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Protein-Inhibitor Interaction Studies Using NMR

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Abstract

Solution-state NMR has been widely applied to determine the three-dimensional structure, dynamics, and molecular interactions of proteins. The designs of experiments used in protein NMR differ from those used for small-molecule NMR, primarily because the information available prior to an experiment, such as molecular mass and knowledge of the primary structure, is unique for proteins compared to small molecules. In this review article, protein NMR for structural biology is introduced with comparisons to small-molecule NMR, such as descriptions of labeling strategies and the effects of molecular dynamics on relaxation. Next, applications for protein NMR are reviewed, especially practical aspects for protein-observed ligand-protein interaction studies. Overall, the following topics are described: (1) characteristics of protein NMR, (2) methods to detect protein-ligand interactions by NMR, and (3) practical aspects of carrying out protein-observed inhibitor-protein interaction studies.

Keywords

Drug; inhibitor; interaction; NMR; protein; relaxation; structure

1. INTRODUCTION AND INSTRUMENTATION

Proteins in solution are not rigid molecules but undergo internal dynamics; hence, in solution, proteins sample an ensemble of conformations [1]. Further, during many biological processes, such as cell signaling, dynamic regulation of enzymes, and molecular recognition, binding between macromolecules is often weak and transient [2–4]. A powerful method to characterize such dynamic, weak, and transient protein interactions and molecules is NMR. Protein NMR is unique in its applicability for this purpose, for several reasons. Since proteins are polypeptides with repeats of similar backbone chemical structures, systematic signal assignment methods can be applied for protein signal identification. In addition, NMR relaxation of protein protons tends to be similar to among protons, due to spin-diffusion by the abundant protons, particularly in large proteins [5–8]. Furthermore, the chemical structure, *i.e.*, the primary amino acid sequence, is often known prior to an NMR experiment, whereas identification of the primary chemical structure is one of the major

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

objectives when applying chemistry NMR (Fig. 1A and 1B). Thus, “how many protons are *J*-coupled to a carbon” may not be an important concern for protein NMR. Instead, since there are many signals in a spectrum, identification of signals, *i.e.*, “knowing their residue numbers and atom names”, through systematic *J*-coupling or dipolar coupling, becomes a main concern. With these unique features, protein NMR is ideal for the study of structure, dynamics, and molecular interactions of proteins.

NMR instrumentation is not within the scope of this review. However, in brief, a typical setting for protein NMR is with (1) three or four channels to excite ^1H , ^{13}C , ^{15}N , and, if possible, ^2H nuclear spins, and (2) a ^1H detection triple-resonance probe with a pulse-field gradient [9]. A low-temperature probe (coils are cooled down to reduce noise) is often used to further gain signal-to-noise ratio, which enables experiments at low protein concentration or samples with a short lifetime, compared to an ambient temperature probe. NMR instruments at > 600 MHz (as ^1H Larmor frequency) are often used to study proteins (M.W. > 10 kDa). An upper limit to the size of the proteins that can be studied by NMR depends on the spectral complexity, the protein concentration, and the purpose of study as well as the magnetic field strength. Sharing NMR instruments that are primarily used for studies of synthesized or purified chemical compounds may be impractical for two reasons: first, protein NMR experiments need continuous NMR machine time for a week or two, to record a series of experiments, and, second, protein NMR uses a proton-detection triple resonance probe.

2. CHARACTERISTICS OF PROTEIN NMR

2.1. Relative Sensitivity Determinants

In natural product NMR, nuclear spin at the natural abundance is typically detected without isotope enrichment. On the other hand, in protein NMR, nuclei that have low natural abundances are enriched with stable isotopes to increase sensitivity. The most common strategy to label proteins with isotopes is over-expression of proteins in *E. coli* in a minimal medium that contains ^{15}N -labeled NH_4Cl and ^{13}C -labeled glucose as their sole nitrogen and carbon sources, respectively [10, 11]. This enables experiments that correlate backbone carbons with amide nitrogen sites, as well as carbon side chains [12–14]. In proteins that are labeled in this way, the concentration of the active nuclear spins can be almost equal to the protein concentration.

In addition to nuclear spin concentrations, transverse relaxation rate, R_2 , practically determines the relative sensitivity of NMR signals in protein NMR, due to the large molecular size of proteins, compared to small molecules [15]. There are two kinds of R_2 contributions to NMR sensitivity. First, an increase in molecular size slows down the molecular tumbling, resulting in a lower signal height with broader line width (*i.e.*, large R_2) in the Fourier-transformed spectrum (Fig. 2A). Second, such large R_2 significantly attenuates detected signal intensity after several magnetization-transfer periods in two- and three-dimensional (2D and 3D) NMR experiments (Fig. 2B). To reduce the R_2 of amide protons and carbons that relax by dipolar interaction with surrounding protons in proteins, deuterium (^2H) labeling of protein hydrogens may be applied (described in section [2.2]) [16–18]. For this, bacteria are grown in a deuterated medium (for example, a minimal

medium in $^2\text{H}_2\text{O}$), and, through the purification of the protein using conventional aqueous buffer without deuteration, labile hydrogen sites (such as $-\text{NH}$, $-\text{NH}_2$, $-\text{NH}_3$, and $-\text{OH}$) are exchanged back to protons.

The longitudinal relaxation rate, R_1 , also *differently* affects the actual signal sensitivity in protein NMR compared to chemistry NMR. In chemistry NMR, the relaxation mechanism of individual nuclei, such as number of dipolar couplings, the magnitudes of chemical shift anisotropy, and molecular size, determined R_1 . In contrast, R_1 in proteins tends to be uniform, because of the repeated chemical structures in peptides and of proton spin-diffusion in protein [5, 19]. Although complete magnetization recovery during the pulse repetition delay (*i.e.*, delay between the experiments to accumulate the number of scans) is ideal, to maximize signal intensity, the delay is often minimized, to increase the number of accumulations, to obtain a net gain of actual sensitivity per unit time. This uniformity of R_1 makes adjustment of total accumulations of data and the pulse repetition delay straightforward in protein NMR.

2.2. Observed Nuclear Spins

Since the design of protein NMR experiments is tightly coupled with the isotopes labeled in the proteins, the experiments are described from the aspect of the type of spins that are labeled.

1. *Uniform ^{15}N labeling.* Proteins that are ^{15}N -labeled are primarily used to record ^1H - ^{15}N heteronuclear single-quantum coherence (HSQC) spectra [20]. Proteins, which are polypeptide chains, contain amides in the backbone peptide bonds. The ^1H - ^{15}N HSQC spectrum, which detects chemical shifts of the amide ^1H and ^{15}N , is sensitive to backbone structure and protein folding. Because of this, the ^1H - ^{15}N HSQC spectrum is often called a protein “finger print”. Similar to HSQC, the transverse relaxation optimized spectroscopy (TROSY) experiment, which selects only the slowly relaxing component from a pair of ^1H - ^{15}N coupled signals, is recorded for larger proteins [21–23]. The TROSY may be used together with cross-correlated relaxation-induced polarization transfer (CRIPT) or cross-correlated relaxation-enhanced polarization transfer (CRINEPT) for extremely large proteins [24–26]. These ^1H - ^{15}N correlation spectra are encoded for various purposes, such as to monitor titration, temperature-dependence, and H-D exchange [27]. ^{15}N relaxation experiments with proton detection are recorded using ^{15}N -labeled samples [28–32].
2. *Uniform ^{15}N and ^{13}C labeling.* Introduction of ^{13}C labels enables one to record ^1H - ^{13}C heteronuclear multi-quantum coherence (HMQC) [33], as well as other ^{13}C correlated experiments, such as HSQC. However, different from ^1H - ^{15}N correlation experiments, ^1H - ^{13}C correlation experiments for uniformly ^{13}C -labeled samples require constant-time, t_1 , evolution to avoid the ^{13}C - ^{13}C coupling evolution. Further, signal correlations needed for backbone and side chain signal assignments are obtained by combining J -magnetization transfers through ^{15}N - ^{13}C and ^{13}C - ^{13}C , as in HNCA, HNCACB, and CBCACONH experiments [12, 34]. These acronyms denote the observed nuclear-spin correlations in the spectra. For

example, HNCA indicates the experiment that correlates nuclear spins between HN of the i^{th} residue to C_{α} s of the i^{th} and the $(i-1)^{\text{th}}$ residues. Similarly, CBCACONH correlates nuclear spins from C_{β} and C_{α} of the $(i-1)^{\text{th}}$ residue to NH of the i^{th} residue through CO. This set of labeling is critical to obtain complete signal assignments in protein NMR.

3. *Perdeuteration*. Expression of proteins in deuterated environments reduces the total proton density in proteins [16]. As described above, this is useful to improve the relative signal sensitivity (by decreasing R_2). This “silencing effect” by perdeuteration is useful for proteins over 30 kDa and may be used in combination with amino acid specific labeling [18]. A drawback is that the perdeuterated proteins cannot be used to detect side-chain protons, such as HCCH-TOCSY (HCCH-total correlation spectroscopy) that detects correlation of the carbons. In addition to applying deuteration for the silencing effect, some protein NMR studies indirectly detect magnetization of deuterated signals through J -coupling with ^{13}C nuclei. A well-known example is spin relaxation of methyl deuteron using partially deuterated proteins in solution [35–37].
4. ^{19}F NMR. In contrast to isotope enrichments of intact protein atoms, there are applications that selectively introduce non-natural amino acids into proteins, by either biosynthesis or chemical modification. ^{19}F NMR for biomolecular application has received particular attention. Because there is no natural ^{19}F in biomolecules, observation of ^{19}F NMR signals gives selective information without any background signals [38–45]. Labeling with NMR active nuclei that do not give background signals is especially important for NMR studies of membrane proteins, for which even ^{13}C signals from detergents or micelles, both of which are used to solubilize the proteins, generate significant background signals in the ^1H - ^{13}C HMQC experiments at natural abundance. For studies of membrane proteins that can be expressed in mammalian or insect cells, ^{19}F is often introduced by chemical modification of a cysteine residue [46–49]. Application of fluorine in medicinal chemistry has been of great interest recently [50, 51]. However, there are two down sides to ^{19}F protein NMR: (1) introduction of ^{19}F may modify the functions of the proteins, [52] and (2) ^{19}F line width increases significantly in high-field NMR, due to the large ^{19}F chemical shift anisotropy [53, 54].

2.3. Effect of Aqueous Solution

Most protein NMR studies are performed in aqueous solution, in conditions close to the physiological condition or conditions in which proteins are folded in their functional forms. In particular, to detect labile proton signals in proteins, such as amide protons, experiments are performed in 90–95% H_2O with 5–10% D_2O environment, but not ~100% D_2O . Because of this, techniques to suppress water signals are required to detect NMR signals of protein protons. Preferably, water suppression is not achieved by water signal saturation, because amide-water proton exchange causes reduction of amide signal intensity; instead, this is achieved by coherence selection and/or using water-flip back pulses that keep water magnetization in the Z -direction [55–57]. The latter is frequently used for experiments that detect ^1H - ^{15}N correlations, because protein signals at the chemical shift of the water proton

are not detected. In contrast, coherence selection is more frequently used for ^1H - ^{13}C correlation experiments in which $^1\text{H}_\alpha$ signals are located at chemical shifts similar to that of the water proton [58, 59]. For better water suppression, an NMR tube that is made with special glass, whose magnetic susceptibility matches that of water [60], is used. The application of such glass tubes is also advantageous for the purpose of reducing the amount of protein sample that is needed to obtain spectra with adequate sensitivity.

Particular care for water-signal handling is needed in experiments conducted at high magnetic field strength (> 800 MHz, as ^1H Larmor frequency) or conducted with a highly-sensitive cryogenic probe [61–65]. An increase in the Q-factor, due to an increase in the magnetic field strength and/or by use of a “cold probe”, may result in significant radiation damping, even when the residual transverse water magnetization is small: a small residual of transverse magnetization induces a current in the coil, which in turn causes -Z-magnetization to return to +Z [66–68]. To avoid this problem, a weak pulsed field gradient is applied during the time that proton magnetization is in the transverse plane [69].

The aqueous solution used for protein studies typically contains salts, buffering chemicals, and/or other additives, such as metal ions, to stabilize the protein. The solution is typically chosen on the basis of the protein's surface charges, hydrophobicity, folding, and stability [70, 71]. However, a buffer well-suited for a protein may not be ideal for NMR experiments: high pH causes rapid proton exchange between amide protons and water protons [72], high chloride reduces signal sensitivity by inductive loss and dielectric loss of the probe [73–76], and low temperature slows down molecular tumbling rate, resulting in broadening of signals. Further, if the solvent buffer and additives contain H-C abundantly, the solute ^1H - ^{13}C correlation, stemming from the natural abundance of ^{13}C fraction covalently bonded to ^1H , may remain in the ^1H - ^{13}C HSQC-based spectra even with coherence selection. Finally, buffering molecules, which can have a relatively long residence time on protein molecules or affect protein conformation, should be avoided. In modern state-of-art NMR experiments, perturbations of protein chemical shifts by buffer molecules are sensitively detected by the chemical exchange effect [77, 78].

3. METHODS TO DETECT PROTEIN-LIGAND INTERACTION BY NMR

Here, three types of protein-ligand interaction studies, using NMR in solution, are reviewed from the aspect of how intrinsic NMR relaxation affects the observed parameters differently between small molecules and proteins. To generalize the description, we use the words “protein” and “ligand” in this section. These sets of molecules can be either an enzyme and an inhibitor, or a protein and a co-factor, or any system in which a protein interacts with a small molecule [79–81].

3.1. Cross Relaxation, Saturation, and Saturation-Transfer Difference Experiment

The saturation-transfer difference (STD) experiment is performed using relatively small amounts of proteins, by recording a set of one-dimensional NMR spectra. Stable-isotope labeling of proteins is not needed for this experiment. STD identifies whether a ligand interacts with a protein even when the binding is weak (for example, when the dissociation constant is above 0.1 mM) and, thus, is suitable as an initial check of the protein-ligand

interaction or for drug-screening purposes [82–88]. Since many reviews describe the advantages of this method, here, the underlying mechanisms are described with regard to protein NMR, for comparison with protein-observed NMR methods in the following sections.

Two major NMR phenomena are involved in the STD experiment designed to detect a ligand interaction on a protein: (1) saturation effect of nuclear spin and (2) proton spin-diffusion. When proton magnetization is irradiated continuously or repeatedly, with a short duration, for a sufficiently long time, the energy levels are equally populated, so that no additional absorption of the transition energy is observed, *i.e.*, it is saturated. Saturation is transferred from one proton (or a portion of a proton) to another proton in a protein, by cross relaxation between a pair of dipolar-coupled nuclei that are in close proximity (Fig. 3A). This cross relaxation occurs when magnetization relaxes by dipolar interaction: longitudinal magnetization of the dipolar coupled nuclei relaxes by auto relaxation, ρ , and by the cross relaxation, σ (Fig. 3A) [89].

The cross relaxation between I and S nuclei is described by,

$$\sigma_{IS} = \frac{d^2}{10} [6J(\omega_I + \omega_S) - J(\omega_I - \omega_S)] \quad (1)$$

Here, $J(\omega_I)$ is a spectral density function that is a Fourier-transform of the autocorrelation function of the fluctuating magnetic dipole interactions. When an autocorrelation function is described by a single-exponential function, with a rotational correlation time, τ , $J(\omega_I)$ is expressed by,

$$J(\omega_I) = \frac{2\tau}{1 + \tau^2 \omega^2} \quad (2)$$

When I and S are homonuclei, the term in the parenthesis in Eq. (1) becomes $6J(2\omega_H) - J(0)$. In a folded protein, the rotational correlation time of the molecule, τ , is larger than the inverse of the resonance frequency, ω (*i.e.*, $\omega\tau \gg 1$), giving the relationship $6J(2\omega_H) < J(0)$. Thus, $\sigma < 0$ in Eq. (1). This negative σ decreases the intensity of the magnetization of the cross-relaxed protons, even when they are not directly irradiated (Fig. 3B). The “decreased intensity” (this is similar to saturation) of I_0 , in turn, decreases the intensity of magnetization of I_1 , and that of I_1 is transferred to the surrounding proton spins, I_2 , I_3 , and so on, by the cross relaxation. This cross relaxation extends throughout the network of protons in a protein, so that saturation of a part of a protein propagates to the entire protein proton population. This networking is called spin-diffusion. This mechanism also tends to make ^1H R_I uniform in a rigid protein [5–8].

When there is a residence time for a ligand on a protein, by a ligand-protein interaction (*i.e.*, the protein bound form), spin-diffusion decreases signal intensities of the ligand protons, as well as protons in the protein, during the interacting interval. The reduced intensity of the ligand proton signals in the bound form is carried over to the free-form and is detected as NMR resonances of the free-form ligand (Fig. 3C) [82–84].

This method can detect a ligand-protein interaction even when the binding is weak. To run this experiment for such a weak binding system, an NMR sample that contains a small amount of protein and an excess amount of ligand is prepared (for example, 0.03 mM and 1 mM, respectively) [82–84]. An optimal ligand:protein ratio for the STD experiment depends on the dissociation constant, *i.e.*, on on and off rates, and the solubility of the ligand. Then, two spectra, one with saturation of protein ^1H signals and the other without the saturation, are recorded for a set of STD experiments. When the saturated protein ^1H magnetization is transferred to the ligand ^1H magnetization, the ligand signal intensity of the two spectra differs. The experiments may be repeated by selecting different irradiation frequencies, to make sure that the protein signals are saturated sufficiently without direct irradiation of ligand signals. Also, recording spectra of individual ligand and protein, as controls, is useful to interpret the STD data [82, 83].

3.2. Ligand Titration by Observing Protein Signals

Protein-detected ligand titration experiments are often performed using isotope-labeled samples, so that many protein signals can be tracked at once. This is useful to determine a ligand-dissociation constant and estimate the ligand-binding site, as described below. Although the minimum protein concentration required for observation of these protein signals depends on various factors, such as magnetic field strength, available machine time, protein size, buffer, and temperature, typically, for a 20 kDa protein, 0.1–0.3 mM of protein may be used when conducting experiments on a 600 MHz NMR instrument [90]. To separate many signals, ^1H - ^{15}N HSQC experiments, which mainly reflect ^1H - ^{15}N correlations of protein backbone, or ^1H - ^{13}C HMQC experiments, which mainly reflect ^1H - ^{13}C correlations of protein side-chains, are often employed. Even when a ligand binds the side chains of a protein, chemical shift perturbation is observed for backbone signals. Indeed, ^1H - ^{15}N HSQC spectra are more frequently recorded than ^1H - ^{13}C HMQC spectra for three reasons: (1) ^1H - ^{13}C correlation signals stemming from the natural abundance ^{13}C of the ligand may overlap with the protein signals, (2) ^{13}C -labeling of proteins is more costly than ^{15}N -labeling, (3) ^1H - ^{15}N HSQC spectra of a uniformly ^{15}N -labeled protein show fewer signals, and less overlap, than the ^1H - ^{13}C HMQC spectra of a uniformly ^{13}C -labeled protein.

As ligand concentration increase, protein NMR signals show one of two titration-spectral patterns: (1) changes in chemical shift positions (*i.e.*, fast exchange régime, Fig. 4A) or (2) a decrease in the free-form signals and an accompanying increase in the bound-form signals (*i.e.*, slow exchange régime, Fig. 4B) [81]. The fast exchange pattern is observed when the exchange rate is larger than the difference in chemical shifts between the free and the bound forms, while the slow exchange pattern is observed when the exchange rate is smaller than the shift difference. Both patterns may be observed in one protein NMR spectrum, because the differences in chemical shifts between the free and ligand-bound forms vary from site to site. The fraction of the bound form of a protein, f , at each ligand concentration is obtained from the relative chemical shift change between the ligand-free and the bound form signals in the fast exchange régimes, or from the relative ratio of the ligand-free and bound form signals in the slow exchange régimes. The ligand-dissociation constant, K_D , for a 1:1

stoichiometric ligand-protein interaction is expressed using f , the total ligand concentration, L_0 , and the total protein concentration, P_0 , by Eq. (3).

$$K_D = \frac{[P][L]}{[PL]} = \frac{[(1-f)P_0][L_0 - fP_0]}{[fP_0]} = \frac{(1-f)(L_0 - fP_0)}{f} \quad (3)$$

Practical aspects of the determination of K_D are described in section [4.2]. When the binding is very weak and the $L_0 \gg P_0$, Eq. (3) becomes

$$K_D = L_0(1-f)/f \quad (4)$$

The ligand concentration at $f = 0.5$ gives a good estimate of K_D [81]. In contrast to the very weak binding case, broadening becomes significant when the exchange rate is close to the difference in chemical shifts between the free- and the bound forms. In such a case, line-shape analysis may be useful, as well as tracking the chemical shift changes, to validate the analysis [91–93].

3.3. Protein Titration by Observing Ligand Signals

In an actual ^1H NMR experiment, background protein signals may interfere with attempts to record the ligand signals. However, protein background signals are assumed not to interfere when (a) the chemical shifts of the ligand protons differ from the chemical shifts of protein protons, or (b) an excess amount of the ligand is added, or (c) the protein is per-deuterated. Alternatively, when an active nuclear spin, such as fluorine, exists only in the ligand but not in the protein, the NMR spectrum of the nuclear spin does not exhibit any background protein signal. In any case, the fraction of the bound form of the ligand, g , at each protein concentration is obtained from the relative chemical shift change between the protein-free and the bound form signals in the fast exchange régimes, or from the relative ratio of the protein-free and bound form signals in the slow exchange régimes. The ligand-dissociation constant, K_D , for the ligand-protein interaction is expressed, using g , by Eq. (5).

$$K_D = \frac{[L][P]}{[PL]} = \frac{[(1-g)L_0][P_0 - gL_0]}{[gL_0]} = \frac{(1-g)(P_0 - gL_0)}{g} \quad (5)$$

Although the equation is analogous to the case of protein detected ligand-titration (Eq. (3)), there are a couple of practical differences. Due to a difference in the molecular tumbling rate of the ligand compared to the protein, the ligand signals show a significant signal broadening upon protein interaction, compared to those in the free form. In the fast exchange régime (Fig. 4C), the ligand signal intensity is attenuated as the concentration of the protein bound form increases. In the slow exchange régime, the protein-bound ligand signals are broad, similar to the protein signals (Fig. 4D). In either case, the ideal experimental condition to detect ligand signals in protein-titration experiments requires sufficient sensitivity to detect ligand signals on the protein, but not necessarily free-ligand signals. Alternatively, in the slow exchange régime, population changes, upon titration, are estimated from changes in the signal intensities of the free-form ligand only, when the each

titration point is recorded exactly in the same conditions [94]. Again, except for the very extreme case (very fast or very slow exchange), line-shape analysis, along with tracking of the chemical shift changes, may be needed for precise data analysis [91–93].

3.4. Protein-Ligand Complex Structure Determination

Using NMR, the structure of a protein-ligand complex can be determined when the protein has a reasonable size (< 30 kDa). For this, both proton observation and ligand observation experiments are comprehensively employed. A traditional example is structure determination of the calmodulin-peptide complex structure (Fig. 5A) [13]. In the peptide free form, the two domains of calmodulin are far from each other in solution (Fig. 5B). Since the two domains of calmodulin are dynamic in both the Ca^{2+} free and the Ca^{2+} bound form [95, 96], how the peptide interacts with the two domains at the same time was questioned. Using NMR, the complex structure was found to be quite compact, with the two domains folded near each other, and the peptide sandwiched between the two. This complex structure, determined by NMR, was consistent with the crystal structure of a calmodulin-protein complex that was determined at almost the same time [97, 98].

Three types of NMR experiments are required to determine the structure of a ligand-protein complex: experiments to determine (1) the conformation of the protein, (2) the conformation of the ligand (here, peptide), and (3) the interface interaction, such as inter-molecular distances, between the protein and the peptide [13, 99]. When a complex of a $^{13}\text{C}/^{15}\text{N}$ -labeled protein and an unlabeled inhibitor are studied, these three types of data are obtained from the following experiments. The conformation of the protein in the complex is determined by a set of NMR experiments that are typically used to determine three-dimensional structures; these include signal assignments experiments, $^{13}\text{C}/^{15}\text{N}$ -edited Nuclear Overhauser Effect Spectroscopy (NOESY) experiments, and if possible, an experiment that determines torsion angles. Here, $^{13}\text{C}/^{15}\text{N}$ -edited-NOESY is used to record ^1H - ^1H NOE connections from any proton to a proton that is covalently attached to $^{13}\text{C}/^{15}\text{N}$. Thus, in a complex of a $^{13}\text{C}/^{15}\text{N}$ -labeled protein with a non-labeled ligand, the edited NOESY selects NOE connectivities from the protons of a non-labeled ligand to the $^{13}\text{C}/^{15}\text{N}$ -labeled protein protons. In contrast, the structure of the ligand is determined by recording filtered-NOESY and filtered-correlation spectroscopy (COSY) experiments, which detect correlations among protons that are *not* attached to ^{15}N or ^{13}C , by filtering out protons that have J -evolution to ^{15}N or ^{13}C [13, 100]. Thus, this method detects only ligand signals. Finally, “half-filtered” NOESY experiments are performed to select NOE transfers from protons that are not attached to either by ^{15}N or ^{13}C to a $^{13}\text{C}/^{15}\text{N}$ -labeled proton. This experiment selects only ligand-protein inter-molecular NOEs [101–103].

In addition to the parameters described above, other NMR parameters can be used to better determine complex structures [104]. When the ligand can be labeled by ^{13}C and/or ^{15}N (such as the case of a short peptide), ^1H - ^1H distance information within the ligand is directly obtained through $^{13}\text{C}/^{15}\text{N}$ -edited NOESY experiment, for a complex of a $^{13}\text{C}/^{15}\text{N}$ -labeled ligand with a unlabeled protein. Similarly, the ligand signals may be selected when a complex with a perdeuterated protein is used [105]. The ligand-protein complex structure is ideally determined when the ligand binds to the protein relatively tightly.

When the off-rate of the ligand is much greater than the inverse of the NOE mixing time, the effective inter-molecular NOE is not observed. However, as long as the protein sample is stable at the ligand-saturated condition, the overall structure of the protein in the ligand-bound form, without detailed structure around the binding site, may be determined by intra-protein proton NOEs. On the other hand, the ligand conformation in the protein-bound form may be investigated by transferred NOE, which detects NOE within the ligand in the bound state [105–112]. Further, the ligand conformation in the protein-bound form may be estimated from the chemical shift differences in the Carr-Purcell-Meiboom-Gill (CPMG) R_2 dispersion experiment, by observing the ligand signals when the exchange between the protein-free and bound forms of the ligand causes chemical exchange in the CPMG R_2 dispersion [113].

Paramagnetic effects, such as changes in chemical shifts and relaxation rates, are used to estimate distances of nuclei from a paramagnetic center and provide long-range structural constraints in protein structure determination [114, 115]. Paramagnetic relaxation enhancement (PRE), which is caused by the magnetic dipolar interaction between a nucleus and unpaired electrons in a paramagnetic molecule, is a powerful tool to determine the structures of protein-DNA complexes, which do not have sufficient inter-molecular NOEs [116, 117]. In this approach, a paramagnetic tag is attached to DNA, and the PRE effects from the tag in the DNA to protein protons are monitored. Since many protons exhibit PRE, analysis of the proton PREs in all the observed residues provides reliable information for the complex structure. Repeating the experiments by adding the paramagnetic spin labeling at different DNA positions increases the accuracy of the structure determination. Further, when the complex consists of an ensemble of conformers, PRE data sets obtained for DNA with the paramagnetic tags at different positions are not explained by assuming a single complex structure but explained by assuming multiple conformations. Thus, PRE is a powerful method to elucidate such multiple complex configurations [117, 118]. PRE is also an effective method to characterize conformational changes in intrinsically disordered proteins (IDPs) that do not exhibit single sets of static structures [119, 120].

Finally, thanks to recent increases in computational power, *in silico* molecular docking is becoming widely available [121–127]. NMR titration experiments illuminate chemical-shift perturbation sites, which include, but are not limited to, the actual ligand-binding site. Note, the magnitudes of the perturbations may not directly indicate the binding site, either. However, the information gained from analyzing the perturbation sites can be used to narrow the region that is searched by computational molecular docking, resulting in a more reliable docking result than would be possible without the NMR information. Application of computational molecular docking based on NMR data is an indispensable approach for weak ligand-binding systems, since NMR determination of ligand-protein complex structures is hampered by the lack of sufficient inter-molecular NOE data. Further, in such weak binding systems, the protein conformation is not expected to change significantly upon inhibitor interaction, thus, the ligand-free protein structure can be safely applied as a starting conformation when carrying out molecular docking.

3.5. Protein-Ligand Complex Dynamics

Experiments that determine the ^{15}N longitudinal relaxation rate (R_1), transverse relaxation rate (R_2), and $\{^1\text{H}\}$ - ^{15}N NOE are widely applied to characterize protein backbone dynamics, for which model-free analysis is often utilized [28–32, 128–131]. The analysis optimizes several model-free parameters: the generalized order parameter, S^2 (or the fast and slow order parameters, S_s^2 and S_f^2); correlation time for internal motion, τ_I ; the chemical exchange term, R_{ex} ; and the rotational correlation time, τ_R , of the entire molecule. The degree of internal motion of a backbone N-H bond vector, which is faster than the motion of the entire molecule, is evaluated from the S^2 , whereas slow processes that make chemical exchange contributions to R_2 are evaluated from the R_{ex} term. The method was optimized and established over several years, to evaluate chemical shift anisotropy, to accommodate domain motions, and to improve model selection strategies [132–146]. To characterize protein dynamics in the milli- to microsecond timescale in more a quantitative fashion than the model-free analysis, relaxation experiments in the rotating frame ($R_{1\rho}$) [147–153] and the Carr-Purcell-Meiboom-Gill (CPMG) R_2 experiments [32, 148, 154–157] are often applied. In the latter, a constant-time (CT) ^{15}N CPMG R_2 dispersion experiment was recently established as a robust method to characterize milli-microsecond motion in proteins [158–160]. $R_{1\rho}$ and R_2 more completely characterize chemical exchange than extraction of the R_{ex} term in the model-free analysis, so that the time scale of exchange and the chemical shifts of the minor conformers are obtained [161–163].

For the ligand-protein complex dynamics, R_1 and $\{^1\text{H}\}$ - ^{15}N NOE will mainly reflect fraction-averaged dynamics of the free and the bound form: if 99% of the relative population is the bound form, these parameters reflect the dynamics of the bound form. However, in either $R_{1\rho}$ or R_2 , it is critical to eliminate the effects of on and off rates of the ligand interaction from the observed relaxation rate. Even when equilibrium is supposed to be 99% shifted to the protein-ligand complex form, $R_{1\rho}$ and R_2 may reflect significant effects from the exchange rate between the free and the bound forms, in addition to the slow internal motion within the complex. For example, in a monomer-dimer equilibrium system, the dimer dissociation effect of the two subunits was observed *via* ^{15}N CPMG R_2 dispersion experiment [164]. Similarly, even buffer solute can increase R_2 by the chemical exchange phenomenon that is induced by transient association of the solute and the protein [77, 155]. The off rate effect on R_2 is detected through the ligand signal [113]. Such a magnitude of the exchange effect on R_2 depends on the difference in chemical shifts in the two forms and the relative populations as well as the off rate.

In addition to these experiments that detect sub-nano second motion and milli- to microsecond motion, exchange spectroscopy of Z-magnetization, such as obtained from EXSY or ZZ-exchange experiments, is used to detect dynamics slower than the millisecond time scale or to identify the slow off-rate of the ligand [165–169]. Since NMR exchange spectroscopy exhibits exchange phenomena at individual sites, the obtained result may be used to gain insight into the molecular interaction. For example, Human Immunodeficiency Virus-1 (HIV-1) protease, which is a target for anti-HIV drug design, is known to form a homodimer in solution (Fig. 6) [170]. Yamazaki [171] applied the NOESY pulse sequence (which is identical to the EXSY sequence) to the protease in the presence of an asymmetric

inhibitor, KNI529, that tightly binds to the protease ($K_D \sim \text{nM}$). At such strong binding, the protease NMR spectrum showed only the bound form signals when a slight excess amount of inhibitor was added. In the fully-bound form, a cross peak from residue 31 in one subunit to 31 in the other subunit was observed in the EXSY spectrum [171]. These two residues are over 20 Å from each other, much too far to show either a direct NOE or too specific to be explained by the indirect NOE caused by spin diffusion (Fig. 6). Such cross peaks between distant proton pairs were observed at specific region, *i.e.*, around the inhibitor and exactly between the two subunits. Based on a model analysis, the authors concluded that the inhibitor flips its orientation without completely dissociating from the protein.

In contrast to relaxation experiments that probe backbone amide signals, relaxation experiments of side chains provide dynamics information at the direct ligand interaction site. This is useful to gain insight into the binding mechanism, such as to estimate conformational entropy changes upon ligand-protein interaction. [172–174] For this purpose, methyl relaxation experiments have frequently been used. These studies were advanced by the development of ^2H relaxation for proteins, which is detected through coherence transfer from ^2H to ^{13}C and from ^{13}C to ^1H , using a fractionally deuterated and uniformly ^{13}C labeled protein [35–37]. Although ^{13}C methyl relaxation has been applied to detect side chain dynamics on a sub-nanosecond time scale [175, 176], ^{13}C relaxation decay of a CH_3 group is complicated by the ^1H - ^{13}C dipole-dipole cross correlation and is not expressed by a single exponential function.[177] The complexity is severe in R_2 relaxation in macromolecular relaxation. Thus, application of ^{13}C R_1 and $\{^1\text{H}\}$ - ^{13}C NOE (although R_1 is slightly affected by the cross correlation effect) [178] or of ^{13}C CPMG R_2 dispersion experiment [162, 179] may be preferred to obtain reliable data for methyl relaxation analysis. In either case, it is important to suppress the ^{13}C - ^{13}C dipolar coupling effect; this is achieved by using alternative or selective ^{13}C labeled carbon sources, such as 1,3- $^{13}\text{C}_2$ (and 2- ^{13}C) glycerol and 3- ^{13}C pyruvate [180, 181].

4. PRACTICAL ASPECTS ON INHIBITOR TITRATION BY OBSERVING PROTEIN SIGNALS

Since NMR can detect even a weak protein-inhibitor interaction, protein-inhibitor studies by NMR are often used at the initial stage and at the optimization phase of drug-development [85, 182–188]. In practical applications for drug-development, the sample conditions required for NMR experiments may not be ideal to study a particular protein-inhibitor interaction of interest. For this reason, careful sample preparation and analysis are required. In this section, practical aspects of the NMR titration experiment to detect *enzyme-inhibitor* interactions are discussed.

4.1. A General Scheme for Inhibitor-Titration Experiments

Many natural products and drug-leads may not be soluble in aqueous solution. In particular, compounds that recognize hydrophobic pockets of proteins tend to have high hydrophobicity and are, thereby, less soluble in aqueous solution. Thus, testing the inhibitor solubility in the same aqueous buffer solution that is used for protein studies is needed. Similarly, when a stock solution of an inhibitor in organic solvent, such as a DMSO or methanol, is added to a

protein solution, protein stability in the organic solvent should be tested. This is required for two reasons. First, the organic solvents may interact with the protein when added to the aqueous solution [189–191]. Thus, even when protein chemical shifts vary upon inhibitor titration, data interpretation will be confounded because the changes may be due to the organic solvent rather than inhibitor interaction to the protein. The former results in a false positive. Second, many proteins are chemically denatured as the organic solvent concentration increases [192–194]. A few percentage DMSO concentration in aqueous solution may be sufficient to denature a fraction of the protein population. Denaturation may cause loss of sensitivity as well as chemical exchange effects between the folded and unfolded protein forms. The latter may also generate a false positive titration profile.

When inhibitor is dissolved in an organic solvent, it is important to properly prepare titration samples to obtain reliable data. Recent literature by Markin and Spyropoulos discuss sample preparation in detail [195, 196]. Adding an inhibitor that is dissolved in an organic solvent to a protein in an NMR tube dilutes total concentration of the protein and changes the solvent concentration in the NMR sample (Fig. 7A). Thus, to keep the protein and buffer concentration uniform throughout the titration, individual mixtures of protein and inhibitor should be prepared, in advance, using both protein and inhibitor stock solutions as well as buffer and organic solvent, by calculating their dilution factors (Fig. 7B). Obviously, this method requires preparation of multiple NMR samples, whose number matches the number of titration points. Although the second method is ideal, when qualitative data is needed, the first method may be used. As a compromise, one may use a hybrid of the two methods, *i.e.*, use a small number of samples and add only small amounts of inhibitor solution to the protein samples. In either case, since the protocol of sample preparation directly affects the data accuracy, as described here, it is important to take into account the protocol for sample preparation when assessing the accuracy of data analysis.

Once the samples are ready, the titration spectra are recorded (Fig. 7C). As described in section (3.2), ^1H - ^{15}N HSQC spectra are often recorded using ^{15}N -labeled proteins. Protein-observed inhibitor titration data are used (1) to determine K_D and (2) to estimate the inhibitor binding sites based on the chemical shift perturbation of sites on the protein, which will be described in the next section.

4.2. Determination of the Dissociation Constant

Inhibitors in the early stage of drug development typically bind to proteins weakly, showing NMR titration data in the fast exchange régime: NMR signals in the ^1H - ^{15}N HSQC spectra change positions as a function of the inhibitor concentration. For the signals that exhibit fast exchange titration-profiles in the ^1H - ^{15}N HSQC spectra, a certain weighted average of ^1H and ^{15}N chemical shift magnitudes, for example as in Eq. (6), is used to express the chemical shift change at each titration point, i (Eq. 1).

$$\Delta\delta_{obs}^i = \sqrt{(\delta_H^i - \delta_H^{free})^2 + \left[(\delta_N^i - \delta_N^{free}) \times \frac{\gamma_N}{\gamma_H}\right]^2} \quad (6)$$

Here, δ_k and γ_k indicates chemical shift (ppm) and a gyromagnetic ratio of k nuclear spin ($k = {}^1\text{H}$ or ${}^{15}\text{N}$ in Eq. (6)), respectively. Although the gyromagnetic ratio is used as a normalization factor in Eq. (6), Schumann's article discusses various normalization factors that account for differences in sensitivity of the ${}^{15}\text{N}$ chemical shift against the ${}^1\text{H}$ chemical shifts [197]. Chemical shift changes of individual types of spins are not usually used, since the magnitudes of the chemical shift changes may differ between the two nuclei. In a comprehensive review article, Williamson discussed the effect of such individual types of spins when monitoring NMR chemical shift changes in ligand-titration studies [198].

In the ligand-titration experiment monitored by recording ${}^1\text{H}$ - ${}^{15}\text{N}$ HSQC spectra, K_D is determined for each resonance that exhibits chemical shift changes upon titration (Eq. (6)). Titration data are firstly analyzed by assuming a two-site exchange model, unless a three-site model or a more complex model is indicated by other experiments, in advance. When more than three-molecules are involved or the binding occurs *via* multiple steps, the above simple model may not yield an accurate dissociation constant. Once data cannot be explained using a two-site exchange model, a more complex model may be tested.

Although efforts to convert a non-linear titration curve to a linear function, such as a Scatchard plot [199], were made, direct parameter fit to the non-linear curve can be easily done using computers. In the fast exchange régime, δ^i at the i^{th} titration point is expressed using the saturated shift, δ_∞ , a K_D , a total protein concentration $[P]_{\text{total}}$, and a current total ligand concentration $[L]_i$,

$$\Delta\delta_{\text{cal}}^i = \Delta\delta_\infty \cdot \frac{([P]_{\text{total}} + [L]_i + K_D) + \sqrt{([P]_{\text{total}} + [L]_i + K_D)^2 - 4 \cdot [P]_{\text{total}} \cdot [L]_i}}{2 \cdot [P]_{\text{total}}} \quad (7)$$

The unknown parameters, K_D and δ_∞ , are optimized by minimizing the difference between the experimentally observed δ_{obs}^i and the calculated δ_{cal}^i for a set of titration curves [197, 198]. The experimental errors, such as uncertainties in chemical shifts and inaccuracy of inhibitor or protein concentration, are taken into account to determine the parameter uncertainty.

Chemical shift changes that are explained using Eq. (6) should show linear changes in the HSQC spectra even though the direction and the magnitudes of the changes depend on each residue (Fig. 8A). In contrast, when multiple processes are involved, the chemical shift changes may not be linear, because sets of chemical shifts of the three forms are involved (Fig. 8B). Note, the difference in chemical shifts between the first and the second step and the second and the third step may not be the same and may vary among NMR signals. Thus, upon a first qualitative examination, the overlay of a series of titration spectra shows the inhibitor-protein interaction profile and whether the two-site exchange is appropriate as an initial model. Consistency of the data may be quantitatively evaluated by further analyzing the data. For example, principal component analysis of chemical shift perturbation data has been applied to analyze multiple-ligand-binding systems [200, 201].

4.3. Estimate the Inhibitor-Binding Site

Is there a direct correlation between the magnitude of observed chemical shift changes and the distances from the inhibitor? This may be answered *yes* based on empirical observation, because many inhibitors have multiple aromatic rings, causing significant chemical shift changes for the proximate protein protons [202]. Also, when inhibitors interact with hydrophobic surfaces that include aromatic side chains in a protein, ordering the aromatic residues upon inhibitor interaction most likely shows significant changes in chemical shifts of protein protons close to the aromatic side chains. However, the correct answer to the above question is *no*. This is because there may be a site that shows chemical shift perturbation due to conformational changes induced by the inhibitor-interaction. Such an indirect effect may not correlate to the distance from the ligand [198]. Similarly, the magnitudes of chemical shift changes upon inhibitor interaction depend on the source of the chemical shift perturbation, such as, dihedral angle changes, hydrogen bond formation, and aromatic ring current effects. In these cases, there is no direct correlation between magnitude of the observed chemical shift changes and distance from the inhibitor. In any case, the inhibitor interaction site will show chemical shift perturbation upon inhibitor titration, allowing estimation of the inhibitor-binding site by searching all the observed chemical shift perturbation sites.

In the past 6 years, I and coworkers have studied inhibitor interactions with the ribonuclease H (RNH) domain of the HIV-1 reverse transcriptase (RT) [90, 203, 204]. Among the activities of HIV-1 encoded enzymes, ribonuclease activity is the only one for which a clinically approved inhibitor does not exist, despite the fact that many compounds have been screened [205–211]. Thus, it is important to know the structural basis of RNH inhibition by the screened compounds, and how binding can be improved based on the structural data. Both the RNH domain in RT and an RNH fragment that is used for screening assays have been used in inhibitor interaction studies. To identify inhibitor binding sites, we have applied NMR to an isolated RNH fragment, validating the results with inhibition assays and molecular docking [90, 204].

We previously conducted inhibitor-titration experiments on the chimeric RNH fragment that was used for inhibitor screening [90]. Prior to the titration experiments, backbone NMR signals were assigned by recording 3D NMR experiments using $^{15}\text{N}/^{13}\text{C}$ -labeled RNH. Titration of an acylhydrazone inhibitor, BHMP07, was monitored by recording ^1H - ^{15}N HSQC spectra. A set of titration spectra showed chemical shift perturbations in the fast exchange regime. In particular, magnified shift changes were observed in a relatively small region that contains a helix-loop-helix (Fig. 9A). Using shift changes (Eq. (6)) and a model (Eq. (7)), K_D was determined to be $\sim 140\ \mu\text{M}$ and $\sim 390\ \mu\text{M}$ in the absence and presence of $20\ \text{mM}\ \text{Mg}^{2+}$, respectively.

The helix-loop-helix region affected by the inhibitor contains three Trp residues, suggesting that the chemical shift changes produced by the inhibitor are enlarged by the ring current effect of the Trp side chains (Fig. 9B). In addition to the helix-loop-helix region, the amide of Thr 71 showed pronounced chemical shift changes upon BHMP07 titration (Fig. 9A). Importantly, a Trp side chain resides near the Thr 71 amide (within $4\text{\AA}$) in the model

structure, thus, the chemical shift perturbation of this residue is also likely more enhanced by the ring current, compared to adjacent residues. These changes may be due to direct inhibitor interaction caused by a favorable hydrophobic interaction with the inhibitor, or due to an indirect effect caused by rearrangement of the Trp side chain orientations.

In addition to these large chemical shift perturbations, weak chemical shift perturbation upon BHMP07 titration was observed in a region (Fig. 9A orange highlight) located more than 15 Å away from the helix-loop-helix region; this remote site includes a residue located at the active site where Mg^{2+} and substrate binding occurs (Fig. 9B). Note, as described above, there may not be direct correlation between magnitude of the observed chemical shift changes and distances from the inhibitor. From the magnitudes of the chemical shift changes, one cannot conclude whether the interaction site is in the helix-loop-helix region or in the active site. Thus, there are two possibilities: the chemical shifts of the amides in the orange-highlighted region were (1) indirectly perturbed through the conformational change induced by the inhibitor interaction with the helix-loop-helix region or (2) directly perturbed by inhibitor interaction with the orange-highlighted region that is close to the active site. Additional information was needed to narrow the possible binding sites. Since experiments for the BHMP07-titration in the presence of Mg^{2+} also showed similar chemical shift changes upon interaction with BHMP07, we concluded that the BHMP07 does not directly compete with the Mg^{2+} interaction. Thus, the (2) case is not likely. Based on these observations, allosteric conformational change that inhibits ribonuclease activity of the enzyme was proposed [90]. Subsequent studies demonstrated that the binding site is basically the same in the wildtype RNH, showing chemical shift perturbation in both sites, but slightly towards the active site region [204].

SUMMARY

As reviewed here, there are various NMR methods to investigate protein-inhibitor interactions for application in medicinal chemistry and protein biochemistry. However, recent the technology advancements, to automate NMR protocols and analysis, have hidden the details of the experimental design and ensuing analysis. To prevent misinterpretation of data, it is important to review and understand the underlying basis for choosing a set of NMR experiments.

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REFERENCES

1. Wüthrich, K. NMR of Proteins and Nucleic Acids. New York: Wiley; 1986.
2. Ohlson S, Jungar C, Strandh M, Mandenius CF. Continuous weak-affinity immunosensing. Trends Biotechnol. 2000; 18(2):49–52. [PubMed: 10652508]
3. Perkins JR, Diboun I, Dessailly BH, Lees JG, Orengo C. Transient protein-protein interactions: structural, functional, and network properties. Structutre. 2010; 18(10):1233–1243.
4. Vinogradova O, Qin J. NMR as a unique tool in assessment and complex determination of weak protein-protein interactions. Top Curr Chem. 2012; 326:35–45. [PubMed: 21809187]

5. Kalk A, Berendsen HJC. Proton magnetic relaxation and spin diffusion in proteins. *J Magn Reson.* 1976; 24:343–366.
6. Sykes BD, Hull WE, Snyder GH. Experimental evidence for the role of cross-relaxation in proton nuclear magnetic resonance spin lattice relaxation time measurements in proteins. *Biophys J.* 1978; 21(2):137–146. [PubMed: 623862]
7. Weiss MA, Olejniczak ET. Are methyl groups relaxation sinks in small proteins? *J Magn Reson.* 1990; 86:148–155.
8. Ishima R, Shibata S, Akasaka K. General Features of Proton Longitudinal Relaxation in Proteins in Solution. *J Magn Reson.* 1991; 91:455–465.
9. Cavanagh, J.; Fairbrother, WJ.; Palmer, AG., 3rd; Skelton, NJ. *Protein NMR Spectroscopy.* San Diego: Academic Press; 1996.
10. McIntosh LP, Dahlquist FW. Biosynthetic Incorporation of ^{15}N and ^{13}C for Assignment and Interpretation of Nuclear Magnetic Resonance Spectra of Proteins. *Quart Rev Biophys.* 1990; 23(1):1–38.
11. Koenig BW, Rogowski M, Louis JM. A rapid method to attain isotope labeled small soluble peptides for NMR studies. *J Biomol NMR.* 2003 Jul; 26(3):193–202. [PubMed: 12766417]
12. Grzesiek S, Bax A. Correlating backbone amide and side-chain resonances in larger proteins by multiple relayed triple resonance NMR. *J Am Chem Soc.* 1992; 114(16):6291–6293.
13. Ikura M, Bax A. Isotope filtered 2D NMR of a protein-peptide complex: Study of a skeletal muscle myosin light chain kinase fragment bound to calmodulin. *J Am Chem Soc.* 1992; 114:2433–2240.
14. Clore, GM.; Gronenborn, AM. *NMR of Proteins.* Fuller, SN.; Cohen, WJS., editors. London: The Macmillan Press Ltd; 1993.
15. Abragam, A. *Principles of Nuclear Magnetism.* Oxford: Oxford University Press; 1961.
16. Venters RA, Huang CC, Farmer BT 2nd, Trolard R, Spicer LD, Fierke CA. High-level $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ labeling of proteins for NMR studies. *J Biomol NMR.* 1995; 5(4):339–344. [PubMed: 7647552]
17. Gardner KH, Konrat R, Rosen MK, Kay LE. An $(\text{H})\text{C}(\text{CO})\text{NH}$ -TOCSY pulse scheme for sequential assignment of protonated methyl groups in otherwise deuterated (^{15}N) , (^{13}C) -labeled proteins. *J Biomol NMR.* 1996; 8(3):351–356. [PubMed: 20686885]
18. Kay LE, Gardner KH. Solution NMR spectroscopy beyond 25 kDa. *Curr Opin Struct Biol.* 1997; 7(5):722–731. [PubMed: 9345633]
19. Stoesz JD, Redfield AG. Cross relaxation and spin diffusion effects on the proton NMR of biopolymers in H_2O : Solvent saturation and chemical exchange in superoxide dismutase. *FEBS Lett.* 1978; 91(2):320–324. [PubMed: 680139]
20. Bodenhausen G, Ruben DJ. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chem Phys Lett.* 1980; 69(1):185–189.
21. Pervushin K, Riek R, Wider G, Wüthrich K. Attenuated T_2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci U S A.* 1997; 94(23):12366–12371. [PubMed: 9356455]
22. Weigelt J. Single scan, sensitivity- and gradient-enhanced TROSY for multidimensional NMR experiments. *J Am Chem Soc.* 1998; 120(41):10778–10779.
23. Pervushin K. Impact of transverse relaxation optimized spectroscopy (TROSY) on NMR as a technique in structural biology. *Q Rev Biophys.* 2000; 33(2):161–197. [PubMed: 11131563]
24. Riek R, Fiaux J, Bertelsen EB, Horwich AL, Wüthrich K. Solution NMR techniques for large molecular and supramolecular structures. *J Am Chem Soc.* 2002; 124(41):12144–12153. [PubMed: 12371854]
25. Frueh DP, Ito T, Li JS, Wagner G, Glaser SJ, Kaneja N. Sensitivity enhancement in NMR of macromolecules by application of optimal control theory. *J Biomol NMR.* 2003; 32(1):23–30. [PubMed: 16041480]
26. Tzakos AG, Grace CR, Lukavsky PJ, Riek R. NMR techniques for very large proteins and rnas in solution. *Annu Rev Biophys Biomol Struct.* 2006; 35:319–342. [PubMed: 16689639]

27. Englander SW, Mayne L. Protein folding studied using hydrogen-exchange labeling and two-dimensional NMR. *Annu Rev Biophys Biomol Struct.* 1992; 21:243–265. [PubMed: 1525469]
28. Kay LE, Torchia DA, Bax A. Backbone dynamics of proteins as studied by nitrogen-15 inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease. *Biochemistry.* 1989; 28:8972–8979. [PubMed: 2690953]
29. Clore GM, Driscoll PC, Wingfield PT, Gronenborn AM. Analysis of the backbone dynamics of interleukin-1 beta using two-dimensional inverse detected heteronuclear ^{15}N - ^1H NMR spectroscopy. *Biochemistry.* 1990; 29(32):7387–7401. [PubMed: 2223770]
30. Palmer AG 3rd, Rance M, Wright PE. Intramolecular motions of a zinc finger DNA-binding domain from Xfin characterized by proton-detected natural abundance ^{13}C Heteronuclear NMR spectroscopy. *J Am Chem Soc.* 1991; 113(12):4371–4380.
31. Schneider DM, Dellwo MJ, Wand AJ. Fast internal main-chain dynamics of human ubiquitin. *Biochemistry.* 1992; 31(14):3645–3652. [PubMed: 1314645]
32. Orekhov VY, Pervushin KV, Arseniev AS. Backbone dynamics of (1–71)bacterioopsin studied by two-dimensional ^1H - ^{15}N NMR spectroscopy. *Eur J Biochem.* 1994; 219(3):887–896. [PubMed: 8112340]
33. Bax A, Griffey RH, Hawkins BL. Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR. *J Magn Reson.* 1983; 55(2):301–315.
34. Grzesiek S, Bax A. Improved 3D triple-resonance NMR techniques applied to a 31 kDa protein. *J Magn Reson.* 1992; 96(2):432–440.
35. Muhandiram DR, Yamazaki T, Sykes BD, Kay LE. Measurement of H-2 T-1 and T-1p Relaxation-Times in Uniformly C-13-Labeled and Fractionally H-2-Labeled Proteins in Solution. *J Am Chem Soc.* 1995 Nov; 117(46):11536–11544.
36. Millet O, Muhandiram DR, Skrynnikov NR, Kay LE. Deuterium spin probes of side-chain dynamics in proteins 1. Measurement of five relaxation rates per deuteron in (^{13}C)-labeled and fractionally (^2H)-enriched proteins in solution. *J Am Chem Soc.* 2002; 124(22):6439–6448. [PubMed: 12033875]
37. Skrynnikov NROM, Kay LE. Deuterium spin probes of side-chain dynamics in proteins 2. Spectral density mapping and identification of nanosecond time-scale side-chain motions. *J Am Chem Soc.* 2002; 124(22):6449–6460. [PubMed: 12033876]
38. Aaron BM, Oikawa K, Reithmeier RA, Sykes BD. Characterization of skeletal muscle calsequestrin by ^1H NMR spectroscopy. *J Biol Chem.* 1984; 259(19):11876–11881. [PubMed: 6480588]
39. Sylvia LA, Gerig JT. Fluorine NMR studies of the metabolism of flumecinol (3-trifluoromethyl-alpha-ethylbenzhydrol). *Drug Metab Dispos.* 1993; 21(1):105–113. [PubMed: 8095202]
40. Percival MD, Withers SG. ^{19}F NMR investigations of the catalytic mechanism of phosphoglucomutase using fluorinated substrates and inhibitors. *Biochemistry.* 1992; 31(2):505–512. [PubMed: 1531026]
41. Danielson MA, Falke JJ. Use of ^{19}F NMR to probe protein structure and conformational changes. *Annu Rev Biophys Biomol Struct.* 1996; 25:163–195. [PubMed: 8800468]
42. Loewen MC, Klein-Seetharaman J, Getmanova EV, Reeves PJ, Schwalbe H, Khorana HG. Solution ^{19}F nuclear Overhauser effects in structural studies of the cytoplasmic domain of mammalian rhodopsin. *Proc Natl Acad Sci.* 2001; 98(9):4888–4892. [PubMed: 11320239]
43. Dalvit C, Flocco M, Veronesi M, Stockman BJ. Fluorine-NMR competition binding experiments for high-throughput screening of large compound mixtures. *Comb Chem High Throughput Screen.* 2002; 5(8):605–611. [PubMed: 12470257]
44. Malet-Martino M, Gilard W, Desmoulin F, Martino R. Fluorine nuclear magnetic resonance spectroscopy of human biofluids in the field of metabolic studies of anticancer and antifungal fluoropyrimidine drugs. *Clinica Chimica Acta.* 2006; 366(1–2):61–73.
45. Kolocouris A, Zikos C, Broadhurst RW. ^{19}F NMR detection of the complex between amantadine and the receptor portion of the influenza A M2 ion channel in DPC micelles. *Bioorg Med Chem Lett.* 2007; 17(14):3947–3952. [PubMed: 17502147]

46. Klein-Seetharaman J, Getmanova EV, Loewen MC, Reeves PJ, Khorana HG. NMR spectroscopy in studies of light-induced structural changes in mammalian rhodopsin: applicability of solution (19)F NMR. *Proc Natl Acad Sci U S A*. 1999; 96(24):13744–13749. [PubMed: 10570143]
47. Liu JJ, Horst R, Katritch V, Stevens RC, Wüthrich K. Biased signaling pathways in β 2-adrenergic receptor characterized by 19F-NMR. *Science*. 2012; 335(2):1106–1110. [PubMed: 22267580]
48. Chung KY, Kim TH, Manglik A, Alvares R, Kobilka BK, Prosser RS. Role of detergents in conformational exchange of a G protein-coupled receptor. *J Biol Chem*. 2012; 287(43):36305–36311. [PubMed: 22893704]
49. Didenko T, Liu JJ, Horst R, Stevens RC, Wüthrich K. Fluorine-19 NMR of integral membrane proteins illustrated with studies of GPCRs. *Curr Opin Struct Biol*. 2013; 23(5):740–747. [PubMed: 23932201]
50. Böhm HJ, Banner D, Bendels S, Kansy M, Kuhn B, Müller K, et al. Fluorine in medicinal chemistry. *Chembiochem*. 2004; 5(5):637–643. [PubMed: 15122635]
51. Hagmann WK. The many roles for fluorine in medicinal chemistry. *J Med Chem*. 2008; 51(15):4359–4369. [PubMed: 18570365]
52. Buer BC, Meagher JL, Stuckey JA, Mars hEN. Structural basis for the enhanced stability of highly fluorinated proteins. *Proc Natl Acad Sci U S A*. 2012; 109(13):4810–4815. [PubMed: 22411812]
53. Griffin RG, Ellett JD, Mehring M, Bullitt JG, Waugh JS. Single Crystal Study of the 19F Shielding Tensors of a Trifluoromethyl Group. *J Chem Phys*. 1972; 57(5):2147–2155.
54. Mehring, M. *Principles of High Resolution NMR in Solids*. Berlin Heidelberg NewYork: Springer-Verlag; 1983.
55. Grzesiek S, Bax A. The Importance of Not Saturating H2o in Protein Nmr - Application to Sensitivity Enhancement and Noe Measurements. *J Am Chem Soc*. 1993 Dec.115(26):12593.
56. Lippens S, Dhalluin C, Weieruszski JM. Use of a water flip-back pulse in the homonuclear NOESY experiment. *J Biomol NMR*. 1995; 5(3):327–331. [PubMed: 22911506]
57. Diercks T, Daniels M, Kaptein R. Extended flip-back schemes for sensitivity enhancement in multidimensional HSQC-type out-and-back experiments. *J Biomol NMR*. 2005; 33(4):243–259. [PubMed: 16341753]
58. Cavanagh J, Palmer AG 3rd, Wright PE, Rance M. Sensitivity improvement in proton-detected two-dimensional heteronuclear relay spectroscopy. *J Magn Reson*. 1991; 91(2):429–436.
59. Kay LE, Keifer P, Saarinen T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J Am Chem Soc*. 1992; 114(26):10663–10665.
60. Takahashi S, Nagayama K. A novel NMR microcell with symmetric geometry. *J Magn Reson*. 1988; 76(2):347–351.
61. Warren WS, Hammes SL, Bates JL. Dynamics of radiation damping in nuclear magnetic resonance. *J Chem Phys*. 1989; 91(10):5895–5904.
62. Mao XA, Guo JX, Ye CH. Competition between radiation damping and transverse relaxation effects on NMR signal intensities. *Chem Phys Lett*. 1994; 218(3):249–253.
63. Augustine MP, Bush SD, Hahn EL. Noise triggering of radiation damping from the inverted state. *Chem Phys Lett*. 2000; 322:111–118.
64. Lin YY, Lisitza N, Ahn S, Warren WS. Resurrection of Crushed Magnetization and Chaotic Dynamics in Solution NMR Spectroscopy. *Science*. 2000; 290:118–121. [PubMed: 11021793]
65. Krishnan VV. Radiation damping in microcoil NMR probes. *J Magn Reson*. 2006; 179(2):294–298. [PubMed: 16427795]
66. Bloembergen N, Pound RV. Radiation damping in magnetic resonance experiments. *Phys Rev*. 1954; 95(1):8–12.
67. Bloom S. Effects of radiation damping on spin dynamics. *J App Phys*. 1957; 28(7):800–804.
68. Sodickson A, Maas WE, Cory DG. The initiation of radiation damping by noise. *J Magn Reson*. 1996; B 110:298–303.
69. Krishnan VV, Murali N. Radiation damping in modern NMR experiments: Progress and challenges. *Proc NMR*. 2013 Spec;68:41–57.

70. Kremer W, Kalbitzer HR. Physiological conditions and practicality for protein nuclear magnetic resonance spectroscopy: experimental methodologies and theoretical background. *Methods Enzymol.* 2001; 339:3–19. [PubMed: 11462818]
71. Bagby S, Tong KI, Ikura M. Optimization of protein solubility and stability for protein nuclear magnetic resonance. *Methods Enzymol.* 2001; 339:20–40. [PubMed: 11462812]
72. Wagner G, Wüthrich K. Nuclear magnetic resonance of labile protons in the basic pancreatic trypsin inhibitor. *J Mol Biol.* 1979; 130(1):1–18. [PubMed: 38342]
73. Hoult DI, Lauterbur PC. The Sensitivity of the Zeugmatographic Experiment Involving Human Samples. *J Magn Reson.* 1979; 34:425–433.
74. Gadian DG, Robinson FNH. Radiofrequency Losses in NMR Experiments on Electrically Conducting Samples. *J Magn Reson.* 1979; 34:449–455.
75. Kelly AE, Ou HD, Withers R, Dotsch V. Low-conductivity buffers for high-sensitivity NMR measurements. *J Am Chem Soc.* 2002; 124(40):12013–12019. [PubMed: 12358548]
76. Horiuchi T, Takahashi M, Kikuchi J, Yokoyama S, Maeda H. Effect of dielectric properties of solvents on the quality factor for a beyond 900 MHz cryogenic probe model. *J Magn Reson.* 2005; 174:34–42. [PubMed: 15809170]
77. Long D, Yang D. Buffer interference with protein dynamics: a case study on human liver fatty acid binding protein. *Biophys J.* 2009; 96(4):1482–1488. [PubMed: 19217864]
78. Wong M, Khirich G, Loria JP. What's in your buffer? Solute altered millisecond motions detected by solution NMR. *Biochemistry.* 2013; 52(37):6548–6558. [PubMed: 23991940]
79. Fielding L. NMR methods for the determination of protein-ligand dissociation constants. *Curr Top Med Chem.* 2003; 3(1):39–53. [PubMed: 12577990]
80. Fielding L, Rutherford S, Fletcher D. Determination of protein-ligand binding affinity by NMR: observations from serum albumin model systems. *Magn Reson Chem.* 2005; 43(6):463–470. [PubMed: 15816062]
81. Fielding L. NMR methods for the determination of protein-ligand dissociation constants. *Prog Nucl Magn Reson Spectrosc.* 2007; 51(4):219–242.
82. Meyer B, Peters T. Characterization of ligand binding by saturation transfer difference NMR spectroscopy. *Angewandte Chemie.* 1999; 38(12):1784–1788.
83. Meyer B, Peters T. NMR Spectroscopy Techniques for Screening and Identifying Ligand Binding to Protein Receptors. *Angewandte Chemie.* 2003; 42(8):864–890. [PubMed: 12596167]
84. Lepre CA, Moore JM, Peng JW. Theory and Applications of NMR-Based Screening in Pharmaceutical. *Research Chem Rev.* 2004; 104(8):3641–3675.
85. Dalvit C, Ardini E, Flocco M, Fogliatto GP, Mongelli N, Veronesi M. A General NMR Method for Rapid, Efficient, and Reliable Biochemical Screening. *J Am Chem Soc.* 2004; 125(47):14620–14625. [PubMed: 14624613]
86. Haselhorst T, Lamerz AC, Itzstein Mv. Saturation transfer difference NMR spectroscopy as a technique to investigate protein-carbohydrate interactions in solution. *Methods Mol Biol.* 2009; 534:375–386. [PubMed: 19277538]
87. Park SJ, Kim KS, Atta-Ur-Rahman Choudhary MI. Investigation of Drug Delivery Behaviors by NMR Spectroscopy. *Structure-Activity Relationship Studies in Drug Development by NMR Spectroscopy.* 2011
88. Mailavaram RP, Devarakonda M, Atta-Ur-Rahman Choudhary MI. NMR-A Gate Way to Drug Discovery. *Structure-Activity Relationship Studies in Drug Development by NMR Spectroscopy:* Bentham Science Publishers. 2011
89. Solomon I. Relaxation Processes in a System of Two Spins. *Physical Review.* 1955; 99(9):559–565.
90. Gong Q, Menon L, Ilina T, Miller LG, Ahn J, Parniak MA, et al. Interaction of HIV-1 Reverse Transcriptase Ribonuclease H with an Acylhydrazide Inhibitor. *Chem Biol Drug Des.* 2011; 77(1):39–47. [PubMed: 21114787]
91. Sudmeier JL, Evelhoch JL, Jonsson NB-H. Dependence of NMR Lineshape Analysis upon Chemical Rates and Mechanisms: Implications for Enzyme Histidine Titrations. *J Magn Reson.* 1980; 40:377–380.

92. Günther UL, Schaffhausen B. NMRKIN: simulating line shapes from two-dimensional spectra of proteins upon ligand binding. *J Biomol NMR*. 2002; 2(3):201–209. [PubMed: 11991351]
93. Kovrigin EL. NMR line shapes and multi-state binding equilibria. *J Biomol NMR*. 2012; 53(3):257–270. [PubMed: 22610542]
94. Shortridge MD, Hage DS, Harbison GS, Powers R. Estimating protein-ligand binding affinity using high-throughput screening by NMR. *J Comb Chem*. 2008; 10(6):948–958. [PubMed: 18831571]
95. Barbato G, Ikura M, Kay LE, Pastor RW, Bax A. Backbone dynamics of calmodulin studied by ¹⁵N relaxation using inverse detected two-dimensional NMR spectroscopy: the central helix is flexible. *Biochemistry*. 1992; 31(23):5269–5278. [PubMed: 1606151]
96. Zhang M, Tanaka T, Ikura M. Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nat Struct Biol*. 1995; 2(9):758–767. [PubMed: 7552747]
97. Meador WE, Means AR, Quijcho FA. Target enzyme recognition by calmodulin: 2.4 angstroms structure of a calmodulin-peptide complex. *Science*. 1992; 257(5074):1251–1255. [PubMed: 1519061]
98. Clore GM, Bax A, Ikura M, Gronenborn AM. Structure of calmodulin-target peptide complexes. *Curr Opin Struct Biol*. 1993; 3(6):838–845.
99. Otting G, Qian YQ, Billeter M, Müller M, Affolter M, Gehring WJ, et al. Protein–DNA contacts in the structure of a homeodomain–DNA complex determined by nuclear magnetic resonance spectroscopy in solution. *EMBO J*. 1990; 9(10):3085–3092. [PubMed: 1976507]
100. Breeze AL. Isotope-filtered NMR methods for the study of biomolecular structure and interactions. *Prog NMR Spectrosc*. 2000; 36(4):323–372.
101. Otting G, Wüthrich K. Extended heteronuclear editing of 2D ¹H NMR spectra of isotope-labeled proteins, using the X(ω₁,ω₂) double half filter. *J Magn Reson*. 1989; 85(3):586–594.
102. Ogura K, Terasawa H, Inagaki F. An improved double-tuned and isotope-filtered pulse scheme based on a pulsed field gradient and a wide-band inversion shaped pulse. *J Biomol NMR*. 1996; 8(4):492–498. [PubMed: 20859780]
103. Zwahlen C, Legault P, Vincent SJF, Greenblatt J, Konrat R, Kay LE. Methods for measurement of intermolecular NOEs by multinuclear NMR spectroscopy: Application to a bacteriophage lambda N-peptide/boxB RNA complex. *J Am Chem Soc*. 1997; 119:6711–6721.
104. Otting G. Experimental NMR techniques for studies protein-ligand interactions. *Curr Opin Struct Biol*. 2008; 3(5):760–768.
105. Gelis I, Bonvin AM, Keramisanou D, Koukaki M, Gouridis G, Karamanou S, et al. Structural basis for signal-sequence recognition by the translocase motor SecA as determined by NMR. *Cell*. 2007; 131(4):756–769. [PubMed: 18022369]
106. Clore GM, Gronenborn AM. Theory of the time dependent transferred nuclear Overhauser effect: Applications to structural analysis of ligand-protein complexes in solution. *J Magn Reson*. 1983; 53(3):423–442.
107. London RE, Perlman ME, Davis DG. Relaxation-matrix analysis of the transferred nuclear overhauser effect for finite exchange rates. *J Magn Reson*. 1992; 97(1):79–98.
108. Lippens RM, Cerf C, Hallenga K. Theory experimental results of transfer-NOE experiments I. The influence of the off rate *versus* cross-relaxation rates. *J Magn Reson*. 1992; 99(2):268–281.
109. Nirmala NR, Lippens GM, Hallenga K. Theory experimental results of transfer NOE experiments II. The influence of residual mobility and relaxation centers inside the protein on the size of transfer NOEs. *J Magn Reson*. 1992; 100(1):25–42.
110. Sykes BD. Determination of the conformations of bound peptides using NMR-transferred nOe techniques. *Curr Opin Biotech*. 1993; 4(4):392–396. [PubMed: 7763968]
111. Pashkov VS, Balashova TA, Zhemaeva LV, Sikilinda NN, Kutuzov MA, Abdulaev NG, et al. Conformation of surface exposed N-terminus part of bacteriorhodopsin studied by transferred NOE technique. *FEBS Lett*. 1996; 381(1–2):119–122. [PubMed: 8641417]
112. Post CB. Exchange-transferred NOE spectroscopy and bound ligand structure determination. *Curr Opin Struct Biol*. 2003; 13(5):581–588. [PubMed: 14568612]

113. Zintsmaster JS, Wilson BD, Peng JW. Dynamics of Ligand Binding from ^{13}C NMR Relaxation Dispersion at Natural Abundance. *J Am Chem Soc.* 2008; 130(43):14060–14061. [PubMed: 18834120]
114. Bertini I, Donaire A, Luchinat C, Rosato A. Paramagnetic relaxation as a tool for solution structure determination: Clostridium pasteurianum ferredoxin as an example. *Proteins.* 1997; 29(3):348–358. [PubMed: 9365989]
115. Bertini I, Donaire A, Jiménez B, Luchinat C, Parigi G, Piccioli M, et al. Paramagnetism-based *versus* classical constraints: an analysis of the solution structure of Ca Ln calbindin D9k. *J Biomol NMR.* 2001; 21(2):85–98. [PubMed: 11727989]
116. Iwahara J, Schwieters CD, Clore GM. Ensemble approach for NMR structure refinement against (^1H) paramagnetic relaxation enhancement data arising from a flexible paramagnetic group attached to a macromolecule. *J Am Chem Soc.* 2004; 126(18):5879–5896. [PubMed: 15125681]
117. Clore GM, Tang C, Iwahara J. Elucidating transient macromolecular interactions using paramagnetic relaxation enhancement. *Curr Opin Struct Biol.* 2007; 17(5):603–616. [PubMed: 17913493]
118. Tang C, Iwahara J, Clore GM. Visualization of transient encounter complexes in protein-protein association. *Nature.* 2006; 444(7117):383–386. [PubMed: 17051159]
119. Gillespie JR, Shortle D. Characterization of long-range structure in the denatured state of staphylococcal nuclease I. Paramagnetic relaxation enhancement by nitroxide spin labels. *J Mol Biol.* 1997; 368(1):158–169. [PubMed: 9149149]
120. Lietzow MA, Jamin M, Dyson HJ, Wright PE. Mapping long-range contacts in a highly unfolded protein. *J Mol Biol.* 2002; 322(4):655–662. [PubMed: 12270702]
121. Goodsell DS, Morris GM, Olson AJ. Automated docking of flexible ligands: applications of AutoDock. *J Mol Recognit.* 1996; 9(1):1–5. [PubMed: 8723313]
122. Smith GR, Sternberg MJE. Prediction of protein-protein interactions by docking methods. *Curr Opin Struct Biol.* 2002; 12(1):28–35. [PubMed: 11839486]
123. Schulz-Gasch T, Stahl M. Binding site characteristics in structure-based virtual screening: evaluation of current docking tools. *J Mol Model.* 2003; 9(1):47–57. [PubMed: 12638011]
124. McGaughey GB, Sheridan RP, Bayly CI, Culberson JC, Kreatsolas C, Lindsley S, et al. Comparison of Topological, Shape, and Docking Methods in Virtual Screening. *J Chem Inf Model.* 2007; 47(4):1504–1519. [PubMed: 17591764]
125. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem.* 2010; 31(2):455–461. [PubMed: 19499576]
126. McGann M. FRED Pose Prediction and Virtual Screening Accuracy. *J Chem Inf Model.* 2011; 51(3):578–796. [PubMed: 21323318]
127. Stark JL, Powers R. Application of NMR and molecular docking in structure-based drug discovery. *Top Curr Chem.* 2012; 326:1–34. [PubMed: 21915777]
128. Lipari G, Szabo A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules 1. Theory and range of validity. *J Am Chem Soc.* 1982; 104(17):4546–4559.
129. Lipari G, Szabo A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules 2. Analysis of experimental results. *J Am Chem Soc.* 1982; 104(17):4559–4570.
130. Nirmala NR, Wagner G. Measurement of C-13 relaxation-times in proteins by two-dimensional heteronuclear H1-C-13 correlation spectroscopy. *J Am Chem Soc.* 1988; 110(2):7557–7558.
131. Mandel AM, Akke M, Palmer AG 3rd. Backbone Dynamics of Escherichia-Coli Ribonuclease Hi - Correlations with Structure and Function in an Active Enzyme. *J Mol Biol.* 1995 Feb 10; 246(1):144–163. [PubMed: 7531772]
132. Lee LK, Rance M, Chazin WJ, Palmer AG 3rd. Rotational diffusion anisotropy of proteins from simultaneous analysis of ^{15}N and ^{13}C alpha nuclear spin relaxation. *J Biomol NMR.* 1997; 9(3):287–298. [PubMed: 9204557]
133. Kroenke CD, Loria JP, Lee LK, Rance M, Palmer AG 3rd. Longitudinal and transverse H-1-N-15 dipolar N-15 chemical shift anisotropy relaxation interference: Unambiguous determination of

rotational diffusion tensors and chemical exchange effects in biological macromolecules. *J Am Chem Soc.* 1998; 120(31):7905–7915.

134. Fushman D, Tjandra N, Cowburn D. An approach to direct determination of protein dynamics from N-15 NMR relaxation at multiple fields, independent of variable N-15 chemical shift anisotropy and chemical exchange contributions. *J Am Chem Soc.* 1999; 121(37):8577–8582.
135. Andrec M, Montelione GT, Levy RM. Estimation of dynamic parameters from NMR relaxation data using the Lipari-Szabo model-free approach and Bayesian statistical methods. *J Magn Reson.* 1999; 139(2):408–421. [PubMed: 10423379]
136. Lee AL, Wand AJ. Assessing potential bias in the determination of rotational correlations times of proteins by NMR. *J Biomol NMR.* 1999; 13:101–112. [PubMed: 10070752]
137. Campbell AP, Spyropoulos L, Irvin RT, Sykes BD. Backbone dynamics of a bacterially expressed peptide from the receptor binding domain of *Pseudomonas aeruginosa* pilin strain PAK from heteronuclear 1H-15N NMR spectroscopy. *J Biomol NMR.* 2000; 17(3):239–255. [PubMed: 10959631]
138. Ishima R, Torchia DA. Protein dynamics from NMR. *Nat Struct Biol.* 2000; 7(9):740–743. [PubMed: 10966641]
139. ruschweiler R. New approaches to the dynamic interpretation and prediction of NMR relaxation data from proteins. *Curr Opin Struct Biol.* 2003 Apr; 13(2):175–183. [PubMed: 12727510]
140. d'Auvergne EJ, Gooley PR. The use of model selection in the model-free analysis of protein dynamics. *J Biomol NMR.* 2003; 25(1):25–39. [PubMed: 12566997]
141. Pelupessy P, Ravindranathan S, Bodenhausen G. Correlated motions of successive amide N-H bonds in proteins. *J Biomol NMR.* 2003; 25(4):265–280. [PubMed: 12766390]
142. Idiyatullin D, Daragan VA, Mayo KH. (NH)-N-15 backbone dynamics of protein GB1: Comparison of order parameters and correlation times derived using various “model-free” approaches. *J Phys Chem B.* 2003; 107(11):2602–2609.
143. Redfield C. Using nuclear magnetic resonance spectroscopy to study molten globule states of proteins. *Methods Mol Biol.* 2004 Sep; 34(1):121–132.
144. Chen J, Brooks CLI, Wright PE. Model-free Analysis of Protein Dynamics: Assessment of Accuracy and Model Selection Protocols Based on Molecular Dynamics Simulation. *J Biomol NMR.* 2004; 29(3):243–257. [PubMed: 15213423]
145. Korchuganov DS, Gagnidze IE, Tkach EN, Schulga AA, Kirpichnikov MP, Arseniev AS. Determination of protein rotational correlation time from NMR relaxation data at various solvent viscosities. *J Biomol NMR.* 2004; 30(4):431–442. [PubMed: 15630563]
146. Spyropoulos L. A suite of Mathematica notebooks for the analysis of protein main chain (15)N NMR relaxation data. *J Biomol NMR.* 2006; 36(4):215–224. [PubMed: 17061025]
147. Szyperski S, Luginbühl P, Otting G, Güntert P, Wüthrich K. Protein dynamics studied by rotating frame. *J Biomol NMR.* 1993; 3:151–164. [PubMed: 7682879]
148. Akke M, Palmer AG 3rd. Monitoring Macromolecular Motions on Microsecond to Millisecond Time Scales by R1ρ-R1 Constant Relaxation Time NMR Spectroscopy. *J Am Chem Soc.* 1996; 118(4):911–912.
149. Mulder FAA, de Graaf RA, Kaptein R, Boelens R. An Off-resonance Rotating Frame Relaxation Experiment for the Investigation of Macromolecular Dynamics Using Adiabatic Rotations. *J Magn Reson.* 1998; 131(2):351–357. [PubMed: 9571112]
150. Kempf JG, Jung JY, Sampson NS, Loria JP. Off-resonance TROSY (R1 ρ - R1) for quantitation of fast exchange processes in large proteins. *J Am Chem Soc.* 2003; 125(40):12064–12065. [PubMed: 14518971]
151. Kim S, Baum J. An on/off resonance rotating frame relaxation experiment to monitor millisecond to microsecond timescale dynamics. *J Biomol NMR.* 2004; 30(2):195–204. [PubMed: 15702526]
152. Massi F, Johnson E, Wang C, Rance M, Palmer AG 3rd. NMR R1 ρ rotating-frame relaxation with weak radio frequency fields. *J Am Chem Soc.* 2004; 126(7):2247–2256. [PubMed: 14971961]
153. Igumenova TI, Palmer AG 3rd. Off-resonance TROSY-selected R-1ρ experiment with improved sensitivity for medium- and high-molecular-weight proteins. *J Am Chem Soc.* 2006; 128(25): 8110–8111. [PubMed: 16787055]

154. Carver JP, Richards RE. General 2-Site Solution for Chemical Exchange Produced Dependence of T2 Upon Carr-Purcell Pulse Separation. *J Magn Reson.* 1972; 6(1):89–105.
155. Davis DG, Perlman ME, London RE. Direct Measurements of the Dissociation-Rate Constant for Inhibitor-Enzyme Complexes *via* the T-1-Rho and T-2 (Cpmg) Methods. *J Magn Reson Ser B.* 1994 Jul; 104(3):266–275. [PubMed: 8069484]
156. Zinn-Justin S, Berthault P, Guenneugues M, Desvaux H. Off-resonance rf fields in heteronuclear NMR. Application to the study of slow motions. *J Biomol NMR.* 1997; 10(4):363–372. [PubMed: 20859782]
157. Mulder FA, van Tilborg PJ, Kaptein R, Boelens R. Microsecond time scale dynamics in the RXR DNA-binding domain from a combination of spin-echo and off-resonance rotating frame relaxation measurements. *J Biomol NMR.* 1999; 13(3):275–288. [PubMed: 10212986]
158. Loria JP, Rance M, Palmer AG 3rd. A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy. *J Am Chem Soc.* 1999 Mar 17;121(10):2331–2332.
159. Tollinger M, Skrynnikov NR, Mulder FA, Forman-Kay JD, Kay LE. Slow dynamics in folded and unfolded states of an SH3 domain. *J Am Chem Soc.* 2001; 123(46):11341–11352. [PubMed: 11707108]
160. Hansen DF, Vallurupalli P, Kay LE. An Improved (15)N Relaxation Dispersion Experiment for the Measurement of Millisecond Time-Scale Dynamics in Proteins. *J Phys Chem B.* 2008; 112(19):5898–5904. [PubMed: 18001083]
161. Mulder FA, Skrynnikov NR, Hon B, Dahlquist FW, Kay LE. Measurement of slow (micros-ms) time scale dynamics in protein side chains by (15)N relaxation dispersion NMR spectroscopy: application to Asn and Gln residues in a cavity mutant of T4 lysozyme. *J Am Chem Soc.* 2001; 123(5):967–975. [PubMed: 11456632]
162. Mulder FA, Hon B, Mittermaier A, Dahlquist FW, Kay LE. Slow internal dynamics in proteins: application of NMR relaxation dispersion spectroscopy to methyl groups in a cavity mutant of T4 lysozyme. *J Am Chem Soc.* 2002; 124(7):1443–1451. [PubMed: 11841314]
163. Kovrigin EL, Kempf JG, Grey MJ, Loria JP. Faithful estimation of dynamics parameters from CPMG relaxation dispersion measurements. *J Magn Reson.* 2006; 180(1):83–104. [PubMed: 16460975]
164. Jee J, Ishima R, Gronenborn AM. Characterization of Specific Protein Association by (15)N CPMG Relaxation Dispersion NMR: The GB1(A34F) Monomer-Dimer Equilibrium. *J Phys Chem B.* 2007; 112(19):6008–6012. [PubMed: 18004837]
165. Jeener J, Meier BH, Bachmann P, Ernst RR. Investigation of exchange processes by two-dimensional NMR spectroscopy. *J Chem Phys.* 1979; 71(11):4546–4553.
166. Montelione GT, Wagner G. 2D chemical exchange NMR spectroscopy by proton-detected heteronuclear correlation. *J Am Chem Soc.* 1989; 111(8):3096–3098.
167. Farrow NA, Zhang O, Forman-Kay JD, Kay LEJ. Slow Dynamics in Folded and Unfolded States of an SH3 Domain. *J Biomol NMR.* 1994; 4(5):727–734. [PubMed: 7919956]
168. Li Y, Palmer AG 3rd. TROSY-selected ZZ-exchange experiment for characterizing slow chemical exchange in large proteins. *J Biomol NMR.* 2009; 45(4):357–360. [PubMed: 19890725]
169. Robson SA, Peterson R, Bouchard LS, Villareal VA, Clubb RT. A heteronuclear zero quantum coherence Nz-exchange experiment that resolves resonance overlap and its application to measure the rates of heme binding to the IsdC protein. *J Am Chem Soc.* 2010; 132(28):9522–9523. [PubMed: 20578738]
170. Wlodawer A, Miller M, Jaskolski M, Sathyanarayana BK, Baldwin E, Weber IT, et al. Conserved Folding in Retroviral Proteases - Crystal-Structure of a Synthetic Hiv-1 Protease. *Science.* 1989 Aug 11;245(4918):616–621. [PubMed: 2548279]
171. Katoh E, Yamazaki T, Kiso Y, Wingfield PT, Stahl SJ, Kaufman JD, et al. Determination of the rate of monomer interchange in a ligand-bound homodimeric protein from NOESY cross peaks: Application to the HIV protease/KNI-529 complex. *J Am Chem Soc.* 1999; 121(11):2607–2608.
172. Akke M, Bruschweiler R, Palmer AG 3rd. Nmr Order Parameters and Free-Energy - an Analytical Approach and Its Application to Cooperative Ca²⁺ Binding by Calbindin-D(9k). *J Am Chem Soc.* 1993 Oct 20; 115(21):9832–9833.

173. Yang DW, Kay LE. Contributions to conformational entropy arising from bond vector fluctuations measured from NMR-derived order parameters: Application to protein folding. *Journal of Molecular Biology*. 1996 Oct 25; 263(2):369–382. [PubMed: 8913313]
174. Li Z, Raychaudhuri S, Wand AJ. Insights into the local residual entropy of proteins provided by NMR relaxation. *Protein Sci*. 1996; 5(12):2647–2650. [PubMed: 8976574]
175. Nicholson LK, Kay LE, Baldisseri DM, Arango J, Young PR, Bax A, et al. Dynamics of methyl groups in proteins as studied by proton-detected ¹³C NMR spectroscopy. Application to the leucine residues of Staphylococcal nuclease. *Biochemistry*. 1992; 31:5253–5263. [PubMed: 1606149]
176. Lee AL, Kinnear SA, Wand AJ. Redistribution and loss of side chain entropy upon formation of a calmodulin-peptide complex. *Nat Struct Biol*. 2000; 7(1):72–77. [PubMed: 10625431]
177. Werbelow LG, Marshall AG. Internal Rotation and Nonexponential Methyl Nuclear Relaxation for Macromolecules. *J Magn Reson*. 1973; 11:299–313.
178. Wand AJ, Urbauer JL, McEvoy RP, Bieber RJ. Internal dynamics of human ubiquitin revealed by ¹³C–relaxation studies of randomly fractionally labeled protein. *Biochemistry*. 1996; 35(19): 6116–6125. [PubMed: 8634254]
179. Ishima R, Louis JM, Torchia DA. Transverse C-13 relaxation of CHD2 methyl isotopomers to detect slow conformational changes of protein side chains. *Journal of the American Chemical Society*. 1999 Dec 15; 121(49):11589–11590.
180. LeMaster DM, Kushlan DM. Dynamical Mapping of E. coli Thioredoxin *via* ¹³C NMR Relaxation Analysis. *J Am Chem Soc*. 1996; 118(39):9255–9264.
181. Lee AL, Urbauer JL, Wand AJ. Improved labeling strategy for ¹³C relaxation measurements of methyl groups in proteins. *J Biomol NMR*. 1997; 9(4):437–440. [PubMed: 9255947]
182. Hajduk PJ, Gerfin T, Boehlen JM, Häberli M, Marek D, Fesik SW. High-throughput nuclear magnetic resonance-based screening. *J Med Chem*. 1999; 42(13):2315–2317. [PubMed: 10395471]
183. Moore JM. NMR techniques for characterization of ligand binding: Utility for lead generation and optimization in drug discovery. *Biopolymers*. 1999; 51(3):221–243. [PubMed: 10516573]
184. Hajduk PJ, Huth JR, Fesik SW. Druggability indices for protein targets derived from NMR-based screening data. *J Med Chem*. 2005; 48(7):2518–2525. [PubMed: 15801841]
185. Wishart D. NMR spectroscopy and protein structure determination: applications to drug discovery and development. *Curr Pharm Biotechnol*. 2005; 6(2):105–120. [PubMed: 15853690]
186. Skinner AL, Laurence JS. High-field solution NMR spectroscopy as a tool for assessing protein interactions with small molecule ligands. *J Pharm Sci*. 2008; 97(11):4670–4695. [PubMed: 18351634]
187. Ziarek JJ, Peterson FC, Lytle BL, Volkman BF. Binding site identification and structure determination of protein-ligand complexes by NMR a semiautomated approach. *Methods Enzymol*. 2011; 493:241–275. [PubMed: 21371594]
188. Akke M. Conformational dynamics and thermodynamics of protein-ligand binding studied by NMR relaxation. *Biochem Soc Trans*. 2012; 40(2):419–423. [PubMed: 22435823]
189. Jóhannesson H, Denisov VP, Halle B. Dimethyl sulfoxide binding to globular proteins: a nuclear magnetic relaxation dispersion study. *Protein Sci*. 1997; 6(8):1756–1763. [PubMed: 9260288]
190. Mattos C, Ringe D. Proteins in organic solvents. *Curr Opin Struct Biol*. 2001; 11(6):761–764. [PubMed: 11751059]
191. Arakawa T, Kita Y, Timasheff SN. Protein precipitation and denaturation by dimethyl sulfoxide. *Biophys Chem*. 2007; 131(1–3):62–70. [PubMed: 17904724]
192. Kuntz ID, Kauzmann W. Hydration of proteins and polypeptides. *Adv Protein Chem*. 1974; 28:239–345. [PubMed: 4598824]
193. Mande SC, Sobhia ME. Structural characterization of protein-denaturant interactions: crystal structures of hen egg-white lysozyme in complex with DMSO and guanidinium chloride. *Protein Eng*. 2000; 13(2):133–141. [PubMed: 10708653]
194. Giugliarelli A, Paolantoni M, Morresi A, Sassi P. Denaturation and preservation of globular proteins: the role of DMSO. *J Phys Chem B*. 2012; 116(45):13361–13367. [PubMed: 23101790]

195. Markin CJ, Spyropoulos L. Accuracy and precision of protein-ligand interaction kinetics determined from chemical shift titrations. *J Biomol NMR*. 2012; 54(4):355–376. [PubMed: 23086713]
196. Markin CJ, Spyropoulos L. Increased precision for analysis of protein-ligand dissociation constants determined from chemical shift titrations. *J Biomol NMR*. 2012; 53(2):125–138. [PubMed: 22534787]
197. Schumann FH, Riepl H, Maurer T, Gronwald W, Neidig KP, Kalbitzer HR. Combined chemical shift changes and amino acid specific chemical shift mapping of protein-protein interactions. *J Biomol NMR*. 2007; 39(4):275–289. [PubMed: 17955183]
198. Williamson MP. Using chemical shift perturbation to characterise ligand binding. *Prog NMR Spectrosc*. 2013; 73(1):1–16.
199. Scatchard G. The attractions of proteins for small molecules and ions. *Ann NY Acad Sci*. 1949; 51:660.
200. Konuma T, Lee YH, Goto Y, Sakurai K. Principal component analysis of chemical shift perturbation data of a multiple-ligand-binding system for elucidation of respective binding mechanism. *Proteins*. 2013; 81(1):107–118. [PubMed: 22927212]
201. Sakurai K, Goto Y. Principal component analysis of the pH-dependent conformational transitions of bovine beta-lactoglobulin monitored by heteronuclear NMR. *Proc Natl Acad Sci U S A*. 2007; 104(39):15346–15351. [PubMed: 17878316]
202. Pople JA. Proton Magnetic Resonance of Hydrocarbons. *J Chem Phys*. 1956; 111(5):1111–1112.
203. Ilina T, LaBarge K, Sarafianos SG, Ishima R, Parniak MA. Inhibitors of HIV-1 Reverse Transcriptase—Associated Ribonuclease H Activity. *Biology*. 2012; 1(3):521–541. [PubMed: 23599900]
204. Christen MT, Menon L, Myshakina NA, Ahn J, Parniak MA, Ishima R. Structural Basis of the Allosteric Inhibitor Interaction on the HIV-1 Reverse Transcriptase RNase H domain. *Chem Biol Drug Des*. 2012; 80(5):706–716. [PubMed: 22846652]
205. Parniak MA, Min KL, Budihas SR, Le Grice S, Beutler JA. A fluorescence-based high-throughput screening assay for inhibitors of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H activity. *Anal Biochem*. 2003; 322(1):33–39. [PubMed: 14705777]
206. Klumpp K, Hang JQ, Rajendran S, Yang Y, Derosier A, Wong Kai In P, et al. Two-metal ion mechanism of RNA cleavage by HIV RNase H and mechanism-based design of selective HIV RNase H inhibitors. *Nucleic Acids Res*. 2003; 31(23):6852–6859. [PubMed: 14627818]
207. Budihas SR, Gorshkova I, Gaidamakov S, Wamiru A, Bona MK, Parniak MA, et al. Selective inhibition of HIV-1 reverse transcriptase-associated ribonuclease H activity by hydroxylated tropolones. *Nucleic Acids Res*. 2005; 33(4):1249–1256. [PubMed: 15741178]
208. Sarafianos SG, Marchand B, Das K, Himmel DM, Parniak MA, Hughes SH, et al. Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. *J Mol Biol*. 2009; 385(3):693–713. [PubMed: 19022262]
209. Davis CA, Parniak MA, Hughes SH. The effects of RNase H inhibitors and nevirapine on the susceptibility of HIV-1 to AZT and 3TC. *Virology*. 2011; 419(2):64–71. [PubMed: 21907380]
210. Chung S, Himmel DM, Jiang JK, Wojtak K, Bauman JD, Rausch JW, et al. Synthesis, Activity, and Structural Analysis of Novel α -Hydroxytropolone Inhibitors of Human Immunodeficiency Virus Reverse Transcriptase-Associated Ribonuclease H. *J Med Chem*. 2011; 54(13):4462–4473. [PubMed: 21568335]
211. Le Grice SF. Human immunodeficiency virus reverse transcriptase: 25 years of research, drug discovery, and promise. *J Biol Chem*. 2012; 287(49):40850–40857. [PubMed: 23043108]
212. Adachi M, Ohhara T, Kurihara K, Tamada T, Honjo E, Okazaki N, et al. Structure of HIV-1 protease in complex with potent inhibitor KNI-272 determined by high-resolution X-ray and neutron crystallography. *Proc Natl Acad Sci U S A*. 2009; 106(12):4641–4646. [PubMed: 19273847]

(A) Chemical structure – known.

(B) Residue number – unknown.

Figure 1.

What is known and unknown in the protein NMR. In a typical case, (A) we know the primary amino acid sequence of the protein, and (B) do experiments that identify the residue numbers and the atom-types of the observed NMR signals, which become a basis for further experiments to understand structure, dynamics, interaction of proteins.

Figure 2.

Two reasons why large R_2 reduces NMR sensitivity: (A) fast signal decay in the acquisition time domain results in broad line width with a low signal height in the Fourier transformed spectrum, and (B) fast signal decay significantly reduces signal intensity in the acquisition dimension in 2D and 3D NMR experiments.

Figure 3.

Saturation and cross relaxation within protein protons. **(A)** When one proton is saturated, the saturation transfers from the proton, I_0 , to a nearby proton, I_1 , by cross relaxation, σ . **(B)** The decreased intensity of I_1 in turn, decreases the intensity of the surrounding proton spins, I_2 , I_3 , ... by the cross relaxation. **(C)** This cross relaxation makes a network in a protein, so that saturation of a part of the protein propagates to all protein protons. When there is interaction between a protein and a ligand, the saturation transfers to the ligand signals during the residence time of the ligand on the protein.

Figure 4.

Typical ligand-titration profiles by monitoring the NMR signal of a small protein in the (A) very fast or (B) very slow exchange régime, and protein-titration profiles by monitoring a ligand NMR signal that interacts with a large protein in the (C) very fast or (D) very slow exchange régime. Note that broadening of a ligand signal upon protein interaction is significant in the ligand-observation experiments.

Figure 5. Ribbon presentation of (A) the calmodulin-peptide complex structure and (B) the free calmodulin (lime color) with cartoon of the peptide (red color) and Ca^{2+} (purple color). Calmodulin structures were generated using PDB IDs 1DMO and 2BBM, for the apo and the peptide bound forms, respectively [13, 96].

Figure 6.

Ribbon presentation of HIV-1 protease homodimer (pink and lime) in complex with an inhibitor, KNI272 (bond at the center), which is an analogue of KNI292. Residue numbers and N- and C-terminal notations are shown with dash for the second subunit in the homodimer. The backbone amides are shown in stick presentation for residues 28 and 28', and 31 and 31' in the two subunits, respectively. A pair of residues with the same residue number are distant from each other in the two subunits, but showed clear cross peaks in the EXSY spectra, suggesting a mechanism in which the inhibitor flips without complete dissociation from the protease [171]. Ribbon structure was generated using PDB ID 3F5X [212].

Figure 7.

Cartoon showing the schemes of the inhibitor-titration experiments that monitor protein NMR signals. **(A)** Titration of the inhibitor from the stock solution to a single NMR sample will cause errors, by dilution of the protein concentration and by buffer composition changes. **(B)** It is ideal to prepare all the samples for the titration points in advance, with uniform protein and buffer concentrations. **(C)** Once a set of spectra is recorded, the data is analyzed to estimate the inhibitor-binding site and/or to determine the dissociation constant.

Figure 8.

Cartoon showing patterns of chemical shift changes upon fast exchange for a system in (A) two-site exchange and (B) three-site exchange. Here, “n-site exchange” is a spectroscopic definition. For example, two-site exchange involves signals from the free and the bound sites but does not mean involvement of two binding-sites. In three-site exchange, non-linear changes in chemical shifts may be observed since the magnitudes of chemical-shift changes between the 1st and the 2nd states may differ from those between the 2nd and the 3rd states.

Figure 9.

Highlight of the chemical shift perturbation sites on the chimeric RNH upon BHMP07 interaction. In (A), regions exhibiting moderate chemical shift changes (orange) as well as large ones (red) are shown on the RNH cartoon structure. In (B), positions of the Trp side chains (blue, sticks) are also presented on top of the figure in (A). A model structure of the chimeric RNH was used to generate the figures (redrawn to show the moderate perturbation sites [90]).