A Dynamic Look Backward and Forward

Arthur G. Palmer, III

Department of Biochemistry and Molecular Biophysics

Columbia University

630 West 168th Street

New York, NY 10032

Abstract

The 2015 Gunther Laukien Prize recognized solution NMR studies of protein dynamics and thermodynamics. This Perspective surveys aspects of the development and application of NMR spin relaxation for investigations of protein flexibility and function over multiple time scales in solution. Methods highlighted include analysis of overall rotational diffusion, theoretical descriptions of $R_{1\rho}$ relaxation, and molecular dynamics simulations to interpret NMR spin relaxation. Applications are illustrated for the zinc-finger domain Xfin-31, the calcium-binding proteins calbindin D_{9k} and calmodulin, and the bZip transcription factor of GCN4.

Keywords: chemical exchange, conformational dynamics; kinetics; ligand binding; molecular dynamics simulation; nuclear magnetic resonance spectroscopy; spin relaxation.

Introduction

The investigation of the dynamics of proteins, nucleic acids, and other biological macromolecules has a long history in NMR spectroscopy. The first nuclear spin relaxation measurement for a protein was a one-dimensional natural-abundance ^{13}C R_1 experiment for ribonuclease A reported in 1971 [1]. Of course, theoretical descriptions of spin relaxation essential for interpreting relaxation measurements already were established by the 1970's with key papers establishing the Bloch-Wangness-Redfield (BWR) [2] and Bloch-McConnell [3] equations, which form the basis for theoretical descriptions of Carr-Purcell-Meiboom-Gill (CPMG) [4], $R_{1\rho}$ [5], and exchange spectroscopy [6]. Indeed, by 1970, both CPMG [7] and $R_{1\rho}$ [5] experiments had been used to determine the kinetic rate constant for the chair-to-boat isomerization of cyclohexane. However, the congestion of one-dimensional NMR spectra and the low sensitivity of natural abundance ^{13}C NMR spectroscopy severely limited applications of what was widely known, at least to NMR spectroscopists, to be a potentially powerful technique for investigating the dynamic properties of biological molecules. Despite these limitations, critical aspects of protein statistical mechanics were elucidated, summarized for BPTI in an influential review by Wagner in 1983 [8]. Notably, ^{13}C relaxation measurements demonstrated that certain aromatic rings in BPTI rotate rapidly, showing that the interiors of proteins have considerable plasticity. As one measure of the fundamental importance of NMR spin relaxation measurements for understanding protein properties, investigations of relaxation of aromatic groups continue to be pursued to this day [9].

The situation changed dramatically in the two-year period from 1988 to 1989. First, “inverse” ^1H -detected techniques for measuring nuclear spin relaxation of ^{13}C and ^{15}N heteronuclei began to be developed. The first methods by Nirmala and Wagner were based on a double DEPT experiment for ^{13}C and were sensitive enough for ^{13}C natural abundance NMR

spectroscopy of small soluble proteins [10, 11]. These initial reports were quickly followed by a seminal study of uniformly ^{15}N enriched staphylococcal nuclease using INEPT-based experiments for ^{15}N R_1 , ^{15}N R_2 , and the heteronuclear $\{^1\text{H}\}$ - ^{15}N steady-state NOE [12]. The approach for data acquisition and analysis presented in this paper, although highly refined over the past two decades, remains the basic framework for contemporary work.

The developments in 1988-1989 revolutionized and invigorated studies of biological systems by spin relaxation and subsequent developments in experimental methods have enabled investigation of numerous spin types in proteins and nucleic acids [13]. With these advances, site-specific information on multiple time scales can be obtained on large molecules and molecular machines, ultimately up to ~one-half megadalton in pioneering work by Kay and co-workers [14]. Impressive studies of complex systems demonstrate that important information can be obtained from these measurements. At the same time, these experimental methods have diffused out of the laboratories of specialists and are being employed increasingly outside of academic settings [15].

The main goal of this Perspective is to summarize my own contributions to the development and application of spin relaxation methods, beginning with my introduction to the field as a postdoctoral scientist with Peter E. Wright at The Scripps Research Institute and continuing throughout my career at Columbia University. The 2015 Gunther Laukien Prize Lecture described some aspects of this work and is available at www.enc-conference.org/LaukienRecipients/PastRecipients/. In addition, recent Perspective [16] and Accounts [17] articles have summarized chemical exchange methods and applications of spin relaxation methods to enzymes, respectively. Accordingly, this Perspective focuses on the role of protein flexibility and dynamics in recognition of target molecules by proteins.

First results: Xfin-31 and Calbindin D_{9k}

Investigations of the Zn-finger domain Xfin-31 [18-20] and the EF-hand protein calbindin D_{9k} [21, 22] provided early experimental insights and illustrate the research concerns and approaches that continue to be central to my research.

Initial studies of the 25-residue Xfin-31 protein ligated with Zn²⁺ used two-dimensional DEPT-based experiments to measure backbone and side chain ¹³C spin relaxation at natural abundance [18]. The first experimental work focused on the backbone ¹³C^α spins, analyzed with the Lipari-Szabo model-free formalism [23, 24]. Values of the ¹³C generalized order parameter, S^2 , are shown in Figure 1. Like the earlier example of staphylococcal nuclease [12], the results showed that bond vectors in secondary structures are highly ordered, with values of the generalized order parameter $S^2 > 0.85$. The termini and loops were more heterogeneously disordered, with the most C-terminal residue having $S^2 = 0.30$. The experimental results subsequently were compared to values calculated from a 100 ps molecular dynamics (MD) simulation in aqueous solution [19]. The MD simulations yielded values of S^2 that were more than 0.10 larger than experimental values only for three residues: Tyr 1, Phe 10, and Lys 24. For the remaining residues, the average difference between experimental and simulated values was 0.013 with a sample deviation of 0.06. The two residues near the N- and C-termini have small secondary ¹³C shifts [25], suggesting that these residues are fairly disordered in solution and over-restrained in the MD simulation, perhaps reflecting the short trajectory length that could be achieved in 1992.

Chemical exchange contributions to R_2 were observed for six of the backbone ¹³C^α resonances, five of these are either ligands to the Zn²⁺ ion or residues or adjacent to such residues. Exchange broadening also was observed for side chain ¹³C^α resonances of the Zn²⁺ ligand His 19 and His 23 and the adjacent residue Val 22. The fitted values of the chemical

exchange broadening R_{ex} are uncorrelated with the squares of the $^{13}\text{C}^\alpha$ secondary chemical shifts, suggesting that the exchange process is not rapid unfolding of the protein following transient loss of the Zn^{2+} ion. In these initial studies, further characterization of chemical exchange was not possible, but these phenomena sparked an interest that developed into a major aspect of subsequent work.

Comparison between apo and holo forms of the calcium-binding EF-hand protein calbindin D_{9k} demonstrated the importance of NMR investigations of conformational flexibility for understanding molecular recognition by proteins. In collaboration with Walter Chazin and coworkers, backbone ^{15}N relaxation rate constants were measured for the apo, $(\text{Cd}^{2+})_1$, and $(\text{Ca}^{2+})_2$ states of the protein [21, 22]. Calbindin D_{9k} contains two metal-binding EF-hand motifs and Cd^{2+} binds to the C-terminal EF-hand, mimicking the unobservable state in which a single Ca^{2+} ion is bound to that site (because Ca^{2+} , but not Cd^{2+} , binding is cooperative). Subsequently Mäler and coworkers generated an Asn56Ala mutant that blocks binding to the C-terminal EF-hand, and thereby obtaining a $(\text{Ca}^{2+})_1$ species with the metal ion occupying the N-terminal EF-hand [26]. As shown in Figure 2, backbone ^{15}N order parameters show that the N-terminal EF-hand is ordered and the C-terminal EF-hand is highly disordered in the apo state. In contrast, both EF-hands are highly ordered in the holo state. In addition, the C-terminal EF-hand is ordered in both the $(\text{Cd}^{2+})_1$ and $(\text{Ca}^{2+})_1$ states. In the former case, rigidification is the direct result of ion binding in the C-terminal EF-hand. In the latter case, binding to the N-terminal site rigidifies the C-terminal site as a consequence of structural changes propagated through the β -strand interface between the two EF-hand motifs. The key conclusion is that one contribution to the allosteric phenomenon in calbindin D_{9k} results from rigidification of the C-terminal binding site regardless of which binding site is occupied by the first Ca^{2+} ion.

Acquisition and Analysis of Relaxation Data

Method developments in my laboratory for fast (ps-ns) time-scale dynamics in proteins have focused on pulse sequence development and optimization [27-29], error analysis and model-selection in relaxation data analysis [30], determination of overall rotational diffusion tensors [31-33], relationships between relaxation and conformational entropy [34, 35], and temperature dependence of intramolecular motions [36-38]. For brevity, only recent results on the analysis of rotational diffusion tensors are discussed herein.

In an early study, the quadric method of Brüschweiler and coworkers [39] was extended to joint analysis of ^{13}C and ^{15}N relaxation rate constants to enable more thorough analysis of the overall rotational diffusion tensors of macromolecules, particularly in cases for which the distribution of orientations of ^{15}N bond vectors is not uniform [31]. More recently, the quadric approach was extended to time-dependent diffusion tensors [32, 33]. For the simplest case of interchange between two conformational states described by unequal, but isotropic, diffusion tensors, the spectral density function is given by [40]:

$$J(\omega) = \frac{2}{5} \left\{ \frac{S_o^2 S_f^2 6D}{(6D)^2 + \omega^2} + \frac{S_f^2 (1 - S_o^2) \tau_1}{1 + \omega^2 \tau_1^2} + \frac{S_o^2 (1 - S_f^2) \tau_2}{1 + \omega^2 \tau_2^2} + \frac{(1 - S_o^2)(1 - S_f^2) \tau_3}{1 + \omega^2 \tau_3^2} \right\} \quad (1)$$

in which:

$$\begin{aligned} k &= \left[k_{ex}^2 + 12(D_A - D_B)(k_1 - k_{-1}) + 36(D_A - D_B)^2 \right]^{1/2} \\ S_o^2 &= \frac{1}{2} \left[1 + \frac{(k_1 - k_{-1})(6D_A - 6D_B + k_1 - k_{-1}) + 4k_1 k_{-1} Y_2^0(\mu_A \bullet \mu_B)}{k k_{ex}} \right] \\ 6D &= 3(D_A + D_B) + (k_{ex} - k) / 2 \\ \tau_1 &= (6D + k)^{-1} \\ \tau_2 &= (6D + 1 / \tau_f)^{-1} \\ \tau_3 &= (6D + k + 1 / \tau_f)^{-1} \end{aligned} \quad (2)$$

D_A and D_B are local isotropic diffusion constants for the two states (*vide infra*); $k_{ex} = k_1 + k_{-1}$, k_1 and k_{-1} are the rate constants for transitions from state A to B, and from state B to A, respectively; and S_f^2 and τ_f are the square of the generalized order parameter and effective internal correlation time for internal motions, respectively (assumed to be identical in states A and B and independent of overall rotational diffusion). In addition, μ_A and μ_B are unit vectors along the principal axis of the interaction tensor for the relaxation mechanism under consideration (most commonly, the bond vector between two nuclei) and $Y_2^0(\Omega)$ is the Legendre polynomial.

The above results are generalized to non-isotropic diffusion tensors by replacing the isotropic diffusion tensors in Eqs. 1 and 2 with the local isotropic diffusion tensors obtained from the quadric analysis [32, 33]. Thus, the local isotropic diffusion constants in each state $j = \{A, B\}$ are given by:

$$D_j = \mathbf{e}_j^T \mathbf{Q}_j \mathbf{e}_j \quad (3)$$

in which $\mathbf{e}_j$ are the directions cosines defining the orientation of μ_i in the principal frame of the diffusion tensor, $\mathbf{Q}_j$ is diagonal with elements $Q_{xx,j} = (D_{yy,j} + D_{zz,j})/2$, $Q_{yy,j} = (D_{xx,j} + D_{zz,j})/2$ and $Q_{zz,j} = (D_{xx,j} + D_{yy,j})/2$, and $D_{kk,j}$ are the principal values of the diffusion tensor for the j th conformational state. For an axially symmetric diffusion tensor, with $D_{xx,j} = D_{yy,j}$, the errors in the quadric approximation are $< 10\%$ for $0.65 \leq D_{zz,j} / D_{xx,j} \leq 1.75$.

If the analysis is limited to molecular sites that are highly ordered in both states, only the first two terms of Eq. 1 are significant and

$$\frac{R_2}{R_1} - \frac{1}{2} \approx \left\{ S_o^2 6D + (1 - S_o^2) \tau_1 \right\} / \left\{ \frac{S_o^2 6D}{(6D)^2 + \omega_x^2} + \frac{(1 - S_o^2) \tau_1}{1 + \omega_x^2 \tau_1^2} \right\} \quad (4)$$

is independent of internal motions. In principle, Eq. 4 can be fit to experimentally determined relaxation rate constants to estimate the diffusion tensors and exchange rates. However, even Eq. 4 contains a large number of parameters, so that data analysis is simplified by prior knowledge, particularly of the putative structures of the exchanging conformations. As an illustration, Figure 3 shows experimental data for calmodulin recorded at 316 K by Chang and coworkers [41]. These data have been fit with Eq. 4 assuming (i) the N- and C-terminal EF-hand domains of calmodulin diffuse independently in state A, (ii) the molecule has a rigid conformation with stable central helix in state B, and (iii) interchange between states is in the fast-exchange limit, $k_{ex} \gg |D_A - D_B|$. The diffusion tensors were calculated from the structures of the intact molecule and the N- and C-terminal domains using HydroPro [42]. With these assumptions, the experimental data are fit with a population of the ordered B state of 0.67.

Method developments in my laboratory for slow (μ s-ms) time-scale dynamics have focused on pulse sequence development and optimization [43], theoretical analyses of spin-locking experiments outside the fast exchange limit [44], and interpretation of chemical exchange parameters [45, 46]. Contributions in these areas have been reviewed recently in this journal [16]. For brevity, only theoretical analysis of the $R_{1\rho}$ relaxation rate constant is discussed herein.

A series of results, beginning in 2002 have yielded a number of expressions of increasing accuracy for the $R_{1\rho}$ relaxation rate constant arising from two-site [44, 47, 48] and n -site [49] chemical exchange between conformational or chemical states. For two-site exchange, assuming that the differences in the intrinsic relaxation rate constants between the two states are small, the most accurate expression is given by the Laguerre approximant [48]:

$$R_{1\rho} = R_1^0 \cos^2 \theta + R_2^0 \sin^2 \theta + \frac{\sin^2 \theta p_A p_B \Delta \omega^2 k_{ex}}{\omega_A^2 \omega_b^2 / \omega_e^2 + k_{ex}^2 - \sin^2 \theta p_A p_B \Delta \omega^2} \left\{ 1 + \frac{2k_{ex}^2 (p_A \omega_A^2 + p_b \omega_B^2)}{\omega_A^2 \omega_b^2 + \omega_e^2 k_{ex}^2} \right\} \quad (5)$$

in which R_1^0 and R_2^0 are the population-average relaxation rate constants in the absence of chemical exchange (dominated by dipole-dipole, chemical shift anisotropy, and quadrupolar relaxation mechanisms in biological macromolecules); ω_1 is the amplitude of the spin-lock radiofrequency field; $\tan \theta = \omega_1 / \bar{\Omega}$; $\bar{\Omega} = p_A \Omega_A + p_B \Omega_B$ is the population-average resonance offset frequency; p_k and Ω_k are the equilibrium population and the resonance offset frequency, respectively, for the k th state; $k_{ex} = k_1 + k_{-1}$; k_1 and k_{-1} are the forward and reverse kinetic rate constants, respectively; $\Delta \omega = \Omega_B - \Omega_A$; $\omega_e^2 = \bar{\Omega}^2 + \omega_1^2$; and $\omega_k^2 = \Omega_k^2 + \omega_1^2$. Other results have been reported for cases in which the differences in transverse relaxation rate constants for different states are not negligible (relative to k_{ex}) [16, 50]. One particularly simple such expression is given by:

$$R_{1\rho} = R_{1,A}^0 \cos^2 \theta + R_{2,A}^0 \sin^2 \theta + \sin^2 \theta k_1 \frac{\left\{ \Delta \omega^2 + (\Delta R_2^0)^2 \right\} k_{-1} + \Delta R_2^0 (\omega_A^2 + k_{-1}^2)}{k_{-1} \left\{ \omega_B^2 + (k_{-1} + \Delta R_2^0)^2 \right\} + \Delta R_2^0 \omega_1^2} \quad (6)$$

in which $\Delta R_2^0 = R_{2,B}^0 - R_{2,A}^0$, R_{2k}^0 is the intrinsic transverse relaxation rate constant for the k th state, and differences between R_1 relaxation rate constants have been neglected (and are usually small). Note that Eq. 6 corrects a typographical error in Eq. 11 of the original publication [16].

A simple derivation is provided below to illustrate the approaches used to obtain approximate expressions for $R_{1\rho}$, because most existing derivations rely on direct expansion of the characteristic polynomial for the evolution matrix [44, 48, 50]. As shown in Eq. 5, $R_{1\rho}$ has the form:

$$R_{1\rho} = R_1^0 \cos^2 \theta + R_2^0 \sin^2 \theta - \lambda \quad (7)$$

in which λ is the largest (least negative) eigenvalue of the evolution matrix:

$$\mathbf{L} + \mathbf{K} = \begin{bmatrix} \mathbf{L}_A & \mathbf{0} \\ \mathbf{0} & \mathbf{L}_B \end{bmatrix} + \begin{bmatrix} -k_1 & k_{-1} \\ k_1 & -k_{-1} \end{bmatrix} \otimes \mathbf{I} \quad (8)$$

in which

$$\mathbf{L}_k = \begin{bmatrix} 0 & -\Omega_k & 0 \\ \Omega_k & 0 & -\omega_1 \\ 0 & \omega_1 & 0 \end{bmatrix} \quad (9)$$

and $\mathbf{0}$ and $\mathbf{I}$ are the zero and identity matrices, respectively, with dimensions set by context (3×3 in Eq. 8). The key step in the derivation is based on the observation that the matrix $\mathbf{K}$ can be factored as:

$$\mathbf{K} = \begin{bmatrix} -k_1 & k_{-1} \\ k_1 & k_{-1} \end{bmatrix} \otimes \mathbf{I} = \begin{bmatrix} \mathbf{I} \\ -\mathbf{I} \end{bmatrix} \begin{bmatrix} -k_1 \mathbf{I} & k_{-1} \mathbf{I} \end{bmatrix} = \mathbf{U} \mathbf{V}^T \quad (10)$$

The eigenvalues of $\mathbf{L} + \mathbf{K}$ are determined by equating the characteristic polynomial to 0:

$$\begin{aligned} 0 &= |\mathbf{L} + \mathbf{K} - \lambda \mathbf{I}| \\ &= |\mathbf{L} - \lambda \mathbf{I} + \mathbf{U} \mathbf{V}^T| \\ &= |\mathbf{I} + \mathbf{V}^T (\mathbf{L} - \lambda \mathbf{I})^{-1} \mathbf{U}| |\mathbf{L} - \lambda \mathbf{I}| \end{aligned} \quad (11)$$

in which $|\mathbf{A}|$ is the determinant of the matrix $\mathbf{A}$ and the last line is obtained using the matrix determinant lemma. A series of formal manipulations that utilize the block structure of $\mathbf{L}$ yields:

$$\begin{aligned}
0 &= \left| \mathbf{I} + \mathbf{V}^T (\mathbf{L} - \lambda \mathbf{I})^{-1} \mathbf{U} \right| |\mathbf{L} - \lambda \mathbf{I}| \\
&= \left| \mathbf{I} + \mathbf{V}^T (\mathbf{L} - \lambda \mathbf{I})^{-1} \mathbf{U} \right| |\mathbf{L}_A - \lambda \mathbf{I}| |\mathbf{L}_B - \lambda \mathbf{I}| \\
&= \left| \mathbf{I} + k_{ex} \left[p_B (\mathbf{L}_A - \lambda \mathbf{I})^{-1} + p_A (\mathbf{L}_B - \lambda \mathbf{I})^{-1} \right] \right| |\mathbf{L}_A - \lambda \mathbf{I}| |\mathbf{L}_B - \lambda \mathbf{I}| \\
&= \left| (\mathbf{L}_A - \lambda \mathbf{I})(\mathbf{L}_B - \lambda \mathbf{I}) + k_{ex} \left[p_A (\mathbf{L}_A - \lambda \mathbf{I}) + p_B (\mathbf{L}_B - \lambda \mathbf{I}) \right] \right| \\
&= \left| (\mathbf{L}_A - \lambda \mathbf{I})(\mathbf{L}_B - \lambda \mathbf{I}) + k_{ex} (\bar{\mathbf{L}} - \lambda \mathbf{I}) \right| \\
&= \left| (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}}) - \lambda (\mathbf{L}_A + \mathbf{L}_B - k_{ex} \mathbf{I}) + \lambda^2 \mathbf{I} \right|
\end{aligned} \tag{12}$$

in which $\bar{\mathbf{L}} = p_A \mathbf{L}_A + p_B \mathbf{L}_B$. Notably, the right-hand side of the last line now is the determinant of a matrix with dimension 3×3 , rather than 6×6 as in the starting expression. When λ is small in magnitude, the determinant can be expanded to first order:

$$\begin{aligned}
0 &= \left| (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}}) - \lambda (\mathbf{L}_A + \mathbf{L}_B - k_{ex} \mathbf{I}) + \lambda^2 \mathbf{I} \right| \\
&= \left| (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}}) \right| \left| \mathbf{I} - \lambda (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}})^{-1} (\mathbf{L}_A + \mathbf{L}_B - k_{ex} \mathbf{I}) + (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}})^{-1} \lambda^2 \mathbf{I} \right| \\
&= \left| \mathbf{I} - \lambda (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}})^{-1} (\mathbf{L}_A + \mathbf{L}_B - k_{ex} \mathbf{I}) + (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}})^{-1} \lambda^2 \mathbf{I} \right| \\
&= 1 - \lambda \text{tr} \left\{ (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}})^{-1} (\mathbf{L}_A + \mathbf{L}_B - k_{ex} \mathbf{I}) \right\}
\end{aligned} \tag{13}$$

in which $\text{tr}\{\mathbf{A}\}$ is the trace of the matrix $\mathbf{A}$ and terms proportional to λ^n for $n \geq 2$ are not considered. The final equation is solved for λ to give:

$$\lambda = \text{tr} \left\{ (\mathbf{L}_A \mathbf{L}_B - k_{ex} \bar{\mathbf{L}})^{-1} (\mathbf{L}_A + \mathbf{L}_B - k_{ex} \mathbf{I}) \right\}^{-1} \tag{14}$$

as a first-order approximation. Straightforward algebra then leads to:

$$-\lambda = R_{ex} = \frac{\sin^2 \theta p_A p_B \Delta \omega^2 k_{ex}}{\omega_A^2 \omega_b^2 / \omega_e^2 + k_{ex}^2} \tag{15}$$

which, when substituted into Eq. 7, yields the result of Trott and Palmer [44]. Equation 14 remains valid if the expressions for $\mathbf{L}_A$ and $\mathbf{L}_B$ are generalized to include intrinsic relaxation rate

constants that differ for the two states (except that Eq. 14 then yields the full expression for $R_{1\rho}$, rather than R_{ex}).

The key insight from Eqs. 7 and 15 is obtained by noting that as $p_b \rightarrow 0$, $\omega_A^2 \rightarrow \omega_e^2$. Therefore, $\omega_A^2 \omega_B^2 / \omega_e^2 \rightarrow \omega_B^2$; that is, the $R_{1\rho}$ experiment depends on the effective field in the rotating frame of reference for the B, usually sparsely populated and unobservable, state. As a result, the $R_{1\rho}$ experiment is sensitive to the signed value of the minor state chemical shifts, rather than the absolute value reported by the related Carr-Purcell-Meiboom-Gill experiment. This property facilitates structure determination of sparsely populated states of biological macromolecules, particularly in the weak rf field limit utilized in CEST [51] and DEST [52] experiments.

The accuracies of different approximate expressions for $R_{1\rho}$ calculated for a minor state population $p_B = 0.03$ are compared in Figure 4. When the differences between intrinsic values of the relaxation rate constants are small, the Laguerre formula (Eq. 5) is very accurate (even for much large values of p_B), while the Kay and Baldwin formula (Eq. 5 in [50]) and the simpler result of Eq. 6 have similar accuracies. When the difference in intrinsic relaxation rate constants is large, Eq. 5 becomes highly inaccurate, while the other two expressions maintain similar levels of accuracy.

Application to Ligand-binding

Dynamics of the bZip domain of the yeast transcription factor GCN4 has been explored by both experimental NMR spin relaxation [53, 54] and MD simulations [55]. The most recent experimental data set, published in 2015 [54], and simulated trajectories, published in 2013, [55] illustrate the technical advances in these fields since the work described above on Xfin-31. Relaxation data were acquired for ^{15}N spins in the bZip domain at four static magnetic fields,

600, 700, 800, and 900 MHz, compared to the single 500 MHz field used for the Xfin-31 investigations. In addition, the MD simulations consisted of four 100 ns trajectories, compared to the single 100 ps trajectory obtained for Xfin-31.

The initial experimental study of the bZip domain used ^{15}N spin relaxation recorded at 500 MHz ^1H Larmor frequency for three temperatures $T = 290, 300, \text{ and } 310 \text{ K}$ [53]. These data only allowed S^2 to be determined in a site-specific manner. Data were analyzed assuming a single average rotational correlation time for residues in the basic region given by the mean correlation time determined for residues in the ordered coiled-coil domain. More recently, relaxation data have been recorded at four static magnetic fields (600, 700, 800, and 900 MHz) at $T = 300 \text{ K}$ [54]. Importantly, spectral density mapping of the data recorded at four static magnetic fields suggested that the motional models required to describe the data would include at most five parameters, because the sets of spectral densities $J(\omega_X)$ measured at the ^{15}N and ^1H Larmor frequencies were linear functions of ω_X^{-2} , in which ω_X is ω_N or $0.87\omega_H$, respectively (with the fifth parameter obtained from $J(0)$). Accordingly, relaxation data were fit with a series of model-free spectral density functions of which the most complex was the two-time-scale model-free function proposed by Clore and coworkers [56]. The values of S^2 determined from the newer data set are shown in Figure 5a and the correlation between the two data sets is shown in Fig. 5b. As proposed previously [36, 57], the temperature dependence of the order parameters are characterized by the parameter $\Lambda = d\{\ln(1 - S)/d\ln T$ and values of Λ are shown in Fig. 5c.

The protein displays four regions with distinct dynamic properties: (i) the most N-terminal residues 3–12 and (ii) residues 13–25 in the basic region; (iii) residues 26–55 in the coiled-coil domain, and the (iv) C-terminal residues 56–58. The N-terminal region (i) shows a strong $\text{N} \rightarrow \text{C}$ increase in both the values of S^2 and Λ , while region (ii) shows a strong $\text{N} \rightarrow \text{C}$ increase in S^2 , but Λ appears relatively constant. The order parameters are large and uniform in

the coiled-coil region, but the temperature variation was too small to be characterized. The C-terminal residues are disordered with a uniform value of Λ .

The role of the change in backbone flexibility in DNA binding was explored by estimating the conformational entropic penalty to binding using a theoretical approach originally developed by Akke and coworkers [58] and extended by Kay and coworkers [59] and by Wand and coworkers [60, 61]. Assuming that the order parameters for the basic region in the protein-DNA complex would be similar to those of the coiled-coil domain, the backbone conformational entropic penalty to binding was estimated to be $-0.6 \text{ kJ mol}^{-1} \text{ K}^{-1}$, which is consistent with estimates derived from isothermal titration calorimetry [53].

The MD simulations of the GCN4 bZip domain were validated by comparison of chemical shifts calculated over the trajectories to the experimental values. Previous work on ribonuclease H had established that even 100 ns simulations were capable of reproducing the averaging of chemical shifts between different conformations [62]. The second of the four acquired trajectories best recapitulated the chemical shifts, and by inference the conformational preferences, of the basic region of the bZip domain, including for example a transiently formed helix-capping interaction between residues Ala 5 to Ala 9, Leu 6 to Arg 10, and Lys 7 to Asn 11. Using Sparta+ [63], the root-mean-square-deviations between calculated and experimental shifts were 0.63, 0.54, 0.13, and 1.38 ppm for $^{13}\text{C}^\alpha$, $^{13}\text{C}'$, $^1\text{H}^\text{N}$, and ^{15}N , respectively.

The population of helical conformations was calculated as an average over the trajectory. The residue-specific populations are shown as a graph in Figure 6a. The two distinct segments of the basic region identified from the patterns of S^2 and Λ contain two transiently populated helices: H1, corresponding to residues 4-8, and H2, corresponding to residues 13-19. Mapping of the helical populations onto the structure of the bZip domain/DNA complex in Figure 6b shows that transient helix H2 is part of the basic region that makes contacts in the major groove of the

DNA ligand in the complex: residues Asn11, Ala14, Ala15, Ser18, and Arg19 are highly conserved among bZip domains and form base-specific DNA interactions in the complex. The good agreement between the calculated and experimental chemical shifts is accompanied by good agreement between calculated and experimental values of S^2 , shown in Figure 6c, further supporting the conclusion that the conformational dynamics observed in the MD simulation accurately reflect dynamics in the solution state of the GCN4 bZip domain.

Investigations by NMR spin relaxation techniques, beginning with calbindin D_{9k}, have addressed both thermodynamic and kinetic aspects of ligand binding by proteins. The observation of a populated helix H2 in the center of the DNA-recognition element of the bZip domain of GCN4 suggests a two-step mechanism for binding: (i) initial formation of an encounter complex through interaction with the pre-formed helix H2 and (ii) subsequent folding of the rest of the basic region to form the high affinity helical structure. This model can be represented as a “selected-fit” initial step followed by an “induced-fit” second step or as “coupled-folding-and-binding” [64, 65]. In general, ligand recognition by proteins can occur through parallel mechanisms with varying aspects of selected- or induced-fit models [66]. In the present case, the thermodynamic entropic penalty to binding is reduced by the presence of helix-capping sequences and transient helical conformations in the apo state of the bZip domain that increase the measured order parameters compared to a random coil peptide (e.g. the most C-terminal residues).

The experimental relaxation data for the basic region residues were fit with the extended model-free formalism, which characterizes internal dynamics on two time scales. For the bZip domain of GCN4, the faster dynamics are characterized by values of $S_f^2 \approx 0.6$ for region (i) (residues 2-12) that increase across region (ii) (residues 13-25) to a maximum value of ~ 0.8 . The average effective internal correlation time for the basic region is $\tau_f \approx 50$ ps. In contrast, the

slower time scale dynamics are characterized by values of S_s^2 that increase dramatically from 0.09 for residue 3 to 0.80 for residue 25 with average internal correlation times of $\tau_f \approx 1.5$ ns. These results suggest that the dominant conformational dynamic process required to reach the fully ordered state involves restriction of the conformational states sampled on the 1.5 ns time scale. This time scale is within the range expected for propagation of an α -helix and faster than the expected dissociation time constant for an encounter complex.

Discussion and Conclusion

The nearly three decades of modern applications of NMR spectroscopy to spin-relaxation measurements in proteins have seen dramatic developments in experimental and theoretical methods and equally dramatic advances in the complexity of biological systems under investigation [13]. Work in my own laboratory has focused on spin-relaxation studies of proteins in solution; however, the methods and experimental approaches pioneered in solution NMR spectroscopy have been adapted to nucleic acids in solution [67-69] and to solid-state NMR studies of biological macromolecules [70-72]. These advances suggest that the renaissance in NMR spin relaxation of biological macromolecules that began in 1988 is still vibrant and is likely to generate additional Gunther Laukien Prize awards in the future.

Acknowledgments

I am grateful to the current and past members of my research group for their invaluable contributions to my career at Columbia University. I thank Mark Rance (Univ. Cincinnati) for a longstanding collaboration that has been crucial to many aspects of my work and Peter E. Wright (The Scripps Research Institute) for his mentorship since I joined his research group as a National Science Foundation Postdoctoral Fellow in 1989. My undergraduate and graduate advisors, Colin F. Mackay (Haverford College) and Nancy L. Thompson (Univ. North Carolina),

respectively, catalyzed my interest in dynamic fluctuations in biology and chemistry, the dominant theme throughout my research career.

References

- [1] A. Allerhand, D. Doddrell, V. Glushko, D.W. Cochran, E. Wenkert, P.J. Lawson, F.R.N. Gurd, Conformation and segmental motion of native and denatured ribonuclease A in solution. Application of natural-abundance carbon-13 partially relaxed Fourier transform nuclear magnetic resonance J. Amer. Chem. Soc., 93 (1971) 544-546.
- [2] A.G. Redfield, The theory of relaxation processes, Adv. Magn. Reson., 1 (1965) 1-32.
- [3] H.M. McConnell, Reaction rates by nuclear magnetic resonance, J. Chem. Phys., 28 (1958) 430-431.
- [4] J.P. Carver, R.E. Richards, A general two-site solution for the chemical exchange produced dependence of T_2 upon the Carr-Purcell pulse separation, J. Magn. Reson., 6 (1972) 89-105.
- [5] C. Deverell, R.E. Morgan, J.H. Strange, Studies of chemical exchange by nuclear magnetization relaxation in the rotating frame, Mol. Phys., 18 (1970) 553-559.
- [6] S. Forsén, R.A. Hoffman, Study of moderately rapid chemical exchange reactions by means of nuclear magnetic double resonance, J. Chem Phys., 39 (1963) 2892.
- [7] A. Allerhand, F.M. Chen, H.S. Gutowsky, Spin-echo NMR studies of chemical exchange. 3. Conformational isomerization of cyclohexane and D_{11} -cyclohexane, J. Chem. Phys., 42 (1965) 3040.
- [8] G. Wagner, Characterization of the distribution of internal motions in the basic pancreatic trypsin inhibitor using a large number of internal NMR probes, Q. Rev. Biophys., 16 (1983) 1-57.

- [9] V. Kasinath, Y. Fu, K.A. Sharp, A.J. Wand, A sharp thermal transition of fast aromatic-ring dynamics in ubiquitin, *Angew. Chem. Int. Ed. Engl.*, 54 (2015) 102-107.
- [10] N.R. Nirmala, G. Wagner, Measurement of ^{13}C relaxation times in proteins by two-dimensional heteronuclear ^1H - ^{13}C correlation spectroscopy, *J. Am. Chem. Soc.*, 110 (1988) 7557-7558.
- [11] N.R. Nirmala, G. Wagner, Measurement of ^{13}C spin-spin relaxation times by two-dimensional heteronuclear ^1H - ^{13}C correlation spectroscopy, *J. Magn. Reson.*, 82 (1989) 659-661.
- [12] L.E. Kay, D.A. Torchia, A. Bax, Backbone dynamics of proteins as studied by ^{15}N inverse detected heteronuclear NMR spectroscopy: Applications to Staphylococcal nuclease, *Biochemistry*, 28 (1989) 8972-8979.
- [13] A.G. Palmer, NMR spectroscopy: NMR relaxation methods, in: H.J. Dyson (Ed.) *Biophysical Techniques for Structural Characterization of Macromolecules*, Academic Press, Oxford, 2012, pp. 216-244.
- [14] T.L. Religa, R. Sprangers, L.E. Kay, Dynamic regulation of archaeal proteasome gate opening as studied by TROSY NMR, *Science*, 328 (2010) 98-102.
- [15] A.H. Phillips, A.J. Schoeffler, T. Matsui, T.M. Weiss, J.W. Blankenship, K. Zobel, A.M. Giannetti, E.C. Dueber, W.J. Fairbrother, Internal motions prime cIAP1 for rapid activation, *Nat. Struct. Mol. Biol.*, 21 (2014) 1068-1074.
- [16] A.G. Palmer, Chemical exchange in biomacromolecules: past, present, and future, *J. Magn. Reson.*, 241 (2014) 3-17.

- [17] A.G. Palmer, Enzyme dynamics from NMR spectroscopy, *Acc. Chem. Res.*, 48 (2015) 457-465.
- [18] A.G. Palmer, M. Rance, P.E. Wright, 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.*, 113 (1991) 4371-4380.
- [19] A.G. Palmer, D.A. Case, Molecular dynamics analysis of NMR relaxation in a zinc-finger peptide, *J. Am. Chem. Soc.*, 114 (1992) 9059-9067.
- [20] A.G. Palmer, R.A. Hochstrasser, D.P. Millar, M. Rance, P.E. Wright, Characterization of amino acid side chain dynamics in a zinc-finger peptide using ^{13}C NMR spectroscopy and time-resolved fluorescence spectroscopy, *J. Am. Chem. Soc.*, 115 (1993) 6333-6345.
- [21] J. Kördel, N.J. Skelton, M. Akke, A.G. Palmer, W.J. Chazin, Backbone dynamics of calcium-loaded calbindin $\text{D}_{9\text{k}}$ studied by two-dimensional proton-detected NMR spectroscopy, *Biochemistry*, 31 (1992) 4856-4866.
- [22] M. Akke, J. Kördel, N.J. Skelton, A.G. Palmer, W.J. Chazin, Effects of ion binding on the backbone dynamics of calbindin $\text{D}_{9\text{k}}$ determined by ^{15}N relaxation., *Biochemistry*, 32 (1993) 9832-9844.
- [23] G. Lipari, A. Szabo, Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. I. Theory and range of validity, *J. Am. Chem. Soc.*, 104 (1982) 4546-4559.
- [24] G. Lipari, A. Szabo, Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 2. Analysis of experimental results, *J. Am. Chem. Soc.*, 104 (1982) 4559-4570.

- [25] M.S. Lee, A.G. Palmer, P.E. Wright, Relationship between ^1H and ^{13}C NMR chemical shifts and the secondary and tertiary structure of a zinc finger peptide, *J. Biomol. NMR*, 2 (1992) 307-322.
- [26] L. Mäler, J. Blankenship, M. Rance, W.J. Chazin, Site-site communication in the EF-hand Ca^{2+} -binding protein calbindin $\text{D}_{9\text{k}}$, *Nat. Struct. Biol.*, 7 (2000) 245-250.
- [27] A.M. Mandel, A.G. Palmer, Measurement of relaxation rate constants using constant-time accordion heteronuclear NMR spectroscopy, *J. Magn. Reson., Ser. A*, 110 (1994) 62-72.
- [28] P.A. Carr, D.A. Fearing, A.G. Palmer, 3D accordion spectroscopy for measuring ^{15}N and ^{13}CO relaxation rates in poorly-resolved NMR spectra, *J. Magn. Reson.*, 132 (1998) 25-33.
- [29] C.D. Kroenke, J.P. Loria, L.K. Lee, M. Rance, A.G. Palmer, Longitudinal and transverse ^1H - ^{15}N dipolar/ ^{15}N chemical shift anisotropy relaxation interference: unambiguous determination of rotational diffusion tensors and chemical exchange effects in biological macromolecules, *J. Am. Chem. Soc.*, 120 (1998) 7905-7915.
- [30] A.M. Mandel, M. Akke, A.G. Palmer, Backbone dynamics of *Escherichia coli* ribonuclease HI: Correlations with structure and function in an active enzyme, *J. Mol. Biol.*, 246 (1995) 144-163.
- [31] L.K. Lee, M. Rance, W.J. Chazin, A.G. Palmer, Rotational diffusion anisotropy of proteins from simultaneous analysis of ^{15}N and $^{13}\text{C}^\alpha$ nuclear spin relaxation, *J. Biomol. NMR*, 9 (1997).
- [32] M.L. Gill, A.G. Palmer, Local isotropic diffusion approximation for coupled internal and overall molecular motions in NMR spin relaxation, *J. Phys. Chem. B*, 118 (2014) 11120-11128.

- [33] M.L. Gill, A.G. Palmer, Correction to "Local isotropic diffusion approximation for coupled internal and overall molecular motions in NMR spin relaxation", *J. Phys. Chem. B*, 119 (2015) 3333.
- [34] M. Akke, R. Brüschweiler, A.G. Palmer, NMR order parameters and free energy: An analytic approach and application to cooperative Ca^{2+} binding by calbindin $\text{D}_{9\text{k}}$, *J. Am. Chem. Soc.*, 115 (1993) 9832-9833.
- [35] N. Trbovic, J.-H. Cho, R. Abel, R.A. Friesner, M. Rance, A.G. Palmer, Protein side-chain dynamics and residual conformational entropy, *J. Am. Chem. Soc.*, 131 (2009) 615-622.
- [36] L. Vugmeyster, O. Trott, C.J. McKnight, D.P. Raleigh, A.G. Palmer, Temperature-dependent dynamics of the villin headpiece helical subdomain, an unusually small thermostable protein, *J. Mol. Biol.*, 320 (2002) 841-854.
- [37] E. Johnson, A.G. Palmer, M. Rance, Temperature dependence of the NMR generalized order parameter, *Proteins*, 66 (2007) 796-803.
- [38] A.M. Mandel, M. Akke, A.G. Palmer, Dynamics of ribonuclease H: temperature dependence of motions on multiple time scales, *Biochemistry*, 35 (1996) 16009-16023.
- [39] R. Brüschweiler, X. Liao, W. P.E., Long-range motional restrictions in a multidomain zinc-finger protein from anisotropic tumbling, *Science*, 268 (1995) 886-889.
- [40] V. Wong, D.A. Case, A. Szabo, Influence of the coupling of interdomain and overall motions on NMR relaxation, *Proc. Natl. Acad. Sci. U. S. A.*, 106 (2009) 11016-11021.

- [41] S.L. Chang, A. Szabo, N. Tjandra, Temperature dependence of domain motions of calmodulin probed by NMR relaxation at multiple fields, *J. Am. Chem. Soc.*, 125 (2003) 11379-11384.
- [42] A. Ortega, D. Amoros, J. Garcia de la Torre, Prediction of hydrodynamic and other solution properties of rigid proteins from atomic- and residue-level models, *Biophys J.*, 101 (2011) 892-898.
- [43] J.P. Loria, M. Rance, A.G. Palmer, A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy, *J. Am. Chem. Soc.*, 121 (1999) 2331-2332.
- [44] O. Trott, A.G. Palmer, $R_{1\rho}$ relaxation outside of the fast-exchange limit, *J. Magn. Reson.*, 154 (2002) 157-160.
- [45] J.-H. Cho, N. O'Connell, D.P. Raleigh, A.G. Palmer, ϕ -value analysis for ultrafast folding proteins by NMR relaxation dispersion, *J. Am. Chem. Soc.*, 132 (2010) 450-451.
- [46] M.J. Grey, C.Y. Wang, A.G. Palmer, Disulfide bond isomerization in basic pancreatic trypsin inhibitor: Multisite chemical exchange quantified by CPMG relaxation dispersion and chemical shift modeling, *J. Am. Chem. Soc.*, 125 (2003) 14324-14335.
- [47] O. Trott, D. Abergel, A.G. Palmer, An average-magnetization analysis of $R_{1\rho}$ relaxation outside of the fast exchange, *Mol. Phys.*, 101 (2003) 753-763.
- [48] V.Z. Miloushev, A.G. Palmer, $R_{1\rho}$ relaxation for two-site chemical exchange: General approximations and some exact solutions, *J. Magn. Reson.*, 177 (2005) 221-227.

- [49] O. Trott, A.G. Palmer, Theoretical study of $R_{1\rho}$ rotating-frame and R_2 free-precession relaxation in the presence of n-site chemical exchange, *J. Magn. Reson.*, 170 (2004) 104-112.
- [50] A.J. Baldwin, L.E. Kay, An $R_{1\rho}$ expression for a spin in chemical exchange between two sites with unequal transverse relaxation rates, *J. Biomol. NMR*, 55 (2013) 211-218.
- [51] P. Vallurupalli, G. Bouvignies, L.E. Kay, Studying "invisible" excited protein states in slow exchange with a major state conformation, *J. Am. Chem. Soc.*, 134 (2012) 8148-8161.
- [52] N.L. Fawzi, J. Ying, D.A. Torchia, G.M. Clore, Probing exchange kinetics and atomic resolution dynamics in high-molecular-weight complexes using dark-state exchange saturation transfer NMR spectroscopy, *Nat. Protoc.*, 7 (2012) 1523-1533.
- [53] C. Bracken, P.A. Carr, J. Cavanagh, A.G. Palmer, Temperature dependence of intramolecular dynamics of the basic leucine zipper of GCN4: Implications for the entropy of association with DNA, *J. Mol. Biol.*, 285 (1999) 2133-2146.
- [54] M.L. Gill, R.A. Byrd, A.G. Palmer, Dynamics of GCN4 facilitate DNA interaction: A model-free analysis of an intrinsically disordered region, *Phys. Chem. Chem. Phys.*, in press (2016).
- [55] P. Robustelli, N. Trbovic, R.A. Friesner, A.G. Palmer, Conformational dynamics of the partially disordered yeast transcription factor GCN4, *J. Chem. Theory. Comput.*, 9 (2013).
- [56] G.M. Clore, A. Szabo, A. Bax, L.E. Kay, P.C. Driscoll, A.M. Gronenborn, Deviations from the simple two-parameter model-free approach to the interpretation of nitrogen-15 nuclear relaxation of proteins., *J. Am. Chem. Soc.*, 112 (1990) 4989-4991.

- [57] F. Massi, C. Wang, A.G. Palmer, Solution NMR and computer simulation studies of active site loop motion in triosephosphate isomerase, *Biochemistry*, 45 (2006) 10787-10794.
- [58] M. Akke, R. Brüschweiler, A.G. Palmer, NMR order parameters and free energy: An analytic approach and application to cooperative Ca²⁺ binding by calbindin D9k, *J. Am. Chem. Soc.*, 115 (1993) 9832-9833.
- [59] D.W. Yang, L.E. Kay, Contributions to conformational entropy arising from bond vector fluctuations measured from NMR-derived order parameters: Application to protein folding, *J. Mol. Biol.*, 263 (1996) 369-382.
- [60] Z. Li, S. Raychaudhuri, A.J. Wand, Insights into the local residual entropy of proteins provided by NMR relaxation, *Protein Sci.*, 5 (1996) 2647-2650.
- [61] K.A. Sharp, E. O'Brien, V. Kasinath, A.J. Wand, On the relationship between NMR-derived amide order parameters and protein backbone entropy changes, *Proteins*, 83 (2015) 922-930.
- [62] P. Robustelli, K.A. Stafford, A.G. Palmer, Interpreting protein structural dynamics from NMR chemical shifts, *J. Am. Chem. Soc.*, 134 (2012) 6365-6374.
- [63] Y. Shen, A. Bax, SPARTA+: a modest improvement in empirical NMR chemical shift prediction by means of an artificial neural network, *J Biomol NMR*, 48 (2010) 13-22.
- [64] T. Kiefhaber, A. Bachmann, K.S. Jensen, Dynamics and mechanisms of coupled protein folding and binding reactions, *Curr. Opin. Struct. Biol.*, 22 (2012) 21-29.
- [65] K.G. Daniels, N.K. Tonthat, D.R. McClure, Y.C. Chang, X. Liu, M.A. Schumacher, C.A. Fierke, S.C. Schmidler, T.G. Oas, Ligand concentration regulates the pathways of coupled protein folding and binding, *J. Am. Chem. Soc.*, 136 (2014) 822-825.

- [66] V.Z. Miloushev, F. Bahna, C. Ciatto, G. Ahlsen, B. Honig, L. Shapiro, A.G. Palmer, Dynamic properties of a type II cadherin adhesive domain: Implications for the mechanism of strand-swapping of classical cadherins, *Structure*, 16 (2008) 1195-1205.
- [67] M. Akke, R. Fiala, F. Jiang, D. Patel, A.G. Palmer, Base dynamics in a UUCG tetraloop RNA hairpin characterized by ^{15}N spin relaxation: Correlations with structure and stability, *RNA*, 3 (1997) 702-709.
- [68] H.M. Al-Hashimi, NMR studies of nucleic acid dynamics, *J. Magn. Reson.*, 237 (2013) 191-204.
- [69] Y. Xue, D. Kellogg, I.J. Kimsey, B. Sathyamoorthy, Z.W. Stein, M. McBairty, H.M. Al-Hashimi, Characterizing RNA excited states using NMR relaxation dispersion, *Methods Enzymol*, 558 (2015) 39-73.
- [70] A.G. Palmer, J. Williams, A. McDermott, Nuclear magnetic resonance studies of biopolymer dynamics, *J. Phys. Chem.*, 100 (1996) 13293-13310.
- [71] J.R. Lewandowski, Advances in solid-state relaxation methodology for probing site-specific protein dynamics, *Acc. Chem. Res.*, 46 (2013) 2018-2027.
- [72] J.R. Lewandowski, M.E. Halse, M. Blackledge, L. Emsley, Direct observation of hierarchical protein dynamics, *Science*, 348 (2015) 578-581.
- [73] D. Frishman, P. Argos, Knowledge-based protein secondary structure assignment, *Proteins*, 23 (1995) 566-579.

[74] C. Camilloni, A. De Simone, W.F. Vranken, M. Vendruscolo, Determination of secondary structure populations in disordered states of proteins using nuclear magnetic resonance chemical shifts, *Biochemistry*, 51 (2012) 2224-2231.

Figure Captions

Figure 1. Conformational flexibility of Xfin-31. (a) Structure of Xfin-31. The protein backbone is depicted in grey. The Zn^{2+} ion is shown as a red sphere; the Zn^{2+} ion is ligated by two Cys and two His residues. The Cys side chains are shown as yellow sticks and the His side chains as cyan sticks. (b) (●) Experimental and (○) simulated values of S^2 for the $^{13}\text{C}^\alpha$ - ^1H bond vector. Experimental uncertainties were determined by Monte-Carlo simulations. (c) Correlation between experimental and simulated order parameters. Reprinted with permission from *J. Am. Chem. Soc.*, **1992**, 114, 9059-9067. Copyright 1992 American Chemical Society.

Figure 2. Effects of metal-ion binding on flexibility of calbindin D_{9k} . Backbone ^{15}N order parameters for (a) (○) apo and (●) $(\text{Ca}^{2+})_2$ states of calbindin D_{9k} . The inset shows the secondary structure of calbindin D_{9k} , with the α -helices and two EF-hand binding sites labeled. Notably, the values of S^2 are reduced in apo compared to Ca^{2+} -loaded calbindin D_{9k} in the C-terminal EF-hand (site II, residues 55-62). Correlation between the values of S^2 for $(\text{Ca}^{2+})_2$ and (b) $(\text{Cd}^{2+})_1$ or (c) $(\text{Ca}^{2+})_1$ states. The values for residues in Site II are shown as open circles and are equally ordered in all three states (in contrast to the apo state). Reprinted with permission from *Biochemistry*, **1993**, 32, 9832-9844. Copyright 1993 American Chemical Society.

Figure 3. Relaxation arising from time-dependent diffusion tensors [32, 33]. (a) Schematic of dynamic parameters for protein in equilibrium between two states with different overall rotational diffusion tensors. The two conformations, denoted “A” and “B”, have equilibrium populations p_A and p_B , respectively. A representative amide bond is depicted in both conformations. The effective internal correlation time is τ_f and the square of the generalized order parameter is S_f^2 for fast intramolecular motions of the amide bond. The amide bond is shown superimposed on the enzyme diffusion axes (dotted), denoted $D_{xx,j}$, $D_{yy,j}$, and $D_{zz,j}$, where j indicates either state A or B. The rate constant for transitions from open to closed conformations

is k_1 , while the reverse rate constant is k_{-1} . (b) Structure of the rigid dumbbell structure from PDB file 1CLL. The N-terminal domain (residues 4–73) is green, and the C-terminal domain (residues 83–148) is reddish-purple. (c) ^{15}N spin relaxation data for calmodulin acquired at 800 MHz and 316 K. (black) Experimental R_2/R_1 ratios reported by Chang and co-workers [41]; (blue) R_2/R_1 ratios calculated for the rigid dumbbell structure; (green) R_2/R_1 ratios calculated for the N-terminal domain; (reddish-purple) R_2/R_1 ratios calculated for the C-terminal domain; and (orange) fitted model in the fast averaging limit between the rigid conformation and a conformation in which the central helix is disordered (consistent with the loss of data for residues in the central helix) and the N- and C-terminal domains reorient independently. The fitted model yields a population of the rigid dumbbell conformation of 0.67 in the fast-exchange limit.

Figure 4. Theoretical calculations of $R_{1\rho}$ relaxation rate constants using (black) exact numerical calculation from the Bloch-McConnell equations, (orange) the Laguerre approximation of Eq. 5, (reddish-purple), the expression in Eq. 6, and green) the expression derived by Baldwin and Kay (Eq. 5 in [50]). (a) The calculations were performed by varying $\omega_1/2\pi$ from 25 to 10,000 Hz with $p_b = 0.03$, $\Omega_A = 0 \text{ s}^{-1}$, $\Omega_B = 1000 \text{ s}^{-1}$, $k_{ex} = 1000 \text{ s}^{-1}$, $R_{1,A}^0 = R_{1,B}^0 = 2.5 \text{ s}^{-1}$, $R_{2,A}^0 = 6 \text{ s}^{-1}$, and $R_{2,B}^0 = 12 \text{ s}^{-1}$. (b) The calculations were performed as in (a) with the value of $R_{2,B}^0 = 200 \text{ s}^{-1}$. (c) The calculations were performed as in (a) except that $\omega_1/2\pi = 100 \text{ Hz}$ and k_{ex} was varied from 25 to 10,000 s^{-1} . (d) The calculations were performed as in (c) with the value of $R_{2,B}^0 = 200 \text{ s}^{-1}$.

Figure 5. Backbone flexibility of the apo bZip domain of GCN4 [33, 54]. (a) ^{15}N S^2 determined from relaxation measurements at four static magnetic fields from 600 to 900 MHz (^1H Larmor frequency) and $T = 300$. (b) Correlation between order parameters measured with a single magnetic field of 500 MHz and with four static magnetic fields at $T = 300$. (c) Temperature dependence of S^2 characterized as $\Lambda = d\{\ln(1 - S)\} / d\ln T$, using data obtained at a single

magnetic field of 500 MHz and $T = 290, 300,$ and 310 K. Values of Λ could not be reliably determined for the highly ordered coil-coil residues. Regions of the protein are colored as follows: (reddish-purple) region (i) of basic region (residues 3–12), (green) region (ii) of basic region (residues 13–25); (black) coiled-coil (residues 26–55); black, and (orange) disordered C-term (residues 56–58). The molecular model shown in (a) is width coded according to the reciprocals of S^2 . The dashed lines in (c) correspond to the mean $\pm$ one standard deviation for the values of Λ in region (ii).

Figure 6. Molecular dynamics simulations of the apo bZip domain of GCN4. (a) Fractional helicity for each residue of GCN4 bZip domain observed in a 100 ns MD trajectory. Helical conformations were assigned for each MD snapshot using the program STRIDE [73]. Helix populations predicted from the experimental chemical shifts using $\delta 2D$ [74] are shown as a dashed line. (b) Fractional helicity of each residue, as shown in (a) is mapped onto the crystal structure of the GCN4 bZip domain bound to DNA (PDB code 1YSA) according to the color bar shown. Side chains are displayed for residues that make contacts with the DNA in the x-ray crystal structure. (c) Comparison of (black) experimental and (orange) simulated order parameters (S^2). Reprinted with permission from *Chem. Theory Comput.*, **2013**, 9, 5190–5200. Copyright 2013 American Chemical Society.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6