

review

Protein dynamics from NMR

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This review surveys recent investigations of conformational fluctuations of proteins in solution using NMR techniques. Advances in experimental methods have provided more accurate means of characterizing fast and slow internal motions as well as overall diffusion. The information obtained from NMR dynamics experiments provides insights into specific structural changes or configurational energetics associated with function. A variety of applications illustrate that studies of protein dynamics provide insights into protein–protein interactions, target recognition, ligand binding, and enzyme function.

Proteins are dynamic molecules that often undergo conformational changes while performing their specific functions, such as an enzyme reaction or ligand binding. The dynamic properties intrinsic to a protein structure may provide information on the location and the energetics of the conformational change process, and are thus the focus of many biophysical studies. Nuclear magnetic resonance (NMR) spectroscopy can be used to monitor the dynamic behavior of a protein at a multitude of specific sites. Moreover, protein movements on a broad range of timescales can be monitored using various types of NMR experiments — nuclear spin relaxation rate measurements report the internal motions on fast (subnanoseconds) and slow (microseconds to milliseconds) timescales as well as the overall rotational diffusion of the molecule (5–50 nanoseconds), whereas rates of magnetization transfer among protons with different chemical shifts and proton exchange report movements of protein domains on the very slow timescales (milliseconds to days). These features make NMR a unique and powerful tool in studying protein dynamics related to protein functions, and there has been a tremendous growth in these applications since the review by Lewis Kay¹ in *Nature Structural Biology* in 1998. In parallel with new applications, there have been important developments in experimental methods that significantly increased the accuracy of the information obtained, and thus expanded the range of questions that can be addressed. In the first section, we will focus in detail on the methods used to analyze conformational changes with NMR spectroscopy. The remainder of the review will provide examples of the usefulness of this technology.

Methodological advances

Fast protein dynamics. Information about site-specific internal dynamics on the subnanosecond timescale derives primarily from model-free analyses^{2,3} (Fig. 1). From such analyses, one obtains a set of parameters for each site (for example, the backbone amides) monitored, the most informative of which is the order parameter (S^2). The analysis is termed model-free because the parameters are derived without the need to invoke a specific model for internal motion. The order parameter measures the magnitude of the angular fluctuation of a chemical bond vector such as the NH bond in a protein, and thus reflects the flexibility of the polypeptide chain at these sites. The magnitude of this parameter depends on interactions responsible for relaxing nuclear spins in proteins, including (i) magnetic dipole–dipole (ii) chemical shift anisotropy (CSA) and (iii) electric quadrupole (from nuclei such as deuterium with spin quantum number $I \geq$

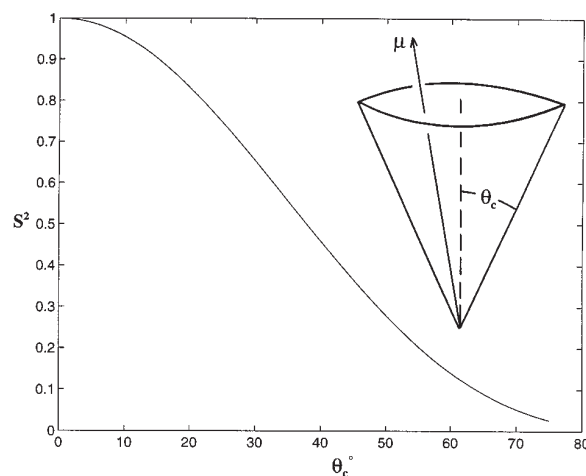


Fig 1 Relationship between internal motion and model-free parameters. The three commonly measured NMR relaxation rates, the spin-lattice relaxation rate, $R_1 = 1/T_1$, the spin-spin relaxation rate, $R_2 = 1/T_2$ and the heteronuclear NOE are all sensitive to internal motions on the subnanosecond timescale. A model-free analysis of these rates provides order parameters (S^2) and correlation times (τ_c) characterizing the subnanosecond internal motions of bond vectors (N–H, C–H, C–C) at numerous sites in the protein sequence. S^2 provides information about the angular amplitude of the internal motion. For example, if the bond vector μ diffuses in a cone as depicted in the inset, S^2 decreases rapidly as the cone angle θ_c increase from 0 to 75°, and remains small for 75° < θ_c < 180°. In general, $S^2 = 1$ when the bond is rigid, and $S^2 = 0$ when internal motion is isotropic.

1) interactions. To obtain quantitative information related to protein functions, such as the amplitude of structural variation or the configurational entropy of the polypeptide chain, it is necessary to determine the order parameter as accurately as possible. Thus, there has been great interest in characterizing these interactions more precisely.

In most NMR studies, backbone dynamics is obtained from the amide ^{15}N - ^1H order parameters, S_{NH}^2 . S_{NH}^2 is very sensitive to the distance between the nitrogen and hydrogen atoms (r_{NH}), and to the ^{15}N chemical shift anisotropy ($\Delta\sigma_{\text{N}}$) used in a model-free analysis. Measurements of ^1H - ^{15}N dipolar couplings and ^{15}N chemical shifts in solids^{4,5}, together with a quantum calculation⁶ show that a consistent set of values — either ($r_{\text{NH}} = 1.02 \text{ \AA}$, $\Delta\sigma_{\text{N}} = -172 \text{ p.p.m.}$)^{7–11} or ($r_{\text{NH}} = 1.04 \text{ m \AA}$, $\Delta\sigma_{\text{N}} = -160 \text{ p.p.m.}$, to remove the contribution of zero-point motions⁶) — should be used to

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extract S_N^2 . Furthermore, related studies indicate that site variations in $\Delta\sigma_N$ are in the 10–20 p.p.m. range^{8,9,11,12}, which may affect the calculated S_N^2 values. Measurements of peptide carbonyl carbon (C') relaxation rates complement those of backbone amides^{13,14}. At high fields ($B_0 \geq 12$ Tesla), CSA is the dominant C' relaxation mechanism, and recent measurements in ubiquitin weakly aligned relative to B_0 ¹¹ show site variations in $\Delta\sigma_C$ of ± 10 p.p.m., similar to those observed in solids. Because S^2 is sensitive to changes in $\Delta\sigma$, small variations in S^2 derived at high fields should be interpreted cautiously until mechanisms affecting $\Delta\sigma$ are better understood. Evidently this caution does not apply to S^2 values for side chains extracted from ^2H methyl relaxation data, because the ^2H methyl quadrupole couplings derived from spectra of CH_2D sites in an aligned SH3 domain¹⁵ are highly uniform and thus are not expected to affect the calculated S^2 values. Recently, a strategy has been devised¹⁶ to obtain S_N^2 values that are independent of variations in $\Delta\sigma_N$.

Determination of the rotational diffusion tensor of a protein provides a means to verify its structure and to determine more accurate values of the model-free parameters. The rotational diffusion tensor is specified by three principal components — the diffusion constants for rotation about the X,Y,Z principal axes — and by the orientation of the principal axes relative to that of the protein. The principal axes, X,Y,Z, are fixed in the protein, and are defined as the coordinate axes in which the diffusion tensor assumes a simple, diagonal form. Both the principal components and orientation of the diffusion tensor in a protein can be reliably derived from an analysis of the ratio of the spin-spin relaxation rate (R_2) and the spin-lattice relaxation rate (R_1)^{2,17–21}. Ideally, only R_2/R_1 from rigid NH sites should be included in the analysis, because this ratio is affected by conformational exchange (discussed in the next section). This restriction can be overcome by using R_2^i/R_1^i , where the superscript indicates that relaxation interference between ^1H – ^{15}N dipolar and ^{15}N CSA interactions is measured. Because R_2^i/R_1^i is independent of conformational exchange, measurement of these quantities greatly increases the number of NH sites that can be reliably used to determine the diffusion tensor components²².

Progress has been made in measuring anisotropic internal motions in proteins by measuring both ^{15}N and ^{13}C relaxation rates^{13,14}. For ubiquitin¹⁴, the motions of the peptide planes have been derived by fitting experimental data to relaxation equations from the three-dimensional Gaussian axial fluctuation model. This model describes physically reasonable anisotropic internal motions of well-ordered portions of the protein backbone and analytical expressions for order parameters. The anisotropies of peptide plane motions derived using this analysis agreed with those predicted by a 1.5 ns molecular dynamics (MD) simulation¹⁴. This study did not utilize measurements of relaxation interference between dipolar and CSA interactions. Inclusion of relaxation interference measurements can provide additional information about the details of internal motion^{23–27}. As the size of the experimental data set increases, Bayesian statistical approaches^{28,29} offer advantages in computational speed over Monte Carlo methods in extracting the values and uncertainties of model-free parameters.

Slow protein dynamics. Conformational changes alter the environment of a nuclear spin and modulate its chemical shift. When this process is on the microsecond to millisecond timescale and the chemical shift changes are large, the NMR signal broadens, leading to an increase in R_2 (ref. 22). This process is most reliably identified by measuring R_2 as a function of the effective radiofre-

quency (ERF) field strength using spin-lock and Carr-Purcell-Meiboom-Gill (CPMG) methods. Recently, it has been shown that deuteration of nonlabile sites allows measurement of R_2 for both amide ^1H and ^{15}N spins at ERF fields 10-fold lower than was previously possible³⁰. Furthermore, an elegant alternative approach³¹ uses a relaxation-compensated CPMG sequence to measure ^{15}N R_2 at low ERF fields. Finally, by combining relaxation compensation and TROSY selection on deuterated protein molecules³², it is possible to measure R_2 of a protein as large as 54 kDa. Thus, CPMG experiments provide R_2 data at low ERF fields whereas spin-lock experiments provide these data at high ERF fields^{33,34}. The R_2 data from such a wide range of ERF fields are useful for determining the timescale of conformational exchange in proteins³⁴, as has been shown for the binding domain of the retinoid receptor³³. Alternatively it has been suggested³⁴ and recently demonstrated³⁵ that measurements of the dependence of R_2 on B_0 is a robust method for determining the time-scale of protein conformational exchange.

Partial deuteration of methyl sites combined with ^{13}C enrichment³⁶ facilitates observation of conformational exchange at protein side chains. Measurement of ^{13}C R_2 values of labeled methyl groups ($^{13}\text{CHD}_2$) in an otherwise highly deuterated protein³⁷ is particularly sensitive for detecting conformational exchange at methyl sites. Methyl $^{13}\text{C}(\text{HD}_2)$ and ^{15}N R_2 measurements are thus well-suited for detecting the presence of rare but biologically significant conformations within a few kcal of the protein native (ground) state.

Target recognition, flexibility and entropy

Proteins have many important functions such as signaling, genome regulation and catalysis; these functions often involve binding to target molecules, including other proteins, nucleic acids or a wide variety of polymeric or small molecule substrates. There is an impressive body of evidence indicating that the target binding sites of many proteins are flexible. NMR relaxation measurements are very useful in identifying which residues in a binding site are flexible, in particular in proteins that bind to nucleic acids^{38–41} and to other proteins^{42–44}. Significantly, these measurements are useful even when a high resolution X-ray structure is available, because crystal contacts may quench local motions. In some instances flexibility at these sites may stabilize the unbound state (see below), while in others it may be necessary for functions. For example, crystal structures of the HIV-inhibitor complexes revealed that the active site is covered by a lid. To perform its function, which is cleaving the viral *gag-pol* polypeptides, the lid must be sufficiently flexible to allow substrate entry and product exit.

Local flexibility within a protein often changes upon target binding. This observation implies that the binding process involves a change of configurational entropy. There is currently great interest in using order parameters to estimate the contribution of configurational entropy changes to the energetics of target binding^{45,46}. Although the detailed relationship between S^2 and configurational entropy (S_p) is complex⁴⁵, formulas relating S^2 to S_p have been derived for simple but plausible physical models of bond reorientation^{47–49}. Applications using these models have indicated that changes in configurational entropy make significant contributions to the free energy of protein binding to hydrophobic inhibitor⁵⁰, peptide⁵¹ and DNA⁵² targets. In work related to protein folding, MD calculations have shown that about a quarter of the total conformational entropy associated with the folding of staphylococcal nuclease is reflected in changes in NH order parameters⁵³. Moreover, the feasibility of obtaining experimental estimates of the contribution of side

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chains to folding entropy (from a nearly complete set of order parameters) has been examined in the case of thioredoxin⁵⁴. Given that it is now possible to obtain order parameters at many backbone and side chain sites in a protein, it seems appropriate that more complete quantitative comparisons of configurational entropy contributions obtained from NMR, calorimetric and molecular dynamics methods should be made in protein systems undergoing conformational transformations. Such studies would reveal the extent to which analyses of order parameters can provide quantitative information about energetics of target binding and protein folding.

Changes in entropy associated with the binding of water molecules to a protein can contribute to free energy in important processes such as protein folding or ligand binding. The rotational entropy differences between bulk water molecules and those bound to the Y35G mutant of pancreatic trypsin inhibitor (BPTI) have been derived from a careful analysis of the B_0 field dependence (relaxation dispersion) of water ^{17}O , ^2H and ^1H relaxation rates⁵⁵. The data showed that, unexpectedly, the average rotational entropy of three ordered bound water molecules, each making 3–4 hydrogen bonds to the protein, was the same as that of free bulk water molecules. This observation suggests that the release of all but the most extensively hydrogen bonded water molecules in a protein may be entropically unfavorable. If this observation can be generalized by further experiments on other systems, the conventional view that ligand binding to proteins is entropically favored by release of bound water molecules will have to be modified.

Conformational exchange and protein function

Protein local stability and redox potentials are related to motions on the microsecond to millisecond timescale. For example, the Y35G mutation of BPTI was found to significantly increase the flexibility of a (Cys 14, Cys 38)-reduced mutant protein compared with wild type. This observation explains the destabilization of the 14–38 disulfide in the mutant protein relative to wild type, and shows how a mutation at one site that affects protein flexibility can also influence the stability of a nearby interaction⁵⁶. Studies of oxidized and reduced cytochrome b_5 isomers also revealed that differences in reduction potentials of this protein correlated with differences in backbone dynamics on the microsecond to millisecond timescale⁵⁷.

Beyond mapping the flexibility of residues in known protein binding sites as discussed above, NMR techniques can identify novel binding sites in proteins. Identification of protein–protein interaction sites is crucial for understanding the basis of molecular recognition. Because residues at the binding interface in the bound state often experience a different environment from that in the free state, the amide NMR signals from the interface residues would be broadened due to exchange between these two environments when the free and bound states are in equilibrium. These residues can thus be identified by relatively simple NMR experiments. This approach has been successfully applied to identify the amino acids at the binding site of a 16 kDa protein that binds to and regulates the 251 kDa hydroxylase of the methane monooxygenase protein system⁵⁸. The free and bound forms of the regulatory protein exchange on the millisecond timescale. In a separate application, specific sites involved in the weak self-association of the N-terminal domain of the rat T-cell adhesion protein CD2 (CD2d1) were identified by the concentration dependence of their R_2 values⁵⁹. This study provided the first evidence for CD2d1 self-association in solution. The sites in the CD2d1 dimer interface are also involved in protein contacts in the

crystal structure of intact CD2, and are thought to be involved in the functional interaction of CD2 with its adhesion partner.

Various cellular targets, such as myosin light chain kinases and calcineurin, are regulated by Ca^{2+} -dependent conformational changes of calmodulin (CaM). Metal ion binding modulates CaM activity and shifts the equilibrium among the open and closed conformational substates of this protein. The rate of conformational exchange between these states has been investigated by NMR dynamics experiments on the C-terminal domain of the E140Q mutant of CaM. The mutant protein displays an interesting phenotype — even in the calcium saturated state, it is in a dynamic equilibrium among the open and closed forms. The NMR experiments show that rate constant for exchange between the open and closed states is $\sim 2.5 \times 10^{-4} \text{ s}^{-1}$ (ref. 60). To separate the effects of Ca^{2+} exchange from those of conformational exchange, additional experiments measuring R_2 as a function of Ca^{2+} concentration in the C-terminal domain of wild type CaM were performed⁶¹. These studies revealed a Ca^{2+} -independent conformational exchange in the apo protein involving an equilibrium between the closed and the open states. Furthermore, a Ca^{2+} -dependent exchange was also observed between the apo and the $(\text{Ca}^{2+})_1$ -bound states (where the subscript indicates that a single Ca^{2+} ion is bound). Upon binding the first Ca^{2+} ion, the C-terminal domain switches from the mostly closed state (in the apo protein) to the mostly open state (in the $(\text{Ca}^{2+})_1$ protein).

The dynamic properties of the HIV-1 protease on the microsecond to millisecond timescales provide insights into the function of this enzyme. Inhibitor binding to the protease enhanced the motions on the millisecond timescale of the β -sheet interface⁶², a region that stabilizes the dimeric structure of the protease. Notably, this region is flanked by the reverse transcriptase (RT) in the *gag-pol* polyprotein. The flexibility of the β -sheet interface may enhance the protease–RT autoprocessing reaction by making the protease–RT junction accessible to cleavage. The flaps covering the active site of the free protease were found to be flexible on the 100 μs timescale. This observation was interpreted as a dynamic equilibrium between major (semi-open) and minor (open) flap conformations; the open conformation could allow substrate access to the active site. In support of this interpretation, the intensities of exchange crosspeaks in NOESY spectra of the inhibited protease indicated that the rate of flap opening was $\sim 10^5$ times slower than in the free protease⁶³.

Dynamics from amide proton exchange

Measurements of amide proton exchange rates in proteins can provide useful information about global stability and the dynamics of local structural fluctuations. Exchange between amide protons in proteins and the bulk solvent can be measured in a variety of ways. Slow exchange lifetimes (from minutes to days) are determined by following the loss of amide proton signal intensity of a protein dissolved in D_2O , and provide information about relative solvent accessibility of various components of protein secondary and tertiary structure⁶⁴. Faster exchange lifetimes (5–500 ms) can be monitored by following the exchange of amide proton magnetization with that of water protons. At high pH the base-catalyzed proton exchange reaction is very fast ($\sim 10^5 \text{ s}^{-1}$ at pH 10), and these magnetization exchange measurements directly measure the timescale of rate limiting protein conformational openings that permit solvent access to the exchanging amide sites. Such measurements have been performed on a highly thermostable protein, rubredoxin from *Pyrococcus furiosus*⁶⁵. Remarkably, at 28 °C solvent access to all amide sites in the protein occurs on the millisecond timescale.

Furthermore, solvent exchange protection factors for all sites are similar to those observed for mesophilic homologs. This surprising observation suggests that the flexibility of thermophilic rubredoxin is similar to that of the mesophilic homologs, and brings into question the general hypothesis that the high stability of a thermophilic protein comes from the rigidity of its native state structure⁶⁵. This observation is further supported by recent work that indicates that the flexibility of a thermophilic protein in the subnanosecond timescale is similar to that of its mesophilic homologs⁶⁶. Clearly additional measurements on a wide range of timescales are required to compare the dynamics of mesophilic and thermophilic proteins; this comparison would provide insights into the relationship between the rigidity of protein structure and its stability.

Concluding remarks

This brief survey discusses current NMR technology that is used to monitor the dynamic properties of proteins. Understanding protein dynamics provides insights into the functions of a variety of important protein systems. With the increasing availability of 750–800 MHz instruments, the development of 900–1000 MHz spectrometers and the implementation of cold probe technology (in which thermal noise is reduced by cooling the detection circuitry — but not the sample — to ~30 K), it should soon be possible to obtain high resolution data in one-tenth the time required at present. These resources together with improved pulse sequences and labeling strategies will make it practical to routinely perform experiments that monitor the full range of accessible timescales. Such information will provide the structural biologist with more realistic views of the ensemble of conformational states sampled by a protein.

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