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Exposing the Moving Parts of Proteins with NMR Spectroscopy

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Abstract

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful tool for investigating the dynamics of biomolecules since it provides a description of motion that is comprehensive, site-specific, and relatively non-invasive. In particular, the study of protein dynamics has benefited from sustained methodological advances in NMR that have expanded the scope and time scales of accessible motion. Yet, many of these advances may not be well known to the more general physical chemistry community. Accordingly, this Perspective provides a glimpse of some of the more powerful methods in liquid state NMR that are helping reshape our understanding of functional motions of proteins.

Keywords

Protein dynamics; Relaxation; RDCs; relaxation dispersion; cross-correlation; order parameters; side chain dynamics; chemical exchange

Since its inception in the late 1940s^{1–2} Nuclear Magnetic Resonance (NMR) spectroscopy has cut across all traditional discipline boundaries. It is now a standard tool for synthetic chemistry, biochemistry, and biomedical science. Indeed, the increasingly “black-box” character of commercial spectrometers continues to broaden the accessibility of NMR, by allowing users to focus more on data throughput, and less on its physical origins. The transformation from a curious set of physics experiments to a widespread, increasingly “turn-key” tool is surely the sign of success. And yet, this success may raise questions as to the “bona fide” physical chemistry credentials of NMR. The more general physical chemist may ask: “Is there anything new in NMR? Is there much physical science left in it?”

The answer to these questions is an unequivocal “yes”. This is obvious in solid-state NMR (see e.g. the review by Renault et al.³) and in research aimed at novel modes for magnetic resonance detection^{4–5}. But what about liquid-state NMR in chemistry? Here, the methodologies are indeed more mature, with the analytical applications pushing more towards automation. But one area of liquid-state NMR that continues to develop new methods is the study of biomolecular dynamics. The goal of this Perspective is to give the more general physical chemist a sense of the power and scope of liquid-state NMR techniques that are reshaping our understanding of biomolecular dynamics, in particular, those of proteins.

Proteins are Nature’s nano-devices and are examples of complex dynamical systems. Indeed, Gregorio Weber famously described them as “kicking screaming stochastic” molecules⁶. A truly predictive understanding of protein function should therefore account for such dynamics. Yet, historically, this assertion was viewed as more of a truism, rather than an imperative. Protein conformational dynamics were often regarded as small perturbations, present to be sure, but usually unnecessary for addressing most aspects of

protein behavior. Cases of conformational change were often cast as sharp transitions between distinct static structures, rather than a consequence of thermal fluctuations.

These views no longer dominate. Mounting evidence shows that conformational dynamics can play a vital role in protein stability, recognition, and catalysis. And a powerful force driving these shifting views has been the abundance of protein dynamics information coming from NMR spectroscopy.

NMR is particularly well suited for investigating the potentially complex conformational dynamics of proteins: it can profile dynamics both site-specifically and globally; it is sensitive to motions over a broad time-scale (10^{-12} – 10^{-3} s); it is also non-invasive (exogenous tags are optional rather than essential). These assets make NMR an excellent companion to other methods for studying protein dynamics, such as fluorescence depolarization, single-molecule spectroscopy, and small-angle x-ray or quasi-elastic neutron scattering. Moreover, the perception that NMR studies are limited to small proteins (< 20 kDa) is increasingly obsolete. Thanks to sustained advances in spin physics and protein isotope labeling methods, NMR studies are now feasible for molecular leviathans exceeding 100 kDa ^{7–8}.

Sources of Dynamic Information in Liquid-state NMR

Figure 1 shows various types of NMR dynamics experiments and the approximate time-scales of motion they sample. In all of these experiments, the dynamics information ultimately stems from internal Hamiltonians that couple the nuclear spin orientations (spin degrees of freedom) with local classical fields that depend on the nuclear coordinates (spatial degrees of freedom). These Hamiltonians decompose naturally into purely isotropic and anisotropic components that convey dynamic information in different ways ^{9–12}. Below we highlight some concepts concerning these Hamiltonians that are relevant for dynamics studies. Our discussion will center on Hamiltonians relevant to non-proton “heteronuclei”, such as ^{15}N , ^{13}C , and ^2D (deuterium), since the vast majority of NMR experiments use these heteronuclei as local probes of motion in appropriately ^{15}N , ^{13}C , and ^2D enriched proteins. These heteronuclei are preferable to protons (^1H) since they more readily admit a site-specific description of dynamics; the protons merely serve as the nucleus of final detection. The biosynthetic methods for protein isotope enrichment are described in various reviews ^{13–14}.

We start with the anisotropic Hamiltonians; these depend on the orientation of the molecular bond vectors with respect to the static external magnetic field, $\mathbf{B}_0$. Important examples include: (i) heteronuclear magnetic dipole-dipole interactions (H_{DD}) in NH and CH bond vectors between ^{15}N and ^{13}C nuclei (spin 1/2) and their directly bonded protons (^1H); (ii) the anisotropic chemical shielding interaction (H_{CSA}) for the same ^{15}N and ^{13}C nuclei; (iii) the nuclear quadrupole interaction (H_{Q}) of a deuterium ^2D (spin 1) with its local electric field gradient. The spatial parts of these Hamiltonians are 2nd rank, symmetric, and traceless tensors that describe the local fields associated with the bond vectors. Such fields include the magnetic dipole fields the ^{15}N and ^{13}C spins experience from their covalently bonded protons, and the local electric field gradient sensed by the ^2D spins. One can visualize these tensors as little coordinate frames that are rigidly attached to the bond vectors and bear a fixed orientation to them ¹⁵. As the bond vector rotates, so do the attached tensors and their associated local fields; the nuclear spin orientations (quantized along $\mathbf{B}_0$) do not. Thus, bond vector rotation (reorientation) modulates the local fields sensed by the resident spins. As a result, an ensemble of immobile bond vectors that point in different directions, such as those in a static powder, give rise to extremely broad line shapes. By contrast, mobile bond

vectors individually sample multiple orientations, thereby causing rotational averaging of the anisotropic interactions and line narrowing.

In isotropic liquids, the rotational averaging is complete: overall molecular tumbling causes the bond vector orientations to fluctuate among all directions with equal likelihood. Accordingly, the attached H_{DD} , H_{CSA} , and H_Q tensors average to zero and have no effect on the NMR resonance positions. Nevertheless, they do cause nuclear spin relaxation. In particular, the tensor fluctuations constitute local fluctuating fields that stimulate spin-state transitions and de-phase coherences, thus enabling relaxation of non-equilibrium bulk spin order (e.g. inverted or transverse magnetization) back to the equilibrium condition (bulk magnetization along B_0). The corresponding experimental observables are relaxation rate constants, such as $R_1 = 1/T_1$, and $R_2 = 1/T_2$, for longitudinal and transverse relaxation, respectively. Thus, heteronuclear spin relaxation rates report on the picosecond (10^{-12} s) – nanosecond (10^{-9} s) orientational dynamics of bond vectors (*vide infra*).

Partial resurrection of the anisotropic Hamiltonians H_{DD} , H_{CSA} , and H_Q , is possible by using methods that induce weak protein alignment, while retaining the narrow line widths of conventional liquid-state NMR^{16–17}. Alignment methods include¹⁸: covalently attaching moieties with large magnetic susceptibility anisotropies that align with B_0 ; co-dissolving the protein with substances that themselves align with B_0 ; and dissolving the protein in porous media with anisotropic cavities that are stretched or compressed along B_0 . The weak alignment produces small perturbations to the isotropic resonances that reflect the residual affects of the anisotropic Hamiltonians. For example, residual dipolar couplings (RDCs) for NH and CH bonds cause additional ^{15}N - ^1H and ^{13}C - ^1H splittings, while residual chemical shift anisotropies (RCSAs) of ^{15}N and ^{13}C cause small resonance shifts. These alignment-induced affects provide information on the average orientations of bond vectors relative to B_0 , which depends both on overall protein tumbling and internal motion. Thus, similar to spin relaxation, the residual anisotropies report on the orientational dynamics of bond vectors, albeit over much broader time scales, picosecond (10^{-12} s) – millisecond (10^{-3} s) (*vide infra*).

What about the isotropic Hamiltonians? They are responsible for the familiar isotropic chemical shifts and scalar J-coupling constants that are heavily exploited by synthetic and biological chemistry. While these Hamiltonians are indifferent to molecular re-orientation, their isotropic parameters are highly sensitive to local conformation. Dynamics involving the exchange of nuclei among different chemical states can render these parameters time-dependent, leading to various spectral effects. These exchange dynamics often occur among states separated by free energy barriers $> k_B T$, leading to exchange time-constants on the order of microseconds (10^{-6} s) – milliseconds (10^{-3} s). These time scales overlap with those of protein binding and enzymatic turnover, and are thus of great interest to biophysics and biophysical chemistry.

Below, we focus on a subset of the experiments in Figure 1 that have proven to be particularly effective within the last fifteen years, including: (i) heteronuclear spin relaxation experiments; (ii) residual dipolar coupling (RDC) measurements; and (iii) chemical exchange experiments. Two caveats deserve mention. First, each of these experiments constitutes a rich and thriving area of NMR research, and has generated critical reviews. This Perspective, which discusses all three, cannot discuss them at a comparable depth or breadth as those reviews. Its more modest goal is to sketch the illuminative power of these methods, in particular, for the more general physical chemist. Along the way, we refer readers seeking more detail to the appropriate critical reviews. Second, the chemical literature often distinguishes “dynamics” from “flexibility”, reserving the former for explicitly time-dependent phenomena, and the latter for equilibrium properties. Yet, both

notions invoke atoms in motion. In this Perspective, we use the word “dynamics” in its most elementary sense from classical mechanics: the motions of particles (atoms).

Nuclear Spin Relaxation and Re-orientational Motions

Heteronuclear (^{15}N , ^{13}C , ^2D) spin relaxation rate measurements provide a powerful means for sequence-specific descriptions of protein dynamics. The relaxation rates of ^{15}N , ^{13}C , and ^2D report on the power spectral density functions $J(\omega)$ that describe the orientational dynamics of NH, CH, and CD bond vectors. Examples are longitudinal and transverse ^2D relaxation rates, $R_1(D_z)$ and $R_2(D_+)$, which probe CD bond motions,

$$R_1(D_z) = \frac{3}{16} \left(\frac{e^2 q Q}{\hbar} \right)^2 (J_{CD}(\omega_D) + 4J_{CD}(2\omega_D)) \quad (1a)$$

$$R_2(D_+) = \frac{1}{32} \left(\frac{e^2 q Q}{\hbar} \right)^2 (9J_{CD}(0) + 15J_{CD}(\omega_D) + 6J_{CD}(2\omega_D)) \quad (1b)$$

and longitudinal and transverse ^{15}N relaxation rates, which probe NH bond motions,

$$R_1(N_z) = \frac{1}{16} (\omega_{NH}^{DD})^2 (J_{NH}(\omega_N - \omega_H) + 3J_{NH}(\omega_H) + 6J_{NH}(\omega_N + \omega_H)) + \frac{1}{3} (\gamma_N B_0 \Delta\sigma)^2 J_{NH}(\omega_N) \quad (2a)$$

$$R_2(N_+) = \frac{1}{32} (\omega_{NH}^{DD})^2 (4J_{NH}(0) + J_{NH}(\omega_N - \omega_H) + 3J_{NH}(\omega_H) + 6J_{NH}(\omega_H) + 6J_{NH}(\omega_N + \omega_H)) + \frac{1}{18} (\gamma_N B_0 \Delta\sigma)^2 (4J_{NH}(0) + 3J_{NH}(\omega_N)).$$

In Equations 1a,b the constant $(e^2 q Q / \hbar)$ reflects the dominance of deuterium relaxation by the nuclear quadrupolar Hamiltonian, H_Q (*vide supra*). eQ is the nuclear quadrupole moment, eq is the electric field gradient, and $\hbar$ is Planck's constant divided by 2π . In

Equations 2a,b the NH dipolar coupling constant $\omega_{NH}^{DD} = 2\gamma_H \gamma_N \hbar / \langle r_{NH}^3 \rangle$ (expressed in cgs units), and the ^{15}N chemical shift anisotropy ($\gamma_N B_0 \Delta\sigma$) reflect the heteronuclear NH dipolar (H_{DD}) and ^{15}N chemical shift anisotropy (H_{CSA}) interactions, respectively. Analogous H_{DD} and H_{CSA} interactions dominate ^{13}C relaxation in CH bonds. In all cases, the relaxation mechanisms localize to the CD, NH, and CH bond vectors, thus allowing site-specific interpretation of the data.

Generally, the key dynamics quantities in nuclear spin relaxation rates are the spectral density functions, $J_{IS}(\omega)$, where we use “IS” to denote an NH, CD, or CH bond vector. $J_{IS}(\omega)$ essentially profiles the component frequencies of the orientational dynamics experienced by an IS bond vector. These dynamics include both conformational dynamics and global protein tumbling (Figure 2a). As seen in Equations 1, 2, the intrinsic dynamics information of the relaxation rates is not $J_{IS}(\omega)$ directly, but rather, samplings of them at discrete frequencies in the MHz range (0, ω_N , ω_D , ω_H , etc) (Figure 2d). Critically, overall protein tumbling puts an upper limit on the time scales of motion sensed by the relaxation rates.

$J_{IS}(\omega)$ is the Fourier Transform of the time- (auto)correlation function for “IS” bond vector reorientation, $C(\tau)$. For example, for a given “IS” bond vector we have

$$J_{IS}(\omega) = 2 \int_0^{\infty} C(\tau) \cos(\omega\tau) d\tau \quad (3)$$

where

$$C(\tau) = \frac{1}{5} \langle P_2 [\cos \Omega_{IS}(\tau)] \rangle = \frac{1}{5} \left\langle \frac{3 \cos^2 \Omega_{IS}(\tau) - 1}{2} \right\rangle \quad (4)$$

In Equation 4, $\Omega_{IS}(\tau)$ is the angle between where the vector is now, versus a time ‘ τ ’ earlier. Hence, $C(\tau)$ describes the decay in auto-correlations between the orientations of the same “IS” bond-vector at two different instants, separated by a waiting time ‘ τ ’ (cf. Figure 2b). $C(\tau)$ begins at (1/5) for all vectors and decays to zero on time scales set by τ_c , the overall rotational correlation time(s), which typically lie(s) in the 10–100 ns range. Orientational fluctuations on longer time scales ($\tau > \tau_c$) are invisible to $C(\tau)$ (since it has already decayed to zero, cf. Figure 2c), and by extension, to $J(\omega)$ and the spin-relaxation rates. Of course, the specific decay of $C(\tau)$ depends on the mobility of the specific bond vector. Vectors with greater or lesser orientational mobility will have $C(\tau)$ functions that decay faster or slower, respectively. This variability translates into site-specific $J_{IS}(\omega)$ and relaxation rates, via Equation 4.

Equations 1,2 show that different kinds of relaxation rates sample $J(\omega)$ in different ways; thus, one can increase the breadth of dynamics information by measuring multiple rate constants for each bond vector. There is now a diverse array of two-dimensional (2-d) proton-detected heteronuclear correlation experiments that measure R_1 , R_2 , and a large variety of other rate constants for numerous bond vectors, on a residue-by-residue basis^{19–21}. The basic procedure involves recording a series of 2-d heteronuclear correlation spectra, which contains a sequence of rf-pulses and delays that isolates the particular relaxation process of interest. The relaxation occurs for a variable delay, such that the resulting 2-d cross-peak intensities depend on the extent of relaxation allowed. One then fits the series of cross-peak intensities to exponential decays to extract the site-specific relaxation rates (cf. Figure 3a, b).

To turn the raw relaxation rates into dynamics information, several options emerge. First, if one measures a set of relaxation rates for each bond vector that equals or exceeds the number of unique spectral density samplings introduced, then one can directly determine the intrinsic dynamics quantities (the spectral density samplings) without any *a priori* model assumptions of the dynamics. This method is the “spectral density mapping” approach^{22–24}. Second, if the relaxation rate is dominated by a single spectral density sampling, then a complete mapping becomes possible via B_0 dispersion studies, using field-cycling methods^{25–26}. However, the data sets are usually too sparse for direct mapping methods. One then pursues the third and most common approach, which is to choose an analytical model form for $J(\omega)$, which contains micro-dynamic parameters that one fits to best reproduce the experimental relaxation rates. The best-known version of this approach is the “model-free” formalism of Lipari and Szabo^{27–28}, which is closely related to the earlier two-step model by Halle and Wennerström²⁹. First applied by Dellwo and Wand to the CH bond motions of cyclosporine³⁰, the Lipari-Szabo formalism has become the most popular approach for analyzing protein NMR relaxation data. In its simplest form, the model-free formalism invokes an orientational time-correlation function

$$C_{MF}(\tau) = \frac{1}{5} e^{-\frac{\tau}{\tau_c}} \left\{ S^2 + (1 - S^2) e^{-\frac{\tau}{\tau_e}} \right\} \quad (5)$$

and corresponding spectral density function

$$J_{MF}(\omega) = \frac{2}{5} \left\{ \frac{S^2 \tau_c}{1 + (\omega \tau_c)^2} + \frac{(1 - S^2) \tau}{1 + (\omega \tau)^2} \right\}, \quad \frac{1}{\tau} = \frac{1}{\tau_e} + \frac{1}{\tau_c} \quad (6)$$

that describes the internal motion of each bond vector with two parameters: (i) a generalized order parameter S^2 , which measures the amplitude of internal re-orientational motions. S^2 ranges between 0 and 1, with completely unrestricted (isotropic) internal motion yielding 0, and no internal motion (rigid limit) yielding 1; (ii) an effective correlation time, τ_e , that gauges the rapidity of the internal motion. These site-specific parameters, along with a global correlation time for isotropic overall tumbling (τ_c), are adjusted to fit the relaxation data. The result is S^2 and τ_e versus sequence, and thus, a profile of internal bond vector motion on time scales up to those of global protein tumbling (picosecond – ns). S^2 is “model-free” because it can be related to more specific models of motion, such as the “Wobbling in a Cone”³¹ or the 1-d and 3-d Gaussian-Axial-Fluctuation (GAF) models (*vide infra*)^{32–34}. Extensions of the original Lipari-Szabo formalism account for chemical exchange contributions (R_{ex}) to transverse relaxation data^{35–37}, anisotropic overall tumbling^{38–40} and additional internal motions^{41–43}. Of course, these extensions carry the cost of introducing additional fitting parameters.

A key assumption of the Lipari-Szabo formalism is that motions due to conformational dynamics (internal motion) are statistically decoupled from global tumbling. This assumption is eminently reasonable if the conformational dynamics occur on time scales much faster than global tumbling, or do not significantly affect the overall structure. But the decoupling assumption is suspect if the internal motions change the overall shape of the protein, thus changing its overall rotational diffusion properties, on a time scale comparable to global tumbling. The latter scenario is conceivable for flexibly linked multi-domain proteins and intrinsically disordered proteins. There are several tactics to evaluate and address the possibility of coupling between internal and global motion. Prompers and Brüschweiler have developed the isotropic re-orientational eigenmode dynamics (iRED) approach⁴⁴. This is a computational approach that evaluates the covariance matrix of bond vector fluctuations over the course of a molecular dynamics (MD) simulations to assess the true extent of (de)coupling. More recently, investigators^{45–46} have developed analytical formalisms in which the protein jumps between conformers with different diffusion tensors. Critically, these formalisms allow for correlations between the bond vector orientational dynamics within a given conformation (diffusion tensor), and the trans-tensor jumping. Another analytical approach is the slowly relaxing local structure (SRLS) model of Freed, Meirovitch, and co-workers⁴⁷. Their model derives from two-body Smoluchowski theory and yields a spectral density function that explicitly includes fitting parameters for the coupling of internal and global motions, as well as descriptors for the local anisotropy (asymmetry) of internal motion. In principle, these are assets. However, ascertaining whether SRLS provides a significantly better description of motional coupling remains an active area of research^{48–49}. Stochastic dynamics studies by Wand and co-workers show that the Lipari-Szabo approach, which is simpler to use, adequately characterizes the amplitudes of local anisotropic motion⁵⁰, although recent work by Meirovitch and co-workers suggests SLRS may give more accurate internal correlation times τ_e ⁵¹. The recent theoretical study of Halle⁵² provides an updated derivation of the model-free and SRLS

approaches as a foundation for comparing their physical bases and inherent assumptions. In any event, formulating a general, analytical treatment of coupled internal and global motions that remains “user-friendly” is an outstanding challenge.

Applications of heteronuclear spin relaxation experiments have decisively demonstrated the importance of protein dynamics in ligand binding, catalysis, and evolution. The initial studies focused on protein backbone dynamics – the motions of methine C α H bonds, as probed by ^{13}C relaxation rates^{36,53}, and the motions of NH bonds as probed by ^{15}N spin relaxation³⁵. Today, NH bond motion studies predominate, due to the relative ease of producing ^{15}N -enriched protein, and the freedom from erroneous relaxation measurements arising from homo-nuclear scalar coupling.

The ^{15}N spin relaxation experiments give a residue-by-residue description of the backbone NH bond motions relative to $\mathbf{B}_0$. These motions reflect the orientational dynamics of the host peptide plane, caused by local ϕ , ψ torsional fluctuations and long-range (e.g. segmental) dynamics. The NH studies have become a regular assay in dynamics-function investigations involving mutation and/or ligand binding. Examples are those by Palmer and co-workers⁵⁴, and Peng and co-workers⁵⁵. Using a Lipari-Szabo analysis of NH relaxation rates, the investigators compared the NH S^2 versus sequence for various loop mutants to investigate sequence-dynamics-binding correlations, and thus, the extent to which sequence encodes recognition-specific fluctuations. NH backbone dynamics studies have also illuminated the recognition mechanisms of intrinsically disordered proteins – a large class of proteins that defy the traditional structure-function dogma, yet, are essential for cell cycle signaling. In particular, NH dynamics show that such proteins recognize their targets, through coupled folding and binding^{56–58}. NH studies have further investigated the interplay between the picosecond-nanosecond fluctuations of NH bond vectors and the slower dynamics associated with enzymatic turnover, as in the comprehensive studies by Kern and co-workers on mesophilic versus thermophilic adenylate kinase^{59–60}. Finally, the growing number of backbone dynamics studies underscores the diversity of dynamic responses of proteins to binding. A common stereotype is that ligand binding invariably reduces the intrinsic flexibility of protein. While this is true in many cases, it is by no means a universal response. For example, backbone dynamics studies by Zidek et al⁶¹ indicated that the backbone flexibility of mouse major urinary protein actually increased upon binding its pheromone ligand. This phenomenon is in other proteins, and frequently in side chain dynamics studies (*vide infra*). Further examples of backbone dynamics studies by spin relaxation are in the comprehensive review by Jarymowycz and Stone⁶².

What kinds of specific atomic motions underlie the model-free S^2 values? To address this question, molecular dynamics simulations (MD) are illuminating. Investigating the interplay between MD-simulations and NMR dynamics experiments is a thriving field, and sustained improvements in MD force-fields have led, in some cases, to near quantitative agreement between NH S^2 calculated from MD trajectories and their experimental counterparts⁶³. For greater depth, we refer the reader to recent work by Brüschweiler and co-workers⁶³ and Palmer and co-workers⁶⁴. Notably, MD simulations show that many S^2 values reflect bond vectors undergoing restricted torsion angle fluctuations. This motivated the development of the 1d and 3d Gaussian Axial Fluctuation (GAF) models^{32–34}. In particular, in the 3D GAF model, the NH S^2 reflect the torsional fluctuations of the peptide plane about three orthogonal axes defined by the equilibrium peptide plane orientation. Typically, the dominant fluctuations occur about an axis defined by C α_{i-1} —C α_i atoms. The appropriateness of the GAF models indicates that, in some cases, a quadratic potential function is a good approximation of the potential of mean force guiding the NH bond motions.

For further insight into the shape of the potentials of mean force, investigators have varied the sample pressure (see e.g. the work of Akasaka and co-workers⁶⁵) or temperature^{66–67}. In particular, measurements of NH S^2 versus temperature by Palmer, Rance and co-workers⁶⁷ revealed that quadratic potentials (axially symmetric or 1d GAF) must be enhanced with temperature-dependent force constants that decrease with increasing temperature; moreover, the force constants for NHs separate into two classes corresponding to bond vectors in α helices versus β -sheets.

For studies of side chain dynamics, deuterium (^2D) relaxation measurements have proven to be particularly powerful^{23,68–69}. The ^2D rates probe the motions of CD bonds of CH_2D and/or CHD_2 methyl isotopomers relative to the external field. These motions include torsional fluctuations within a rotamer state, transitions between rotamer states, or both. Typically, one uses Lipari-Szabo model-free formalism to get S^2 values for the CD bonds, which factor as $S^2 = 0.111S_{\text{axis}}^2$. The 0.111 reflects the common rapid rotation of the CD bond about the methyl symmetry axis, whereas S_{axis}^2 reflects the site-specific motion of the methyl symmetry axis itself. S_{axis}^2 is thus the central object of interest. The abundance of methyls in protein hydrophobic cores ensures a broad sampling of internal motions.

Side chain dynamics studies are less prevalent than those of the backbone, yet they are equally powerful probes of protein functional dynamics. Their importance is well illustrated by the comprehensive work of Wand and co-workers on the side chain dynamics of calmodulin (CaM)¹⁹. Their work shows that side chain studies can expose functional dynamics, such as those sensitive to ligand binding, that would otherwise be missed by a purely NH backbone investigation^{19,70–71}. Moreover, their variable temperature studies show that the S_{axis}^2 values follow a “trimodal” distribution, reflecting three classes of motion: “J”, “ α ”, and “ ω ”, which correspond to decreasing amplitudes of side chain motion owing to decreased rotamer-state hopping. Remarkably, the S_{axis}^2 values lacked significant correlation with many intuitive structural parameters, such as burial depth, local packing density, or solvent accessible surface area¹⁹. Thus, defining the structural determinants of S_{axis}^2 requires further investigation.

Methyl side chain motions have also highlighted the importance of protein conformational entropy in binding thermodynamics. These results derive from formalisms introduced by Akke et al⁷² for free energy, and subsequently Li and Wand⁷³ and Yang and Kay⁷⁴ that use changes in Lipari-Szabo order parameters to relate binding free energy to site-specific changes in conformational dynamics. In particular, S^2 is an equilibrium average over the ensemble of orientations adopted by a bond vector. Changes in S^2 upon ligand binding thus reflect changes in the ensemble breadth and thus, conformational entropy. While these methods are applicable for both the backbone (e.g. NH) and side chain bond vectors, the side chain studies have been most effective for mapping binding-induced changes in conformational entropy. In particular, Wand and co-workers have analyzed the changes CaM S_{axis}^2 in parallel with thermodynamic studies for a series of peptide ligands. Their results show that changes in protein conformational entropy, sensed by changes in S_{axis}^2 , play a highly significant role in modulating the binding affinity of CaM toward its various targets⁷⁵. This result is highly significant for the design and modification of protein-ligand interactions (e.g. drug design), since such entropy cannot be calculated from static structures. This work well complements recent computational efforts to correlate conformational entropy and NMR order parameters⁷⁶.

Methyl side chain dynamics studies have also provided tantalizing evidence for coupled changes in sub-nanosecond dynamics as mechanisms for site-to-site communication within proteins^{77–78}. In particular, Figure 3c,d illustrate the changes in the methyl side-chain S^2 for the mitotic regulator Pin1, upon binding saturating amounts of a known phosphopeptide

substrate. The red and blue spheres indicate increased or decreased S_{axis}^2 , and thus, losses or gains of internal motion, upon substrate interaction. Notably, the sites that lose motion are highly conserved hydrophobic residues that connect the known functional sites of Pin1. These results support the notion of dynamically driven allostery, as proposed by Cooper and Dryden⁷⁹.

Methyl side chain dynamics are also appealing for studying the internal motions of supramolecular systems. More specifically, the methyl Transverse Relaxation Optimized Spectroscopy (TROSY) methods developed by Kay and co-workers, which exploit cross-correlated relaxation to select for the slowly relaxing coherences of the methyl spin system, now enable extraction of methyl S_{axis}^2 in systems exceeding 80 kDa^{8,69,80–81}. Another attractive feature of the methyl-TROSY experiments is that it permits sensitive measurement of both ^{13}C and ^2D relaxation rates for the same methyl groups, thus enhancing accuracy and broadening the scope of the resulting dynamics information. More recently, these methods have been applied to 345kDa KaiC⁸², and the 670kDa 20S proteasome⁸³ and its substrates⁸⁴. These systems are truly Brobdingnagian by NMR standards, yet, common enough in biology. Thus, methyl-TROSY opens the possibility of understanding, for the first time, the internal moving parts of these biomolecular machines.

Residual Dipolar Couplings

The most commonly measured residual anisotropies stem from heteronuclear dipolar couplings (H_{DD}), or residual dipolar couplings (RDCs)^{16–17}. For an IS bond vector with vibrationally averaged interspin distance r_{IS} , the RDC (in hertz) is

$$J_{\text{IS}}^{\text{RDC}} = \frac{-\omega_{\text{IS}}^{\text{DD}}}{2\pi} \langle P_2(\cos\Omega_{\text{IS}}) \rangle \quad (7)$$

where

$$\omega_{\text{IS}}^{\text{DD}} = \frac{2\gamma_{\text{I}}\gamma_{\text{S}}\hbar}{\langle r_{\text{IS}}^3 \rangle}, \quad \langle P_2(\cos\Omega_{\text{IS}}) \rangle = \left\langle \frac{3\cos^2\Omega_{\text{IS}} - 1}{2} \right\rangle \quad (8)$$

As depicted in Figure 4a, the angle Ω_{IS} specifies the orientation of the IS bond vector relative to the external field $\mathbf{B}_0$. Thus, the RDC is the “full-blown” dipolar-coupling constant $\omega_{\text{IS}}^{\text{DD}}$ (cgs units), scaled down by the orientational average, $\langle P_2(\cos\Omega_{\text{IS}}) \rangle$. The average is over the ensemble of orientations sampled by the bond vector. This average is zero under conventional isotropic tumbling conditions, but becomes non-zero under conditions of weak alignment, and is tuned by judicious adjustments of alignment media parameters¹⁸.

The most common RDCs are those of the backbone NH and CH bond vectors, and CH bond vectors of methyl side-chains. The measurements typically involve recording proton-detected 2-d heteronuclear correlation spectra that maintain the I-S spin-spin coupling during the indirect dimension. Spectra are recorded under isotropic conditions, and under conditions of partial alignment. Differences in the apparent J_{IS} splitting between the two conditions give $J_{\text{IS}}^{\text{RDC}}$ parameters^{18,85–86} (Figure 4c).

The dynamic information from the RDCs lies in the ensemble average of Equations 7,8. That average encompasses all orientations sampled by the IS bond vector over the time-course of the NMR signal, and is therefore sensitive to motions ranging from picosecond-millisecond time scales. Such motions include overall rotational diffusion and internal

conformational dynamics. To disentangle these effects, one expresses the RDC in terms of the coordinates of the IS bond with respect to coordinate frame fixed in the molecule that reflects the overall molecular ordering, as schematized in Figure 4b. Specifically, the overall protein alignment is expressed by a second-rank, symmetric, and traceless order tensor S_{ij} .^{87–88} S_{ij} has five independent elements in an arbitrary molecular-fixed frame, such as that implicit in a PDB file. Determining these elements requires measuring at least five distinct RDCs per rigid molecule, per alignment medium. The symmetry of S_{ij} means it can be diagonalized to produce three principle values (eigenvalues) and three principles axes (eigenvectors). These axes constitute the principle axes system (PAS) of the ordering tensor, in which a given RDC simplifies to

$$J_{IS}^{RDC} = -\frac{\omega_{IS}^{DD} S_{ZZ}}{2\pi} \left\{ \left(\frac{3z^2 - 1}{2} \right) + \left(\frac{S_{XX} - S_{YY}}{3S_{ZZ}} \right) \left\{ \frac{3x^2 - 1}{2} - \frac{3y^2 - 1}{2} \right\} \right\} \quad (9)$$

S_{ZZ} , S_{YY} , and S_{XX} are the three principle values of the S_{ij} tensor; they measure the average alignment of the corresponding principle axes via

$$S_{ii} = \left\langle \frac{3\cos^2\Theta_{ii} - 1}{2} \right\rangle, \quad i \in X, Y, Z \quad (10)$$

where Θ_{ii} is the angle that the i^{th} principle axis makes with respect to $\mathbf{B}_0$. The lower-case x, y, and z are the coordinates of the unit vector representing the IS bond (its direction cosines), with respect to the PAS of S_{ij} . The convention $|S_{ZZ}| \geq |S_{YY}| \geq |S_{XX}|$ means that the PAS Z-axis is the direction within the protein that has greatest alignment along $\mathbf{B}_0$. In this context, Equation 9 essentially says that the variation in RDCs among the bond vectors reflects their projection along this Z-axis. The PAS thus provides a common molecular reference frame whereby the average orientations of widely separated bond vectors can be compared. To determine the principle values and PAS from the raw RDC data, one can start from a 3-d structural model, and then apply algorithms that effectively rotate the molecular coordinates to diagonalize S_{ij} while reproducing the experimental RDCs^{89–90}.

Equation 9 suggests two main strategies for extracting dynamic information from the RDCs¹⁸. The first subdivides the protein into approximately rigid fragments; the order tensor is derived for each using Equation 9. For a totally rigid protein, a common order tensor should fit all fragments; thus RDCs from the separate fragments should each produce the same order tensor (same principle values and axes). Failure to comply with this expected agreement is evidence of inter-fragment mobility. This strategy works well for inter-domain dynamics, in which the domains are approximated as rigid structures⁹¹.

The second approach relates the RDCs to Lipari-Szabo order parameters (*vide supra*)^{92–93}. Here, one interprets the x, y, and z expressions in Equation 9 as ensemble averages over the bond vector orientations sampled within the PAS system, and thus;

$$J_{IS}^{RDC} = -\frac{\omega_{IS}^{DD} S_{zz}}{2\pi} \left\{ \left\langle \frac{3z^2 - 1}{2} \right\rangle + \left(\frac{S_{xx} - S_{yy}}{3S_{zz}} \right) \left\{ \left\langle \frac{3x^2 - 1}{2} \right\rangle - \left\langle \frac{3y^2 - 1}{2} \right\rangle \right\} \right\} \quad (11)$$

The key assumption of Equation 11 is that the internal motions are statistically decoupled from those determining the overall molecular alignment. In effect, it says that sub-millisecond fluctuations in bond vector orientation within the PAS will tend to reduce J_{IS}^{RDC} from its static value. One can extract the ensemble averages of the x, y, and z coordinates by

measuring RDCs from at least five independent alignment media^{18,92}. From these averages, and with the aid of a 3-d structural model, one can construct S_{RDC}^2 , the Lipari-Szabo generalized order parameter. More specific versions of this approach, such as the 3-d GAF, give analytical expressions for the fluctuations in terms of axial fluctuations of the peptide plane axis⁹⁴. Since RDCs sense motions over a broader range of time scales than spin relaxation, we expect $S_{RELAX}^2 \geq S_{RDC}^2$; hence, the former can serve as upper limits to scale the latter. In this context, an important breakthrough is the structure-free Gaussian-Axial-Fluctuation approach (SF GAF) from Blackledge and co-workers⁹⁵. This approach enables independent fits of S_{RDC}^2 without reference to a structural model or S_{RELAX}^2 . Critically, their S_{RDC}^2 results are consistent with those determined by previously established methods, and reproduce motions with correlation times ~ 400 ns (gleaned from MD simulations). Of course, such motions cannot be detected from spin-relaxation, which are inherently blind to re-orientational dynamics beyond the overall rotational correlation time, τ_c .

A practical challenge of the S_{RDC}^2 approach is finding at least five independent media that are congenial to the protein's well being. Nevertheless, the information gained is impressive, and has begun reshaping our appreciation of the importance of conformational transitions in molecular recognition. The recent work on ubiquitin provides an example⁹⁶. Analysis of the RDCs showed that the recognition loop samples a range of conformers that coincide with those collectively covered by known ubiquitin-ligand complexes (Figure 4d). Thus, different binding partners select from a manifold of inter-converting structures inherently sampled by the free protein. Similarly, Al-Hashimi and co-workers used RDCs to demonstrate an equally impressive range of motion for tar-RNA⁹⁷. The resulting manifold of loop conformations was also consistent with conformational selection by its various binding targets (Figure 4e). In the case of RNA, a novel extension method was used to isolate the internal motion affects on the RDCs⁹⁸. Both examples of RDC-derived dynamics highlight the complementary power of RDCs of detect "supra- τ_c " dynamics⁹⁹ that are invisible to spin-relaxation, and central to biomolecular recognition.

Chemical Exchange and Spin-locked Relaxation Dispersion

Chemical exchange studies based on the isotropic chemical shift are "vintage" NMR methods for studying equilibrium dynamics. Nevertheless, their impact on protein dynamics has never been more powerful. Thanks to parallel advances in spin-physics, isotope-labeling, and computational chemistry, chemical exchange studies are revealing the importance of protein dynamics on microsecond-millisecond time-scale affecting receptor binding, folding-unfolding transitions, and enzyme catalysis.

The most common exchange experiments focus on the isotropic chemical shift. In the simplest case, a nucleus hops between two states with chemical shifts, δ_a and δ_b , equilibrium populations p_a , and p_b , and forward and backward rate constants $k_{ab} = k_{ex}p_b$ and $k_{ba} = k_{ex}p_a$.

The exchange makes the chemical shift a fluctuating quantity, with consequences to the line shape and relaxation parameters that depend on how $k_{ex} = k_{ab} + k_{ba}$ compares to the frequency difference in chemical shifts, $\Delta\omega = \gamma B_0(\delta_a - \delta_b)$ ^{100–101}. One speaks of fast, intermediate, or slow exchange on the chemical shift time scale for k_{ex} that is $>$, $\sim$, or $<$ $\Delta\omega$, respectively.

In fortuitous cases, the exchange rates are sufficiently slow, and the populations comparable, such that one observes separate, state-specific resonances. One can then apply 2-d exchange spectroscopy (EXSY)¹⁰² to determine both the lifetimes and relative populations. 2-d heteronuclear exchange experiments on isotope-enriched samples are particularly effective for mapping slow exchange in proteins¹⁰³, and recent improvements in sensitivity and resolution have expanded their applicability to larger proteins^{104–105}.

More often, however, the exchange occurs between lopsided populations. This can cause excessive line broadening and decreased intensity of the minor state resonances, such that they, and the conformations they represent, are “invisible”^{100,106–108}. In these cases, heteronuclear (¹⁵N, ¹³C) transverse relaxation dispersion experiments, optimized for the relaxation properties of proteins^{20,109–110} are extremely powerful. These experiments measure the dependence of ¹⁵N or ¹³C transverse relaxation on the strength of an applied radio-frequency spin-lock, quoted in frequency units. Nuclei with exchange rates comparable to the spin-lock frequencies show a “dispersion” curve – transverse relaxation rates that decrease with increasing spin-lock frequency (this dispersion is not to be confused with the **B**₀ dispersion studies carried out with field-cycling). These dispersion curves are essentially the spectral density functions that describe the fluctuations in isotropic chemical shifts that result from the exchange dynamics. Fits of these curves to those predicted by exchange models yields the lifetimes of the exchange-coupled states, and, in optimal situations, the fractional populations and chemical shift differences. Examples of dispersion curves are in Figure 5a. Thus, the intrinsic chemical shift of the “invisible minor” state can be deduced from the dispersion of the observed major state. By far, the simplest models of two-state exchange^{111–112} dominate the fitting of dispersion curves. Studies that go beyond the confines of the two-state model^{113–114} are fewer, and in a younger stage of development, owing to the increased number of fitting parameters required relative to the typical sensitivity and abundance of dispersion data.

Traditionally, identifying the inter-converting conformations that give rise to the relaxation dispersion has been difficult. But this has changed. Retrospective analyses of protein chemical shift databases, paralleled by advances in quantum chemistry calculations (e.g. density functional theory) have dramatically increased our ability to predict conformation from chemical shifts and vice versa^{115–118}. As a consequence, the modest chemical shift has gone beyond a mere resonance index, to become a pre-eminent probe of structural dynamics. Notably, because the dispersion profiles from major state resonances can give the chemical shifts of minor “invisible” states, one can begin to model the conformations of the latter. This has opened the door to characterizing rare conformational excursions on the microsecond – millisecond time scale relevant for catalysis and folding (*vide infra*).

A growing number of relaxation dispersion studies implicate the role of millisecond conformational fluctuations with enzyme catalysis. Notable examples include Ribonuclease A (RNase A)¹¹⁹, *E. coli* DHFR¹²⁰, and adenylate kinase^{59,121}. For RNase A, conformational exchange rates k_{ex} from dispersion agreed well with k_{cat} and the rate constant for product release. Solvent kinetic isotope studies gave similar reductions of k_{ex} and k_{cat} . Structural interpretations of the dispersion data suggested RNase A samples the “invisible” (< 5%) conformation of the following state. For DHFR, extensive dispersion studies showed similar behavior: within a given intermediate state of its catalytic cycle, the enzyme samples “invisible” conformations characteristic of the ground states of the preceding and following states. Furthermore, DHFR mutations that perturb millisecond exchange dynamics also perturb hydride transfer, but not substrate binding¹²². Collectively, these findings suggest that the millisecond conformational fluctuations sensed by NMR influence enzyme catalysis. Yet, it is also well accepted that the atomic motions for the chemical step (crossing of the transition state free energy barrier) are orders of magnitude

faster than the millisecond processes. Indeed, recent reviews^{123–124} emphasize that millisecond enzyme motions cannot and do not influence the chemical step. The inconsistency of these two views is likely only apparent¹²⁵. The millisecond dynamics sensed by relaxation dispersion correspond to the motions of multiple moieties, whose individual motions might be quite fast (10^{-12} – 10^{-9} sec), but whose *allied* motion – an alliance essential for progressing along the reaction coordinate – is far less frequent. The relative infrequency of these cooperative motions manifests as longer millisecond dynamics sensed by NMR dispersion. Such motions are not the actual chemical step. Nevertheless, they reflect essential conformational changes (e.g. those associated with substrate binding and product release) that can be rate-limiting. As such, their characterization by NMR will continue to be important for understanding the overall catalytic process.

An exciting new method consists of relaxation dispersion studies carried out under conditions of weak protein alignment. These experiments can reveal invisible state properties beyond the chemical shift, including RDCs and RCSAs^{126–127}. In turn, this has enabled the determination of invisible state structures. In particular, Kay and co-workers demonstrated the proof-of-concept by determining the structure of an invisible state of SH3 domain¹²⁸; that corresponded to its ligand-bound form. They then applied a similar strategy to a *de novo* problem, in which they solved the structure of a transient folding intermediate of the FF domain¹²⁹, which was present at only 3% (cf. Figure 5b). Most recently, they have determined a transient state of a mutant T4 lysozyme¹³⁰.

Finally, the methyl-TROSY methods described above⁸⁰ have granted access to the millisecond chemical exchange dynamics of supra-molecular machines. Kay and co-workers have used relaxation dispersion⁸³ along with Paramagnetic Relaxation Enhancement (PRE) experiments⁸⁴ to characterize the functional motions of the 20S core-particle proteasome (MW ~ 670 kDa), responsible for timely protein degradation in the cell. More recently, they have characterized the folding-unfolding dynamics of small protein substrates within the proteasome¹³¹. Many key biological processes in the cell involve supra-molecular machines, such as the proteasome. The methyl-TROSY dispersion methods now enable a detailed investigation of their inner moving parts.

Future Challenges

The above passages tried to give a sense of the means and power by which liquid-state NMR can illuminate functional protein dynamics. Of course, the field itself remains dynamic, with ample room for methods development and novel applications. What are some future “challenge areas” for dynamic NMR? Of course, the answers will vary violently from one person to the next; nevertheless, here are a few possibilities.

Characterizing correlated or collective motions in proteins is of great interest, since they are likely critical for the long-range site-to-site communication that support allosteric communication and signal transduction. Yet, characterizing these motions by NMR has been historically challenging. In particular, the spin-relaxation experiments above report on auto-correlation functions, which measure the orientational fluctuations of the same bond vector. But to detect *correlated* motions, we need cross-correlation functions that compare the fluctuations of *pairs* of distinct bond vectors. Most NMR relaxation experiments that are sensitive to cross-correlation functions exploit pairs of vectors that share a common origin, and thus naturally undergo correlated re-orientations. Such experiments include the aforementioned TROSY methods that have dramatically increased the sensitivity of NMR for larger biomolecules^{80,132}, and provided more accurate means for detecting chemical exchange^{133–134}. But from a correlated motion perspective, these experiments are not sufficient; greater interest lies in correlated motions between more distal vector pairs. Toward this end, multiple quantum relaxation measurements have proven most

effective^{135–139}. Transverse relaxation limits the spatial range of these methods to NH/CH bond vectors separated by one or two bonds. Apparent correlated changes in methyl side-chain S^2 suggest collective picosecond-nanosecond motions related to allostery^{140–141}. Other dynamics experiments probing slower time scale processes, including RDCs and hydrogen-bond scalar couplings¹⁴² or relaxation dispersion^{143–144}, also point to correlated motion and site-to-site communication. Nevertheless, improved methods for the direct detection of longer range correlated motions, while retaining comprehensive site-by-site description of more standard NMR dynamics experiments, is an area waiting for further advances.

The challenge of detecting correlated motion draws attention to another area that will certainly intensify: the synergy between computation and NMR dynamics. Computational studies can explore how correlated or collective motions might leave distinct signatures on experimental observables. In this context a recent breakthrough is the work by Griesinger, Salvatella, and co-workers,¹⁴⁵ that generates an ensemble of structures consistent with conventional auto/cross-correlated spin-relaxation and RDC data as a means to identify long-range correlated motions that are not (yet) directly available from experiment. Additionally, the increase in the trajectory lengths of all-atom simulations¹⁴⁶, and newer “coarse-grained” methods for sampling conformational space^{147–148} opens the door for understanding the slow time scale motions detected by NMR in far richer detail and with far less model bias. Longer trajectories, coupled with experimental data¹⁴⁹ should also help clarify the extent the functional motions can be attributed to a few “essential degrees of freedom”.

A third challenge is that of molecular context: how does the protein dynamics information gained from *in vitro* NMR translate to the cellular environment? Of course, if *in vitro* dynamics-function relationships correlate with cellular assays, then this lends biological credence to the former. Nevertheless, it is desirable to systematically investigate how phenomena such as cellular crowding, local variations in viscosity, and diffusion, might assist or interfere with protein functional motions. The burgeoning “in cell” NMR studies^{150–151} raise the possibility of carrying out those investigations. At the moment, such studies must cope with technical difficulties associated with inhomogeneous broadening and comparatively low sensitivity. Conceivably, liquid-state in-cell methods, in alliance with the recent development of cellular solid-state NMR experiments¹⁵², may provide substantial progress.

A fourth challenge area is to continue investigations of the interplay between protein evolution and conformational dynamics. Thanks to the advances in bioinformatics, there is now an abundance of tools to identify and analyze conserved protein sequences, and correlated changes therein. To understand the evolution of these sequences, it is reasonable to consider any and all properties pertaining to protein function and stability, including conformational dynamics. Specifically, it makes sense to investigate whether and how protein sequences might reflect the selection of functional dynamics, or promote better adaptability under conditions of stress. Such studies could be particularly important for understanding protein evolution related to drug-resistance, and thus better anticipate sites of drug-resistant mutations.

Finally, of interest to both fundamental and applied research is defining how protein dynamics information might benefit molecular design. The examples above clearly show the involvement of protein dynamics with binding and catalysis. So it might seem that protein dynamics investigations would be a mainstay in the design of novel therapeutics. But, historically, this has been a rarity. One bottleneck is that a detailed dynamics analysis often requires time investments beyond those accepted by high-throughput programs. Moreover,

the information gained does not easily translate into metrics that can immediately guide synthetic chemistry. To become truly integrated in the design process, the dynamics information must go beyond an inventory of protein motions, to further describe concretely how it can enhance traditional methods for defining and predicting structure-activity relationships and pharmacokinetic properties. Parallel dynamics studies of the ligand are also valuable, as the ligands are the central objects of structure-activity studies. Efforts along these lines are growing,¹⁵³ but more systematic efforts are needed.

The study of protein dynamics is an effort to understand the behavior of complex dynamical systems. NMR has been an illuminating force in this effort, due to research efforts that maintain a deep interest in the physical basis of NMR. The techniques discussed in this Perspective are only a subspace within the larger space of dynamics experiments available from NMR. Nevertheless, it is hoped that this Perspective conveys a sense of the excitement and opportunities dynamic NMR holds for physical chemists. Indeed, in the study of biomolecular motion, liquid-state NMR is “alive and kicking”, like the dynamic molecules they study.

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Biography

Biography. Jeffrey (Jeff) W. Peng received his PhD in Biophysics from the University of Michigan in 1993, developing methods for studies of protein dynamics under Prof. Gerhard Wagner. Afterwards, he trained with Prof. Dr. Richard Ernst at ETH-Zürich, as a Damon Runyon Walter Winchell postdoctoral fellow. He then joined the NMR/Biophysics group at

Vertex Pharmaceuticals Inc. Cambridge MA. In 2003, he joined the faculty at the University of Notre Dame, where he is now Associate Professor of Chemistry and Biochemistry, and a Concurrent Associate Professor in Physics. <http://chemistry.nd.edu/faculty/detail/jpeng/>

Figure 1. Schematic of popular liquid-state NMR experiments for studying protein dynamics, and the relevant time scales of motion. This perspective focuses on the methods in bold: (heteronuclear) spin relaxation, residual dipolar couplings (RDCs), and spin-locked relaxation dispersion.

Figure 2.

Schematic of dynamics information available from heteronuclear spin relaxation. **(a)** Relaxation probes the orientational dynamics of bond vectors relative to the external static magnetic field, B_0 . **(b)** The orientational dynamics are characterized by a time correlation function $C(\tau)$. **(c)** The $C(\tau)$ decay depends on the individual bond vector motion, but always decays to zero due to isotropic overall tumbling. **(d)** The measured relaxation rates sample the spectral density function, which is the Fourier Transform of $C(\tau)$. The panel depicts the cases of deuterium R_1 and R_2 relaxation rates, which sample the $J_{CD}(\omega)$ spectral density at 0, ω_D , and $2\omega_D$.

Figure 3.

Examples of protein spin relaxation measurements. **(a)** A typical contour plot of a 2d $^{13}\text{C}_{\text{methyl}}\text{—}^1\text{H}_{\text{methyl}}$ heteronuclear correlation spectrum, for methyl side-chain 2D relaxation of CD bonds in CH_2D methyl groups. The protein is the mitotic regulator, Pin1. Each cross peak corresponds to a particular side-chain CH_2D methyl group. **(b)** The various cross-peak decays give site-specific 2D R_1 and R_2 . **(c)** The rates sample the spectral densities that describe the sub-nanosecond orientational dynamics of the CD bond (which is essentially that of the methyl symmetry axis). **(d)** Changes in Pin1 methyl side-chain motions upon interaction with a phosphopeptide substrate. Each sphere is a side-chain methyl. The changes are in terms of Lipari-Szabo order parameters, S^2_{axis} . Red/Blue spheres indicate increases/decreases of S^2_{axis} upon substrate interaction.

Figure 4.

Conformational dynamics from residual dipolar couplings (RDCs). **(a)** The RDC reports on the average orientation of bond vectors (typically NH, CH) with respect to the external static field B_0 . **(b)** The overall orientation is decomposed into the orientation with respect to a molecular-fixed frame (the order tensor), and the orientation of that tensor with respect to B_0 . **(c)** RDCs are typically measured by comparing apparent heteronuclear splittings under isotropic versus aligned conditions. **(d)** Examples of the manifold of conformations sampled by ubiquitin (red region, upper left), derived from extensive RDCs followed by order parameter analysis. The manifold is reprinted from Lange, O. F.; Lakomek, N. A.; Fares, C.; Schroder, G. F.; Walter, K. F.; Becker, S.; Meiler, J.; Grubmuller, H.; Griesinger, C.; de Groot, B. L. *Science* **2008**, *320*, 1471-5, with permission. Copyright 2008 AAAS.. **(e)** Example of the manifold of conformations sampled by the TAR-RNA loops, derived from RDC data. The figure is reprinted from Zhang, Q.; Stelzer, A. C.; Fisher, C. K.; Al-Hashimi, H. M. *Nature* **2007**, *450*, 1263-7, with permission. Copyright 2007 Nature Publishing Group. In both cases **(d)** and **(e)**, these orientational motions occur on time scales ($\tau > \tau_c$) that are invisible to spin relaxation. The manifolds encompass the conformations observed in structures of complexes, indicating that the ligands select from a pre-existing dynamic ensemble of conformers.

Figure 5.

Example of spin-locked relaxation dispersion for characterizing “invisible” protein states. **(a)** ^{15}N relaxation dispersion curves, $R_{2,eff}$ versus ν_{CPMG} , for residues of the FF domain, using Carr-Purcell-Meiboom-Gill (CPMG) spin-locking. The numbers in the upper refer to specific residues. Critically, only one set of NMR resonances are observed, that of the major “N” state (97%). **(b)** The curves are fit to functions based on two-state chemical exchange, to extract populations, rate constants, and conformational parameters of the invisible state, including chemical shifts, RDCs, and RCSAs. Together they enable the determination of the “invisible” conformation, the folding intermediate “I”. Figure 5a reprinted from Korzhnev, D. M.; Religa, T. L.; Banachewicz, W.; Fersht, A. R.; Kay, L. E. *Science* **2010**, 329, 1312-6 with permission, and Figure 5b reprinted from Al-Hashimi in *Science* **2010**, 329, 129. Copyright 2010 AAAS.