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Recent advances in measuring the kinetics of biomolecules by NMR relaxation dispersion spectroscopy

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ABSTRACT

Protein function can be modulated or dictated by the amplitude and timescale of biomolecular motion, therefore it is imperative to study protein dynamics. Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful technique capable of studying timescales of motion that range from those faster than molecular reorientation on the picosecond timescale to those that occur in real-time. Across this entire regime, NMR observables can report on the amplitude of atomic motion, and the kinetics of atomic motion can be ascertained with a wide variety of experimental techniques from real-time to milliseconds and several nanoseconds to picoseconds. Still a four orders of magnitude window between several nanoseconds and tens of microseconds has remained elusive. Here, we highlight new relaxation dispersion NMR techniques that serve to cover this “hidden-time” window up to hundreds of nanoseconds that achieve atomic resolution while studying the molecule under physiological conditions.

1. Introduction

In the last decades, knowledge on the relationship between structure and function has been greatly expanded owing to significant improvements in experimental and computational approaches. Our ability to determine the three-dimensional (3D) structures of biomolecules at atomic resolution using Nuclear Magnetic Resonance (NMR) spectroscopy, X-ray crystallography, and cryo-electron microscopy (cryo-EM) has provided immense insight to understand the functions of biomolecules (based on the nearly 125,000 deposited structures deposited in the PDB [1]). Particularly, NMR provides powerful unique tools to permit the studies of biomolecules under near physiological environments while maintaining atomic resolution. Conventional structure determination by NMR has been used to solve protein structures with a molecular weight up to 45 kDa and has successfully defined good atomic models of proteins up to 80 kDa [2–4]. Furthermore, near complete backbone assignments and partial side chain assignments for proteins up to 200 kDa [5] have been accomplished and importantly experiments that measure dynamic parameters of proteins and their complexes with molecular weights over ~700 kDa [6–9] have been performed. However, for some proteins their function is induced or changed upon interaction with a ligand which can cause subtle or large conformational changes. This requires the atoms to displace from their original position and produces an induced variety of structural states. Many examples of proteins adapting to their binding partners in the so called “induced fit” scenario have been presented [10], which indicates that proteins are malleable. These examples reveal that upon interaction with ligands such as small molecules, or other proteins, the target protein adapts to its binding partner to elicit responses [11,12]. However, many proteins exist in solution as a structural ensemble, *i.e.* the variety of structures is their intrinsic property. These ensembles often include at least several bound states even in the absence of the ligand [10,13]. Not only is it important to determine the conformational states that a particular protein can sample, but understanding how the energy landscape is linked to different conformational states is also required. Linking the different conformational states can deliver important aspects to function as was illustrated early on how the intrinsic conformational diversity of antibodies allows them to adapt to different antigens [14]. In that study a collection of X-ray structures displayed the heterogeneity between binding competent states

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Abbreviations

3D	three-dimensional
Cryoprobe	Cryogenically Cooled Probehead
CCR	Cross Correlated Relaxation
cryo-EM	Cryo-Electron Microscopy
COLD	Cryogenic Optical Localization in 3D
EST	Exchange Saturation Transfer
EXSY	Exchange Spectroscopy
FRET	Förster Resonance Energy Transfer
GB3	Third IgG Domain of Protein G
HEROINE	Heteronuclear Invasive Nuclear Exchange
NMR	Nuclear Magnetic Resonance
MD	Molecular Dynamics
PELDOR	Pulsed Electron-Double Resonance
RD	Relaxation Dispersion
RDC	Residual Dipolar Coupling
SAXS	Small Angle X-ray Scattering
XFEL	X-ray Free Electron Laser

however, only upon ascertaining their kinetic differences [14] was the "true" binding competent state identified, explaining an antibody's ability to bind to a diversity of antigens. The evaluation of kinetics to connect different conformations has demonstrated to be of importance for other types of protein systems that exert different cellular functions as well [15,16]. Therefore, determining the kinetics still remains a vital necessity in assembling a complete picture for protein function [11,14–16].

Richard Feynman, said, "Everything that living things do can be understood in terms of 'jigglings' and 'wigglings' of atoms" [17]. For biomolecules such as proteins, nuclei acids, and so on, this "jiggling" and "wiggling" can be interpreted as hopping among minima in terms of thermal energy. These minima are separated by energy barriers, which define the kinetics of going from minimum to minimum. These unique minima define distinct conformational states that a biomolecule can explore via their thermal energies. Since many biological processes are transient, it is not surprising that conformational states controlled by their kinetics are linked to important biological processes including molecular recognition [10,13,18,19], transcriptional regulation [20], signal transduction [21,22], protein folding [23,24] and catalysis [25,26].

The aforementioned examples of biological processes and their associated references contain further examples of how protein motion can be linked to or affect function. However, several important examples that pertain to the techniques discussed within this review are as follows. Some proteins rely on the intrinsic sampling of different bound conformations [13,27–29] in the absence of ligand. Understanding this sampling can allow modulating the selectivity for particular binding partners [30] and identifying stronger inhibitors by finding additional binding competent conformers which are transient in nature [31]. Transcriptional regulation can be modulated by the concentration of small molecule effectors within the cell. For a particular transcriptional activator [32], it was shown that a transient on-pathway intermediate is abolished and consequently cannot bind DNA, when an allosteric inhibitor is bound. Catalysis is a critical cellular process for which NMR based dynamic studies have illuminated the importance of protein motion. For the enzyme dihydrofolate reductase, its energy landscape maintains a variety of lowly populated states that mimic the successive step in the catalytic cycle [26], and its product release mechanism is linked to a transient conformational shift [33]. It has also been demonstrated that disease related proteins have various conformational states, which sometimes differ in their healthy or diseased states [34–37]. Therefore, it is of no surprise that through the past several decades the study of proteins and their dynamics, has been paramount in understanding their function.

2. Investigation on dynamics at atomic resolution

A variety of structural biology techniques (Fig. 1) such as X-ray crystallography or cryo-EM are capable of attaining high resolution images of biomolecules, however, they must be studied in the crystalline or frozen state. Therefore, they do not capture the complete conformational landscape that a protein may exhibit in solution, but only represent a static snapshot of the protein at some temperature below physiological temperature. X-ray crystallography using time resolved Laue diffraction [38] or the X-ray free-electron-laser (XFEL) [39] have been effectively used to determine the kinetics and the structural intermediates at atomic resolution for processes that happen within pico- or nanoseconds. Yet, such studies have been limited to proteins involved in photo-activated processes. Small Angle X-ray Scattering (SAXS) provides an avenue for studying biomolecules in solution using X-ray sources with [40] a time resolution from picoseconds to milliseconds, however atomic resolution has not been reached and mainly information about changes in the overall shape are accessible. Normally, atomic models of the protein are required for interpreting the obtained scattering curves. Other scattering techniques like small angle neutron scattering, which has been used to attain information about proteins in the solution state [7,41,42], also reports on the overall shape and dimensions of a molecule. These types of experiments have also been performed in a time dependent manner, but the primary systems of application have been focused on polymer materials and not proteins [43]. A complementary technique has also been the use of dielectric relaxation spectroscopy [18] which covers timescales of motion from picoseconds to milliseconds; however no structural information at the atomically resolved level can be determined, yet, if structures are available, they can be cross validated against the data [18].

Cryogenic single particle electron microscopy (cryo-EM) has also emerged as a powerful tool to study proteins with a molecular weight on the order of ~100 kDa and greater. Modern detectors have enabled an amazing number of structures to be solved at a resolution comparable to conventional X-ray based approaches [44,45]. However, the ability to explore the conformational landscape that a biomolecule can sample by cryo-EM still poses a challenge due to having to work at approximately 90 K or lower where kinetic information is lost. Similar to NMR, slow processes (greater than minutes timescale) can be studied directly. For example, cryo-EM has been applied to the study of how the tRNA moves through the ribosome and a trajectory has been derived by time resolved single particle reconstructions. This allowed for the deconvolution of different states of the tRNA/ribosome complex and provided the overall kinetics for the displacement of tRNA and the equilibrium free energy difference for the process [46]. The ribosome/t-RNA complexes undergo large amplitude conformational changes while subtle smaller changes that are observed in many other systems cannot be determined.

The advent of single molecule fluorescent based techniques has attained significant acclaim in the biophysical community which has found application in studying fundamental features of proteins like protein folding, the nature of intrinsically disordered proteins and the liquid-like phase transition properties of certain proteins [7,47–49]. In the case of single molecule Förster resonance energy transfer (FRET) fluorescent intensities are counted over a large

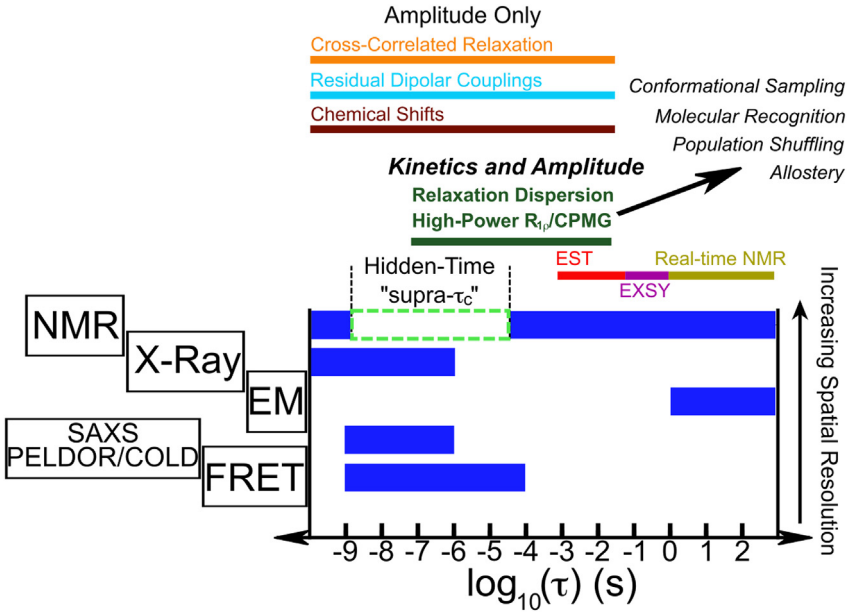

Fig. 1. Accessing protein motions by different techniques frequently employed in structural biology. The temporal regime that is accessible by different techniques ranges from picoseconds to real time. However, given that the system of study is tractable, NMR is able to cover the entire temporal range by using a variety of different techniques. In particular, the hidden-time window between several nanoseconds to tens of microseconds has seen recent methodological development that has increased its resolution to faster motions within that time range. High-power relaxation dispersion has closed the gap by four fold [95,115] as what was previously accessible. Importantly, this approach provides kinetics and amplitude information unlike several other observables like cross-correlated relaxation, residual dipolar couplings, and chemical shifts which only report on amplitudes of motion from within this time window. Recent studies [18,115–117], have been able to quantitate previously undetected features of a protein's energy landscape.

number of observations for a given molecule in which the emitted signal can be related to distances between a donor/acceptor fluorophore pair [50], but temporal dynamic information is also accessible by these experiments. Fluorescent intensity measurements can be conducted with the attachment of a single fluorophore to a biomolecule. This permits the anisotropy of the single probe to be determined; however, the corresponding information will only report on the local tumbling properties of the fluorophore [50]. Interestingly for FRET measurements, if different conformational states posing unique diffusional properties are sampled, their associated anisotropy relaxation rates can be related to the underlying kinetics of the process. This is done by calculating a correlation function for a single molecule's fluorescence intensity over time [48,51]. An inherent issue is that one to two fluorophores must be attached to the protein requiring chemical modification to the system of interest and thus, runs the risk of altering a protein's intrinsic dynamics. Also, the linkers required to attach the dyes reduce the possible resolution since the position of the dye on the protein is not known in most cases. Therefore, in cases of single molecule measurements only an averaged distance between two sites can be measured with a FRET pair. Therefore, normally, as for SAXS high resolution structures are required to interpret the FRET effects with high resolution structures [48].

Pulsed electron-electron double resonance (PELDOR) in Electron Paramagnetic Resonance spectroscopy provides similar structural information as FRET, but does not require two different dyes. This dramatically reduces the requirement for protein modification. Since PELDOR is done at cryogenic temperatures, kinetics cannot be measured for processes that are faster than what was described above for the ribosome. PELDOR is a bulk method and thus requires appreciable quantities of protein. An exciting single particle technique is Cryogenic Optical Localization in 3D (COLD) which localizes identical dyes with a resolution down to 5 Å and can even deal with several dyes [52,53].

Owing to advances in computing power and computational efficiency, Molecular Dynamics (MD) simulations can be performed up to milliseconds [54,55]. The capability to run highly extended MD simulations has had important impact in providing atomistic insight into protein folding [54,56,57], membrane protein behavior (including G-protein coupled receptors and ion channels) [58–61], and drug binding mechanisms [62]. However, in some cases MD is still insufficient to recapitulate experimental observables, such as side chain order parameters [19], motions slower than the overall rotational correlation time (τ_c) [20], and rotameric populations [63], possibly due to the imperfection of utilized force fields [22,23]. Therefore, solution NMR spectroscopy given that the system is amenable for study, poses to be the ideal tool to ascertain functional dynamics of proteins because all observables (Fig. 1) directly report on motion. Furthermore, the technique requires no chemical modification to the protein, and the systems can be studied under near physiological conditions while maintaining absolute atomic resolution.

3. NMR accesses a broad range of timescales for dynamics studies of proteins

NMR relaxation methods are ideal tools for studying motions faster than the overall tumbling time (τ_c) (several nanoseconds to picosecond motions) of a protein (Fig. 1). The amplitudes of these motions are quantified in terms of the Lipari-Szabo order parameter [64]. It has been found that this motional regime serves as an excellent proxy for the conformational entropy of a protein and has found application in understanding the role of conformational entropy during molecular recognition [65,66] and functional activity for a transcriptional activator [67]. Slower time scale motions (ca. 40 μs to several ms) can be addressed by NMR based Relaxation Dispersion (RD) spectroscopy (Fig. 1).

NMR RD has emerged as a powerful technique that is capable of providing kinetic, thermodynamic, and structural information regarding the conformational landscape of a protein at atomic

resolution. In brief, these experiments function by exploiting the phenomenon of chemical exchange, in which structural features modulate the isotropic chemical shift of nuclei when the protein samples distinct conformational states. This modulation causes a phase difference (difference in chemical shifts; $\Delta\omega$) between differentially populated states that are magnetically inequivalent and interconvert with a particular exchange lifetime (kinetics) (Fig. 2). This manifests as a contribution to NMR line widths in addition to the spin-spin or spin-lattice relaxation effects that depend exclusively on pico-nanosecond motion. NMR RD can quantitate this contribution by monitoring a change in the effective relaxation rate as a function of the strength of an applied radio frequency field which acts to "spin-lock" the magnetization (Fig. 2). Increased radio frequency field strengths (ν_{RF}; Fig. 2) function to limit deviations of the magnetization from the spin-lock field, and if chemical exchange exists, causes the effective relaxation rate to decrease (Fig. 2; increased semi-angle of the cone indicates improved spin-locking of the magnetization). This dependence can be curve fitted to yield parameters that describe the kinetics and structural features between the exchanging conformations of the structural ensemble for a biomolecule (Fig. 2). The extraction of parameters from NMR RD depends on the chemical shift timescale of the system which is defined as the ratio between the exchange lifetime of the slowest exchanging states and chemical shift difference between those states. When this ratio is less than one, population and structural information can be separated permitting all parameters to be determined. However, and most pertinent for this review, motion within the supra-τ_c range is in the fast regime (i.e. the aforementioned ratio is greater than one) and thus, only kinetic and chemical shift variances (product between the populations and square of the chemical shift differences) can be measured. More complex models that maintain more than one state require that the processes should differ by almost an order of magnitude in order for them to be clearly identified during the analysis of NMR RD data [68]. Our focus here is not to discuss the intricacies of complex RD data fitting as the theoretical framework for RD experiments has been presented in theory and application for simple two-state exchange and multi-state scenarios thus, we direct the reader to the following publications [68–76] for in-depth discussions. Rather, here we discuss recent advances to measure kinetics for biomolecules using NMR RD spectroscopy.

NMR RD has yielded insight to a variety of proteins [10,23,25,71,77,78] and nucleic acids [29,79,80]. Motions that are slower than tens of milliseconds are usually not averaged out in the chemical shift time scale and thus, exhibit distinct peaks. The interconversion of these slow processes can be studied using EXchange SpectroscopY (EXSY) [81,82] and Exchange Saturation Transfer (EST) [83–85] experiments which also provide kinetic and structural information at atomic resolution (Fig. 1). In particular EST experiments have had tremendous application in ascertaining the structural features of slowly exchanging processes such as the misfolding of proteins [37,83,86] and in investigations related to amyloid formation permitting insight into how small proteins can interact with large (>1 MDa) substrates [84,87]. These experiments have seen recent strides in methodological development in which now different nuclei types can be targeted [88–91], acquisition times can be decreased [92,93], and artifacts due to decoupling can be reduced [94]. However, until recently, the time range between the rotational diffusion correlation time τ_c (several ns) to forty microseconds (four orders of magnitude large time window) remained "hidden" to NMR experimentalists because of a lack of tools required to make observations within this time range [13,18,95,96]. This "hidden-time" window (also referred to as the supra-τ_c range) has been resolved by extending the time resolution amenable to NMR RD experiments. Therefore, now protein motional amplitudes and kinetics from this time window can be observed at atomic resolution by NMR.

4. Amplitude of motions from the hidden-time window

For the past decade, it has been realized that several NMR observables have direct access to dynamics within the hidden-time window and thus, a variety of experiments have been developed to measure and quantify their behavior. These experiments cover the amplitude of motions that cover the hidden-time window and measure the following interactions: residual dipolar couplings (RDC) [71,96–98], cross-correlated relaxation (CCR) [99–106], cross-relaxation [107], scalar couplings [102,108,109], paramagnetic relaxation enhancement [110], and chemical shifts [111] (Fig. 1). Particularly, the development of ensemble refinement procedures that included multiple orthogonal RDC datasets had revealed that ubiquitin, in the free form, samples structural configurations that resemble ubiquitin in bound complex structures, but in the absence of any binding partner [13]. This extensive

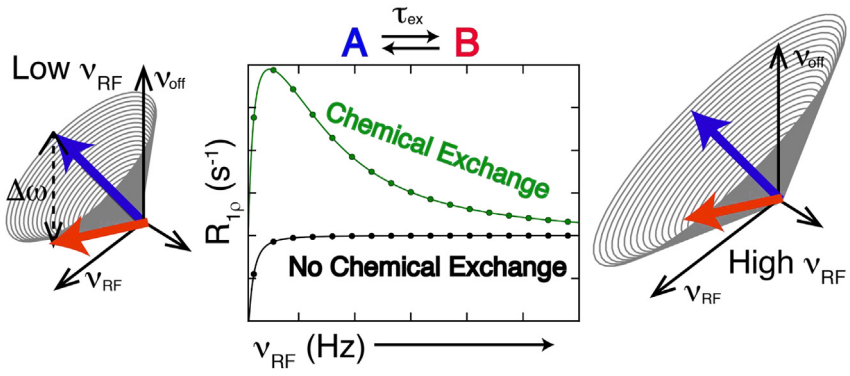

Fig. 2. Illustration describing NMR RD as measured by transverse rotating frame ($R_{1\rho}$) experiments. In a two-state exchange scenario two magnetization vectors states A (blue vector arrow) and B (red vector arrow) will interconvert with a kinetic lifetime given by τ_{ex} and whose magnetization will maintain a phase difference of $\Delta\omega$. $R_{1\rho}$ RD experiments function by monitoring the relaxation rate as a function of either the frequency difference between the queried resonance and the placement of the spin-lock field (ν_{off}) or via the amplitude of the radio-frequency field (ν_{RF}). The cone indicates the "spin-lock" field. For the RD experiments used to measure kinetics and amplitude information from the supra-τ_c range, typically ν_{RF} is changed and the offset is kept constant. The $R_{1\rho}$ curve maintains contributions from longitudinal relaxation, transverse relaxation, and chemical exchange. At low ν_{RF} values longitudinal relaxation can dominate the relaxation rate; therefore, it is important to use large enough ν_{RF} values where the transverse components dominate the $R_{1\rho}$ rate. If exchange occurs (green curve) a Lorentzian type profile will manifest in the dependence of $R_{1\rho}$ in cases where exchange is non-existent the dependence in the curve will be flat across values where the longitudinal relaxation rate no longer contributes (black curve).

collection of RDC datasets, in conjunction with CCR rates, revealed low amplitude inter β-strand correlations across the interaction surface of ubiquitin [102]. Motional amplitudes from the hidden-time window has also been observed in nucleic acids and used to successfully understand large domain structural plasticity in RNA [28]. Although, invaluable information has been obtained from these NMR parameters, these methods can only provide amplitude information and do not report on the energetics or kinetics of these processes.

5. Kinetic information from high-power RD accesses protein kinetics down to ~3 μs

RD experiments can provide the necessary kinetic information. However, a prime limitation in RD experiments is the minimum accessible timescale. The fastest motion that is accessible is limited to the rate at which the magnetization can be refocused as only processes slower than the refocusing rate are detected due to a loss in magnetization which translates to increased effective relaxation rates. One such experiment that permits the generation of stronger refocusing fields is the transverse rotating frame ($R_{1\rho}$) experiment [70,71]. These experiments create refocusing fields based on the amplitude of the employed radio frequency field (ν_{RF}) and at what frequency it is applied with respect to a nuclei's resonance frequency (ν_{off}) to which acts to "spin-lock" the queried magnetization. An effective relaxation rate is then calculated based on the amount the signal has attenuated with respect to the duration of the spin-lock pulse (Fig. 2). Within these experiments the fastest exchange lifetime (τ_{ex}) that is observable is proportional to the reciprocal of the amplitude of the refocusing field ($\tau_{ex} \propto 1/(2\pi\sqrt{\nu_{RF}^2 + \nu_{off}^2})$). Up until several years ago, the fastest observable kinetic lifetime for a conventional RD experiment was around 40 μs [95,112]. Recently, we have shown that these previous limitations could be surpassed through the use of cryogenically cooled probes (cryoprobe), which are found widely throughout NMR laboratories [95]. It was found that modern cryoprobes could safely withstand spin-lock pulses up to a length of 120 ms with a field strength of 6.4 kHz for ^{15}N nuclei. This improved the fastest detectable timescale for ^{15}N nuclei by a factor of three permitting exchange lifetime events down to 25 μs to be detected and is called "high-power" RD. RD measured using the high-power approach matched earlier RD measurements from a study in which the rate of conformational sampling was determined from super-cooled RD with the protein ubiquitin [18]. This also highlighted the ability to now monitor small contributions of conformational exchange with high precision [95].

By being able to generate larger refocusing fields, the RD curve can be sampled to faster time scales, i.e. contributions to the line-width originating from fast motion can be quenched. Therefore, an exchange free transverse relaxation rate ($R_{2,0}$; relaxation due to fast sub-τ_c motions) can be measured, i.e. a rate that does not contain contributions due to exchange down to the limit determined by the maximum $\gamma B_1/2\pi$ used. This was demonstrated for ubiquitin where a reduced variance across backbone ^{15}N nuclei was observed [95] when going from conventional relaxation dispersion to high-power relaxation dispersion by a variance of 40%. Since, $R_{2,0}$ is required for fitting of all RD data it is practical to have an experiment in which the most accurate exchange free $R_{2,0}$ can be determined. The **He**te**r**onuclear **I**nvasive **N**uclear **E**xchange (HEROINE) [113] experiment was created to supplement conventional RD data in which the same averaged relaxation rate was monitored, but acquired with ν_{RF} and could be used as a known parameter during fitting routines. It was found that for an anti-HIV lectin [114] conventionally determined $R_{2,0}$ underestimated the true kinetic values by 66% [113] compared to HEROINE determined $R_{2,0}$. Given that modern cryoprobes were shown to be able to handle large ν_{RF}, it is obvious that moving to nuclei with a larger gyromagnetic ratio (γ) would lead to even larger ν_{RF} with the added advantage of requiring less power to be deposited in the probe ($\sqrt{P} \propto B_1 = 2\pi\nu_{RF}/\gamma$). High-power RD has been applied to both ^{13}C and ^{1}H nuclei [115–117] and increased the time resolution to 13 and 3 μs for ^{13}C and ^{1}H nuclei, respectively. Irradiation durations of up to 125 ms (Fig. 3) are feasible. Recording RD on different nuclei has several key advantages: *1)* Increased data size to provide better definition of the global kinetic parameters, *2)* the collection of different nuclei provides greater coverage over the entirety of a biomolecule, since some chemical shifts may not be affected by certain kinetic processes, *3)* provides the ability to acquire RD data from different chemical moieties and *4)* moving to higher γ nuclei increases the detectable contribution of exchange to RD. These advantages have provided useful to two recent applications [115,116] of multi-nuclear high-powered RD. However, for systems which have even faster motion, what can be done to detect their dynamics?

6. Nanosecond motions detected by high-power RD under super-cooled conditions (Fig. 4)

The observable exchange contribution to RD is dictated by the kinetics and conformational amplitude, which within the fast

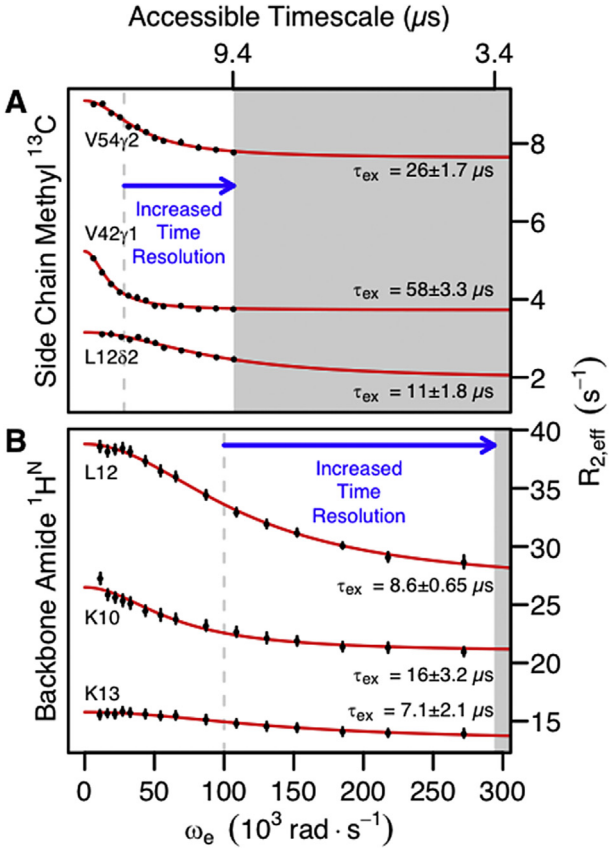

Fig. 3. High-power RD can measure kinetics as fast as ~3 μs at atomic resolution. [115] The timescales accessible in RD experiments are determined by the maximum field strength (ω_e). Through high-power RD experiments, the accessible timescale is extended from the previous limits (dashed lines) to 9.4 and 3.4 μs with (A) ^{13}C and (B) ^{1}H nuclei, respectively. Shaded areas indicate inaccessible timescales. The utility of high-power RD is shown with (A) side chain methyl ^{13}C and (B) backbone amide $^{1}H^{N}$ nuclei (Reprinted with permission from publication [115] copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with Permission).

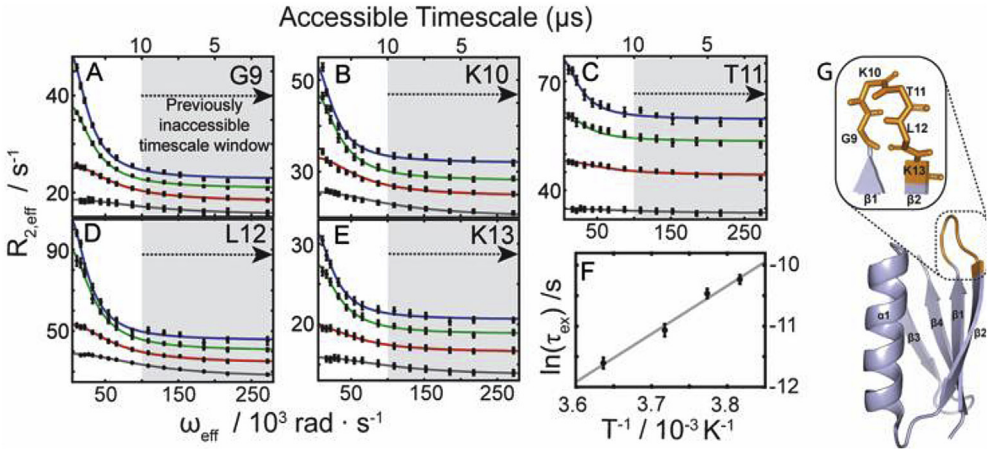

Fig. 4. Temperature dependence of the observed hidden time dynamics in GB3. [95] High-power $R_{1\rho}$ RD experiments on backbone amide protons of GB3 revealing previously undetected dynamics within the first β-turn for residues (A) G9, (B) K10, (C) T11, (D) L12, and (E) K13. Shaded areas indicate the recently developed high-power $R_{1\rho}$ RD experiment, which can be used to investigate motion within the previously inaccessible dynamics window. Solid lines represent fits of the experimental data utilizing a second-order Akaike's Information Criterion, where all RD data at a single temperature were fitted to a single global exchange lifetime (τ_{ex}). Experimental temperatures are denoted by the following colors: gray, 275 K; red, 269 K; green, 265 K; and blue, 262 K. (F) Plot of τ_{ex} versus temperature. The solid gray line indicates the fit of the experimental data to the Arrhenius equation where τ_{ex} is extrapolated to physiological temperature (310 K) as 371 ± 115 ns. (G) The residues showing significant RD are depicted on a structure of GB3 (PDB ID: 2OED) with orange color. This β-turn is involved in antibody binding, exhibiting the potential link of the observed motion with molecular recognition. Reprinted with permission from Ref. [117] copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with Permission.

regime is the product between the populations and chemical shift differences between distinct states (chemical shift variance) and frequently for small contributions of conformational exchange it is favorable to try to increase its contribution to the effective transverse relaxation rate. The chemical shift variance can be increased using paramagnetic tags with lanthanides that have an anisotropic g-tensor which via the pseudo-contact shift effect will increase the chemical shift variance [118,119] or through increasing the static magnetic field [120]. Another method is to lower the temperature thereby slowing down the kinetic process (super-cooled conditions) [18,121]. By performing high-power RD measurements under super-cooled temperatures, a recent study found significant global exchange events in the β-turn of the third IgG-binding domain of protein G (GB3) at 262 K [117]. A temperature dependence of RD data from 262 K to 275 K allowed for a global τ_{ex} value to be determined at each temperature for these β-turn residues. An Arrhenius extrapolation of the temperature dependent super-cooled high-power RD determined τ_{ex} values rendered this kinetic process to be 371 ± 115 ns at physiological temperature (310 K). The motion was ascribed to a change in hydrogen bonding within the β-turn. This demonstrates the ability of high-power RD in conjunction with super-cooled measurements to detect nanosecond fluctuations. Without the aforementioned combination of techniques this kinetic process would have been beyond detection. Furthermore, the same residues show plasticity in the model-free RDC order parameters and in an ensemble encoding the hidden time dynamics [117].

7. Application of high-power RD

For the proteins, ubiquitin and GB3, high-power RD experiments were performed on methyl ^{13}C methyl ^{1}H, and backbone $^{1}H^{N}$ nuclei [115] (Fig. 5). The methyl ^{13}C and ^{1}H RD data for both proteins revealed an interesting result. A total of ten methyl carbon atoms reported RD while their corresponding ^{1}H atoms did not. Considering all sources that can modulate isotropic chemical shifts to induce detectable RD for methyl carbons, but that does not modulate the attached proton chemical shift could only be explained by the γ−effect. The γ−effect refers to the difference in chemical shift of a carbon which is antiperiplanar or synclinal to another carbon. Thus, the γ−effect sensitively reports on the rotameric state of side chains. Furthermore, an asymmetry in the amplitude of RD was discovered for amino acids that have two methyl groups. This difference in the observed amplitudes extracted from the methyl carbon RD data, allowed to suggest a sophisticated kinetic model that incorporated all possible rotameric transitions (highlighting the existence of gauche^{-} rotamers in solution) for a given methyl group (Fig. 5). This was formulated to become the “population shuffling” model in which slower microsecond fluctuations of the backbone and sidechain moieties of the protein caused transitions of the ground state (“macrostates”) that shuffle, or redistribute the populations of rotamers for a given methyl group whose transitions still occur on a timescale orders of magnitude faster (ps-ns) than what is detected by the RD experiments (Fig. 5). Population shuffling has also been found in a recent XFEL study of cyclophilin A [122] where the multitemperature X-ray data could be explained in light of solid state relaxation data. The population shuffling model has also been applied to methyl groups for proteins that sample sparsely populated states (<5%) and whose macrostate interconversion kinetics are much slower than that of ubiquitin or GB3 [63].

Another recent application of high-power RD revealed an allosteric switch in ubiquitin [116] (Fig. 6). Intermolecular interactions are one of the key mechanisms by which proteins mediate their biological functions. For many proteins, these interactions are enhanced or suppressed by allosteric networks that couple distant regions together. The mechanisms by which these networks function are just starting to be understood [123,124], and many of the important details have yet to be uncovered. In particular, the role of intrinsic protein motion and kinetics remains elusive. Although a number of structural ensembles representing the breadth of ubiquitin's conformational diversity have been recently proposed [13,18,30,102], the mechanism behind ubiquitin's microsecond fluctuations have remained elusive. It has been suggested that through motion at the binding interface, its free state visits the same conformations found in complex with its many binding partners [13,18]. An unanswered question that remained was whether ubiquitin's multi-specificity for its variety of binding

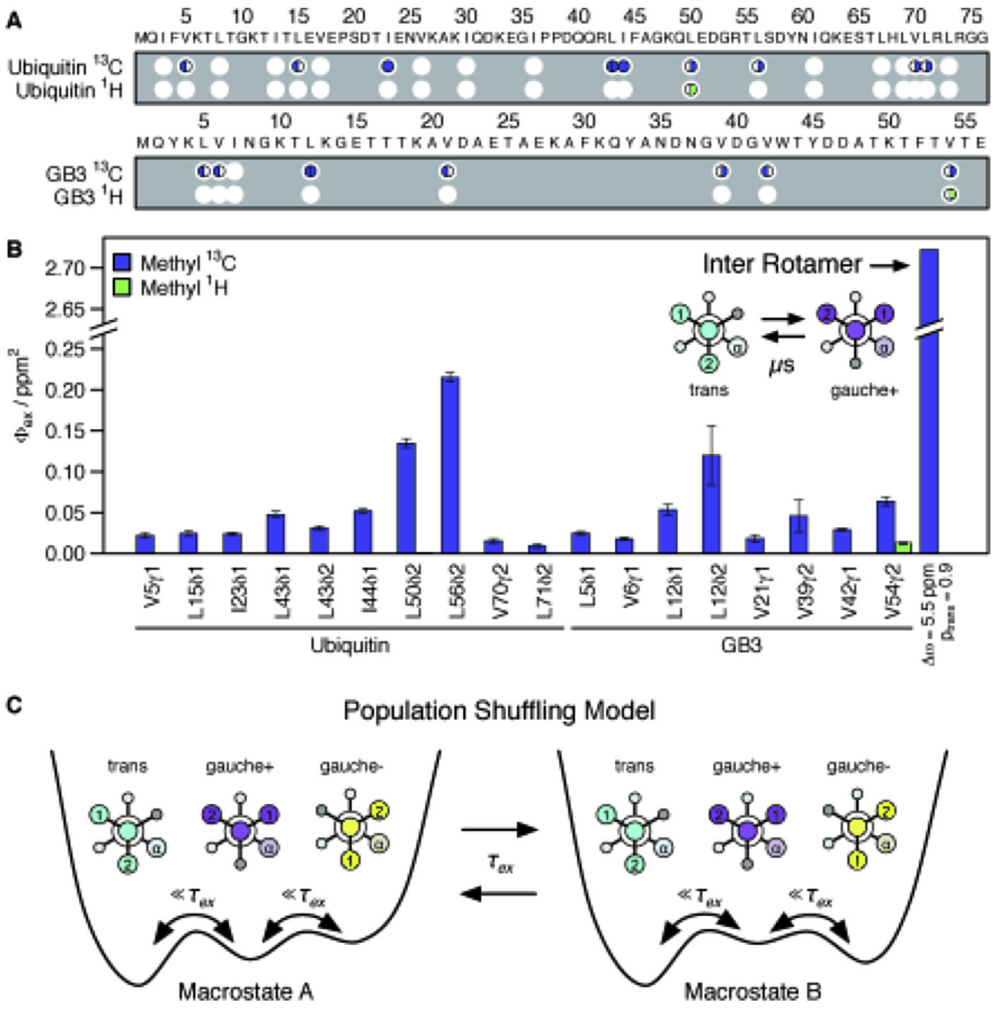

Fig. 5. Populations of protein conformations shuffle on the microsecond timescale. A) Significant side-chain motions in both ubiquitin and GB3 at the microsecond timescale. White circles indicate the residues for which RD data was collected. Residues showing motions at the microsecond timescale are indicated with filled circles. Where two methyl groups were present in the same residue (i.e. valine and leucine), shaded semicircles indicate the respective methyl group(s) showing microsecond motion (left: γ1 or δ1, right: γ2 or δ2). B) Amplitude of the observed microsecond motions, monitored by the chemical shift variance (Φ_{ex}) in RD. The last bar represents the theoretical Φ_{ex} value for an inter-rotamer model, which is incompatible with the observed microsecond motions (see the text). In the Newman projections of leucine, carbon atoms are colored and hydrogen atoms shown in gray. Delta carbon atoms are numbered and the alpha carbon atom is labeled. C) Population shuffling model for methyl side-chain motions at the microsecond timescale. In the population shuffling model, inter-rotamer conversion occurs at a faster timescale ($\ll\tau_{ex}$) than the observed microsecond motion ($\tau_{ex} \approx 55$ μs for ubiquitin and 27 μs for GB3). Microsecond motions shuffle the populations of rotamers and lead to population weighted chemical shift changes significantly less than 5.5 ppm. Reprinted with permission from Ref. [115] copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with Permission.

partners stems only from its canonical binding interface or whether the process is global and allosterically coupled through the rest of the protein. This is of keen interest given the presence of motion sites distal to the canonical binding site. To address this question the high-power RD experiments were carried out for multiple nuclei (^{1}H, ^{13}C, and ^{15}N) and yielded a nearly eightfold increase in the number of nuclei where RD had been previously observed [116]. The full set of backbone and side chain nuclei, which were spread throughout the structure, showed a consistent timescale of motion ($\tau_{ex} = 55$ μs at 277 K). This suggested that the motions were not independent but shared a common molecular mechanism (Fig. 6). The RD data was modeled assuming a single mode of motion that was collective in nature. A computational method was developed that took a large collection of MD simulations of ubiquitin in order to derive a weighted linear mode to describe the motion which was optimized against the experimental RD data. The RD weighted motional mode that fulfilled the experimental data represents a detailed structural model for the reaction coordinate along which much of the microsecond motion of ubiquitin took place (Fig. 6). This is one of the first atomic models for a fast exchange transition in the ground state [116], in contrast with other models where the interconversion between states involved motion in a slow exchange regime from which distinct chemical shifts could be extracted [24,125] between the ground and excited states. The optimized RD weighted linear mode highlighted a structural change that involved a peptide flip that causes a change in the hydrogen bond patterning of G53 within ubiquitin [116]. An analysis of the atomic models derived from the RD weighted motional mode identified a correlated allosteric motion that caused contraction and expansion of ubiquitin's binding surface which enables binding to ubiquitin-specific proteases (USP). In order to test whether the correlated motion stemmed from the peptide flip, two mutant constructs of ubiquitin were created one in which G53 was mutated to alanine and another where E24 was mutated to alanine. These mutants both revealed not only the disruption of RD observed in wild-type ubiquitin but diminished competent binding states that resulted in weakened affinity for a USP [116].

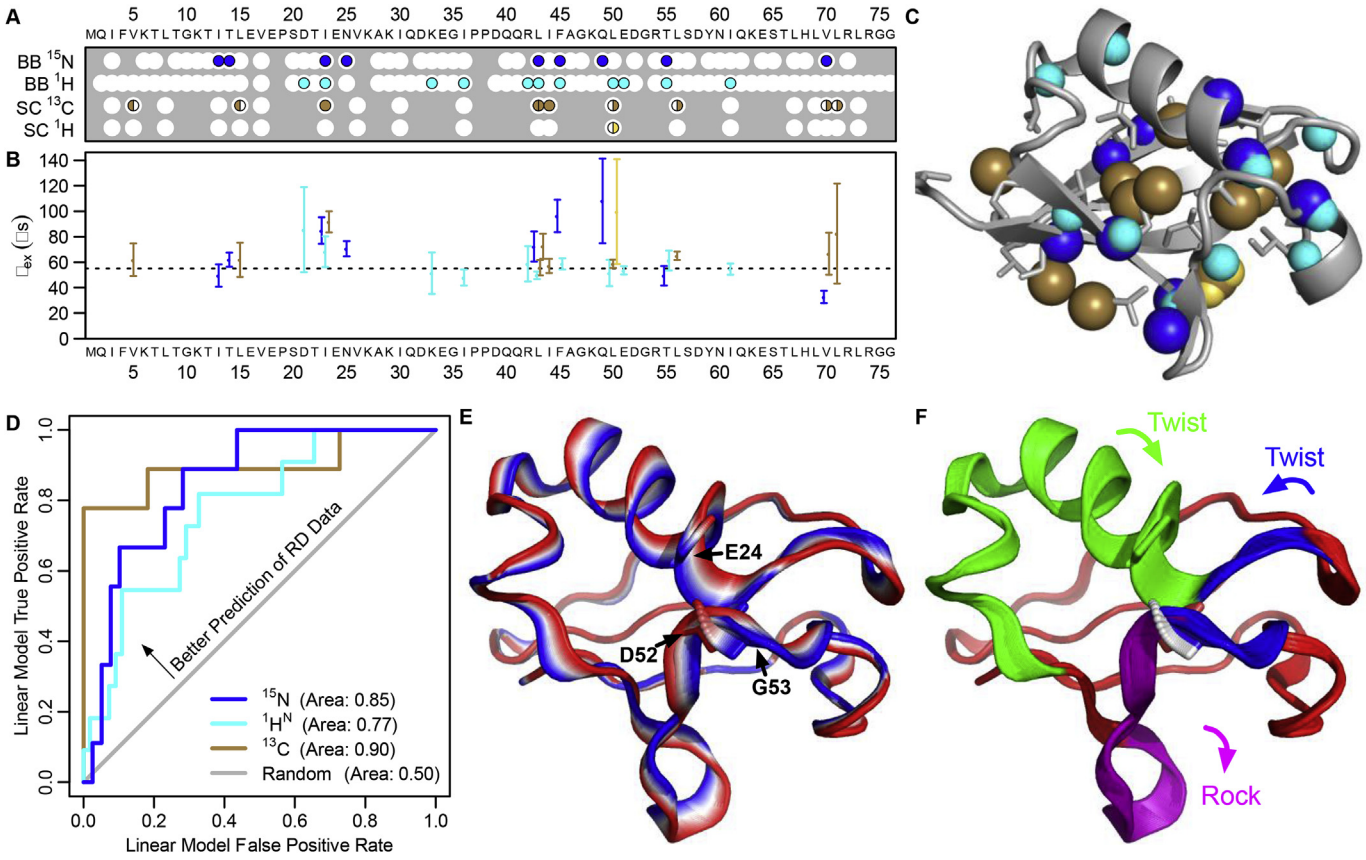

Fig. 6. A single collective mode based on kinetic information. [116] (A) 31 different nuclei show statistically significant RD at 277 K and are spread across the primary sequence of ubiquitin. White circles indicate residues for which measuring a RD curve was possible. Where two labeled methyl groups were present in the same residue, shaded semicircles indicate the respective methyl(s) showing RD. (B) When fit individually, most nuclei show a similar exchange lifetime (τ_{ex}) that is consistent with the globally fit value of 55 μs (dotted line). (C) The nuclei showing RD are distributed throughout the structure, suggesting concerted motion of the whole structure. (D) Receiver Operator Characteristic (ROC) curves show that an optimized collective mode can predict a significant fraction of the ^{15}N, and ^{1}H^{N}, and ^{13}C RD data. The straight gray line indicates a random prediction. (E) Interpolation of the backbone from one extreme of the concerted motion vector (blue) to the other (red). The rotating peptide bond between D52 and G53 is shown with a stick representation, along with the Cα-Cβ vector of E24. (F) The optimized motional mode contains several semi-rigid substructures, each indicated by different colors. Reprinted with permission from the Proceedings of the National Academy of Science [116].

8. Conclusion

Understanding protein dynamics is required to evaluate a biomolecule's conformational landscape which in turn can translate into understanding a systems function and dysfunction. NMR RD has enabled the study of these systems and has provided important insight for a variety of systems. With the use of multinuclear high-power RD, motions including lifetimes of states down to 100s of nanoseconds are now accessible by solution NMR spectroscopy [18,115]. Recent application of high-power RD has brought forth insight into a novel allosteric switch which could be modulated by mutation, but when combined with a novel computational approach revealed a structural model for motion in the fast exchange [116]. New phenomenological knowledge as to the relationship between slower macrostate fluctuations and rapid ps-ns motions in sidechains have also been formulated in the population shuffling model [115]. The combination of super-cooled RD with high-power also identified nanosecond motion which may have implications for antibody recognition of GB3 [117]. Further opportunities may exist to further extend high-power RD not only by hardware development, but methods of analysis from advanced computational techniques like extended molecular dynamics trajectories [54,126] will have continued impact in the understanding of protein dynamics and their underlying energy landscapes. With this new tool, it is very likely that surprising motions in other systems will be discovered. However, a still formidable goal is to completely close the hidden time window.

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