficult to foresee is reflected by an editorial aside in that work: “The hope held out for a complete determination of the structure of proteins and other flexible biomolecules in solution has not materialized and will not do so in the immediate future”. By the mid 1980’s Wüthrich and others determined the structures of a number of small proteins.^{4–6} This was enabled by the development of two-dimensional NMR methods, early computers and software, innovation, and nascent molecular modeling. A good deal of the focus of NMR during the following 20 years was on the development and application of NMR as a tool for structural determination of proteins and nucleic acids. During that time period, NMR was extended to >2 dimensions (usually with multiple nuclei involved)^{7, 8}, while the tools of preparative molecular biology enabled both routine high level production

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and NMR isotopic labeling of numerous previously inaccessible protein and RNA targets. The use of relaxation measurements to illuminate biomolecular motions also advanced dramatically during this time period.

By the year 2000, it became clear that NMR could not keep pace with X-ray crystallography as an approach to routine structural determination for a majority of proteins and nucleic acids, particularly large molecules and complexes. This is despite the development of residual dipolar coupling measurements, pulsed field gradients, pulse shaping, magnets in which ^1H NMR frequencies of 1 GHz are approached, exploitation of the TROSY phenomenon, and sophisticated pulse sequences. Even for small proteins, crystallography usually provides a shorter route to structural determination and, frankly, one that is more accessible to non-specialists than NMR, thanks in part to the admirable efforts of crystallographers to make their field novice-friendly. Moreover, hopes for new technical breakthroughs comparable in broad impact with the introduction of the Fourier transform, 2-D spectroscopy, or ^1H -detected multidimensional heteronuclear methods have not been realized. Of course, in addition to holding its position as the pre-eminent experimental method for studying biomolecular dynamics, NMR continued to occupy an important niche in structural determination of molecules that prove recalcitrant to the formation of well-diffracting crystals. Nevertheless, there has been much soul-searching the past decade about whether NMR represents an approach that is on the decline as a contributor of high impact results to basic biological and biomedical research.

It is the thesis of this review that, in fact, biomolecular solution NMR is undergoing a quiet renaissance. As in the Renaissance Age of western Europe, which was spurred in part by a renewed appreciation for Classical form and philosophy, recent progress is based partly on a renewed appreciation for the classical applications of NMR, in which total structural determination is only sometimes the goal, and the emphasis is on a wide range of problem-solving applications. And, just as the Renaissance was also driven by transformative humanism and accompanying advances in science and technology, NMR is being transformed by a series of innovations. While no single one of these technical developments may rise to the landmark status of, say, the introduction of the pulsed excitation methods, these innovations are in the process of enhancing and broadening the impact of NMR in biological research. In this paper, we highlight some of these technical innovations and offer examples of important applications. The focus is on *solution* NMR, with the authors noting that recent progress in solid state NMR has been spectacular, but beyond the scope of this review. The emphasis is also on protein-related work, but we hope the reader will seek out impressive recent applications of NMR to nucleic acids and glycans, work that relies on the same technical advances that are summarized here.^{9–16}

The Ability of NMR to Access Dilute Samples Has Been Dramatically Enhanced

Since the first NMR experiments over 50 years ago, there has been an impetus to obtain improved signal to noise (S/N) ratios (increased “sensitivity”). Even in the late 90s, the inherently low sensitivity of NMR spectroscopy dictated long acquisition times and large quantities of sample — typically at least 200 microliters of >0.5 mM protein. However, NMR has seen dramatic improvements in sensitivity during the past 15 years. One factor in this development has been the emergence of very high field (>600 MHz ^1H frequency) magnets, as NMR sensitivity is proportional to (field strength)^{3/2}. The largest currently-available NMR magnet suitable for use in biomolecular NMR is now 23.4 Tesla (1000 MHz ^1H frequency).

The emergence of superior probes for excitation and signal detection has also dramatically improved S/N in biomolecular NMR. Advances have been based on changing probehead/sample sizes and/or chilling key probe components. The sensitivity of an NMR probe is determined by its “quality factor” (Q), with the resulting S/N ratio being directly proportional to $Q^{1/2}$. The Q -factor for a probe is determined by the resonance frequency ω , the inductance L , and the resistance R of the probe¹⁷:

$$Q = \omega L / R \quad (1)$$

Q can be increased either by increasing inductance or by lowering resistance. The inductance is determined by the size and geometry of the coils, with the easiest way of increasing L being to reduce the size of the probehead. This has been exploited in the development of microcoil probes that, for a fixed concentration, allow improved sensitivity for dramatically reduced sample volumes.¹⁸ Decreasing the resistance has been accomplished by the development of “cryogenic probes” in which the probe detection coil and preamplifier are chilled to a very low temperature with helium gas. Cryogenic probes have the added benefit that cooling the preamplifier reduces the thermal noise in the system, allowing for even greater increases in sensitivity.¹⁷ Here we outline the capabilities of both microcoil and cryogenic probes and show examples of how they have improved NMR data collection.

Microcoil probes enhance NMR S/N and allow collection of data on samples with volumes as small as 5 μ L and only nanomoles of sample for $^{15}\text{N}/^{13}\text{C}$ -labeled proteins.^{19, 20} The use of microcoil technology also confers two distinct advantages besides low sample concentration and volume. The first is the ability to generate novel pulse sequences that exploit the enhanced radiofrequency power handling of solenoid coils relative to the saddle configuration.²¹ Another capability of microcoil probes is that they can be adapted for flow-through mode for use as an analytical detector in conjunction with liquid chromatography. An example of the use of microcoil probes is provided by NMR measurement of the translational diffusion coefficients of the β 2-adrenergic receptor, a G protein-coupled receptor (GPCR), in a variety of different micelles and mixed micelles.²² For these studies a 1 mm sample diameter microcoil probe was used, for which the sample volume was a mere 6 μ L.

The underpinning theory for cryogenic probe technology was presented the late 1970s by Hoult and Richards²³ and the first such probe was constructed in 1984.²⁴ Widespread access to cryogenic probes became common by the mid-2000s. Commercial cryogenic probes are now typically the “default” probe installed in spectrometers dedicated to biomolecular studies. For any given sample, cryogenic probes allow for a 3–4 fold increase in the S/N relative to a same-generation conventional probe. Since NMR experiments are based on averaging of the signals from accumulated scans, and the spectral S/N is proportional to the square root of the number of scans, this 3–4 fold increase in sensitivity correlates to a 9–16 fold decrease in the time required to achieve a desired S/N ratio.¹⁷

Many of the NMR-based advances in biological research during the past decade could not have been accomplished without the use of cryogenic probes. Shown in Figure 1 are ^1H , ^{15}N -TROSY spectra of the spectrum of the human visual arrestin protein, which binds to light-activated phosphorylated rhodopsin to shut off photo-signaling.²⁵ Rhodopsin is the GPCR that serves as the photoreceptor of mammalian vision. Spectra are shown for free monomeric v-arrestin (45 kDa) as a 10 μ M solution (Figure 1A), as well as for the complex of 30 μ M v-arrestin with a saturating concentration of light-activated and phosphorylated bovine rhodopsin (P-Rh*) in bicelles (ca. 200 kDa complex, Figure 1B). These 200

microliter samples contained only 0.1–0.3 mg of v-arrestin. It can be seen that many of the ^{15}N -v-arrestin resonances disappear following complex formation with unlabeled P-Rh*. Based on other NMR data it was concluded that this peak disappearance results from transition of the bound v-arrestin structure into a partially disordered structural state, resulting in extensive exchange line broadening (and peak disappearance). It has been hypothesized that this transition to a partially disordered state represents the structural change underlying activation of arrestins to initiate non-canonical signaling pathways, which are now known to occur when arrestins are activated for signaling upon engagement with phosphorylated active state GPCRs.^{25, 26} This result was absolutely dependent on access to very high field magnets and use of modern cryogenic probes.

Solution NMR studies of larger proteins have traditionally been limited to only those proteins that can be generated in uniformly ^{15}N - and ^{13}C -labeled forms. This excludes the many mammalian proteins that cannot be produced in functional form using methods that allow uniform isotopic enrichment (*E. coli*, methylotrophic yeast, and cell free expression). Cryogenic probes now enable the acquisition of ^1H -detected multinuclear NMR data using only natural abundance ^{13}C (present at 1%) and ^{15}N (0.4%).^{17, 27} While this currently requires high concentrations of samples, this is likely to gradually relax as probe technology continues to develop. For example the first combined cryogenic/micro-coil probe has recently become commercially available. The possibility that the routine need for NMR isotopic enrichment of proteins could be obviated is an attractive notion.

There Has Been a Resurgence in Use of Simple 1-D NMR Experiments to Tackle Biological Problems, Such As Signaling by GPCRs

While a number of recent advances are based on using NMR methods of formidable complexity, simple 1-D solution NMR methods can be used to address highly significant biological questions. A variation of this approach involves fluorine NMR. ^{19}F is a spin- $\frac{1}{2}$ nucleus with an NMR sensitivity that approaches that of protons, making it easy to obtain satisfactory signal-to-noise at short acquisition times even for dilute samples of large proteins and complexes. Moreover, ^{19}F NMR chemical shifts are highly sensitive to local environment, such that ^{19}F NMR generally yields well-resolved 1-D NMR spectra,^{28, 29} making ^{19}F an excellent reporter probe in binding studies or in studies of protein folding or conformational changes. ^{19}F probes can be attached to proteins by chemical modification of cysteine thiol sites³⁰ or via incorporation of labeled amino acids.^{29, 31, 32}

Recent collaboration between the Wüthrich and Stevens labs utilized ^{19}F NMR to probe biased signaling pathways of the β_2 -adrenergic receptor ($\beta_2\text{AR}$), a G protein coupled-receptor (GPCR). This work built on earlier ^{13}C NMR studies of this same system by the Kobilka lab.³³ Binding of various agonists to the $\beta_2\text{AR}$ induces a conformational change in the receptor that activates G protein association on the cytosolic face of the receptor. The G protein binding site includes the cytosolic end of transmembrane segment 6 (TM6). While agonists also induce changes in structure that impact the cytosolic end of TM7 and the membrane proximal region of the adjacent C-terminus, these segments are thought *not* to be directly involved in G-protein binding. Instead, agonists stimulate phosphorylation of juxtamembrane residues of the C-terminus that result in activation of association with β -arrestin, which can block G-protein association, leading to endocytosis and downregulation of the receptor. Induced changes in the structure of the bound β -arrestin can also activate various pathways of signal transduction in the cell. It is known that certain agonists stimulate G protein-based signaling vs. β -arrestin-based effects to different degrees, with those agonists that preferentially stimulate β -arrestin-binding/signaling being known as “biased agonists”.³⁴ To probe the underlying structural biology of biased agonism, certain $\beta_2\text{AR}$ cysteines (C265, C327, and C341) were labeled with an ^{19}F -containing thiol-reactive

reagent (Figure 2A).³⁵ C265 and C327 are located at the cytoplasmic ends of TM6 and TM7 respectively. C341 served as a control site, being located on the membrane-distal C-terminus where its local environment is insensitive to the β 2AR signaling state. Ca. 200 μ L samples were used, containing 10–20 μ M β 2AR in mixed micelles. The ^{19}F NMR spectra of both C265 and C327 of the unstimulated receptor each exhibited a pair of peaks that are believed to correspond to slowly interconverting activated and inactive states (Figure. 2B).³⁵ Not surprisingly, classical agonists shifted the population of the peaks for ^{19}F probes at both C265 (TM6) and C327 (TM7) to increase the active: inactive ratio (Figures 2B,C). Biased agonists induced a greater shift of the inactive-to-active ^{19}F peaks for C327 (at TM7) than was observed for C265. Remarkably, it can be seen that the ratios of the two states as judged by the peaks from ^{19}F at C265 vs. C327 are, for most compounds, not equal. These results suggest that there are at least three functional states: inactive, G-protein-coupling activated (reflected by a change in chemical shift for ^{19}F at C265), and β -arrestin binding-activated (reflected by a change of chemical shift at C327). Different compounds alter the relative populations between these states in distinct ways. This exemplifies the profound insight into protein dynamics and function that can sometimes be gleaned from even the most simple of NMR measurements.

Shimada and co-workers used ^{13}C to address the nature of “partial agonism”, whereby G-protein coupled receptors are activated by compounds that stimulate a level of signaling that is significantly lower (“reduced efficacy”) than that induced by “full agonists”.³⁶ For their work, β 2AR was expressed in insect cells using culture supplemented with $^{13}\text{CH}_3$ -*e*-methionine. 2-D ^1H , ^{13}C -HMQC spectra of 5–40 μ M receptor in its ligand-free state were compared to spectra acquired when the receptor was saturated by an inverse agonist, a neutral antagonist, a pair of partial agonists, or a full agonist. These spectra revealed that saturation of β 2AR by partial agonists tipped the equilibrium between the active state and a pair of inactive states towards the active state, but not to the extent of full agonists. No support was generated in this study for the notion that partial agonists result in lower efficacy because they convert the receptor into an active state that is both structurally and functionally distinct from the active state generated by full agonists.

In related work Kobilka and co-workers used a nearly identical NMR approach in studies of β 2AR, but focused on a somewhat different mechanistic question.³⁷ Their work showed a difference in the nature of the conformational state induced by binding of a full agonist relative to the case where the receptor was saturated with both a full agonist and a nanobody that mimics binding of β 2AR’s cognate G protein (G_s). They interpreted their data to indicate that full activation occurs only in the presence of both agonist and the G_s mimic. When saturated by the agonist alone, the receptor appears to be in a conformationally heterogeneous state that is distinct from the fully activated state. It may be this former state that is stabilized to various degrees by various partial agonists in the Shimada study.³⁶

The Use of NMR to Monitor Protein-Ligand and Protein-Protein Interactions Has Been Extended

NMR spectroscopy has long been used to characterize protein/ligand or protein-protein interactions^{38–40}, being well-suited for studies of weak interactions (K_d in the micro- to milli-molar range) where the lifetime of the complex formed is too short to isolate the complex via pull-down or other methods. The simplest and most commonly used method is to monitor changes in protein ^1H , ^{15}N -HSQC (or TROSY) resonance positions induced by titration of a ligand or another protein. This “differential chemical shift perturbation method” is based on the notion that NMR-active nuclei located at the binding interface usually undergo larger changes in chemical shift than resonances for distal sites. In ideal cases it is possible to map the location of the binding site on the protein based on shift

perturbation patterns and to determine binding affinity by monitoring the ligand concentration-dependence of observed changes in peak positions.

Our lab recently demonstrated how monitoring chemical shift perturbation can be an effective means of investigating the interaction between the membrane protein C99 and cholesterol. C99 is the single transmembrane span C-terminal domain of the amyloid precursor protein (APP) that is released by β -secretase cleavage of the full length APP. Cleavage of C99 by γ -secretase releases the amyloid- β polypeptides that are closely related to the etiology of Alzheimer's disease. Elevated levels of cholesterol promote amyloid- β production. While the mechanism for this effect is not well understood, it is thought that both β - and γ -secretase preferentially associate with cholesterol-rich membrane domains often referred to as "lipid rafts".⁴¹ Structural studies of C99 in detergent micelles^{42, 43} led us to hypothesize that this protein might have a cholesterol binding site. Following early studies in micelles that employed a water soluble cholesterol analog⁴² we switched to the use of isotropic lipid/detergent bicelles, which can solubilize *bona fide* cholesterol up to a concentration of 20 mol% (1 cholesterol for every 4 molecules of phospholipid and detergent). NMR experiments were used to monitor C99 in bicelles upon titration with cholesterol, revealing that it forms a 1:1 complex with C99 characterized by a K_d of 5 mol %⁴³, a value well within the physiological range of cholesterol in mammalian membranes. In addition to determining K_d , site-specific chemical shift perturbations mapped the location of the binding pocket, subsequently verified by mutagenesis studies (Figure 3A). The discovery that C99 is a cholesterol binding protein led the hypothesis that this binding event results in partitioning of C99 into lipid rafts, where the amyloidogenic β - and γ -secretase are preferentially localized⁴¹, thereby activating amyloid- β production. This offers a compelling explanation for how elevated cholesterol levels promote amyloidogenesis.

We have also used NMR to illuminate the mechanisms by which a family of potential anti-Alzheimer's drugs, known as γ -secretase modulators (GSMs), exert their potentially therapeutic effect—namely to selectively and favorably alter the γ -secretase production ratio between less toxic short forms of amyloid- β (such as A β 40) and much more toxic long forms, such as A β 42.⁴⁴ While it has been proposed that GSMs act by first forming a complex with C99 prior to association with γ -secretase^{45, 46}, a series of NMR experiments failed to yield any evidence that GSMs form a complex with monomeric or dimeric forms of C99 in micelles or model membranes^{47, 48}, an observation now supported by a number of biochemical studies from other labs.^{49, 50}

In addition to monitoring protein/small molecule interactions, NMR spectroscopy can monitor protein/protein interactions. We recently conducted a study of whether the transmembrane C99 can form a complex in model membranes with another single span membrane protein, CD147⁵¹, representative of a difficult problem in membrane biophysics.^{52, 53} Through mechanisms that are not well understood, CD147 is believed to reduce levels of amyloid- β in cell cultures.^{54, 55} In our studies a simple set of experiments were developed to classify intermolecular interactions as either (i) a specific and stoichiometric association, (ii) a non-specific association, or (iii) "forced co-habitation" whereby two molecules are forced to interact by virtue of being entrapped in the same model membrane unit (i.e., within the same micelle) (Figure 3B). These methods allowed us to establish that CD147 associates more avidly with monomeric C99 than C99 self-associates to form homodimers. Heterodimerization between C99 and CD147 does appear to be structurally specific, but is weak and may not be physiologically relevant. A final and groundbreaking example of using NMR to study a membrane protein complex is determination by the Ulmer and Qin labs of the structure of the heterodimeric transmembrane domain of the platelet integrin $\alpha_{IIb}\beta_3$ in bicelles.^{56, 57}

NMR Paramagnetic Relaxation Enhancement (PRE) Extends the Capabilities of NMR to Probe Protein Structure and Dynamics

Early in the development of NMR, the physics of nuclear relaxation enhancement arising from the presence of proximal paramagnetic species was explored.⁵⁸ NMR signals are broadened to a degree that is proportional to $1/r^6$, where r is the distance between the paramagnet and the NMR nucleus. This broadening stems from the “paramagnetic relaxation enhancement” (PRE) phenomenon. In biological NMR, paramagnets can be added to samples as free probes (such as Mn(II) and Gd(III) and their chelates) or can be attached to proteins either through metal ion coordination or by modifying free cysteines with thiol-reactive nitroxide spin labeled compounds such as MTSL ((*S*-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanethiosulfonate)).^{59–65} PRE of NMR sites can be quantitated using methods as simple as recording 1- or 2-D NMR spectra of matched samples, one containing a paramagnet and the other in which the paramagnet is absent or quenched to a diamagnetic form. The PRE effect can be detected over a broad range from approximately 0 to 25 Å for a proton experiencing relaxation enhancement from a nitroxide spin label. The distance can be extended to approximately 35 Å if a more powerful paramagnetic species is used (e.g. Mn²⁺).⁶⁶

The most common PRE measurements involve use of 2-D ¹H,¹⁵N-HSQC or TROSY to measure the distances between a nitroxide spin label fixed at a specific protein site and the backbone amide protons of the same protein, an experiment that can generate dozens of distances from a single pair of NMR spectra. The derived distances can be used as a source of restraints for structure determination.^{59, 67} Such measurements have proven especially important for structural studies of challenging molecules where only the backbone chemical shift assignments are available, as is commonly the case for integral membrane proteins.^{43, 62, 67–70}

Most recently, the use of the PRE effect has been extended to: aid in the NMR assignment process^{71, 72}, increase the sensitivity of NMR experiments⁷³, serve as a route to map intermolecular binding surfaces of macromolecular complexes^{74, 75}, elucidate the active/binding sites of proteins⁶², map the topology of membrane proteins⁴², and probe dynamic and sparsely populated states of macromolecules.^{63, 76, 77}

α-Synuclein is under intense study because of its propensity to aggregate and form Parkinson's disease-related fibrils. For many proteins involved in aggregation-based diseases such as Parkinson's and Alzheimer's, the mechanism of protein fibril formation is currently not well understood. The Baum group recently utilized PRE based experiments to probe the interchain interactions of the intrinsically disordered protein α-synuclein, leading to a new hypothesis for its mechanism of amyloid formation.⁷⁸ α-Synuclein has an uneven distribution of charged amino acids across its sequence. NMR-based PRE experiments were used to show that the transient encounter complexes of α-synuclein have a non-random distribution and that the properties of the transient encounter complex are pH dependent. Under acidic conditions, α-synuclein is estimated to be relatively charge-neutral and was shown to favor relatively strong parallel (tail-to-tail) interchain interactions, factors that may explain why fibril formation is faster at acidic pH values. On the other hand, at more neutral pH values α-synuclein is more highly charged and was shown to hetero-associate only weakly and with an anti-parallel (head-to-tail) interaction. The results from these PRE studies suggest both a potential mechanism for increased kinetics of α-synuclein fibrillization occurring at low pH (as in endosomes) and a qualitative mechanism for how the normally soluble protein may be rendered insoluble. This study highlights the high sensitivity of PREs to transient interactions and how they can provide insight into intrinsically disordered proteins that is hard to obtain using other methods.

Clore and coworkers have been instrumental in the exploration of sparsely populated macromolecular states with PREs.⁷⁷ Tang et al. probed the apo- and sugar-bound holo state of the maltose-binding protein (MBP) with PREs and compared the results to existing X-ray crystallography structures of these states.⁶³ The authors found that PRE measurements for sugar-bound MBP were consistent with the crystal structure of the protein-carbohydrate complex (Figure 4A). On the other hand, the PRE data for apo-MBP were characteristic of a rapidly exchanging mixture between an open state (captured in the apo-MBD crystal structure), and a rare and transient (ca. 5% population) structure (Figure 4B). The rare partially closed apo-MBP was characterized using the PRE data and shown to be a partially closed state that is distinct from either the open or closed (holo) crystal structures, but instead more closely resembles the conformation of the maltose-complexed holoprotein. It is likely that it is to this rare excited state that maltose initially binds, enabling induced fit to the stable holo form. These data expressly support the existence of a dynamic equilibrium that samples conformational space beyond the bound and apo structural forms of MBP and shows that carbohydrate binding results in an induced fit complex.

Methyl TROSY NMR Allows NMR to Probe Structural and Mechanistic Questions for Protein Complexes in Excess of 200 kDa

The $^1\text{H}/^{13}\text{C}$ NMR signals from protein side chain methyl groups provide a powerful approach to probe the structures, dynamics, and interactions of very large proteins or complexes, often providing a route to useful data under conditions where others (such as $^1\text{H},^{15}\text{N}$ -TROSY) fail.^{79–81} This stems from (i) the favorable relaxation properties and motions associated with side chain methyl ^1H and ^{13}C , (ii) the more favorable gyromagnetic ratio of ^{13}C compared to ^{15}N , (iii) the fact that there are three protons per methyl group, and (iv) the existence of favorable (slow) relaxation pathways that can be spectroscopically selected for using the classic HMQC pulse sequence originally developed in the late 70s.^{82–84} These properties have led to the HMQC-based “methyl-TROSY” family of multidimensional $^1\text{H}, ^{13}\text{C}$ -NMR experiments.

To take maximal advantage of methyl-TROSY-based approaches, it is necessary to label proteins with ^{13}C and preserve methyl protons for selected amino acids within a protein while at the same time perdeuterating all other hydrocarbon sites.⁷⁹ Methods for this are now available for all methyl-containing amino acid types.^{85–87} Along with these labeling methods a series of multidimensional NMR experiments have been developed that allow side chain methyl groups to be assigned based on correlation of their signals with already assigned backbone resonances.^{88–90} For very large proteins where backbone resonance assignments may not be available or feasible, mutagenesis can sometimes be used as a route to assign methyl peaks.^{91–93}

Methyl-TROSY methods have been applied to large proteins and complexes, such as malate synthase G (81.4 kDa)⁹⁴, aspartate transcarbamoylase (306 kDa)⁹⁵, SecA (204 kDa)⁹⁶, p53 tetramer/DNA complex (210 kDa)⁹⁷ and sensory rhodopsin in micelles (70 kDa protein-detergent complex).⁹⁸ Besides providing a route to structure determination, methyl-TROSY-based data can provide powerful insight into protein-protein interactions⁹⁹, protein-ligand interactions, protein dynamics⁹¹, and protein-DNA interaction.⁸⁶

In perhaps the most impressive example, Sprangers and Kay successfully applied methyl-TROSY methods to the 670 kDa 20S proteasome core particle, which consists of four heptameric rings arranged as $\alpha_7\beta_7\beta_7\alpha_7$.⁹¹ They were able to assign the $^1\text{H}-^{13}\text{C}$ resonances for ca. 90 of 97 ILV methyl groups present in the α subunits of this complex. A set of residues within the antechamber that separates the entrance from the catalytic site were seen to undergo correlated millisecond motion that may be related to threading of the substrate

into the proteolytic site. These studies also suggested that the N-terminal 12 amino acids can exchange between the outside of the protein assembly and the lumen of the antechamber, likely acting as an access gate (Figure 5).⁹¹ This was confirmed in a follow-up study in which the alpha subunit was labeled at its N-terminus with methyl-¹H/¹³C-methionine. The methionine methyl-labeled alpha subunit was also spin-labeled at various sites and PREs were measured to yield spin label/methionine distances, which confirmed that the N-terminus does indeed populate slowly exchanging (seconds time scale) outside and luminal configurations. Additional experiments established that when all 7 alpha N-termini are in the outside configuration, the proteasome is fully active, whereas activity is reduced when N-termini extend into the lumen of the proteasome, occluding substrate access to the active site.¹⁰⁰ Studies were also conducted of binding of the 11S activator to the 670 kDa $\alpha_7\beta_7\beta_7\alpha_7$ to form a 1100 kDa $\alpha_7\beta_7\beta_7\alpha_7$ -11S complex that mapped the 11S binding sites on the α_7 rings and also provided a measurement of binding affinity.⁹¹

Methyl-TROSY has also been successfully applied in membrane protein structure determination. The phototaxis receptor sensory rhodopsin II (pSRII) from *natronomonas pharaonis*, a seven transmembrane helix blue light phototactic receptor, functions via trans-cis isomerization of its retinal group, enabling its host to seek the dark when respiratory substrates are plentiful. As a membrane protein, Ile, Leu, and Val residues account for 32% of the pSRII sequence and are distributed throughout all seven helices. Using methyl-TROSY-based NMR, Nietlispach and co-workers successfully assigned numerous aliphatic methyl ¹H and ¹³C resonances and went on to measure a large number of distance restraints based on methyl-methyl and methyl-amide ¹H, ¹H NOEs.⁹⁸ This led to determination of the pSRII structure, which shows an rmsd for the backbone residues (1–221) of only 0.48 Å and is in excellent agreement with the crystal structure. Methyl-TROSY methods have also been applied in studies of other multi-span transmembrane proteins, including VDAC-1¹⁰¹¹⁰², KcsA¹⁰³ and KpOmpA.¹⁰⁴

Increased Use of NMR in Drug Discovery

The 2010 Pharma R&D Annual Review reported over 9000 drug candidates in various phases of the drug development pipeline (http://www.pharmaprojects.com/therapy_analysis/annual-review-2010.htm). However, despite the vast resources being devoted to drug development, only 30 novel new drugs were approved by the FDA in 2011. Moreover, the average cost of bringing a drug to the stage of approval approaches one billion dollars. An important early stage of drug development is lead compound discovery, a process that usually involves screening of large compound libraries. NMR methods have been developed for high throughput screening of compounds directed towards validated drug targets as a route to identifying leads.

Perhaps the highest impact of NMR in drug discovery has been the seminal role this method played in establishing an approach to lead compound development now usually referred to a “fragment based drug/lead discovery” (FBDD or FBLD).¹⁰⁵ The first practical implementation of this approach was dubbed “SAR by NMR” by Fesik and co-workers.^{106, 107} The original NMR-centric form of this approach requires that the structure of the target protein and its NMR resonance assignments be known. By monitoring protein resonances NMR is used to identify lead molecules that bind to spatially adjacent sites (often with only very modest efficiency), followed by synthesis of 2nd generation molecules in which two lead molecules are chemically tethered to each other with a linker of rationally chosen length. This generates a new lead compound whose affinity reflects the partial additivity of the binding energies of the original unlinked pair of ligands. (Figure 6A). The FBDD approach is being vigorously pursued in many drug discovery labs (sometimes using NMR, sometimes not) and has been used to develop at least 1 approved drug and 26

compounds in various stages of clinical trials.^{108,109} ABT-263 (Navitoclax, in phase II clinical trials) is an inhibitor of the anti-apoptotic (pro-survival) proteins Bcl-2 and Bcl-X_L and that was the outcome of NMR-based FBDD of two lead compounds that bind to the hydrophobic BH3-binding region of Bcl-X_L (Figure 6B). 4'-Fluoro-biphenyl-4-carboxylic acid and 5,6,7,8-tetrahydro-naphthalene were found by NMR to bind with modest affinity to different but proximal sites in a functionally significant domain of Bcl-X_L (K_d 0.3 mM and 4 mM, respectively). Covalently linking the two lead compounds resulted in dramatically increased binding affinity for Bcl-X_L (K_i = 36 nM). This was followed by more traditional medicinal chemical optimization to generate the final ABT-263, which has a K_i of 1 nM towards both Bcl-2 and Bcl-X_L.^{110, 111}

NVP-AUY922 is an inhibitor of the ATPase activity of the chaperone Hsp90 and is currently in Phase II clinical trials for the treatment of cancer. This compound was developed using FBDD relying on a second class of NMR screening methods based on detection of signal from the candidate ligands being screened against a target (Figure 6A).³⁸ Ligand-detected NMR screening can employ a variety of readouts, including simple observation of ligand peak disappearance (indicating tight binding to a very large or even insoluble target), peak shifts or line-broadening, magnetization transfer from a possibly NMR-invisible target to a ligand, or observation of a change in the translational diffusion coefficient of a molecule. In developing NVP-AUY922 over 1000 fragments were initially tested for binding to 10uM Hsp90 using ligand-based NMR screening experiments, resulting in a number of resorcinol- or phenol-containing candidate fragments. Subsequent determination of the crystal structure of the complexes between the fragments and Hsp90, followed by fragment linkage and further optimization resulted in NVP-AUY922.^{112, 113}

An interesting recent variation of ligand-detected NMR screening is target immobilized NMR screening (TINS), which has proven to be suitable for fragment-based drug discovery targeting membrane proteins. In TINS, a target and a negative control reference protein (both bathed in model membranes such as nanodiscs) are immobilized on a solid support in separate flow-through NMR tubes. The tubes are then loaded into a dual cell, flow-injection sample holder located within a special NMR probe.¹¹⁴ The mixture of compounds to be screened is simultaneously injected into both tubes. 1D ¹H NMR spectra of the small molecule mixture from both NMR tubes are obtained by performing space-selective spectroscopy.¹¹⁵ If a small molecule binds to the immobilized target, its peak amplitude decreases (relative to the reference sample) because the peak from the bound state became undetectably broad due to association with the immobilized target. It is possible to repeatedly use the same immobilized membrane protein sample to screen an entire fragment collection (>1000 compounds), such that only ~25 nmoles of membrane protein may be required to complete an initial screen (1 mg for a 40 kDa protein).¹¹⁶

NMR Can Now Be Used to Probe Protein Structure and Interactions in Living Cells

Recently, successful experiments to study proteins under truly physiological conditions have been reported.^{117, 118} “In-cell” NMR spectroscopy utilizes isotopically labeled proteins to selectively observe the protein of interest over numerous other cellular components. Various protein labeling and/or delivery methodologies have been developed. One method is the delivery of isotopically labeled recombinant proteins into oocytes using microinjection^{119–122} of up to ca. 20 nanoliters per egg. This method allows direct control of the concentration of the delivered protein. Another approach for introducing proteins into eukaryotic cells is to tag the protein of interest with a “cell penetrating peptide” (CPP) sequence. This enables the transfer of the protein from the extracellular medium to the cell interior via penetration of the cellular membrane.^{122–124} Often, pore forming toxins are

used, such as the CPP sequences.¹²⁵ Another approach for introducing labeled protein into living cells is known as “single protein production” (SPP). SPP is based on first growing *E. coli* in unlabeled medium, followed by triggering degradation of all the mRNA in the cells except for that encoding the target protein. The cells are then transferred to a medium containing NMR-active isotopes and protein expression is induced, resulting in isotopic labeling the target protein in a cellular background of unlabeled proteins.¹²⁶ Expression of isotopically labeled proteins in yeast and subsequent collection of NMR spectra of these proteins *in situ* have recently been reported.¹²⁷

The first structure of a protein determined in live cells was reported by Ito and co-workers^{128, 129} The putative heavy metal binding protein TTHA1718 was expressed in *E. coli* using the SPP method. The intact cells containing labeled TTHA1718 were then transferred to NMR tubes, followed by resonance assignments and structure determination. Rapid data collection using non-linear sampling and selective protonation of aliphatic methyl groups (in otherwise deuterated protein) was crucial for the success of this effort. The structure determined in cells was similar to that of the purified protein, although modest differences were seen in the conformation of solvent-exposed loops.

In the case of eukaryotic cells, TROSY-based NMR spectra of reasonably high quality have been obtained from labeled protein that was microinjected into *Xenopus* oocytes. Selenko et al.¹¹⁹ compared the NMR spectra of the recombinant ¹⁵N-GB1 domain in isolation, in oocyte cell extracts, and injected inside oocytes, revealing similar spectra. Chemical shifts of this protein in oocytes were very similar to those of purified protein but some of the peaks were distorted or split, indicating some structural heterogeneity generated by the cellular milieu.

The ability of NMR to study the specific interactions of drugs with proteins in cells has also been documented. The interaction between the immunosuppressants FK-506 and rapamycin with the protein FKBP12 has been observed using in-cell NMR.¹²² ¹⁵N-labeled FKBP12 was introduced into HeLa cells using a CPP construct. Changes in its ¹H, ¹⁵N-HSQC NMR spectra were observed when FK506 or rapamycin were administered to the cells that were similar to the changes induced when purified FKBP12 was titrated with these compounds. This work serves as a prototype for future studies of protein-ligand interactions using NMR spectroscopy of mammalian cells.

In-cell NMR remains in its infancy, but clearly provides a powerful approach to study proteins and their interactions in a truly native environment. Interesting applications are already beginning to be reported. For example, the Pielak lab has reported the surprising observation that the crowded cell cytosol does not provide stabilization of a marginally stable protein relative to its stability as a pure protein in standard buffer solutions.¹³⁰ In another example, Shekhtman and co-workers expressed the FKBP and FRB proteins in *E. coli*. In higher organisms these proteins form a ternary complex with rapamycin that leads to cell cycle arrest. NMR was then used to screen a 289 member dipeptide library to discover compounds that could both permeate the cells and mimic rapamycin by forming a ternary complex with FKBP and FRB. One of the dipeptides identified through this screen was then shown to be able to inhibit growth of yeast in a rapamycin-like manner by interacting with the yeast homologs to FKBP AND FRB.¹³¹

Cell Free Expression Methods Expand the Applicability and Efficiency of NMR

Cell free (CF) expression systems are based on use of extracted or purified transcription and translation machinery in cellular extracts from wheat germ or *E. coli*.^{132–135} The most

important impact of these methods on NMR spectroscopy has been to enable isotopic labeling schemes that are impossible or impractical using cellular expression methods.^{136–140} Access to these advanced labeling methods can be critical to tackling difficult problems in protein structure and function.

One of the common hurdles to backbone resonance assignments for helical MPs is that their transmembrane sequences are typically dominated by only six types of amino acids, which can result in near-degeneracy in the spin connectivity patterns of the multidimensional NMR data sets used to make resonance assignments. To overcome this obstacle, an optimized combinatorial dual-isotope labeling method has been developed in conjunction with CF expression to facilitate rapid resonance assignments¹³⁷ (as an extension of a previous site-selective screening method).¹⁴⁰ In this method, each sample is prepared using a subset of site-selectively carbonyl ¹³C- and amide ¹⁵N-labeled amino acids using CF expression. The backbone residues can then be identified according to peak patterns in HSQC and HNCOSY spectra.¹³⁷ Software (MCCL; <http://sbl.salk.edu/combipro>) is available to optimize the labeling schemes and to minimize both the number of required samples and the complexity of the spectra.¹³⁷ The combination of this labeling method and CF expression system was initially employed by Choe and co-workers to determine the membrane domain backbone structures of three receptor histidine kinases (ArcB, QseC and KdpD)¹³⁷ containing multiple transmembrane helices. This led to the now plausible, but once unthinkable, suggestion that high throughput structural determination may be possible for small and medium-sized multispan integral membrane proteins. More recently, this approach resulted in determination of the backbone structures of six additional human integral membrane proteins.¹⁴¹

Work from the Dötsch lab shows how CF expression can be used to further enhance the study of MP's when roadblocks are reached with traditional methods. Proteorhodopsin is a retinal-binding heptahelical membrane protein that functions as a proton pump in marine bacterium. Although almost all backbone NH resonances of proteorhodopsin were detected in diheptanoylphosphatidylcholine micelle conditions, this protein still yielded poorly resolved NMR spectra, making resonance assignments difficult and hindering measurements of NOE-derived distance restraints. Employment of the SAIL (Stereo-Array Isotope Labeling) method¹³⁹ that uses synthetic stereoisotopically-labeled amino acids in combination with CF expression provided significantly simplified spectra and facilitated partial completion of side-chain assignments for residues in the transmembrane domain. Together with measured long-range NOEs, PREs and RDCs, the assignments enabled determination of the first ever proteorhodopsin structure¹⁴².

Relaxation Dispersion Provides Unprecedented Access to Cryptic Structural and Dynamic States

Many biomacromolecules are intrinsically flexible and characterizing their dynamics is key to understanding their mechanisms of function in diverse biological processes. NMR is unparalleled as a tool to study macromolecular dynamics because it can provide atomic resolution information on motions occurring over all timescales.^{143–145}

Among the most important recent developments in NMR has been the widespread application of relaxation dispersion experiments that can simultaneously probe both structure and dynamics.¹⁴⁶ In relaxation dispersion experiments contributions to the NMR spin-spin relaxation time (T_2) from conformational transitions occurring on the micro- to millisecond timescale are modulated as a function of the frequency of the field generated by Carr–Purcell–Meiboom–Gill (CPMG) refocusing or spin-lock pulse trains. Information about these conformational transitions can be determined from plots of T_2 versus field

strength for each peak.¹⁴⁴ In cases where two (and sometimes more) conformations are in equilibrium, relaxation dispersion experiments can provide the exchange rate, the relative populations, and the chemical shift differences between exchanging states of the molecule. In favorable cases, it is also possible to measure residual dipolar coupling¹⁴⁷ and residual chemical shift anisotropies¹⁴⁸ for each state. Such data can illuminate the kinetics of the transitions between states, the equilibrium constant, and the structures of the molecule in each state, even when one is only marginally populated. NMR relaxation dispersion methods have been widely used in studies of RNA.^{9, 10}

Relaxation dispersion methods have contributed tremendously to the recent recognition of how dynamics critically impact enzyme mechanisms, including development of the concepts of “dynamic coupling” of steps along reaction pathways, and “conformational selection” by ligands of dynamically-sampled excited states that correspond to the structure of the protein in the complex to be formed.^{146, 149} A classic example is provided by studies of dihydrofolate reductase (DHFR) by the labs of Wright, Dyson, Lee, and others.^{150–152} DHFR catalyzes the reduction of 7,8-dihydrofolate (DHF) to 7,8-tetrahydrofolate (THF) by the cofactor NADPH and is a key enzyme in the folate biosynthetic pathway that is important for cell growth and proliferation. DHFR’s reaction pathway contains 5 distinct complexes with different combinations of bound substrates and products (see Figure 7). Relaxation dispersion NMR studies have shown that DHFR dynamics on the μ s-ms timescale are closely coupled to the progression of its catalytic pathway. It has been observed that for each complex along the reaction cycle, the enzyme dynamically samples “excited” states that have conformations resembling those of the adjacent complex states. Thus, substrate recognition and binding occurs primarily via the conformational selection mechanism (rather than by lock-and-key or induced-fit). Moreover, product dissociation is promoted by the fact that the free state conformations of the product binding sites are dynamically sampled even while the product is still bound. The rates of both chemical and binding steps typically correlate well with the rates of interconversion between the relevant dynamically coupled states (see Figure 7).

Another pioneering application of NMR relaxation dispersion is the study of the intermediates in protein folding and aggregation. Accessing the structures of these intermediates has always been difficult, with NMR-based amide H-D exchange rate measurements being perhaps the most powerful of the classical approaches for probing the structures of these states.¹⁵³ Relaxation dispersion methods have recently taken studies of folding intermediates to a higher level of insight. The Kay group determined the structure of a folding intermediate for a mutant form of the Fyn SH3 domain using NMR relaxation dispersion spectroscopy.¹⁵⁴ Under the equilibrium conditions of the NMR experiments, this intermediate is populated at only 2%. The backbone chemical shift assignments ($^1\text{H}_\text{N}$, $^1\text{H}_\alpha$, ^{15}N , ^{13}CO , $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$), the ^{13}CO residual chemical shift anisotropies, and the backbone amide ^{15}N - ^1H residual dipolar couplings of this folding intermediate state of mutant Fyn SH3 domain were extracted from a set of relaxation dispersion data. The structure of the intermediate was then calculated based on these data. Native and intermediate state structures and their propensities to aggregate^{154, 155} are illustrated in Figure 8. In the folded ground state, the mutant Fyn SH3 domain shows an incomplete β barrel structure with 5 β strands, among which the C-terminal β 5 strand undergoes hydrogen bonding with the N-terminal β 1 strand. In the folding intermediate state, the C-terminus (including the residues that comprise β 5) is disordered, which potentially makes the β 1 strand available as a template for aggregation via formation of inappropriate intermolecular β -sheet formation. However, this normally occurs only rarely because of the short lifetime and low population of this intermediate state. The role of the C-terminal β 5 strand in the long-lived folded state therefore seems to be to prevent the formation of the aggregates. This was confirmed by the finding that removal of 4 residues from the C-terminus prevents

formation of the $\beta 5$ strand and leads to spontaneous formation of fibrillar aggregates. This work illustrates both how folding rates are tuned so that aggregation is avoided in an otherwise susceptible folding intermediate state, as well as how the conformation of the folded state has been optimized to include structural elements that play roles to suppress aggregation.

NMR Has Provided Seminal Insight into Intrinsically Disordered Proteins and Tethered Multidomain Proteins

NMR spectroscopy has proven to be a powerful tool for characterizing intrinsically-disordered proteins (IDPs), partly because of its ability to interrogate protein structure and dynamics at both very local (residue-specific) and global scales.¹⁵⁶ The finding that many peptide hormones normally adopt fully or partially disordered conformations was demonstrated by ¹H NMR spectroscopy in early the 1970s.¹⁵⁷ However, that numerous larger proteins are intrinsically disordered long escaped recognition, reflecting the implicit assumption that disordered proteins must represent non-native and non-functional forms. Seminal work in the 1990s^{158, 159} led to the realization that many proteins are designed by nature to be intrinsically disordered in a way that is closely linked to native function and intermolecular interactions.^{160–163} That these domains and proteins often have very specific functions and propensities to form ordered structures upon complex formation with proteins or other molecules is now a well-established paradigm in molecular biology.¹⁶⁴

Because of its ability to inform on both macromolecular conformations and dynamics, NMR has been the primary technique used to discover and study IDPs, both in their free state and after complex formation. It has been revealed that IDPs and proteins with natively-disordered domains play a dazzling number of biological roles and that their conformational behavior is rich and varied. For example, it is now appreciated that even complex formation between two proteins may not reflect only a single mode of interaction, but may instead represent a composite of exchanging binding modes, each of which in isolation is of only low affinity, but which sum as a dynamic equilibrium to yield much higher overall affinity. Forman-Kay, Mittag, and co-workers have shown this to be the case for complex formation between the multi-phosphorylated Sic1 protein (a cyclin-dependent kinase inhibitor) and the WD40 domain of the Cdc4 component of the SCF^{Cdc4} ubiquitin ligase (Figure 9).^{165–167} There is only a single primary phosphate binding site in Cdc4/WD40 and it has been shown that this site can engage serially with different phosphates on Sic1, with the unliganded phosphates possibly contributing additional electrostatic stabilization. The resulting complex is dynamic both in the sense that the composite affinity between Sic1 and Cdc4 reflects the partial summation of the affinities for the various exchanging binding modes and also because Sic1 remains largely disordered even after complex formation with Cdc4. By altering the extent and patterns of phosphorylation, cell biology may be able to tune binding affinity and specificity to achieve a dynamic range and complexity of binding/response behavior that extends far beyond what is possible with simple two-state regulation.¹⁶⁸ It seems likely that there are other entirely new paradigms involving protein disorder and biological complexity waiting to be discovered.¹⁶⁹ The importance of IDPs in biology and the role that NMR has played in discovering and characterizing these proteins are difficult to overstate.

In the case of multi-domain proteins, disordered regions between domains facilitate modular domain behavior and inter-domain flexibility. High quality NMR spectra can often be obtained even for very large multi-domain proteins because of tether-enabled domain motions. For example, in studies of the replication protein A, Chazin and co-workers used NMR to pinpoint the sequential locations of tethered and folded domains and to characterize the dynamics and relative orientations of the tethered domains.¹⁷⁰ Veenhoff and her

coworkers used 1D ^1H spectra and 2D ^1H - ^{13}C HSQC spectra in their investigation of the pathway traveled by the Heh2 integral membrane protein from the outer nuclear membrane to reach the inner nuclear membrane.¹⁷¹ This study revealed that Heh2 remains membrane-associated and diffuses from one membrane domain to the other through the linking membrane that lines the orifice of the nuclear pore complex. The transmembrane domain of Heh2 is connected to membrane-distal domains that include its nuclear localization sequences (NLS) by a 150 residue linker (L) domain. Simple NMR methods were used to show that the L domain is an intrinsically disordered protein. The L domain appears to play a critical role in the transport of Heh2 through the nuclear pore by providing a flexible tether to the membrane-distal domains that allows these domains to clear the core scaffold of the pores and to be recognized by trafficking partner proteins. L domain flexibility also allows these distal Heh2 domains and their associated partner proteins to bob and weave as they associate with successive FG-Nup proteins in the process of making their way through the jungle-like matrix of the nuclear pore.

Conclusions

We have surveyed technical advances that, in concert with progress in sample preparation, are transforming the applicability of NMR to proteins. This has led to recent NMR application to problems of great significance, such as studies of GPCR and proteasome function-structure relationships. Other applications have thus far been prototype in nature, such as the first applications of NMR to specific proteins in living cells. Other emerging methods not covered in this review, such as non-classical ways of collecting and processing pulsed NMR data^{172–174} can also be expected to further expand the problem-solving capability of solution NMR, particularly if such approaches can be widely implemented in user-friendly form. While soothsaying is perilous, all indicators point to a bright future for NMR as a tool for studying protein structure, folding, dynamics, interactions, and function.

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References

1. Saunders M, Wishnia A, Kirkwood JG. THE NUCLEAR MAGNETIC RESONANCE SPECTRUM OF RIBONUCLEASE1. *Journal of the American Chemical Society*. 1957; 79:3289–3290.
2. Ernst RR. Nuclear Magnetic Resonance Fourier Transform Spectroscopy (Nobel Lecture). *Angewandte Chemie International Edition in English*. 1992; 31:805–823.
3. Jardetzky, OR.; Kenmure, Gordon Carl. *Nuclear Magnetic Resonance in Molecular Biology*. Academic Press; New York: 1981.
4. Wuthrich, K. *NMR of Proteins and Nucleic Acids*. John Wiley & Sons; New York: 1986.
5. Bax A. Two-Dimensional NMR and Protein Structure. *Annu Rev Biochem*. 1989; 58:223–256. [PubMed: 2673010]
6. Wagner G, Hyberts SG, Havel TF. NMR Structure Determination in Solution: A Critique and Comparison with X-Ray Crystallography. *Annual Review of Biophysics and Biomolecular Structure*. 1992; 21:167–198.
7. Clore GM, Gronenborn AM. Two-, Three-, and Four-Dimensional NMR Methods for Obtaining Larger and More Precise Three-Dimensional Structures of Proteins in Solution. *Annual Review of Biophysics and Biophysical Chemistry*. 1991; 20:29–63.

8. Ikura M, Kay LE, Bax A. A novel approach for sequential assignment of proton, carbon-13, and nitrogen-15 spectra of larger proteins: heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin. *Biochemistry*. 1990; 29:4659–4667. [PubMed: 2372549]
9. Dethoff EA, Petzold K, Chugh J, Casiano-Negroni A, Al-Hashimi HM. Visualizing transient low-populated structures of RNA. *Nature*. 2012; 491:724–728. [PubMed: 23041928]
10. Bothe JR, Nikolova EN, Eichhorn CD, Chugh J, Hansen AL, Al-Hashimi HM. Characterizing RNA dynamics at atomic resolution using solution-state NMR spectroscopy. *Nat Meth*. 2011; 8:919–931.
11. Lu K, Heng X, Garyu L, Monti S, Garcia EL, Kharytonchyk S, Dorjsuren B, Kulandaivel G, Jones S, Hiremath A, Divakaruni SS, LaCotti C, Barton S, Tummlillo D, Hosic A, Edme K, Albrecht S, Telesnitsky A, Summers MF. NMR Detection of Structures in the HIV-1 5'-Leader RNA That Regulate Genome Packaging. *Science*. 2011; 334:242–245. [PubMed: 21998393]
12. Latham MP, Brown DJ, McCallum SA, Pardi A. NMR Methods for Studying the Structure and Dynamics of RNA. *ChemBioChem*. 2005; 6:1492–1505. [PubMed: 16138301]
13. Rinnenthal J, Buck J, Ferner J, Wacker A, Urtig FB, Schwalbe H. Mapping the Landscape of RNA Dynamics with NMR Spectroscopy. *Accounts of Chemical Research*. 2011; 44:1292–1301. [PubMed: 21894962]
14. Zhang Q, Kim N-K, Feigon J. Architecture of human telomerase RNA. *Proceedings of the National Academy of Sciences*. 2011; 108:20325–20332.
15. Dominguez C, Schubert M, Duss O, Ravindranathan S, Allain FHT. Structure determination and dynamics of protein–RNA complexes by NMR spectroscopy. *Progress in Nuclear Magnetic Resonance Spectroscopy*. 2011; 58:1–61. [PubMed: 21241883]
16. Barb AW, Prestegard JH. NMR analysis demonstrates immunoglobulin G N-glycans are accessible and dynamic. *Nature chemical biology*. 2011; 7:147–153.
17. Kovacs H, Moskau D, Spraul M. Cryogenically cooled probes—a leap in NMR technology. *Progress in Nuclear Magnetic Resonance Spectroscopy*. 2005; 46:131–155.
18. Webb A. Nuclear magnetic resonance of mass-limited samples using small RF coils. *Analytical and Bioanalytical Chemistry*. 2007; 388:525–528. [PubMed: 17340087]
19. Li Y, Webb AG, Saha S, Brey WW, Zachariah C, Edison AS. Comparison of the performance of round and rectangular wire in small solenoids for high-field NMR. *Magnetic Resonance in Chemistry*. 2006; 44:255–262. [PubMed: 16477681]
20. Aramini JM, Rossi P, Anklin C, Xiao R, Montelione GT. Microgram-scale protein structure determination by NMR. *Nature methods*. 2007; 4:491–493. [PubMed: 17496898]
21. Peti W, Norcross J, Eldridge G, O'Neil-Johnson M. Biomolecular NMR Using a Microcoil NMR Probe – New Technique for the Chemical Shift Assignment of Aromatic Side Chains in Proteins. *Journal of the American Chemical Society*. 2004; 126:5873–5878. [PubMed: 15125680]
22. Horst R, Stanczak P, Stevens RC, Wuthrich K. β 2-Adrenergic Receptor Solutions for Structural Biology Analyzed with Microscale NMR Diffusion Measurements. *Angewandte Chemie International Edition*. 2013; 52:331–335.
23. Hoult DI, Richards RE. The signal-to-noise ratio of the nuclear magnetic resonance experiment. *Journal of Magnetic Resonance*. 2011; 213:329–343. [PubMed: 22152352]
24. Styles P, Soffe NF, Scott CA, Cragg DA, Row F, White DJ, White PCJ. A high-resolution NMR probe in which the coil and preamplifier are cooled with liquid helium. *Journal of Magnetic Resonance*. 2011; 213:347–354. [PubMed: 22152353]
25. Zhuang T, Chen Q, Cho M-K, Vishnivetskiy SA, Iverson TM, Gurevich VV, Sanders CR. Involvement of distinct arrestin-1 elements in binding to different functional forms of rhodopsin. *Proceedings of the National Academy of Sciences*. 2013; 110:942–947.
26. Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by β -arrestins. *Science*. 2005; 308:512–517. [PubMed: 15845844]
27. Tian F, Valafar H, Prestegard JH. A Dipolar Coupling Based Strategy for Simultaneous Resonance Assignment and Structure Determination of Protein Backbones. *Journal of the American Chemical Society*. 2001; 123:11791–11796. [PubMed: 11716736]
28. Danielson MA, Falke JJ. Use of ^{19}F NMR to Probe Protein Structure and Conformational Changes. *Annual Review of Biophysics and Biomolecular Structure*. 1996; 25:163–195.

29. Gerig J. Fluorine NMR of proteins. *Progress in Nuclear Magnetic Resonance Spectroscopy*. 1994; 26:293–370.
30. Kitevski-LeBlanc JL, Prosser RS. Current applications of ^{19}F NMR to studies of protein structure and dynamics. *Progress in Nuclear Magnetic Resonance Spectroscopy*. 2012; 62:1–33. [PubMed: 22364614]
31. Jackson JC, Hammill JT, Mehl RA. Site-Specific Incorporation of a ^{19}F -Amino Acid into Proteins as an NMR Probe for Characterizing Protein Structure and Reactivity. *Journal of the American Chemical Society*. 2007; 129:1160–1166. [PubMed: 17263397]
32. Shi P, Wang H, Xi Z, Shi C, Xiong Y, Tian C. Site-specific ^{19}F NMR chemical shift and side chain relaxation analysis of a membrane protein labeled with an unnatural amino acid. *Protein Science*. 2011; 20:224–228. [PubMed: 21080424]
33. Bokoch MP, Zou Y, Rasmussen SGF, Liu CW, Nygaard R, Rosenbaum DM, Fung JJ, Choi H-J, Thian FS, Kobilka TS, Puglisi JD, Weis WI, Pardo L, Prosser RS, Mueller L, Kobilka BK. Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature*. 2010; 463:108–112. [PubMed: 20054398]
34. Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, Mailman RB. Functional Selectivity and Classical Concepts of Quantitative Pharmacology. *Journal of Pharmacology and Experimental Therapeutics*. 2007; 320:1–13. [PubMed: 16803859]
35. Liu JJ, Horst R, Katritch V, Stevens RC, Wuthrich K. Biased Signaling Pathways in β_2 -Adrenergic Receptor Characterized by ^{19}F -NMR. *Science*. 2012; 335:1106–1110. [PubMed: 22267580]
36. Kofuku Y, Ueda T, Okude J, Shiraishi Y, Kondo K, Maeda M, Tsujishita H, Shimada I. Efficacy of the β_2 -adrenergic receptor is determined by conformational equilibrium in the transmembrane region. *Nat Commun*. 2012; 3:1045. [PubMed: 22948827]
37. Nygaard R, ZY, Dror RO, Mildorf TJ, Arlow DH, Manglik A, Pan AC, Liu CW, Fung JJ, Bokoch MP, Thian FS, Kobilka TS, Shaw DE, Mueller L, Prosser RS, Kobilka BK. The Dynamic Process of β_2 -Adrenergic Receptor Activation. *Cell*. 2013 (in Press).
38. Lepre CA, Moore JM, Peng JW. Theory and Applications of NMR-Based Screening in Pharmaceutical Research. *Chemical Reviews*. 2004; 104:3641–3676. [PubMed: 15303832]
39. Zuiderweg ERP. Mapping Protein–Protein Interactions in Solution by NMR Spectroscopy. *Biochemistry*. 2001; 41:1–7. [PubMed: 11771996]
40. Skinner AL, Laurence JS. High-field solution NMR spectroscopy as a tool for assessing protein interactions with small molecule ligands. *Journal of Pharmaceutical Sciences*. 2008; 97:4670–4695. [PubMed: 18351634]
41. Beel AJ, Sakakura M, Barrett PJ, Sanders CR. Direct binding of cholesterol to the amyloid precursor protein: An important interaction in lipid–Alzheimer’s disease relationships? *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2010; 1801:975–982.
42. Beel AJ, Mobley CK, Kim HJ, Tian F, Hadziselimovic A, Jap B, Prestegard JH, Sanders CR. Structural Studies of the Transmembrane C-Terminal Domain of the Amyloid Precursor Protein (APP): Does APP Function as a Cholesterol Sensor? *Biochemistry*. 2008; 47:9428–9446. [PubMed: 18702528]
43. Barrett PJ, Song Y, Van Horn WD, Hustedt EJ, Schafer JM, Hadziselimovic A, Beel AJ, Sanders CR. The Amyloid Precursor Protein Has a Flexible Transmembrane Domain and Binds Cholesterol. *Science*. 2012; 336:1168–1171. [PubMed: 22654059]
44. Weggen S, Eriksen JL, Sagi SA, Pietrzik CU, Ozols V, Fauq A, Golde TE, Koo EH. Evidence That Nonsteroidal Anti-inflammatory Drugs Decrease Amyloid β_2 Production by Direct Modulation of γ -Secretase Activity. *Journal of Biological Chemistry*. 2003; 278:31831–31837. [PubMed: 12805356]
45. Kukar TL, Ladd TB, Bann MA, Fraering PC, Narlawar R, Maharvi GM, Healy B, Chapman R, Welzel AT, Price RW, Moore B, Rangachari V, Cusack B, Eriksen J, Jansen-West K, Verbeeck C, Yager D, Eckman C, Ye W, Sagi S, Cottrell BA, Torpey J, Rosenberry TL, Fauq A, Wolfe MS, Schmidt B, Walsh DM, Koo EH, Golde TE. Substrate-targeting gamma-secretase modulators. *Nature*. 2008; 453:925–929. [PubMed: 18548070]

46. Botev A, Munter L-M, Wenzel R, Richter L, Althoff V, Ismer J, Gerling U, Weise C, Koksche B, Hildebrand PW, Bittl R, Multhaup G. The Amyloid Precursor Protein C-Terminal Fragment C100 Occurs in Monomeric and Dimeric Stable Conformations and Binds γ -Secretase Modulators. *Biochemistry*. 2010; 50:828–835. [PubMed: 21186781]
47. Beel AJ, Barrett P, Schnier PD, Hitchcock SA, Bagal D, Sanders CR, Jordan JB. Nonspecificity of Binding of γ -Secretase Modulators to the Amyloid Precursor Protein. *Biochemistry*. 2009; 48:11837–11839. [PubMed: 19928774]
48. Barrett PJ, Sanders CR, Kaufman SA, Michelsen K, Jordan JB. NSAID-Based γ -Secretase Modulators Do Not Bind to the Amyloid- β Polypeptide. *Biochemistry*. 2011; 50:10328–10342. [PubMed: 21995415]
49. Czirr E, Leuchtenberger S, Dorner-Ciossek C, Schneider A, Jucker M, Koo EH, Pietrzik CU, Baumann K, Weggen S. Insensitivity to A β 42-lowering Nonsteroidal Anti-inflammatory Drugs and γ -Secretase Inhibitors Is Common among Aggressive Presenilin-1 Mutations. *Journal of Biological Chemistry*. 2007; 282:24504–24513. [PubMed: 17573346]
50. Takahashi Y, Hayashi I, Tominari Y, Rikimaru K, Morohashi Y, Kan T, Natsugari H, Fukuyama T, Tomita T, Iwatsubo T. Sulindac Sulfide Is a Noncompetitive γ -Secretase Inhibitor That Preferentially Reduces A β 42 Generation. *Journal of Biological Chemistry*. 2003; 278:18664–18670. [PubMed: 12637581]
51. Zhuang T, Jap BK, Sanders CR. Solution NMR Approaches for Establishing Specificity of Weak Heterodimerization of Membrane Proteins. *Journal of the American Chemical Society*. 2011; 133:20571–20580. [PubMed: 22084929]
52. Wu Y, Shih SCC, Goto NK. Probing the structure of the Ff bacteriophage major coat protein transmembrane helix dimer by solution NMR. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 2007; 1768:3206–3215.
53. Kobus FJ, Fleming KG. The GxxxG-containing transmembrane domain of the CCK4 oncogene does not encode preferential self-interactions. *Biochemistry*. 2005; 44:1464–1470. [PubMed: 15683231]
54. Zhou S, Zhou H, Walian PJ, Jap BK. CD147 is a regulatory subunit of the γ -secretase complex in Alzheimer's disease amyloid β -peptide production. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:7499–7504. [PubMed: 15890777]
55. Vetrivel KS, Zhang X, Meckler X, Cheng H, Lee S, Gong P, Lopes KO, Chen Y, Iwata N, Yin K-J, Lee J-M, Parent AT, Saido TC, Li Y-M, Sisodia SS, Thinakaran G. Evidence That CD147 Modulation of β -Amyloid (A β) Levels Is Mediated by Extracellular Degradation of Secreted A β . *Journal of Biological Chemistry*. 2008; 283:19489–19498. [PubMed: 18456655]
56. Lau T-L, Kim C, Ginsberg MH, Ulmer TS. The structure of the integrin α IIb β 3 transmembrane complex explains integrin transmembrane signalling. *EMBO J*. 2009; 28:1351–1361. [PubMed: 19279667]
57. Yang J, Ma YQ, Page RC, Misra S, Plow EF, Qin J. Structure of an integrin α IIb β 3 transmembrane-cytoplasmic heterocomplex provides insight into integrin activation. *Proceedings of the National Academy of Sciences*. 2009; 106:17729–17734.
58. Solomon I. Relaxation Processes in a System of Two Spins. *Physical Review*. 1955; 99:559–565.
59. Battiste JL, Wagner G. Utilization of Site-Directed Spin Labeling and High-Resolution Heteronuclear Nuclear Magnetic Resonance for Global Fold Determination of Large Proteins with Limited Nuclear Overhauser Effect Data. *Biochemistry*. 2000; 39:5355–5365. [PubMed: 10820006]
60. Kosen, PA. [5] Spin labeling of proteins. In: Norman, JO.; Thomas, LJ., editors. *Methods in Enzymology*. Academic Press; 1989. p. 86-121.
61. Donaldson LW, Skrynnikov NR, Choy W-Y, Muhandiram DR, Sarkar B, Forman-Kay JD, Kay LE. Structural Characterization of Proteins with an Attached ATCUN Motif by Paramagnetic Relaxation Enhancement NMR Spectroscopy. *Journal of the American Chemical Society*. 2001; 123:9843–9847. [PubMed: 11583547]
62. Berardi MJ, Shih WM, Harrison SC, Chou JJ. Mitochondrial uncoupling protein 2 structure determined by NMR molecular fragment searching. *Nature*. 2011; 476:109–113. [PubMed: 21785437]

63. Tang C, Schwieters CD, Clore GM. Open-to-closed transition in apo maltose-binding protein observed by paramagnetic NMR. *Nature*. 2007; 449:1078–1082. [PubMed: 17960247]
64. Otting G. Protein NMR Using Paramagnetic Ions. *Annual Review of Biophysics*. 2010; 39:387–405.
65. Rodriguez-Castaneda F, Haberz P, Leonov A, Griesinger C. Paramagnetic tagging of diamagnetic proteins for solution NMR. *Magnetic Resonance in Chemistry*. 2006; 44:S10–S16. [PubMed: 16921533]
66. Iwahara J, Tang C, Marius Clore G. Practical aspects of ¹H transverse paramagnetic relaxation enhancement measurements on macromolecules. *Journal of Magnetic Resonance*. 2007; 184:185–195. [PubMed: 17084097]
67. Liang B, Bushweller JH, Tamm LK. Site-Directed Parallel Spin-Labeling and Paramagnetic Relaxation Enhancement in Structure Determination of Membrane Proteins by Solution NMR Spectroscopy. *Journal of the American Chemical Society*. 2006; 128:4389–4397. [PubMed: 16569016]
68. Van Horn WD, Kim H-J, Ellis CD, Hadziselimovic A, Sulistijo ES, Karra MD, Tian C, Sonnichsen FD, Sanders CR. Solution Nuclear Magnetic Resonance Structure of Membrane-Integral Diacylglycerol Kinase. *Science*. 2009; 324:1726–1729. [PubMed: 19556511]
69. Zhou Y, Cierpicki T, Jimenez RHF, Lukasik SM, Ellena JF, Cafiso DS, Kadokura H, Beckwith J, Bushweller JH. NMR solution structure of the integral membrane enzyme DsbB: functional insights into DsbB-catalyzed disulfide bond formation. *Molecular Cell*. 2008; 31:896–908. [PubMed: 18922471]
70. Chen H, Ji F, Olman V, Mobley CK, Liu Y, Zhou Y, Bushweller JH, Prestegard JH, Xu Y. Optimal mutation sites for PRE data collection and membrane protein structure prediction. *Structure*. 2011; 19:484–495. [PubMed: 21481772]
71. Zhuang T, Vishnivetskiy SA, Gurevich VV, Sanders CR. Elucidation of Inositol Hexaphosphate and Heparin Interaction Sites and Conformational Changes in Arrestin-1 by Solution Nuclear Magnetic Resonance. *Biochemistry*. 2010; 49:10473–10485. [PubMed: 21050017]
72. Venditti V, Fawzi N, Clore G. Automated sequence- and stereo-specific assignment of methyl-labeled proteins by paramagnetic relaxation and methyl–methyl nuclear overhauser enhancement spectroscopy. *Journal of Biomolecular NMR*. 2011; 51:319–328. [PubMed: 21935714]
73. Theillet F-X, Binolfi A, Liokatis S, Verzini S, Selenko P. Paramagnetic relaxation enhancement to improve sensitivity of fast NMR methods: application to intrinsically disordered proteins. *Journal of Biomolecular NMR*. 2011; 51:487–495. [PubMed: 22008951]
74. Sivakolundu SG, Nourse A, Moshiah S, Bothner B, Ashley C, Satumba J, Lahti J, Kriwacki RW. Intrinsically Unstructured Domains of Arf and Hdm2 Form Bimolecular Oligomeric Structures In Vitro and In Vivo. *Journal of Molecular Biology*. 2008; 384:240–254. [PubMed: 18809412]
75. Madl T, Guttler T, Gorlich D, Sattler M. Structural Analysis of Large Protein Complexes Using Solvent Paramagnetic Relaxation Enhancements. *Angewandte Chemie International Edition*. 2011; 50:3993–3997.
76. Tang C, Louis JM, Aniana A, Suh J-Y, Clore GM. Visualizing transient events in amino-terminal autoprocessing of HIV-1 protease. *Nature*. 2008; 455:693–696. [PubMed: 18833280]
77. Clore GM. Exploring sparsely populated states of macromolecules by diamagnetic and paramagnetic NMR relaxation. *Protein Science*. 2011; 20:229–246. [PubMed: 21280116]
78. Wu K-P, Baum J. Detection of Transient Interchain Interactions in the Intrinsically Disordered Protein α -Synuclein by NMR Paramagnetic Relaxation Enhancement. *Journal of the American Chemical Society*. 2010; 132:5546–5547. [PubMed: 20359221]
79. Gardner KH, Kay LE. The use of ²H, ¹³C, ¹⁵N multidimensional NMR to study the structure and dynamics of proteins. *Annu Rev Biophys Biomol Struct*. 1998; 27:357–406. [PubMed: 9646872]
80. Kreishman-Deitrick M, Egile C, Hoyt DW, Ford JJ, Li R, Rosen MK. NMR analysis of methyl groups at 100–500 kDa: model systems and Arp2/3 complex. *Biochemistry*. 2003; 42:8579–8586. [PubMed: 12859205]
81. Religa TL, Ruschak AM, Rosenzweig R, Kay LE. Site-Directed Methyl Group Labeling as an NMR Probe of Structure and Dynamics in Supramolecular Protein Systems: Applications to the

- Proteasome and to the ClpP Protease. *Journal of the American Chemical Society*. 2011; 133:9063–9068. [PubMed: 21557628]
82. Tugarinov V, Kay LE. Methyl Groups as Probes of Structure and Dynamics in NMR Studies of High-Molecular-Weight Proteins. *ChemBioChem*. 2005; 6:1567–1577. [PubMed: 16075427]
83. Prestegard JH, Grant DM. Characterization of anisotropic motion in fatty acid micelles by analysis of transverse relaxation in an AX2 nuclear spin system. *Journal of the American Chemical Society*. 1978; 100:4664–4668.
84. Mueller L. Sensitivity enhanced detection of weak nuclei using heteronuclear multiple quantum coherence. *Journal of the American Chemical Society*. 1979; 101:4481–4484.
85. Fischer M, Kloiber K, Hausler J, Ledolter K, Konrat R, Schmid W. Synthesis of a ¹³C-methyl-group-labeled methionine precursor as a useful tool for simplifying protein structural analysis by NMR spectroscopy. *Chembiochem*. 2007; 8:610–612. [PubMed: 17328009]
86. Sinha K, Jen-Jacobson L, Rule GS. Specific labeling of threonine methyl groups for NMR studies of protein-nucleic Acid complexes. *Biochemistry*. 2011; 50:10189–10191. [PubMed: 22039762]
87. Sprangers R, Velyvis A, Kay LE. Solution NMR of supramolecular complexes: providing new insights into function. *Nat Meth*. 2007; 4:697–703.
88. Venditti V, Fawzi NL, Clore GM. Automated sequence- and stereo-specific assignment of methyl-labeled proteins by paramagnetic relaxation and methyl-methyl nuclear overhauser enhancement spectroscopy. *Journal of Biomolecular NMR*. 2011; 51:319–328. [PubMed: 21935714]
89. Gill ML, Palmer AG 3rd. Multiplet-filtered and gradient-selected zero-quantum TROSY experiments for (¹³C (1)H (3) methyl groups in proteins. *J Biomol NMR*. 2011; 51:245–251. [PubMed: 21918814]
90. Tugarinov V, Hwang PM, Kay LE. Nuclear magnetic resonance spectroscopy of high-molecular-weight proteins. *Annu Rev Biochem*. 2004; 73:107–146. [PubMed: 15189138]
91. Sprangers R, Kay LE. Quantitative dynamics and binding studies of the 20S proteasome by NMR. *Nature*. 2007; 445:618–622. [PubMed: 17237764]
92. Kay LE. Solution NMR spectroscopy of supra-molecular systems, why bother? A methyl-TROSY view. *J Magn Reson*. 2011; 210:159–170. [PubMed: 21458338]
93. Yu L, Sun C, Song D, Shen J, Xu N, Gunasekera A, Hajduk PJ, Olejniczak ET. Nuclear magnetic resonance structural studies of a potassium channel-charybdotoxin complex. *Biochemistry*. 2005; 44:15834–15841. [PubMed: 16313186]
94. Tugarinov V, Kay LE. Ile, Leu, and Val methyl assignments of the 723-residue malate synthase G using a new labeling strategy and novel NMR methods. *Journal of the American Chemical Society*. 2003; 125:13868–13878. [PubMed: 14599227]
95. Velyvis A, Yang YR, Schachman HK, Kay LE. A solution NMR study showing that active site ligands and nucleotides directly perturb the allosteric equilibrium in aspartate transcarbamoylase. *Proc Natl Acad Sci U S A*. 2007; 104:8815–8820. [PubMed: 17502625]
96. Gelis I, Bonvin AMJJ, Keramisanou D, Koukaki M, Gouridis G, Karamanou S, Economou A, Kalodimos CG. Structural Basis for Signal-Sequence Recognition by the Translocase Motor SecA as Determined by NMR. *Cell*. 2007; 131:756–769. [PubMed: 18022369]
97. Bista M, Freund SM, Fersht AR. Domain–domain interactions in full-length p53 and a specific DNA complex probed by methyl NMR spectroscopy. *Proceedings of the National Academy of Sciences*. 2012; 109:15752–15756.
98. Gautier A, Mott HR, Bostock MJ, Kirkpatrick JP, Nietlispach D. Structure determination of the seven-helix transmembrane receptor sensory rhodopsin II by solution NMR spectroscopy. *Nat Struct Mol Biol*. 2010; 17:768–774. [PubMed: 20512150]
99. Kodama Y, Reese ML, Shimba N, Ono K, Kanamori E, Dotsch V, Noguchi S, Fukunishi Y, Suzuki E, Shimada I, Takahashi H. Rapid identification of protein-protein interfaces for the construction of a complex model based on multiple unassigned signals by using time-sharing NMR measurements. *J Struct Biol*. 2011; 174:434–442. [PubMed: 21501688]
100. Ruschak AM, Religa TL, Breuer S, Witt S, Kay LE. The proteasome antechamber maintains substrates in an unfolded state. *Nature*. 2010; 467:868–871. [PubMed: 20944750]

101. Hiller S, Garces RG, Malia TJ, Orekhov VY, Colombini M, Wagner G. Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. *Science*. 2008; 321:1206–1210. [PubMed: 18755977]
102. Strop P, Brunger AT. Refractive index-based determination of detergent concentration and its application to the study of membrane proteins. *Protein Sci*. 2005; 14:2207–2211. [PubMed: 16046633]
103. Imai S, Osawa M, Takeuchi K, Shimada I. Structural basis underlying the dual gate properties of KcsA. *Proc Natl Acad Sci U S A*. 2010; 107:6216–6221. [PubMed: 20212150]
104. Renault M, Saurel O, Czaplicki J, Demange P, Gervais V, Lohr F, Reat V, Piotto M, Milon A. Solution state NMR structure and dynamics of KpOmpA, a 210 residue transmembrane domain possessing a high potential for immunological applications. *J Mol Biol*. 2009; 385:117–130. [PubMed: 18952100]
105. Scott DE, Coyne AG, Hudson SA, Abell C. Fragment-based approaches in drug discovery and chemical biology. *Biochemistry*. 2012; 51:4990–5003. [PubMed: 22697260]
106. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW. Discovering High-Affinity Ligands for Proteins: SAR by NMR. *Science*. 1996; 274:1531–1534. [PubMed: 8929414]
107. Hajduk PJ, Meadows RP, Fesik SW. Discovering high-affinity ligands for proteins. *Science*. 1997; 278:497, 499. [PubMed: 9381145]
108. Baker M. Fragment-based lead discovery grows up. *Nature Reviews Drug Discovery*. 2013; 12:5–7.
109. Erlanson, D. Fragments in the clinic: 2013 Edition. 2013. <http://practicalfragments.blogspot.com/2013/01/fragments-in-clinic-2013-edition.html>
110. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature*. 2005; 435:677–681. [PubMed: 15902208]
111. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, Johnson EF, Marsh KC, Mitten MJ, Nimmer P, Roberts L, Tahir SK, Xiao Y, Yang X, Zhang H, Fesik S, Rosenberg SH, Elmore SW. ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. *Cancer Research*. 2008; 68:3421–3428. [PubMed: 18451170]
112. Roughley SD, Hubbard RE. How Well Can Fragments Explore Accessed Chemical Space? A Case Study from Heat Shock Protein 90. *Journal of Medicinal Chemistry*. 2011; 54:3989–4005. [PubMed: 21561141]
113. Brough PA, Barril X, Borgognoni J, Chene P, Davies NGM, Davis B, Drysdale MJ, Dymock B, Eccles SA, Garcia-Echeverria C, Fromont C, Hayes A, Hubbard RE, Jordan AM, Jensen MR, Massey A, Merrett A, Padfield A, Parsons R, Radimerski T, Raynaud FI, Robertson A, Roughley SD, Schoepfer J, Simmonite H, Sharp SY, Surgenor A, Valenti M, Walls S, Webb P, Wood M, Workman P, Wright L. Combining Hit Identification Strategies: Fragment-Based and in Silico Approaches to Orally Active 2-Aminothieno[2,3-d]pyrimidine Inhibitors of the Hsp90 Molecular Chaperone. *Journal of Medicinal Chemistry*. 2009; 52:4794–4809. [PubMed: 19610616]
114. Marquardsen T, Hofmann M, Hollander JG, Loch CMP, Kiihne SR, Engelke F, Siegal G. Development of a dual cell, flow-injection sample holder, and NMR probe for comparative ligand-binding studies. *Journal of Magnetic Resonance*. 2006; 182:55–65. [PubMed: 16814582]
115. Murali N, Miller WM, John BK, Avizonis DA, Smallcombe SH. Spectral unraveling by space-selective Hadamard spectroscopy. *Journal of Magnetic Resonance*. 2006; 179:182–189. [PubMed: 16364668]
116. Fruh V, Zhou Y, Chen D, Loch C, Ab E, Grinkova YN, Verheij H, Sligar SG, Bushweller JH, Siegal G. Application of Fragment-Based Drug Discovery to Membrane Proteins: Identification of Ligands of the Integral Membrane Enzyme DsbB. *Chemistry & biology*. 2010; 17:881–891. [PubMed: 20797617]
117. Robinson KE, Reardon PN, Spicer LD. In-cell NMR spectroscopy in *Escherichia coli*. *Methods Mol Biol*. 2012; 831:261–277. [PubMed: 22167679]

118. Maldonado AY, Burz DS, Shekhtman A. In-cell NMR spectroscopy. *Progress in Nuclear Magnetic Resonance Spectroscopy*. 2011; 59:197–212. [PubMed: 21920217]
119. Selenko P, Serber Z, Gadea B, Ruderman J, Wagner G. Quantitative NMR analysis of the protein G B1 domain in *Xenopus laevis* egg extracts and intact oocytes. *Proceedings of the National Academy of Sciences*. 2006; 103:11904–11909.
120. Serber Z, Selenko P, Hansel R, Reckel S, Lohr F, Ferrell JE, Wagner G, Dotsch V. Investigating macromolecules inside cultured and injected cells by in-cell NMR spectroscopy. *Nat Protocols*. 2007; 1:2701–2709.
121. Sakai T, Tochio H, Tenno T, Ito Y, Kokubo T, Hiroaki H, Shirakawa M. In-cell NMR spectroscopy of proteins inside *Xenopus laevis* oocytes. *Journal of Biomolecular NMR*. 2006; 36:179–188. [PubMed: 17031531]
122. Inomata K, Ohno A, Tochio H, Isogai S, Tenno T, Nakase I, Takeuchi T, Futaki S, Ito Y, Hiroaki H, Shirakawa M. High-resolution multi-dimensional NMR spectroscopy of proteins in human cells. *Nature*. 2009; 458:106–109. [PubMed: 19262675]
123. Derossi D, Chassaing G, Prochiantz A. Trojan peptides: the penetratin system for intracellular delivery. *Trends in Cell Biology*. 1998; 8:84–87. [PubMed: 9695814]
124. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In Vivo Protein Transduction: Delivery of a Biologically Active Protein into the Mouse. *Science*. 1999; 285:1569–1572. [PubMed: 10477521]
125. Ogino S, Kubo S, Umemoto R, Huang S, Nishida N, Shimada I. Observation of NMR Signals from Proteins Introduced into Living Mammalian Cells by Reversible Membrane Permeabilization Using a Pore-Forming Toxin, Streptolysin O. *Journal of the American Chemical Society*. 2009; 131:10834–10835. [PubMed: 19603816]
126. Mao L, Tang Y, Vaiphei S, Shimazu T, Kim S-G, Mani R, Fakhoury E, White E, Montelione G, Inouye M. Production of membrane proteins for NMR studies using the condensed single protein (cSPP) production system. *Journal of Structural and Functional Genomics*. 2009; 10:281–289. [PubMed: 19856129]
127. Bertrand K, Reverdatto S, Burz DS, Zitomer R, Shekhtman A. Structure of Proteins in Eukaryotic Compartments. *Journal of the American Chemical Society*. 2012; 134:12798–12806. [PubMed: 22758659]
128. Sakakibara D, Sasaki A, Ikeya T, Hamatsu J, Hanashima T, Mishima M, Yoshimasu M, Hayashi N, Mikawa T, Walchli M, Smith BO, Shirakawa M, Guntert P, Ito Y. Protein structure determination in living cells by in-cell NMR spectroscopy. *Nature*. 2009; 458:102–105. [PubMed: 19262674]
129. Ikeya T, Sasaki A, Sakakibara D, Shigemitsu Y, Hamatsu J, Hanashima T, Mishima M, Yoshimasu M, Hayashi N, Mikawa T, Nietlispach D, Walchli M, Smith BO, Shirakawa M, Guntert P, Ito Y. NMR protein structure determination in living *E. coli* cells using nonlinear sampling. *Nat Protocols*. 2010; 5:1051–1060.
130. Schlesinger AP, Wang Y, Tadeo X, Millet O, Pielak GJ. Macromolecular crowding fails to fold a globular protein in cells. *Journal of the American Chemical Society*. 2011; 133:8082–8085. [PubMed: 21534566]
131. Xie J, Thapa R, Reverdatto S, Burz DS, Shekhtman A. Screening of small molecule interactor library by using in-cell NMR spectroscopy (SMILI-NMR). *Journal of Medicinal Chemistry*. 2009; 52:3516–3522. [PubMed: 19422228]
132. Endo Y, Sawasaki T. Cell-free expression systems for eukaryotic protein production. *Current Opinion in Biotechnology*. 2006; 17:373–380. [PubMed: 16828277]
133. Katzen F, Chang G, Kudlicki W. The past, present and future of cell-free protein synthesis. *Trends in Biotechnology*. 2005; 23:150–156. [PubMed: 15734558]
134. Rajesh S, Knowles T, Overduin M. Production of membrane proteins without cells or detergents. *New Biotechnology*. 2011; 28:250–254. [PubMed: 20654746]
135. Schwarz D, Dotsch V, Bernhard F. Production of membrane proteins using cell-free expression systems. *Proteomics*. 2008; 8:3933–3946. [PubMed: 18763710]

136. Yokoyama J, Matsuda T, Koshiba S, Kigawa T. An economical method for producing stable-isotope labeled proteins by the *E. coli* cell-free system. *Journal of Biomolecular NMR*. 2010; 48:193–201. [PubMed: 21052777]
137. Maslennikov I, Klammt C, Hwang E, Kefala G, Okamura M, Esquivies L, Mors K, Glaubitz C, Kwiatkowski W, Jeon YH, Choe S. Membrane domain structures of three classes of histidine kinase receptors by cell-free expression and rapid NMR analysis. *Proceedings of the National Academy of Sciences*. 2010; 107:10902–10907.
138. Isaacson RL, Simpson PJ, Liu M, Cota E, Zhang X, Freemont P, Matthews S. A New Labeling Method for Methyl Transverse Relaxation-Optimized Spectroscopy NMR Spectra of Alanine Residues. *Journal of the American Chemical Society*. 2007; 129:15428–15429. [PubMed: 18041839]
139. Kainosho M, Torizawa T, Iwashita Y, Terauchi T, Mei Ono A, Guntert P. Optimal isotope labelling for NMR protein structure determinations. *Nature*. 2006; 440:52–57. [PubMed: 16511487]
140. Trbovic N, Klammt C, Koglin A, Lohr F, Bernhard F, Dotsch V. Efficient Strategy for the Rapid Backbone Assignment of Membrane Proteins. *Journal of the American Chemical Society*. 2005; 127:13504–13505. [PubMed: 16190707]
141. Klammt C, Maslennikov I, Bayrhuber M, Eichmann C, Vajpai N, Chiu EJC, Blain KY, Esquivies L, Kwon JHJ, Balana B, Pieper U, Sali A, Slesinger PA, Kwiatkowski W, Riek R, Choe S. Facile backbone structure determination of human membrane proteins by NMR spectroscopy. *Nat Meth*. 2012; 9:834–839.
142. Reckel S, Gottstein D, Stehle J, Lohr F, Verhoeven M-K, Takeda M, Silvers R, Kainosho M, Glaubitz C, Wachtveitl J, Bernhard F, Schwalbe H, Guntert P, Dotsch V. Solution NMR Structure of Proteorhodopsin. *Angewandte Chemie International Edition*. 2011; 50:11942–11946.
143. Boehr DD, Dyson HJ, Wright PE. An NMR perspective on enzyme dynamics. *Chem Rev*. 2006; 106:3055–3079. [PubMed: 16895318]
144. Mittermaier A, Kay LE. New tools provide new insights in NMR studies of protein dynamics. *Science*. 2006; 312:224–228. [PubMed: 16614210]
145. Kleckner IR, Foster MP. An introduction to NMR-based approaches for measuring protein dynamics. *Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics*. 2011; 1814:942–968.
146. Palmer AG, Massi F. Characterization of the Dynamics of Biomacromolecules Using Rotating-Frame Spin Relaxation NMR Spectroscopy. *Chemical Reviews*. 2006; 106:1700–1719. [PubMed: 16683750]
147. Vallurupalli P, Hansen DF, Stollar E, Meirovitch E, Kay LE. Measurement of bond vector orientations in invisible excited states of proteins. *Proc Natl Acad Sci U S A*. 2007; 104:18473–18477. [PubMed: 18006656]
148. Vallurupalli P, Hansen DF, Kay LE. Probing structure in invisible protein states with anisotropic NMR chemical shifts. *J Am Chem Soc*. 2008; 130:2734–2735. [PubMed: 18257570]
149. Eisenmesser EZ, Bosco DA, Akke M, Kern D. Enzyme dynamics during catalysis. *Science*. 2002; 295:1520–1523. [PubMed: 11859194]
150. Bhabha G, Lee J, Ekiert DC, Gam J, Wilson IA, Dyson HJ, Benkovic SJ, Wright PE. A dynamic knockout reveals that conformational fluctuations influence the chemical step of enzyme catalysis. *Science*. 2011; 332:234–238. [PubMed: 21474759]
151. Boehr DD, McElheny D, Dyson HJ, Wright PE. The dynamic energy landscape of dihydrofolate reductase catalysis. *Science*. 2006; 313:1638–1642. [PubMed: 16973882]
152. Carroll MJ, Mauldin RV, Gromova AV, Singleton SF, Collins EJ, Lee AL. Evidence for dynamics in proteins as a mechanism for ligand dissociation. *Nat Chem Biol*. 2012; 8:246–252. [PubMed: 22246400]
153. Englander SW. Protein folding intermediates and pathways studied by hydrogen exchange. *Annu Rev Biophys Biomol Struct*. 2000; 29:213–238. [PubMed: 10940248]
154. Neudecker P, Robustelli P, Cavalli A, Walsh P, Lundstrom P, Zarrine-Afsar A, Sharpe S, Vendruscolo M, Kay LE. Structure of an intermediate state in protein folding and aggregation. *Science*. 2012; 336:362–366. [PubMed: 22517863]

155. Tartaglia GG, Pawar AP, Campioni S, Dobson CM, Chiti F, Vendruscolo M. Prediction of aggregation-prone regions in structured proteins. *J Mol Biol.* 2008; 380:425–436. [PubMed: 18514226]
156. Dyson HJ, Wright PE. Intrinsically unstructured proteins and their functions. *Nat Rev Mol Cell Biol.* 2005; 6:197–208. [PubMed: 15738986]
157. Boesch C, Bundi A, Oppliger M, Wuthrich K. 1H Nuclear-Magnetic-Resonance Studies of the Molecular Conformation of Monomeric Glucagon in Aqueous Solution. *European Journal of Biochemistry.* 1978; 91:209–214. [PubMed: 720338]
158. Kriwacki RW, Hengst L, Tennant L, Reed SI, Wright PE. Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity. *Proceedings of the National Academy of Sciences.* 1996; 93:11504–11509.
159. Daughdrill GW, Chadsey MS, Karlinsey JE, Hughes KT, Dahlquist FW. The C-terminal half of the anti-sigma factor, FlgM, becomes structured when bound to its target, sigma 28. *Nature structural biology.* 1997; 4:285–291.
160. Uversky VN. Natively unfolded proteins: A point where biology waits for physics. *Protein Science.* 2002; 11:739–756. [PubMed: 11910019]
161. Dyson, JH.; Wright, PE. Insights into the structure and dynamics of unfolded proteins from nuclear magnetic resonance. In: George, DR., editor. *Advances in Protein Chemistry.* Academic Press; 2002. p. 311–340.
162. Dunker K, Brown C, Lawson D, Iakoucheva L, Obradovi Z. Intrinsic Disorder and Protein Function. *Biochemistry.* 2002; 41:6573–6582. [PubMed: 12022860]
163. Tompa P. Intrinsically unstructured proteins. *Trends in Biochemical Sciences.* 2002; 27:527–533. [PubMed: 12368089]
164. Wright PE, Dyson HJ. Linking folding and binding. *Current Opinion in Structural Biology.* 2009; 19:31–38. [PubMed: 19157855]
165. Mittag T, Marsh J, Grishaev A, Orlicky S, Lin H, Sicheri F, Tyers M, Forman-Kay JD. Structure/Function Implications in a Dynamic Complex of the Intrinsically Disordered Sic1 with the Cdc4 Subunit of an SCF Ubiquitin Ligase. *Structure.* 2010; 18:494–506. [PubMed: 20399186]
166. Tang X, Orlicky S, Mittag T, Csizmok V, Pawson T, Forman-Kay JD, Sicheri F, Tyers M. Composite low affinity interactions dictate recognition of the cyclin-dependent kinase inhibitor Sic1 by the SCFCdc4 ubiquitin ligase. *Proceedings of the National Academy of Sciences.* 2012; 109:3287–3292.
167. Mittag T, Orlicky S, Choy WY, Tang X, Lin H, Sicheri F, Kay LE, Tyers M, Forman-Kay JD. Dynamic equilibrium engagement of a polyvalent ligand with a single-site receptor. *Proceedings of the National Academy of Sciences.* 2008; 105:17772–17777.
168. Mittag T, Kay LE, Forman-Kay JD. Protein dynamics and conformational disorder in molecular recognition. *Journal of Molecular Recognition.* 2009; 23:105–116. [PubMed: 19585546]
169. Mitrea DM, Yoon MK, Ou L, Kriwacki RW. Disorder-function relationships for the cell cycle regulatory proteins p21 and p27. *Biological Chemistry.* 2012; 393:259–274. [PubMed: 23029651]
170. Brosey CA, Chagot M-E, Ehrhardt M, Pretto DI, Weiner BE, Chazin WJ. NMR Analysis of the Architecture and Functional Remodeling of a Modular Multidomain Protein, RPA. *Journal of the American Chemical Society.* 2009; 131:6346–6347. [PubMed: 19378948]
171. Meinema AC, Laba JK, Hapsari RA, Otten R, Mulder FAA, Kralt A, van den Bogaart G, Lusk CP, Poolman B, Veenhoff LM. Long Unfolded Linkers Facilitate Membrane Protein Import Through the Nuclear Pore Complex. *Science.* 2011; 333:90–93. [PubMed: 21659568]
172. Hyberts S, Arthanari H, Wagner G. Applications of non-uniform sampling and processing. *Novel Sampling Approaches in Higher Dimensional NMR.* 2012:125–148.
173. Szyperski T, Atreya HS. Principles and applications of GFT projection NMR spectroscopy. *Magnetic Resonance in Chemistry.* 2006; 44:S51–S60. [PubMed: 16826541]
174. Shimba N, Kovacs H, Stern AS, Nomura AM, Shimada I, Hoch JC, Craik CS, Dotsch V. Optimization of 13 C direct detection NMR methods. *Journal of Biomolecular NMR.* 2004; 30:175–179. [PubMed: 15557804]

Figure 1.

800 MHz ^1H - ^{15}N TROSY spectra of low concentrations of human arrestin-1 at 308 K in the absence and presence of bicelle-associated rhodopsin. **(A)** ^1H , ^{15}N -TROSY spectrum of 10 uM wild-type ^2H , ^{15}N -labeled arrestin-1 in pH 6.5 buffer. **(B)** ^1H , ^{15}N -TROSY spectra of 30 uM ^2H , ^{15}N -labeled F85A/F197A-arrestin-1 (black) in the presence of a saturating level (60 uM) of light-activated, phosphorylated rhodopsin P-Rh* in bicelles (red) at pH 6.5. *Panel B is adapted from ²⁵ and used with permission of the publisher.*

Figure 2.

(A) Crystal structure of the inactive state of β_2 AR, showing the locations of the cysteines that were ^{19}F labeled for studies of classical versus biased-agonism by the Stevens and Wüthrich labs.³⁵ (B) ^{19}F NMR spectra of C265- (left) and C327- ^{19}F -labeled (right) β_2 AR at 280°K in β -dodecylmaltoside micelles containing 17 mol% cholesterol hemisuccinate. Each spectrum has been deconvoluted into two spectral components, blue arising from the inactive state and red representing the activated state. It can be seen that the ratios of the two states (as judged by spectra from C265-label vs. C327-label) are, for most compounds, not equal. The simplest explanation for this observation is that there are actually two active states—one being the classical G-protein-coupling activated state, the other being the C-terminal phosphorylation-activated state that leads to binding of β -arrestin. (C) The series of compounds examined in this work classified according to their known pharmacological effects on β_2 AR. Inverse agonists preferentially stabilize the inactive receptor state. Neutral antagonists bind to the receptor without altering the basal inactive/active state ratio. Partial agonists result in sub-maximal conversion into the activated state, while full agonists result in maximal conversion. Carvedilol and isotherine are considered biased agonists in that they preferentially promote activation of complex formation with β -arrestin, although they differ with regards to the extent to which they also stimulate classic agonism. Each compound is color coded to indicate the helices of the receptor that each moiety interacts with (see color coding in panel A). *Figure adapted from*³⁵ *and used by permission of the publisher.*

Figure 3.

(A) Binding of cholesterol to the C99 domain of the amyloid precursor protein. Binding is believed to involve docking of the flat and rigid cholesterol to the flat surface provided by tandem GXXXG motifs on the surface of C99's upper transmembrane helix. Formation of hydrogen bonds between the hydroxyl headgroup of cholesterol and C99 involves a conformational change (or conformational selection) centering on a flexible loop that connects a short surface-associated helix and C99's transmembrane domain. (B) Distinct classes of interaction between two transmembrane proteins that can now be distinguished using NMR methods. *Panel B from*^{43, 51} *and used by permission of the publisher.*

Figure 4.

The NMR observed (red) and crystal structure back-calculated (black) PRE values for the maltose binding protein (MBP) in the holo (maltose-associated) (A) and apo (B) states. Red bars on the top of the plots indicate regions where signal intensities are broadened beyond detection as a result of relaxation enhancements. The holo-MBP PRE values show excellent agreement with the crystal structure indicating that the maltose-bound holo (closed) state protein is relatively rigid when complexed with maltose under both NMR and crystal conditions. The discrepancies in (B) between the observed and back-calculated PRE values for the apo state indicate a rapidly-exchanging mixture of a pair of structural states. These were determined to be the open state (similar to the apo crystal structure) and a 5% partially closed conformer. This latter structure is distinct from both the apo and holo state crystal structures but may represent the excited state conformer that initially binds maltose, which then induces a transition to the stable holo state. The inset in panel B is a surface representation of MBP with the green surface showing the conformational space explored by the paramagnetic nitroxide label and the red surface highlighting the regions where observed and back-calculated PREs don't agree. The electrostatic surface of the open state of apo MBP (C) highlights the sugar binding pocket, with (D) illustrating the differences in the MBP-CTD between the partially closed apo-MBP (green cylinders) and closed holo-MBP states (red cylinders). Thus the data in this figure show the utility of the PRE to detect and

probe minor protein populations that are challenging to observe with other techniques. In this case the minor population observed in the apo state is thought to be critical for ligand recognition and induced fit transition to the stable holo maltose-MBP conformation. *This figure is a composite from those in*⁶³ *and used by permission of the publisher.*

Figure 5.

Structure and dynamics of the 670-kDa $\alpha_7\beta_7\beta_7\alpha_7$ proteasome core particle probed by methyl-TROSY. **(A)** Cross-section view of the proteasome revealing its lumen. The residues in red were shown to undergo concerted motion and are located in the antechamber near the entrance to the catalytic chamber (where active site threonine residues are blue). The resonances from V14 (shown in yellow) were observed to be highly exchanged-broadened, reflecting the even more severe broadening of (invisible) resonances from the adjacent residues 1–12 as a result of msec timescale motion. **(B)** Cross-section highlighting residues that change methyl TROSY chemical shifts upon truncation of the first 12 residues of the α subunit (see scale and color coding at bottom). The largest changes are seen for sites located at the narrowest point of the substrate entrance channel (V129) and inside the antechamber. This suggests that the 12 N-terminal residues missing in the crystal structure populate states in which they are reversibly folded into the antechamber through the entrance to the channel, where they act as a gate. The location of residues 13–18 in the crystal structure are shown in green. (Figure from⁹¹ and used by permission of the publisher. Caption is also adapted from that some reference).

Figure 6.

(A) Illustration of fragment-based drug design (FBDD) and both protein- and ligand-detected screening. The binding of small molecule fragments to a protein target can be detected by NMR even when affinity is low (100 μM–10 mM). High-affinity ligands can be created by linking together low affinity fragments that bind to adjacent sites. In the protein-detected mode, peaks from nuclei located at the binding interface shift when a candidate molecular fragment binds, an approach that has the advantage of suggesting the location of the binding site in the target. In the ligand-detected mode, small molecules that bind to a target protein are identified based on peak shifts or peak broadening/disappearance as a result of binding. (B) Chemical evolution of ABT-263.

Figure 7.

Reaction cycle for dihydrofolate reductase. Highlighted in this figure are excited states confirmed by NMR to be dynamically sampled by each ground state complex along the reaction pathway. NADPH and NADP⁺ are shown in gold, while substrate, product, and analogs are shown in magenta. Note the approximate matches between the NMR-determined rates for conformational changes and the rates for the adjacent chemical/binding steps. From ¹⁵¹, used by permission of the publisher.

Figure 8.

Comparison of the folding intermediate and natives states of mutant Fyn SH3 as determined by NMR. Sites for both native and the folding intermediate states are color-coded according to their predicated Zagg (Zagg > 1—orange to red—indicates significant propensity to aggregate).¹⁵⁴ The high propensity of the $\beta 1$ strand to aggregate is effectively blocked by the adjacent $\beta 5$ strand in the folded native state, but is a source of peril for this protein in the intermediate state. From ¹⁵⁴, used by permission of the publisher.

Figure 9. Dynamic complex of multiply-phosphorylated pSic1 with Cdc4. *From*¹⁶⁷, used by permission of the publisher.