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Applications of NMR Spin Relaxation Methods for Measuring Biological Motions

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Introduction

The activities of biological macromolecules are typically dependent on their conformational flexibility. A recent example is the activation by blue light of the oat plant phototropin, a multidomain protein involved in regulating phototropism, stomatal opening, chloroplast relocation, and leaf opening.¹ Activation of the phototropin involves a kinase domain and arises from light-induced conformational changes in α -helix J of the preceding domain that are propagated across the domains to switch the kinase from an inactive (dark) to an active (light) state. This example illustrates the importance of conformational flexibility to biological activity, which has clearly been appreciated for some time,^{2,3} however, little quantitative information on these dynamic processes is available at atomic resolution.

Nuclear magnetic resonance (NMR) spectroscopy is particularly well-suited to address this problem for two reasons. First, NMR experiments can provide atomic resolution of dynamic processes. The power to characterize dynamic processes of proteins at the atomic level by NMR spectroscopy was demonstrated in 1971 by Allerhand and co-workers.⁴ They estimated the differences in the rotational correlation times of bovine pancreatic ribonuclease A in the folded and unfolded states by measuring NMR spin relaxation of the naturally abundant ^{13}C signal. These studies illustrated the ability of NMR to characterize biomolecular dynamics, but were limited in their general utility because of the difficulty in obtaining biological samples at the high concentrations required for detection of the ^{13}C signal at natural abundance. Now, however, NMR spin relaxation is widely applicable to biomolecular dynamics because of the ability to incorporate stable, NMR-active isotopes such as ^{15}N and ^{13}C , either uniformly or selectively, into biomolecules.^{5–9} Thus, nearly every atom in a biological macromolecule could potentially serve as a probe for dynamic processes. Additionally, the NMR-active nuclei (^1H , ^{15}N , ^{13}C , ^{31}P) do not introduce perturbations to the structure or dynamics.¹⁰

The second reason that NMR is uniquely poised to provide quantitative information on dynamic processes is the wide range of time scales (from picoseconds to several hours) that is accessible by NMR methods. The application of NMR methods for quantitative descriptions of biomolecular dynamic processes that occur on the picosecond to nanosecond time scale is well known.^{10–18} The physical basis for these experiments is that molecular motions cause weak, fluctuating magnetic fields that result in magnetization decay (or recovery) commonly referred to as spin relaxation. In principle, by measuring the rate constants for spin relaxation (R_1 , R_2 , and cross-relaxation), the magnitude and frequency of molecular motions can be extracted.^{10–12,15,19} While such spin relaxation data analysis is by no means trivial, the application of these methods to both proteins and nucleic acids has become routine.^{13,17,18,20,21} These studies have identified that the backbone of proteins is fairly rigid as expected. Furthermore, in some cases, such experiments have led to the identification of flexible regions of biomolecules important for function. In HIV-1 protease, for example, two symmetric flaps of the enzyme have been shown to undergo motions on

the picosecond to nanosecond time scale that are thought to facilitate substrate binding and product release.^{22,23}

Additionally, NMR spin relaxation methods have revealed that picosecond to nanosecond time scale motions contribute to the thermodynamics of biomolecular folding and structure.^{24–33} For example, Akke *et al.*³⁴ developed an analytical expression that relates the changes in backbone mobility derived from NMR spin relaxation data to the Gibbs free energy of a process. The approach was applied to the cooperative Ca^{2+} binding to the protein calbindin $\text{D}_{9\text{k}}$ in which significant contributions to the free energy of cooperativity were demonstrated to arise from motions of the protein backbone on the nanosecond to picosecond time scale. Another important example was provided by Yang and Kay to the folding–unfolding transition of an SH3 protein domain. In this work, they demonstrated picosecond to nanosecond time scale changes in backbone motion correlate with changes in the conformational entropy of folding.³⁵

The NMR methods and applications directed at measuring such picosecond to nanosecond time scale motions have been the subject of a number of thorough reviews.^{10,21,36–38} While the majority of these studies have focused on protein backbone motions, NMR spin relaxation methods also provide the ability to measure protein side chain dynamics.^{16,39–44} Lee and Wand have used ^2H NMR spin relaxation experiments to probe the temperature-dependent dynamics of methyl-containing side chains of the Ca^{2+} -signalling protein calmodulin in association with a target peptide.^{45,46} They found three classes of motion that were suggestive of the heterogeneous glass-like nature of proteins proposed by Frauenfelder and coworkers.^{47–49} Furthermore, the temperature dependence of the methyl dynamics of the calmodulin–peptide complex revealed a heterogeneous distribution of motions that suggests local contributions to the heat capacity of binding. Subsequent molecular dynamics simulations have corroborated these results.⁵⁰ Thus, NMR coupled with molecular dynamics simulations is a powerful tool for exploring the energy landscape of fast biomolecular processes.^{11,50–53}

Many important biological processes such as allosteric transitions, enzyme catalysis, and protein folding occur on the microsecond to millisecond time scale.^{54,55} These so-called slower time scale motions have been known for at least 45 years to affect the observed chemical shift and line-shape of the NMR signal.⁵⁶ Theoretical and experimental approaches were subsequently developed to measure these processes in small molecules, including motions, that arise from chemical kinetics.^{57–64} More recently, developments in heteronuclear (^{13}C and ^{15}N) NMR techniques have led to experiments that have greatly extended the utility of the approaches in two regards. First, the newer methods are typically applied to biomolecules uniformly labeled with ^{15}N or ^{13}C providing dynamics data for each labeled atom. Second, these newer methods significantly extend the range of measurable time scales.^{65–68} For processes on the fast microsecond time scale ($\sim 10^3\text{--}10^6\text{ s}^{-1}$), the transverse relaxation rate will be affected and can be measured using dynamic lineshape analysis⁶⁹ or $R_{1\rho}$ experiments.⁷⁰ For example, Raleigh and co-workers recently reported the folding rate constant for the 36-residue villin headpiece subdomain to be $0.5\text{--}2.0 \times 10^5\text{ s}^{-1}$ from dynamic lineshape analysis.⁷¹ In another important application that laid the foundation for subsequent work, Davis *et al.* measured the dissociation rate constant for tubercidin, an inhibitor of the enzyme purine nucleoside phosphorylase from *Escherichia coli*, to be 2400 s^{-1} using $R_{1\rho}$ experiments.⁶²

Motions on the microsecond to millisecond time scale ($\sim 10^6\text{--}10^3\text{ s}^{-1}$) increase the transverse relaxation rate, R_2 . These motions can be detected indirectly during the spin relaxation data analysis using the model free approach introduced by Lipari and Szabo.^{72,73} In this manner, Volkman *et al.* measured backbone motions of the phosphorylation-driven

signaling protein NtrC in three functional states: the inactive unphosphorylated⁷⁴ state, the phosphorylated active state, and a mutant NtrC that is in a partially active state.⁷⁵ The comparison of the backbone motions for these states led to the conclusion that microsecond to millisecond time scale motions correlated with activation by phosphorylation. This work is particularly significant in the demonstration that a single-domain signaling protein, NtrC, is capable of undergoing allosteric activation by a population-shift mechanism and not an induced-fit mechanism.

More recently, Loria and co-workers have shown a correlation between catalysis and backbone motion for ribonuclease A (RNase A) by directly measuring the contributions of microsecond to millisecond time scale motions to the transverse relaxation rates using Carr–Purcell–Meiboom–Gill (CPMG)-based experiments.⁷⁶ They found that the backbone motion was identical for all residues (k_{ex} 1640 s⁻¹) and was similar to the k_{cat} value for RNase A (1900 s⁻¹). Temperature-dependent CPMG-based experiments allowed extraction of activation energy for microsecond to millisecond motion and was found to be similar to the activation barrier for enzyme catalysis. For processes on the high millisecond to second time scale (0.1–10 s⁻¹ for ¹⁵N), ZZ-exchange spectroscopy can measure the affect of these motions on the transverse relaxation rate constant, R_2 . Farrow *et al.* used ¹⁵N ZZ-exchange experiments to measure the folding and unfolding rate constants for the drk SH3 domain.⁷⁷ These results clearly illustrate the utility of NMR-based methods in linking conformational flexibility to biological function.

This chapter presents data analysis considerations for these new methods that are directed at measuring conformational flexibility or chemical kinetics on the microsecond to millisecond time scale. While the data discussed in this review are primarily from CPMG-based experiments, the principles presented are also applicable to data collected by $R_{1\rho}$ relaxation and ZZ-exchange experiments, even though these latter cases are treated with a slightly different formalism. Several excellent reviews have recently appeared that detail theoretical and experimental aspects of all of these methods, in addition to recent applications.^{38,78–81} While this chapter draws from examples in the literature applied to proteins, the principles presented are also applicable to other biomolecules.⁸² The discussion is aimed at the biologist or biochemist without formal NMR training, however, our goal is that specialists will also benefit from the data analysis considerations.

Conformational Exchange (Flexibility) Gives Rise to NMR Chemical Shift Exchange

Even though biological macromolecules access a continuum of conformational states, many biological processes are known to behave in a two-state manner where the macromolecule predominantly populates two distinct conformational states. Examples of such two-state processes include enzyme allostery (active and inactive states), ligand binding (bound and free states), and the folding of many small proteins (folded and unfolded states). This chapter will consider only cases of interconversion between two conformational states, however, formalisms for treatment of cases that adopt multiple states have been developed and are a generalization of the limited two-state case discussed herein.^{57,83}

Interconversion of an atom or molecule between two distinct conformational states involves molecular motion that almost always results in that atom or molecule experiencing two magnetically distinct environments. The consequence is a dramatic impact on the intensity, line width, and chemical shift of the NMR signal. To aid the discussion, consider the case in which the macromolecule does not undergo exchange on the microsecond to millisecond time scale. For this case, a single NMR signal is observed with an intensity that is proportional to the population. The line width of the NMR signal is proportional to R_2 , the transverse relaxation rate constant, which is a measure of the lifetime of the signal in the so-

called transverse plane (orthogonal to the static magnetic field, B_0). The chemical shift of the NMR signal is governed by the distinct chemical environment experienced by a nucleus.

When a nucleus experiences the presence of conformational flexibility on the microsecond to millisecond time scale, all three of these observables are affected (Fig. 1). Specifically, each state gives rise to a distinct NMR chemical shift. The difference in these chemical shifts is $\Delta\omega$ (in rad s^{-1}), and reports on the difference in chemical environment of each state. Thus, the interconversion between two states, or conformational exchange, gives rise to what is formally known as NMR chemical shift exchange, which is namely the exchange between two distinct NMR chemical shifts. Note that chemical shift exchange could also refer to a chemical kinetic process, such as hydrogen–deuterium exchange,^{84,85} but is not considered herein.

Conformational exchange almost always gives rise to NMR chemical shift exchange and affects the NMR signal. The rate constant that governs the chemical shift exchange process is k_{ex} , and is related to the conformational exchange rate constants depending on the type of motion involved as detailed in Eqs. (1–3). NMR chemical shift exchange causes phase decoherence of the NMR signal in the transverse plane resulting in an increase in the transverse relaxation rate constant, R_2 , relative to the R_2 in the absence of exchange (called R_2^0). The effect is reflected in the lineshape but also impacts the observed chemical shift (Fig. 1).

The observed NMR signal, therefore, depends upon the transverse relaxation rate in the absence of chemical shift exchange, R_2^0 , the chemical shift difference between the two states, $\Delta\omega$, the rate constant for chemical shift exchange, k_{ex} , and the populations of the two states, p_A and p_B . Consider the case in which two conformational states are equally populated. In the event that chemical shift exchange is much slower than the difference in chemical shift (slow chemical shift exchange limit, $k_{\text{ex}} \ll \Delta\omega$), one observes two peaks in the NMR spectrum, at the chemical shift of each state. In the event that chemical shift exchange is much faster than the difference in chemical shift (fast chemical shift exchange limit, $k_{\text{ex}} \gg \Delta\omega$), one observes a single peak in the NMR spectrum at the average chemical shift of the two states. The so-called intermediate chemical shift exchange limit is defined as when $k_{\text{ex}} \approx \Delta\omega$, and results in a single broad peak at the average of their chemical shifts. The time scale for NMR chemical shift exchange, therefore, is defined in part by the difference between the chemical shifts. Thus, a biological process that undergoes conformational exchange described by a single rate constant for the entire macromolecule actually could give rise to individual residues that undergo either slow, intermediate, or fast chemical shift exchange, because the NMR chemical shift time scale is governed not only by the k_{ex} of the overall process but also the chemical shift differences of those residues ($\Delta\omega$).

For many biological processes of interest, the populations of each state will be highly skewed. Consider these examples where $k_{\text{ex}} = k'_1 + k_{-1}$ and k'_1 is the pseudo-first-order reaction rate constant:

Protein folding or allosteric case:

Ligand or macromolecular binding case:

Homooligomerization case:

In each case, typically one species will strongly predominate at a protein concentration suitable for NMR spectroscopy. Therefore, the effect of skewed populations is also illustrated in Fig. 1. By contrast to the case in which each state is equally populated, when conformational exchange gives rise to populations that are skewed the presence and time scale of exchange are difficult to detect. Thus, the observation of a single resonance in the NMR spectrum does not always indicate the presence of fast chemical shift exchange⁶⁸; a single resonance is also consistent with two other situations: (1) slow exchange with highly skewed populations, and (2) no exchange whatsoever. We discuss below a strategy to differentiate these situations.

In summary, conformational exchange gives rise to changes in three observables in the NMR signal. These three observables depend upon six parameters as dictated by Eq. (4),^{57,59,62} the complexity of which prevents one from readily identifying the relative contributions of each parameter to the NMR signal. One goal of this chapter is to demonstrate how these parameters affect the observed NMR signal.

$$R_2(1/\tau_{cp}) = \frac{1}{2} \left(R_a + R_b + k_{ex} - \frac{1}{\tau_{cp}} \cosh^{-1} [D_+ \cosh(\eta_+) - D_- \cos(\eta_-)] \right) \quad (4)$$

where $D_{\pm}$ and $\eta_{\pm}$ both functions of p_A and p_B , R_{2A}^0 , R_{2B}^0 , $\Delta\omega$ and k_{ex} as depicted elsewhere.^{59,62}

Is Exchange Present?—How then might the investigator determine the presence and time scale of exchange for such cases with skewed populations? The answer lies in determining R_{ex} , the contribution to the observed transverse relaxation rate constant (R_2) that arises from chemical shift exchange:

$$R_2(\tau_{cp}) = R_2^0 + R_{ex} \quad (5)$$

where R_2^0 is the transverse relaxation rate constant in the absence of exchange and $R_2(\tau_{cp})$ is written in this fashion to underscore the τ_{cp} dependence on R_2 as discussed in the following. Methods for indirectly determining R_{ex} are well known and include lineshape analysis⁸⁶ and model free analysis of spin relaxation data.⁸⁷ Here the discussion focuses on a class of recently developed NMR experiments that allow a more direct measurement of the R_{ex} contribution to $R_2(\tau_{cp})$.

Perhaps the most experimentally accessible methods for $R_2(\tau_{cp})$ determination are spin echo-based methods—both Carr–Purcell spin echoes with the Meiboom–Gill enhancement (CPMG) and Hahn echoes.^{65,77,81,88–90} If a nucleus undergoes a chemical shift exchange

event during the spin echo delay (the τ_{cp} delay), the chemical shift exchange leads to phase decoherence that is reflected in an increase in $R_2(\tau_{cp})$ (Fig. 2). The more chemical shift exchange events during the τ_{cp} delay, the greater the contribution to $R_2(\tau_{cp})$. Therefore, if chemical shift exchange is present, then longer values of τ_{cp} will result in an increase of $R_2(\tau_{cp})$ when compared to $R_2(\tau_{cp})$ measured with shorter values of τ_{cp} . If chemical shift exchange is absent, then the $R_2(\tau_{cp})$ measured has no dependence on the length of τ_{cp} delay. In summary, one can determine if R_{ex} is contributing to $R_2(\tau_{cp})$ by comparing the values of $R_2(\tau_{cp})$ derived from the shortest and longest possible values of τ_{cp} [$\Delta R_2(\tau_{cp}) > 0$].⁷⁸

The experiment is run in a manner similar to measuring R_2 in that a single τ_{cp} delay is selected and the total duration of the CPMG period is then varied. The resulting data are processed and the volume of the NMR signal is plotted as a function of total duration of the CPMG period. The data are fit using a nonlinear least-squares routine to a single exponential and the transverse relaxation rate constant, R_2 , is obtained for that value of τ_{cp} from the exponential fit of the data.⁹¹ A second value of τ_{cp} is then selected and data are once again collected as a function of the duration of the CPMG period. From these data, R_2 is obtained for the second value of τ_{cp} . Any difference in the values of $R_2(\tau_{cp})$ at the different τ_{cp} values indicates the presence of exchange [$\Delta R_2(\tau_{cp}) > 0$].^{78,92}

What is the Time Scale of Exchange?—Once the presence of exchange is confirmed the question of the time scale of chemical shift exchange arises. As noted earlier, residues involved in biological processes such as loop opening that are governed by identical kinetic rate constants can give rise to chemical shift exchange on different time scales because the NMR chemical shift time scale is defined not only by the rate constant, k_{ex} , but also by the chemical shift difference, $\Delta\omega$. Analysis of the relaxation dispersion data by curve-fitting to Eq. (4) can be used, in principle, to determine whether chemical shift exchange is slow or fast on the NMR chemical shift time scale.⁹³ However, as illustrated by Palmer and co-workers for the protein bovine pancreatic trypsin inhibitor, relaxation dispersion data at one static magnetic field strength do not always accurately define the NMR chemical shift time scale for exchange.⁹³ In their analysis, R_{ex} data collected at one static magnetic field strength demonstrated that residues Cys-38 and Ala-40 fit well to a limiting form of Eq. (4) that is valid only for fast exchange. However, these residues were actually determined to be undergoing slow chemical shift exchange when data collected at two static magnetic field strengths were analyzed. This observation illustrates that relaxation dispersion data collected at a single static magnetic field strength might not distinguish between slow and fast time scales.^{62,93}

To define the NMR chemical shift time scale, therefore, one must collect relaxation dispersion data at different static magnetic field strengths. To rigorously determine the NMR chemical shift time scale requires the determination of the scaling parameter α , which reflects the R_{ex} dependence upon the static magnetic field strength⁹³:

$$\alpha = d \ln \Delta R_{ex} / d \ln \Delta \omega \quad (6)$$

The evaluation of α requires a wide range of τ_{cp} values in order to fully define the relaxation dispersion curve at a minimum of two different static magnetic field strengths, a procedure that consumes considerable instrument time. Fortunately, a reasonable approximation to the scaling parameter α is to measure relaxation dispersion using the very longest value of τ_{cp} at two static magnetic field strengths, and the very shortest value of τ_{cp} at only the lower strength of the two fields.⁷⁸ For ^{15}N , typical long and short values for τ_{cp} for this purpose are 10 and 1 ms, respectively. From these measurements, one can calculate α' :

$$\alpha' = d \ln \Delta R_2(\text{long} \tau_{\text{cp}}, \text{short} \tau_{\text{cp}}) / d \ln \Delta \omega \quad (7)$$

$$\approx \{(B_0^{\text{hi}} + B_0^{\text{lo}}) / (B_0^{\text{hi}} - B_0^{\text{lo}}) \bullet (\Delta R_2^{\text{hi}} - \Delta R_2^{\text{lo}}) / (\Delta R_2^{\text{hi}} + \Delta R_2^{\text{lo}})\} \quad (8)$$

where

$$\begin{aligned} \Delta R_2^{\text{hi}} &= [R_2^{\text{hi}}(\text{long} \tau_{\text{cp}}) - R_2^{\text{lo}}(\text{short} \tau_{\text{cp}})] \\ \Delta R_2^{\text{lo}} &= [R_2^{\text{lo}}(\text{long} \tau_{\text{cp}}) - R_2^{\text{hi}}(\text{short} \tau_{\text{cp}})] \end{aligned}$$

As discussed by Millet *et al.*⁹³ a value of parameter $\alpha' < 1$ always indicates slow exchange, whereas a value of $\alpha' > 1$ could arise from either slow or fast exchange.

Data Analysis

The goal of this section is to discuss important considerations for data analysis. While significant issues surround data collection, two excellent reviews have recently covered this important topic.^{78,81} The application of the CPMG-based techniques to study chemical exchange of a process can be done in a systematic way, and is delineated into a series of steps as detailed in the following.

- Step 1** Simulation of exchange—Computer simulation of the relaxation dispersion curve for the process under study.
- Step 2** Confirmation of exchange—Initial set of NMR experiments at two τ_{cp} values to determine the presence of exchange in the microsecond to millisecond time scale.
- Step 3** Time scale of exchange—Determine whether the chemical shift exchange is slow or fast by determining the scaling parameter α as discussed previously.
- Step 4** k_{ex} determination—Determine the exchange rate constant by fitting to the appropriate expression for slow [Eq. (4)] or fast [Eq. (9)] exchange.

Step 1: Simulation of Exchange—The first step for an investigator interested in quantifying conformational exchange is to study the feasibility of the CPMG-based approach for their system. In general, an equilibrium process of exchange between two biological states in the microsecond to millisecond time scale could be studied using the CPMG-based methods. However, before using the CPMG-based methods to determine the chemical shift exchange-based parameters, it is helpful to simulate the predicted relaxation dispersion-based equations for the phenomenological rate constants [Eq. (4)]. Because Eq. (4) depends on k_{ex} , p_A , $\Delta \omega$ and R_2^0 , a priori knowledge of an approximate value for these parameters is necessary to simulate the relaxation dispersion curve. In many cases, such knowledge is known or reasonable estimates can be inferred. For example, k_{ex} can be obtained from global kinetic studies of the exchange process, p_A from equilibrium studies, $\Delta \omega$ from NMR correlation experiments of the two biological states, and R_2^0 can be estimated based on the molecular weight of the system. The simulations will aid the investigator in determining the feasibility of a CPMG-based approach. In addition, the simulations provide a guideline for choosing optimal τ_{cp} values necessary to define the relaxation dispersion curve.

Chymotrypsin Simulation Example: The following example of catalysis of *N*-acetyl tyrosine ethyl ester by chymotrypsin is illustrative of the simulation process. One needs reasonable estimates of k_{ex} , $\Delta\omega$, p_A , and, R_2^0 . For k_{ex} , we assume that k_{cat} is the rate-limiting step and the step of interest. A value of $k_{\text{cat}} = 190 \text{ s}^{-1}$ has been reported. The value of R_2^0 used in the simulation does not have to be very accurate as it does not define the overall shape of the curve but does define the offset from the x -axis (Fig. 3). Thus, R_2^0 can be assumed to be about 5 s^{-1} for proteins under 10 kDa and about 10 s^{-1} for larger ones around 25–30 kDa. If a slightly more rigorous estimate of R_2^0 is desired, it can be obtained using the Stokes–Einstein relation and the relationship that governs how the transverse relaxation rate varies with the τ_c for an isotropically rotating spherical macromolecule (the spectral density functions).⁹⁴ For example, the molecular weight of chymotrypsin is 25.1 kDa giving a rotational correlation time of 8.9 ns assuming a spherical shape and a 3 Å shell of water at 298 K. Using this value and assuming typical values for ^{15}N -labeled proteins including common backbone flexibility ($S^2 \approx 0.85$), then the R_2^0 is calculated to be 8.3 s^{-1} at 600 MHz. To determine the expected population (of the ES complex) at NMR concentrations, we estimate a value of 0.1 given the K_M of $6.6 \times 10^{-4} \text{ M}$ reported for chymotrypsin.⁹⁵ For the simulation a value of $\Delta\omega$ is also needed and could be obtained readily from an HSQC correlation spectrum of chymotrypsin in the free form as well as bound to substrate. If the value of $\Delta\omega$ is not known a priori, the simulation could still be performed with the typical values for $\Delta\omega$ (~ 1 to 10 ppm for ^{15}N chemical exchange). Using these values in Eq. (4), the relaxation dispersion curve is then simulated (Fig. 3). Both the curves show appreciable relaxation dispersion with a value of ΔR_2 of $\sim 5 \text{ s}^{-1}$ (for longest and shortest τ_{cp} values). The folding of the small protein chymotrypsin inhibitor C12 is also simulated using data obtained from the literature⁹⁶ (Fig. 4). In this simulation, the sensitivity of the CPMG-based methods to small populations is demonstrated by varying p_A between extreme values (99%, 90%, and 50% folded) at $\Delta\omega$ values of 1 and 10 ppm. Thus, the folding rates for this protein could be determined by CPMG-based methods if the unfolded state were populated.

Step 2: Confirmation of Exchange—If exchange occurs in the appropriate time scale for a CPMG-based approach, then the next step would be to experimentally confirm the presence of chemical shift exchange by measuring $R_2(\tau_{\text{cp}})$ at two extrema for τ_{cp} , short and long.^{38,78,93} $\Delta R_2(\tau_{\text{cp}})$ is calculated as the difference in $R_2(\tau_{\text{cp}})$ at the two τ_{cp} values and plotted against residue number. The presence of significant nonzero values indicates the presence of chemical shift exchange on the time scale that could be studied using CPMG-based experiments.

Step 3: Time Scale of Exchange—How does one then proceed to determine the NMR chemical shift time scale? In practice, the investigator should measure α' as described previously [Eqs. (7)–(8)]. If $\alpha' < 1$ then the slow time scale regime is unambiguously determined, and analysis of the system requires full relaxation dispersion data only at a single static magnetic field strength. If $\alpha' > 1$, the chemical shift time scale for exchange is still ambiguous. The investigator must collect full relaxation dispersion data at a minimum of two static magnetic field strengths to differentiate between slow and fast exchange.⁷⁸

Step 4: k_{ex} Determination—For quantitative determination of chemical exchange, the transverse relaxation rate constant, $R_2(\tau_{\text{cp}})$, must be determined at enough values of τ_{cp} to fully define the relaxation dispersion curve. The appropriate number of τ_{cp} values to measure depends upon the chemical shift exchange time scale, which dictates the shape of the dispersion curve (e.g., Fig. 4). A minimum of six to eight values (not including duplicate measurements for error analysis) is necessary, but more values of τ_{cp} will increase the accuracy of the k_{ex} determination.

For each value of τ_{cp} , one needs to determine the transverse relaxation rate constant, $R_2(\tau_{cp})$. This determination is straightforward: The duration of the CPMG period (at a single value of τ_{cp}) is varied with time and the resulting crosspeak volumes fit to an exponential decay with a rate constant defined as R_2 using a nonlinear least-squares routine.^{97,98} The uncertainties in the rate constants should be determined as discussed by Mandel *et al.*⁹⁹ The number of time points necessary to fully define the exponential decay of transverse signal is an important consideration that has been discussed elsewhere.^{78,81,98,99} An important aspect of this consideration is to collect more data at the shorter and longer time points as these data are more useful in restraining the fit. In fact, Jones recently has proposed that only two time points, short and long extrema, are required, although the application of this approach to CPMG-based data has not been reported.¹⁰⁰

Once values of R_2 have been determined at different τ_{cp} delays, these values are plotted as a function of $1/\tau_{cp}$ and the resulting curve is fit to Eq. (4) [or a limiting case of Eq. (4)]. For slow chemical shift exchange ($\alpha' < 1$), values of $R_2(\tau_{cp})$ from a single static magnetic field strength should be fit to Eq. (4). If $\alpha' > 1$, then the chemical shift time scale is ambiguous and multiple field data are necessary to define the time scale for exchange. The multiple field data can then be globally fit to Eq. (4). If the resulting analysis indicates that the fast exchange ($k_{ex} > \Delta\omega$), then significant improvements in the uncertainty of the data analysis can be achieved by fitting to the fast-limit form derived by Luz and Meiboom that has one less fitting variable than Eq. (4)⁶⁰:

$$R_2(1/\tau_{cp}) = R_2(1/\tau_{cp} \rightarrow \infty) + R_{ex} \left[1 - \frac{2 \tanh\left(\frac{k_{ex}\tau_{cp}}{2}\right)}{(k_{ex}\tau_{cp})} \right] \quad (9)$$

For the curve fitting, two shareware programs have been developed that are well documented. The software program CPMGFit developed by Palmer interfaces UNIX-based shell scripts with the XMGR program for curve fitting and plotting.¹⁰¹ CPMGFit features the ability to fit R_{ex} data to Eq. (4) and various limiting forms. CPMGFit also provides several options for estimating the uncertainties in the fitted parameters including covariance matrix analysis, Monte Carlo simulations, and jackknife simulations.¹⁰² The second shareware option for data analysis is the software NMRView,¹⁰³ which contains an R_{ex} data analysis module that has been contributed by Mueller and others.¹⁰⁴

Sensitivity Analysis for Eq. (4)

How does R_{ex} depend upon the populations of states (p_A), the transverse rate constant in the absence of exchange (R_2^0), the exchange rate constant (k_{ex}), and the chemical shift difference ($\Delta\omega$)? The sensitivity of each of these four variables of Eq. (4) is shown by simulations of experimental relaxation dispersion data collected for the $^{13}\text{C}_\alpha$ of Leu-6 of the *de novo* designed protein $\alpha_2\text{D}$.^{105, 106} This protein is a dimeric four-helix bundle that has all the hallmarks of a native-like protein.^{107–110} $\alpha_2\text{D}$ undergoes conformational exchange between an unfolded monomer and a folded dimer species.¹⁰⁸ Thus the chemical shift exchange contributions to the NMR resonances of $\alpha_2\text{D}$ arise from its folding; the rate constants of which were determined from global analysis of relaxation dispersion data collected at $B_0 = 11.7\text{ T}$ and 14.1 T .¹⁰⁵ In the simulations presented here, three of the four parameters described in Eq. (4) are held constant at the value reported from the global analysis of $\alpha_2\text{D}$. The fourth parameter is also held constant at different variables to illustrate how sensitive the data are to this parameter.

Not surprisingly, the simulations indicate that the experimental data are very sensitive to the transverse relaxation rate constant in the absence of exchange, R_2^0 (Fig. 5). As expected, the dependence of $R_2(\tau_{cp})$ on the τ_{cp} delay is independent of R_2^0 as illustrated by the invariant shape of the curves at different values of R_2^0 . Based on this analysis, the uncertainty in determining the transverse relaxation rate constant in the absence of exchange is less than 5%. Jackknife simulations performed on the α_2D data indicated the uncertainty at 2.3%.¹⁰⁵

A small change in the population of the minor species dramatically affects the relaxation dispersion (Fig. 6). In the case for α_2D , detectable contributions to R_{ex} are possible even with chemical shift exchange to an unfolded state that is less than 1% populated. At the smaller values of τ_{cp}^{-1} (longer τ_{cp} delays), the population differences have a more pronounced effect as expected because more exchange events are occurring during the longer delay period. This pattern is also observed for the other two variables (k_{ex} and $\Delta\omega$) as expected.

The change in chemical shifts between the exchanging states ($\Delta\omega$) also strongly affects the fits to the experimental data as expected (Fig. 7). Note that the change in chemical shifts is inextricably linked to the populations of the exchanging sites as illustrated in Fig. 1 and governed by Eq. (4). Thus, R_{ex} measurements at only one static magnetic field strength will not allow one to determine both the populations and the change in chemical shifts. However, these parameters can be determined if R_{ex} measurements are made at more than one magnetic field strength, which constrains the value for $\Delta\omega$. In contrast, simulations are less sensitive to k_{ex} (Fig. 8). For measurements at only one static magnetic field strength, k_{ex} can be determined within ~25–30% accuracy. However, the accuracy can be significantly improved by increasing the number of data points collected or global analysis of data collected at multiple static magnetic field strengths.

The previous examples illustrate the exquisite dependence of the relaxation dispersion on R_2^0 , pM , k_{ex} , and $\Delta\omega$. If a priori knowledge of R_2^0 , pM , and $\Delta\omega$ is possible, then these parameters can be fixed in the fitting procedure and tightly restrain values for the rate constant k_{ex} . Is such knowledge possible? Indeed it is for the cases considered here. For example, in ligand binding the R_2^0 in the absence of exchange is readily determined from NMR spin relaxation experiments of the unliganded macromolecule. In the case of protein allostery this might not be possible, however, experiments with enhanced sensitivity to chemical exchange allow the explicit determination of R_2^0 .⁶⁷ The populations of the species (p_A) is typically available from other biophysical measurements such as fluorescence binding assays in the case of ligand binding and chemical or thermal denaturation in the case of protein folding. The change in chemical shift between the exchanging states ($\Delta\omega$) can sometimes be determined independently in many systems.

How important is a priori knowledge of Eq. (4) variables in obtaining reasonable fits to experimental R_{ex} data? While knowledge of the transverse relaxation rate constant in the absence of exchange, R_2^0 , can improve the fits; the differences in the fits when R_2^0 is fixed at its known value or when it is allowed to float are similar if a fully relaxed dispersion curve is possible to obtain experimentally. However, for some systems this is not the case and measurable dispersion is still evident even at the shortest values of τ_{cp} (long τ_{cp}^{-1}). For these cases, a priori knowledge of R_2^0 will significantly reduce the uncertainty in the fits to experimental data.

Limitations of CPMG-Based Methods

The time scale of exchange events that are measurable by CPMG-based methods ranges from 100 s^{-1} to 3000 s^{-1} depending on whether $\Delta\omega$ is sufficiently large. For these cases, fitting Eq. (4) to experimental data to obtain all four parameters can result in large uncertainties for k_{ex} , $\Delta\omega$, and p_A because of the multiple combinations of these parameters that still gives reasonable fits to the data. Furthermore, at a single magnetic field strength it is impossible to separate the populations of the exchanging species from their chemical shift differences. However, several strategies allow the investigator to overcome these obstacles.

First, independent determination of R_2^0 , $\Delta\omega$, and p_A is often possible. In the cases in which such data are not available, then global analysis of data collected at multiple magnetic field strengths can be used to improve the accuracy of the fits. Finally, the exchange equilibrium can be manipulated in some manner (e.g., temperature, mutagenesis, inhibitors, or substrate analogues) to affect the change of some of the fitting parameters without affecting others. Subsequent global analysis of all data can yield acceptable fits to the data.

Conclusion

In the past few years, the tools for measuring microsecond to millisecond time scale motions by NMR spectroscopy have been developed. These tools combine the great strengths of NMR: the abilities to probe molecular motions in a site-specific manner and to study dynamic processes. In the future, the wide application of these methods to important biological problems will have a huge impact. The traditional notion of biological structure will be replaced with a more realistic view of fluctuating conformational substates, some of which are biologically active. The judicious application of the methods discussed herein, coupled with biochemical, biophysical, and molecular dynamics simulations, will be essential for identifying the active substates from the inactive ones. These studies will ultimately reveal how conformational flexibility is related to biological activity.

References

1. Harper SM, Neil LC, Gardner KH. *Science*. 2003; 301:1541. [PubMed: 12970567]
2. Linderstrom-Land KU, Schellman JA. *Enzyme*. 1959; 1:443.
3. Wuthrich K, Wagner G. *Experientia*. 1975; 31:726.
4. Allerhand A, Doddrell D, Glushko V, Cochran DW, Wenkert E, Lawson PJ, Gurd FRN. *J Am Chem Soc*. 1971; 93:544. [PubMed: 5541522]
5. Marley J, Lu M, Bracken C. *J Biomol NMR*. 2001; 20:71. [PubMed: 11430757]
6. Lee AL, Urbauer JL, Wand AJ. *J Biomol NMR*. 1997; 9:437. [PubMed: 9255947]
7. Rance M, Wright PE, Messerle BA, Field LD. *J Am Chem Soc*. 1987; 109:1591.
8. Goto NK, Kay LE. *Curr Opin Struct Biol*. 2000; 10:585. [PubMed: 11042458]
9. Lemaster DM. *Prog NMR Spectrosc*. 1994; 26:371.
10. Kay LE. *Biochem Cell Biol Biochim Biol Cell*. 1998; 76:145.
11. Palmer AG. *Annu Rev Biophys Biomol Struct*. 2001; 30:129. [PubMed: 11340055]
12. Dayie KT, Wagner G, Lefevre JF. *Annu Rev Phys Chem*. 1996; 47:243. [PubMed: 8930100]
13. Peng JW, Wagner G. *Methods Enzymol*. 1994; 239:563. [PubMed: 7830599]
14. LeMaster DM, Kushlan DM. *J Am Chem Soc*. 1996; 118:9255.
15. Idiyatullin D, Daragan VA, Mayo KH. *J Magn Reson*. 2003; 161:118. [PubMed: 12660119]
16. Tjandra N, Szabo A, Bax A. *J Am Chem Soc*. 1996; 118:6986.
17. Mayer KL, Earley MR, Gupta S, Pichumani K, Regan L, Stone MJ. *Nat Struct Biol*. 2003; 5:5.
18. Mayer KL, Stone MJ. *Proteins*. 2003; 50:184. [PubMed: 12486712]
19. London RE. *Methods Enzymol*. 1989; 176:358. [PubMed: 2811693]
20. Palmer AG. *Curr Opin Struct Biol*. 1997; 7:732. [PubMed: 9345634]

21. Ishima R, Torchia DA. *Nat Struct Biol.* 2000; 7:740. [PubMed: 10966641]
22. Nicholson LK, Yamazaki T, Torchia DA, Grzesiek S, Bax A, Stahl SJ, Kaufman JD, Wingfield PT, Lam PYS, Jadhav PK, Hodge CN, Domaille PJ, Chang CH. *Nat Struct Biol.* 1995; 2:274. [PubMed: 7796263]
23. Yamazaki T, Hinck AP, Wang YX, Nicholson LK, Torchia DA, Wingfield P, Stahl SJ, Kaufman JD, Chang CH, Domaille PJ, Lam PY. *Protein Sci.* 1996; 5:495. [PubMed: 8868486]
24. Spyropoulos L, Sykes BD. *Curr Opin Struct Biol.* 2001; 11:555. [PubMed: 11785755]
25. Mulder FA, Mittermaier A, Hon B, Dahlquist FW, Kay LE. *Nat Struct Biol.* 2001; 8:932. [PubMed: 11685237]
26. Yang DW, Shortle D, Muhandiram DR, Forman-Kay JD, Kay LE. *Biophys J.* 1998; 74:A137.
27. Kay LE, Muhandiram DR, Wolf G, Shoelson SE, Forman-Kay JD. *Nat Struct Biol.* 1998; 5:156. [PubMed: 9461082]
28. Gardner KH, Kay LE. *Annu Rev Biophys Biomol Struct.* 1998; 27:357. [PubMed: 9646872]
29. Bracken C, Carr PA, Cavanagh J, Palmer AG. *J Mol Biol.* 1999; 285:2133. [PubMed: 9925790]
30. Stone MJ. *Acc Chem Res.* 2001; 34:379. [PubMed: 11352716]
31. Stone MJ, Gupta S, Snyder N, Regan L. *J Am Chem Soc.* 2001; 123:185. [PubMed: 11273620]
32. Seewald MJ, Pichumani K, Stowell C, Tibbals BV, Regan L, Stone MJ. *Protein Sci.* 2000; 9:1177. [PubMed: 10892810]
33. Zidek L, Novotny MV, Stone MJ. *Nat Struct Biol.* 1999; 6:1118. [PubMed: 10581552]
34. Akke M, Bruschweiler R, Palmer AG. *J Am Chem Soc.* 1993; 115:9832.
35. Yang DW, Kay LE. *J Mol Biol.* 1996; 263:369. [PubMed: 8913313]
36. Daragan VA, Mayo KH. *Prog NMR Spectrosc.* 1997; 31:63.
37. Fischer MWF, Majumdar A, Zuiderweg ERP. *Prog NMR Spectrosc.* 1998; 33:207.
38. Kempf JG, Loria JP. *Cell Biochem Biophys.* 2003; 37:187. [PubMed: 12625627]
39. Nicholson LK, Kay LE, Baldisseri DM, Arango J, Young PE, Bax A, Torchia DA. *Biochemistry.* 1992; 31:5253. [PubMed: 1606149]
40. Nicholson LK, Kay LE, Delaglio F, Bax A, Torchia DA. *Biophys J.* 1993; 64:A182.
41. Wand AJ, Urbauer JL, McEvoy RP, Bieber RJ. *Biochemistry.* 1996; 35:6116. [PubMed: 8634254]
42. LeMaster DM. *J Am Chem Soc.* 1999; 121:1726.
43. Flynn PF, Bieber RJ, Urbauer JL, Zhang H, Lee AL, Wand AJ. *Biochemistry.* 2001; 40:6559. [PubMed: 11380250]
44. Lee KH, Xie D, Freire E, Amzel LM. *Proteins.* 1994; 20:68. [PubMed: 7824524]
45. Lee AL, Kinnear SA, Wand AJ. *Nat Struct Biol.* 2000; 7:72. [PubMed: 10625431]
46. Lee AL, Wand AJ. *Nature.* 2001; 411:501. [PubMed: 11373686]
47. Frauenfelder H, Petsko GA, Tsernoglou D. *Nature.* 1979; 280:558. [PubMed: 460437]
48. Frauenfelder H, Parak F, Young RD. *Annu Rev Biophys Biophys Chem.* 1988; 17:451. [PubMed: 3293595]
49. Frauenfelder H, Sligar SG, Wolynes PG. *Science.* 1991; 254:1598. [PubMed: 1749933]
50. Prabhu NV, Lee AL, Wand AJ, Sharp KA. *Biochemistry.* 2003; 42:562. [PubMed: 12525185]
51. Tsui V, Radhakrishnan I, Wright PE, Case DA. *J Mol Biol.* 2000; 302:1101. [PubMed: 11183777]
52. Daragan VA, Mayo KH. *Biochemistry.* 1993; 32:11488. [PubMed: 8218215]
53. Smith PE, Vanschaik RC, Szyperski T, Wuthrich K, Vangunsteren WF. *J Mol Biol.* 1995; 246:356. [PubMed: 7532721]
54. Kumar S, Ma B, Tsai CJ, Wolfson H, Nussinov R. *Cell Biochem Biophys.* 1999; 31:141. [PubMed: 10593256]
55. Daggett V, Fersht A. *Nat Rev Mol Cell Biol.* 2003; 4:497. [PubMed: 12778129]
56. McConnell HM. *J Chem Phys.* 1958; 28:430.
57. Jen J. *J Magn Reson.* 1978; 30:111.
58. Gerig JT, Stock AD. *Org Magn Reson.* 1975; 7:249.
59. Carver JP, Richards RE. *J Magn Reson.* 1972; 6:89.

60. Luz Z, Meiboom S. J Chem Phys. 1963; 39:366.
61. Swift TJ, Connick RE. J Chem Phys. 1962; 37:307.
62. Davis DG, Perlman ME, London RE. J Magn Reson B. 1994; 104:266. [PubMed: 8069484]
63. Allerhand A, Gutowsky HS. J Chem Phys. 1965; 42:1587. [PubMed: 14291612]
64. Allerhand A, Gutowsky HS. J Chem Phys. 1964; 41:2115.
65. Loria JP, Rance M, Palmer AG. J Am Chem Soc. 1999; 121:2331.
66. Ishima R, Torchia DA. J Biomol NMR. 2003; 25:243. [PubMed: 12652136]
67. Wang CY, Grey MJ, Palmer AG. J Biomol NMR. 2001; 21:361. [PubMed: 11824755]
68. Ishima R, Torchia DA. J Biomol NMR. 1999; 14:369. [PubMed: 10526408]
69. Huang GS, Oas TG. Proc Natl Acad Sci USA. 1995; 92:6878. [PubMed: 7624336]
70. Peng JW, Wagner G. Biochemistry. 1992; 31:8571. [PubMed: 1390643]
71. Wang M, Tang Y, Sato S, Vugmeyster L, McKnight CJ, Raleigh DP. J Am Chem Soc. 2003; 125:6032. [PubMed: 12785814]
72. Lipari G, Szabo A. J Am Chem Soc. 1982; 104:4559.
73. Lipari G, Szabo A. J Am Chem Soc. 1982; 104:4546.
74. Clore GM, Szabo A, Bax A, Kay LE, Driscoll PC, Gronenborn AM. J Am Chem Soc. 1990; 112:4989.
75. Volkman BF, Lipson D, Wemmer DE, Kern D. Science. 2001; 291:2429. [PubMed: 11264542]
76. Cole R, Loria JP. Biochemistry. 2002; 41:6072. [PubMed: 11994002]
77. Farrow NA, Zhang OW, Formankay JD, Kay LE. J Biomol NMR. 1994; 4:727. [PubMed: 7919956]
78. Palmer AG, Kroenke CD, Loria JP. Methods Enzymol. 2001; 339:204. [PubMed: 11462813]
79. Desvaux H, Berthault P. Prog NMR Spectrosc. 1999; 35:295.
80. Daragan VA, Mayo KH. Prog NMR Spectrosc. 1997; 31:63.
81. Kempf, JG.; Loria, JP. Meth Mol Biol: Protein NMR Techniques. Vol. 278. Humana Press; Totowa, NJ: 2004.
82. Schurr JM, Fujimoto BS, Diaz R, Robinson BH. J Magn Reson. 1999; 140:404. [PubMed: 10497047]
83. Allerhand A, Thiele E. J Chem Phys. 1966; 45:902.
84. Bai YW, Englander JJ, Mayne L, Milne JS, Englander SW. Methods Enzymol. 1995; 259:344. [PubMed: 8538461]
85. Hernandez G, LeMaster DM. Magn Reson Chem. 2003; 41:699.
86. Sandstrom, J. Dynamic NMR Spectroscopy. Academic Press; London: 1982.
87. Cole R, Loria JP. J Biomol NMR. 2003; 26:203. [PubMed: 12766418]
88. Mulder FAA, Skrynnikov NR, Hon B, Dahlquist FW, Kay LE. J Am Chem Soc. 2001; 123:967. [PubMed: 11456632]
89. Mulder FA, Hon B, Muhandiram DR, Dahlquist FW, Kay LE. Biochemistry. 2000; 39:12614. [PubMed: 11027141]
90. Skrynnikov NR, Mulder FAA, Hon B, Dahlquist FW, Kay LE. J Am Chem Soc. 2001; 123:4556. [PubMed: 11457242]
91. Mandel AM, Akke M, Palmer AG. J Mol Biol. 1995; 246:144. [PubMed: 7531772]
92. Palmer AG, Akke M, Mandel AM. Abstr Pap Am Chem Soc. 1997; 214:165-PHYS.
93. Millet O, Loria JP, Kroenke CD, Pons M, Palmer AG. J Am Chem Soc. 2000; 122:2867.
94. Cavanagh, J.; Fairbrother, WJ.; Palmer, AG.; Skelton, NJ. Protein NMR Spectroscopy: Principles and Practice. Academic Press; San Diego: 1996.
95. Voet, D.; Voet, JG. Biochemistry. John Wiley & Sons; New York: 1995.
96. Killick TR, Freund SM, Fersht AR. FEBS Lett. 1998; 423:110. [PubMed: 9506851]
97. Press, WH.; Flannery, BP.; Teukolsky, SA.; Vetterling, WT. Numerical Recipes in Fortran. Cambridge University Press; Cambridge: 1992.

98. Viles JH, Duggan BM, Zaborowski E, Schwarzingier S, Huntley JJA, Kroon GJA, Dyson HJ, Wright PE. *J Biomol NMR*. 2001; 21:1. [PubMed: 11693564]
99. Mandel AM, Akke M, Palmer AG. *J Mol Biol*. 1995; 246:144. [PubMed: 7531772]
100. Jones JA. *J Magn Reson*. 1997; 126:283.
101. Palmer, AG, III.
<http://cpmcnet.columbia.edu/dept/gsas/biochem/labs/palmer/software/cpmgfit.html>
102. Mosteller, F.; Tukey, JW. *Data Analysis and Regression A Second Course in Statistics*. Pearson Education; Essex: 1977.
103. Johnson BA, Blevins RA. *J Biomol NMR*. 1994; 4:603.
104. <http://dir.niehs.nih.gov/dirnmr/scripts/>
105. Hill RB, Bracken C, DeGrado WF, Palmer AG. *J Am Chem Soc*. 2000; 122:11610.
106. Hill RB, Raleigh DP, Lombardi A, DeGrado WF. *Acc Chem Res*. 2000; 33:745. [PubMed: 11087311]
107. Raleigh DP, Betz SF, Degrado WF. *J Am Chem Soc*. 1995; 117:7558.
108. Hill RB, Degrado WF. *J Am Chem Soc*. 1998; 120:1138.
109. Hill RB, Degrado WF. *Struct Fold Des*. 2000; 8:471.
110. Hill RB, Hong JK, Degrado WF. *J Am Chem Soc*. 2000; 122:746.

Fig. 1.

Conformational exchange between two states gives rise to NMR chemical shift exchange. The left panel depicts chemical shift exchange for equal populations at various values of the exchange rate constant, k_{ex} . The right panel depicts skewed populations $p_A = 0.1, p_B = 0.9$. The spectra were simulated $R_{2A}^0 = R_{2B}^0 = 5 \text{ s}^{-1}$ and $\Delta\omega = 314 \text{ rad s}^{-1}$ using Eq. (2) from Huang and Oas.⁶⁹

Fig. 2.

Exchange during the τ_{cp} delay increases the observed $R_2(\tau_{cp})$. An increase in the τ_{cp} delay (A–C) increases the number of exchange events that occur between each CPMG 180° radiofrequency pulses, thereby leading to more phase decoherence and increased R_2 relaxation (faster decay of intensity versus time). For each value of τ_{cp} , a single value of $R_2(\tau_{cp})$ is determined and then plotted as a function of τ_{cp}^{-1} . The presence of chemical shift exchange results in a dispersion in the plot that is not observed in the absence of exchange. This relaxation dispersion curve (D) can be fit using Eq. (4) (or variations thereof) to determine R_2^0 , p_A , k_{ex} , and $\Delta\omega$.

Fig. 3.

The catalysis of *N*-acetyl tyrosine ethyl ester by chymotrypsin will give rise to relaxation dispersion. Simulation of Eq. (4) using expected parameters for chymotrypsin as discussed in the text. Note the sensitivity of the simulations to $\Delta\omega$.

Fig. 4. The folding of chymotrypsin inhibitor 2 will give rise to relaxation dispersion. Simulation of Eq. (4) using data obtained from the literature.⁹⁶ Note the sensitivity of the simulations to the population of the minor species.

Fig. 5.

Sensitivity of R_2^0 to fitting relaxation dispersion data at 11.7 T for $^{13}\text{C}_\alpha$ of Leu-6 of $\alpha_2\text{D}$. The solid line depicts the best fit to the data from a global analysis of R_{ex} data collected at 11.7 and 14.1 T.¹⁰⁵ The other lines depict simulations using an R_2^0 value held constant at 50, 75, 125, and 150% of the best fit value. Values of $p_M = 0.038$, $\Delta\omega = 838.38 \text{ rad s}^{-1}$, and $k_{\text{ex}} = 510 \text{ s}^{-1}$ were also held constant for each simulation.

Fig. 6. Sensitivity of p_M to fitting relaxation dispersion data at 11.7 T for $^{13}\text{C}_\alpha$ of Leu-6 of $\alpha_2\text{D}$. The solid line depicts the best fit to the data from a global analysis of R_{ex} data collected at 11.7 and 14.1 T.¹⁰⁵ The other lines depict simulations using a p_M value held constant at 50, 75, 125, and 150% of the best fit value. Values of $R_2^0 = 22.6 \text{ s}^{-1}$, $\Delta\omega = 838.38 \text{ rad s}^{-1}$, and $k_{\text{ex}} = 510 \text{ s}^{-1}$ were also held constant for each simulation.

Fig. 7.

Sensitivity of $\Delta\omega$ to fitting relaxation dispersion data at 11.7 T for $^{13}\text{C}_\alpha$ of Leu-6 of $\alpha_2\text{D}$. The solid line depicts the best fit to the data from a global analysis of R_{ex} data collected at 11.7 and 14.1 T.¹⁰⁵ The other lines depict simulations using a $\Delta\omega$ value held constant at 50, 75, 125, and 150% of the best fit value. Values of $R_2^0=22.6 \text{ s}^{-1}$, $p_A=0.038$, and $k_{\text{ex}}=510 \text{ s}^{-1}$ were also held constant for each simulation.

Fig. 8.

Sensitivity of k_{ex} to fitting relaxation dispersion data at 11.7 T for $^{13}\text{C}_\alpha$ of Leu-6 of $\alpha_2\text{D}$. The solid line depicts the best fit to the data from a global analysis of R_{ex} data collected at 11.7 and 14.1 T.¹⁰⁵ The other lines depict simulations using a k_{ex} value held constant at 50, 75, 125, and 150% of the best fit value. Values of $R_2^0=22.6 \text{ s}^{-1}$, $p_A = 0.038$, and $\Delta\omega = 838.38 \text{ rad s}^{-1}$ were also held constant for each simulation.