Elucidating the mechanisms underlying protein conformational switching using NMR spectroscopy

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1. Introduction

Biological processes such as signal transduction and enzyme catalysis are orchestrated by structural transitions of protein molecules between states appropriately designed to accommodate ligands, cofactors and other partner biomolecules. An understanding of the mechanisms underlying conformational switching is important not only as a central question in molecular biophysics, but also because functionally relevant protein conformations can serve as drug targets [1].

The mechanism of allosteric regulation at physically remote sites has been actively debated for over six decades [2]. The framework for this debate has been assembled from two contrasting viewpoints; conformational selection (CS), where the functional conformation coexists with the native state of the protein and is selected for binding by the cognate ligand or protein [1,3-6], and induced fit (IF), where ligands induce the protein molecule to adopt the conformation that is structurally best-suited to accommodate them (Fig. 1A) [7-9]. While a discussion of CS and IF pertains to bimolecular (and higher molecularity) events that are typically encountered in molecular recognition, mechanistic questions also arise in purely unimolecular reactions like protein folding and biomolecular complex formation. In addition, recent methodological developments in the areas of saturation transfer and relaxation dispersion have expanded the scope of NMR for probing the mechanics of transitions in systems where one or more states constituting the exchange process are sparsely populated and ‘invisible’ in NMR spectra. In this review, we highlight some of the strategies available from NMR spectroscopy for examining the nature of multi-site conformational exchange, using five case studies that have employed NMR, either in isolation, or in conjunction with other biophysical tools.

How proteins switch between various ligand-free and ligand-bound structures has been a key biophysical question ever since the postulation of the Monod-Wyman-Changeux and Koshland-Nemethy-Filmer models over six decades ago. The ability of NMR spectroscopy to provide structural and kinetic information on biomolecular conformational exchange places it in a unique position as an analytical tool to interrogate the mechanisms of biological processes such as protein folding and biomolecular complex formation. In addition, recent methodological developments in the areas of saturation transfer and relaxation dispersion have expanded the scope of NMR for probing the mechanics of transitions in systems where one or more states constituting the exchange process are sparsely populated and ‘invisible’ in NMR spectra. In this review, we highlight some of the strategies available from NMR spectroscopy for examining the nature of multi-site conformational exchange, using five case studies that have employed NMR, either in isolation, or in conjunction with other biophysical tools.

1. Introduction

Biological processes such as signal transduction and enzyme catalysis are orchestrated by structural transitions of protein molecules between states appropriately designed to accommodate ligands, cofactors and other partner biomolecules. An understanding of the mechanisms underlying conformational switching is important not only as a central question in molecular biophysics, but also because functionally relevant protein conformations can serve as drug targets [1].

The mechanism of allosteric regulation at physically remote sites has been actively debated for over six decades [2]. The framework for this debate has been assembled from two contrasting viewpoints; conformational selection (CS), where the functional conformation coexists with the native state of the protein and is selected for binding by the cognate ligand or protein [1,3-6], and induced fit (IF), where ligands induce the protein molecule to adopt the conformation that is structurally best-suited to accommodate them (Fig. 1A) [7-9]. While a discussion of CS and IF pertains to bimolecular (and higher molecularity) events that are typically encountered in molecular recognition, mechanistic questions also arise in purely unimolecular reactions like protein folding. For example, when folding occurs by a three-state mechanism involving an unfolded (U), a native (N) and an intermediate state (I), it is of importance to decipher whether the intermediate is on-pathway or off-pathway [10,11]. An on-pathway intermediate requires folding to occur through I, while off-pathway intermediates could represent kinetic traps that slow down protein folding or cause aggregation (Fig. 1B).

Fundamentally, the question of mechanism is a question about pathways and addressing it requires knowledge of the kinetics of interconversion between different protein conformational states [12-16]. Does the formation of a ligand-protein complex occur via a pre-existing structurally similar conformation? Does the folding pathway of a protein take it through a partially folded intermediate? These questions generally cannot be unequivocally resolved merely by making measurements on equilibrium properties such as populations and dissociation equilibrium constants. For instance, the existence of a free protein conformation that structurally resembles a ligand-bound form is necessary but not sufficient evidence for the operation of a CS mechanism, as ligand binding may not proceed via this conformation at all [17,18]. Additionally, there is an aspect of biomolecular structure that is superimposed on the kinetics, because it is critical to know the structures of the protein between which a particular rate constant has been measured. Once the rate constants coupling different states of a protein are known, simple flux-based calculations can help in elucidating which mechanism prevails in facilitating conformational switching [12,19].

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NMR spectroscopy is ideally suited for elucidating reaction mechanisms because it can simultaneously provide information on protein structure and reaction kinetics. Structural information is available primarily in the form of chemical shifts [20,21], but also through residual dipolar couplings [22], chemical shift anisotropy [23–25] and paramagnetic relaxation enhancement [26,27]. On the other hand, the rate constants governing conformational interconversion can be accessed through a variety of experiments such as magnetization exchange, saturation transfer and relaxation dispersion [28]. In particular, we have witnessed rapid progress in chemical exchange saturation transfer (CEST) [29–35], and Carr-Purcell-Meiboom-Gill (CPMG) [36–39] and R$_{1p}$ relaxation dispersion (RD) methods [40–42] in the last two decades, and these are now well-established sources of dynamical information on proteins and nucleic acids. The key advantage provided by these methods is that they can also detect higher energy conformations that have sparse populations and transient ms-µs lifetimes [43]. Moreover, the applicability of these methods to proteins has tremendously benefited from improved selective isotope labeling strategies that serve to simplify the spin system under consideration [44–50], as well as the addition of TROSY ([51,52]) and methyl-TROSY [53–55] to the biomolecular toolbox for studying large macromolecular complexes [56–58].

In this manuscript, we highlight five case studies [59–63] which have used various NMR methods to dissect the mechanisms underlying protein conformational transitions. Descriptions of the principles underlying each NMR method are provided wherever they occur for the first time, under 'Methodology' sections. While studies 1–4 employ chemical exchange saturation transfer (CEST), 2 additionally uses magnetization exchange and 5 leverages the benefits of CPMG RD NMR. Case Study 5 synergistically combines the capabilities of NMR spectroscopy with stopped-flow fluorescence spectrophotometry to arrive at a comprehensive picture of the molecular mechanism. In Case Study 4, Vallurupalli and coworkers push the boundaries of CEST by developing a novel double resonance-based scheme dedicated towards discriminating between various pathways available for a protein to transition between distinct conformational states. All these studies illustrate the potential of NMR spectroscopy for deciphering how populations of different protein conformations change in response to environmental conditions or to accommodate the presence of partner proteins and ligands.

2. Case studies that derive mechanistic information

2.1. Case study 1: binding of DHFR to folinic acid

An early example of the use of NMR spectroscopy for eliciting mechanistic information on proteins came from the group of Arnold Burgen in 1980 [59], where the authors used chemical exchange saturation transfer (CEST, Fig. 2A–C) to study the binding of folinic acid to dihydrofolate reductase (DHFR) (Fig. 2D). CEST works by perturbing spins using a weak radiofrequency (RF) field and relying on chemical exchange to transfer the perturbation to a different conformation, as described in detail below.

2.1.1. Defining typical parameters for conformational exchange

Consider a protein molecule exchanging between states A and B with forward and backward rate constants of $k_{AB}$ and $k_{BA}$ (Fig. 2A). Let $(\varsigma_{A}, R_{1A}, R_{2A})$ and $(\varsigma_{B}, R_{1B}, R_{2B})$ be the chemical shifts, and longitudinal and transverse relaxation rate constants, of a target nucleus in the two states. The fractional populations of states A and B can be written in terms of the forward and backward rate constants as:

\[ p_B = \frac{k_{AB}}{k_{AB} + k_{BA}} \quad (1.1) \]
\[ p_A = 1 - p_B \quad (1.2) \]

The exchange rate constant $k_{ex}$ is defined as:

\[ k_{ex} = k_{AB} + k_{BA} \quad (1.3) \]

These expressions can be suitably adapted for three-site exchange, oligomerization or ligand binding [64,65].

![Fig. 1. Conformational transitions in proteins.](image-url)
2.1.2. Methodology: chemical exchange saturation transfer (CEST)

Fig. 2B shows the simulated $^15$N NMR spectrum of a molecule undergoing two-state exchange between a major state A and a minor state B, with $k_{ex} = 100 \text{ s}^{-1}$ and $p_B = 2\%$. The minor state is not observable in the NMR spectrum because of its low population and large exchange broadening. The simulated CEST profile for this system is shown in Fig. 2C. CEST data are acquired by applying a weak RF field of amplitude $B_1$ for an exchange duration $T_{ex}$ at various offset positions and subsequently measuring the intensity (I) of the major state resonance. $I_0$ is the intensity of the major state peak in the absence of the exchange period (and the weak RF field), and the CEST profile is a graph of I/I_0 at each offset position.

The Bloch-McConnell equations [64,66], which describe the trajectories of the x, y- and z-components of magnetization ($M_x$, $M_y$ and $M_z$) in the rotating frame during the exchange duration, are given by:

$$\frac{d}{dt} \begin{bmatrix} E/2 \\ M_x \\ M_y \\ M_z \\ M_A \\ M_B \\ M_A' \\ M_B' \end{bmatrix} = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 \\ 0 & -R_{AB} - k_{AB} & -\Omega_A & 0 & k_{BA} \\ 0 & \Omega_A & -R_{AB} - k_{AB} & -\omega_1 & 0 \\ 0 & 0 & R_{AB} & 0 & -R_{AB} - k_{BA} \\ 2R_{AB}p_A M_B & 0 & 0 & 0 & 0 \\ 2R_{AB}p_B M_B & 0 & 0 & k_{AB} & 0 \end{bmatrix} \begin{bmatrix} E/2 \\ M_x \\ M_y \\ M_z \\ M_A \\ M_B \\ M_A' \\ M_B' \end{bmatrix}$$

(1.4)

where $\omega_1 = 2\pi B_1$ is the amplitude of the weak $B_1$ field applied along the x-axis in rad/s and $M_B$ is the equilibrium magnetization. $\Omega_A$ and $\Omega_B$ are the chemical shift offsets of states A and B (rad/s), related to the chemical shifts as:

$$\Omega_i = -\gamma B_0 (\sigma_i - \sigma_{cor}), i \in \{A, B\}$$

(1.5)

where $\sigma_{cor}$ is the carrier position in ppm at which the weak RF field is applied, $\gamma$ is the gyromagnetic ratio of the nucleus and $B_0$ is the magnetic field strength of the spectrometer. The magnetization at the start of the exchange duration is aligned along the z-axis. While the RF field serves to nutate this z-magnetization in the y-z plane, the exchange rate constants $k_{BA}$ and $k_{AB}$ couple the evolution of corresponding magnetization components from states A and B.

Let us consider the case where $\Delta \Omega = \Omega_A - \Omega_B \gg \omega_1$ and follow $M_{A'B'}$, which is a detected component, through the exchange duration. Since the RF radiation is weak, only peaks that are on-resonance or near-resonance to the offset position of the $B_1$ field are affected by it. When the $B_1$ field is applied far off-resonance, $M_A$ decays because of longitudinal relaxation with the rate constant $R_{1A}$, resulting in a baseline at a value

$$I/I_0 = \exp(-R_{1A}T_{ex}).$$

(1.6)

When the $B_1$ field is on-resonance to the major state, the magnetization of state A gets saturated and the $I/I_0$ value is close to 0. Factors contributing to this reduction in intensity include RF inhomogeneity, chemical exchange and a saturation effect that operates because $\omega_1^2 \gg R_1R_2$ (the typical case) as well as because of the sufficiently long lifetime of state A. When the $B_1$ field is on-resonance to the minor state, there is a reduction in $M_{A'}$ because of the stochastic transitions of molecules from state A to state B, and then back to state A. When a molecule in state A that initially contributes to $M_{A'}$ jumps to state B during $T_{ex}$, it is nutated by the $B_1$ field till it jumps back to state A. Though the average time spent by such molecules in state B is

$$\tau_B = \frac{1}{k_{BA}}$$

(1.7)

the actual time spent by a particular molecule is randomly distributed. Since the extent of nutation depends on this time, the phase angle for each molecule when it returns back to state A is different, and there is overall dephasing of the magnetization of state A; this results in a lowering of the $I/I_0$ value. CEST profiles quantifying $M_{A'}$ for a molecule in two-state exchange thus show two dips in intensity, a major dip at the chemical shift offset of state A, as well as a second minor dip in intensity at the chemical shift offset of state B (Fig. 2C). A key feature of CEST is that it amplifies the signatures of low populated conformations that are invisible in NMR spectra. This ability stems from the efficient dephasing of the major state magnetization by the weak RF field when it is on-resonance to the minor state chemical shift, which occurs to a much greater extent than the ratio $p_B/p_A$ [30].

Quantifying the intensity of an NMR-observable state while sweeping the offset of the weak RF field is a convenient way of carrying out CEST experiments; indeed, it is the only way available for the case of skewed populations, where only the major state is visible. Plots of $I/I_0$ vs offset generated from this mode of data acquisition are powerful probes of ‘invisible’ conformations (Fig. 2C). However, when states A and B are both observable in the NMR spectrum, CEST experiments can also be implemented by irradiating at a single offset frequency on-resonance to either state A or state B, and detecting the entire NMR spectrum. In this second method of performing CEST, the intensities of both states A and B are expected to decrease in the presence of the weak RF field (Fig. 2E). In general, single-offset irradiation is a quick way to ascertain the presence of conformational exchange, while acquiring complete CEST profiles for all the NMR-observable states is crucial for extracting populations and lifetimes of the states participating in the exchange process.

2.1.3. Only one conformation of folinic acid is binding-competent

Folic acid exists in two conformations, I and II, that differ in the orientations of the proton and the carbonyl oxygen about the N-C bond, which has partial double bond character that restricts free rotation (Fig. 2D). The aldehydic proton resonates at 4.88 and 4.16 ppm in forms I and II, respectively (Fig. 2E, top), and the exchange between these two forms is slow on the NMR chemical shift timescale [59]. As a consequence, distinct peaks are seen for each of the forms in the $^1$H 1D spectrum. When DHFR is added to folinic acid, the aldehydic proton of the enzyme-bound form shows a distinct resonance at 5.19 ppm (Fig. 2E, top) and the DHFR-folinic acid complex is also in slow exchange with the two free forms.

In order to determine which form of folinic acid binds DHFR (or if
First, saturation transfer is carried out at a single offset on-resonance to identify the bound state peak and the effects are directly detected through a 1D NMR difference spectrum (Fig. 2E, bottom). In turn, the results of the saturation transfer experiment imply that binding of folinic acid to the bound state only to form I on the timescale of the exchange duration. Accordingly, the perturbation of the bound form by the weak RF field is transferred (Fig. 2E, middle). Intriguingly, there is a concomitant reduction in the intensity of free form I, but not of free form II, clearly demonstrating that only form I of folinic acid binds DHFR (see panel D also).

There are a number of noteworthy features in this pioneering study that have a bearing on the use of NMR methods to dissect mechanism. First, saturation transfer is carried out at a single offset on-resonance to the bound state peak and the effects are directly detected through a 1D NMR difference spectrum. Such a strategy works only because both the free conformations, as well as the bound form, are visible in the NMR spectrum and their intensities can be reliably and independently quantified. In a protein-protein or protein-ligand interaction such as the DHFR-folinic acid interaction. This ensures that the perturbation of the bound-state magnetization is selectively transferred only to the conformation that is in direct exchange with it. If the two forms of folinic acid interconvert at rates comparable to or faster than binding to DHFR, the effect of RF irradiation on the bound state would be observed as reduction in intensities of the peaks of both forms I and II. While such a scenario complicates the interpretation of the saturation transfer experiment, it is possible to modulate the exchange duration so that interconversion between the two free conformations occurs only to a limited extent, as long as $k_{on} > k_{off}$ DHFR, where $k_{on}$ is the association rate constant for the DHFR-folinic acid interaction. This ensures that the perturbation of the bound-state magnetization is selectively transferred only to the conformation that is in direct exchange with it. If the two forms of folinic acid interconverted at rates comparable to or faster than binding to DHFR, the effect of RF irradiation on the bound state would be observed as reduction in intensities of the peaks of both forms I and II. While such a scenario complicates the interpretation of the saturation transfer experiment, it is possible to modulate the exchange duration so that interconversion between the two free conformations occurs only to a limited extent, as long as $k_{on} < k_{off}$. The result of a complete loss of intensity of that peak, as well as partial loss of intensity of the free form folinic acid from DHFR is shown in a complete loss of intensity of that peak, as well as partial loss of intensity of the free form folinic acid from DHFR form folinic acid form I. (Bottom) The saturation transfer difference spectrum shows only the peaks from free form I and DHFR-bound folinic acid, clearly demonstrating that only form I of folinic acid interacts with DHFR (see panel D also).

both forms bind equally well), the authors irradiated the resonance of the bound form using a weak RF field. Upon irradiation, the resonance from the bound state is saturated and disappears from the spectrum (Fig. 2E, middle). Intriguingly, there is a concomitant reduction in the intensity of free form I, but not of free form II, clearly demonstrating that the perturbation of the bound form by the weak RF field is transferred only to form I on the timescale of the exchange duration. Accordingly, only the resonances from the bound form and free form I are observable in the difference spectrum (Fig. 2E, bottom). In turn, the results of the saturation transfer experiment imply that binding of folinic acid to DHFR occurs primarily via conformation I.

There are a number of noteworthy features in this pioneering study that have a bearing on the use of NMR methods to dissect mechanism. First, saturation transfer is carried out at a single offset on-resonance to the bound state peak and the effects are directly detected through a 1D NMR difference spectrum. Such a strategy works only because both the free conformations, as well as the bound form, are visible in the NMR spectrum and their intensities can be reliably and independently quantified. In a protein-protein or protein-ligand interaction such as the DHFR-folinic acid complex studied here, it is usually possible to adjust the concentrations of the protein and the ligand such that the bound form has comparable intensity to the free form. However, if one of the conformations of the free ligand has higher free energy (invisible state, excited state) and is not visible in the NMR spectrum, we would have to first detect the invisible state of the ligand via a CEST profile, and then utilize the changes in the CEST profile upon addition of the protein to elucidate the mechanism. The second pivotal factor crucial to the success of this strategy is that the two free folinic acid conformations interconvert slowly between two conformations I and II that differ in the stereochemistry about the N-CHO bond and each conformation could, in principle, bind the enzyme DHFR. E) Schematic representation of the NMR results from the study of Burgen and coworkers. (Top) 1D $^1$H NMR spectrum of a sample containing a mixture of folinic acid and DHFR. Only the aldehydic proton in folinic acid forms I and II, as well as in the DHFR-bound state of folinic acid are shown at their approximate resonance positions. (Middle) Saturation of the DHFR-bound state resonance of folinic acid (green arrow) results in a complete loss of intensity of that peak, as well as partial loss of intensity of the peak from folinic acid form I. (Bottom) The saturation transfer difference spectrum shows only the peaks from free form I and DHFR-bound folinic acid, clearly demonstrating that only form I of folinic acid binds DHFR (see panel D also).
the limiting case where $k_{\text{int}} >> k_{\text{ex}}[L]$, the free states of the ligand exchange much faster than their rate of binding to the protein, with the result that the protein sees only an ‘average’ conformation of the ligand and it becomes impossible to determine which form of the ligand binds. Finally, the saturation transfer experiment itself does not provide information about the conformation of folinic acid in the DHFR-bound state and cannot be used in isolation to determine whether the mechanism of binding is conformational selection or induced fit. However, if structural information is available on the ligand-protein complex, it will be possible to state with certainty whether the flux of folinic acid from form I to the DHFR-bound form entails a change in ligand conformation.

2.2. Case study 2: conformational selection by the Hsp70 chaperone system

More recently, Kay and coworkers have examined the mechanism by which the Hsp70 chaperone binds substrates using CEST and magnetization transfer experiments [60]. The Hsp70 chaperone system is a quality control module that is ubiquitously present and highly conserved in organisms ranging from bacteria to humans [67–70]. Hsp70 is a 70 kDa protein that interacts with misfolded and unfolded substrate proteins, and helps them to reach their native folded conformation. Structures of the Hsp70-substrate complex have shown that the substrates are in an unfolded form when bound to Hsp70 [71–75].

2.2.1. Choice of model substrates for studying the Hsp70-client interaction

In this study, the authors explored whether Hsp70 directly binds to the unfolded conformation (U) of its substrates (the holo- or CS mechanism), or if it binds to the native conformation (N) and unfolds the substrate (the unfoldase or IF mechanism) (Fig. 3A). Two proteins, the L90A mutant of the 17th domain of chicken-brain α-spectrin (referred to as R17) [76], and the SH3 domain of the Enhancer of sevenless 2B protein from Drosophila melanogaster (referred to as SH3) [77], were chosen as model substrates for three reasons. First, both proteins are marginally stable and populate the unfolded state significantly at equilibrium; this facilitates the reliable quantification of the resonance intensities of both the folded and unfolded substrate conformations in NMR spectra. Second, both substrates fold slowly ($k_{\text{on}} < 1 \text{ s}^{-1}$), while binding to Hsp70 occurs on the timescale of 10–100 of milliseconds [78]. Since binding occurs much faster than interconversion between U and N, it will be possible to use these substrates to discriminate whether the native state or the unfolded state (or both) binds Hsp70. Third, R17 and SH3 have very different native structures; R17 is a three-helix β-barrel fold [80]. The choice of model substrates with disparate structures helps the authors to evaluate whether the mechanism can vary depending on the structure of the native conformation.

2.2.2. Conformational selection of the unfolded state of SH3 by Hsp70

Fig. 3B shows the $^{13}$C–$^1$H HMQC spectrum of 250 µM IM$^{13}$CH$_3$ labeled SH3 acquired in the presence of 500 µM U–$^2$H Hsp70. Deuteration and methyl labeling were employed to leverage the methyl-TROSY effect [54], which is immensely beneficial for NMR studies of large macromolecular systems such as the 77 kDa Hsp70-substrate complex [56]. Distinct resonances are observed for Ile27 in the native, unfolded and Hsp70-bound conformations of SH3 in the $^{13}$C–$^1$H HMQC spectrum of this sample (Fig. 3B), while the methyl-$^1$H CEST profiles for N and U are shown in Fig. 3C and 3D, respectively. Both CEST profiles exhibit a major dip in intensity located at the methyl-$^1$H chemical shift of Ile27 in that conformation. The CEST profile for the native state shows a small additional dip in intensity at the chemical shift of the unfolded state (~0.75 ppm, green dotted line) (Fig. 3C) that arises from exchange between U and N ($k_{\text{ex},\text{UN}} = 1.6 \text{ s}^{-1}$). The presence of exchange between U and N is confirmed from the CEST profile of U, which shows a small dip in intensity at the chemical shift of N (~0.55 ppm, red dotted line) (Fig. 3D). Importantly, the CEST profile of U shows a third prominent dip in intensity at the chemical shift of Ile27 in the Hsp70-bound state (~0.2 ppm, blue dotted line), indicating that there is conformational exchange between the unfolded form of SH3 and the Hsp70-bound form. The presence of this third dip in the CEST profile of U but not in the CEST profile of N (cyan arrow in Fig. 3C) confirms that Hsp70 binds its substrate through its unfolded and not its native conformation. Taken together with the observation that the substrate is unfolded in the chaperone-bound complex, the results from CEST demonstrate that the Hsp70–substrate interaction occurs via a conformational selection or the unfoldase mechanism.

The mechanism of complex formation between Hsp70 and R17 is probed through a different NMR experiment called magnetization exchange [81], in which the interconversion of conformations results in cross-peaks in 2D correlation spectra (Fig. 3E, 3F). A brief description of the spin dynamics underlying the magnetization transfer experiment is provided below.

2.2.3. Methodology: magnetization transfer

Consider a system in two-state exchange (Fig. 2A) with parameters defined as in Section 2.1.1. In the magnetization transfer experiment shown in Fig. 3E, $^1$H coherences are excited and transferred to $^{13}$C by chemical shift labeling during the $t_1$ period. At the end of $t_1$, product operators of the form

$$C_{\text{ex}} \cos(\Omega_{\text{ex}}t_1) + C_{\text{ex}} \cos(\Omega_{\text{ex}}t_1)$$

(1.8)

are generated where $\Omega_{\text{ex}}$ and $\Omega_{\text{ex}}$ are the carbon chemical shift offsets of states A and B, respectively. Subsequent to the $t_1$ period, carbon magnetization is stored along the $z$-axis as $C_z$ (though two-spin order $2H_C$ is also occasionally used) for an exchange duration $T_{\text{ex}}$. During $T_{\text{ex}}$, $C_z$ decays back to equilibrium with a rate constant $R_1$. In the presence of exchange, a fraction of $C_{\text{ex}}$ ($f(T_{\text{ex}})$, which is a function of the exchange duration), gets converted to $C_{\text{ex}}$. At the end of $T_{\text{ex}}$, we get from the first term derived from Eq. (1.8):

$$C_{\text{ex}} \cos(\Omega_{\text{ex}}t_1) \frac{T_{\text{ex}}}{1 - f(T_{\text{ex}})} C_{\text{ex}} \cos(\Omega_{\text{ex}}t_1) + f(T_{\text{ex}})C_{\text{ex}} \cos(\Omega_{\text{ex}}t_1)$$

(1.9)

Crucially, the $C_{\text{ex}}$ magnetization that is generated during $T_{\text{ex}}$ as a consequence of conformational exchange is labeled with the chemical shift of A during $t_1$. At this juncture, carbon magnetization is converted to $^1$H coherence for detection. During the detection period $t_2$, the first term will evolve with the $^1$H chemical shift of A ($\Omega_{\text{ex}}$) and result in a diagonal peak at chemical shifts ($\Delta t_1$, $\Delta t_2$), while the second term of Eq. (1.9) will evolve with the $^1$H chemical shift of B ($\Omega_{\text{ex}}$) and generate a cross-peak between states A and B at chemical shifts ($\Delta t_1$, $\Delta t_2$). Similar diagonal and cross-peaks at chemical shifts of ($\Omega_{\text{ex}}$, $\Omega_{\text{ex}}$) and ($\Omega_{\text{ex}}$, $\Omega_{\text{ex}}$) respectively will be formed from the second term of Eq. (1.8).

The fingerprint of conformational exchange in a magnetization transfer experiment thus lies in the observation of cross-peaks between correlations belonging to distinct molecular conformations. The intensities of the diagonal and cross-peaks (as well as the fraction $f(T_{\text{ex}})$) depend upon the longitudinal relaxation rate constants of the two states as well as the rate constants governing their interconversion during $T_{\text{ex}}$, through the Bloch-McConnell equations:

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Fig. 3. Hsp70 binds to its substrates R17 and SH3 via a process of conformational selection [60]. (A) Hsp70-substrate complexes (Bound, blue) maintain the substrate protein in an unfolded conformation. These complexes can be formed either by a direct interaction of the unfolded client protein with Hsp70 (CS, called the holdase model in Hsp70 literature), or the binding of Hsp70 to the native protein followed by its unfolding (IF, called the unfoldase model). (B) The Ile region of the $^{13}$C-$^1$H HMQC spectrum of a sample containing 250 µM IM-$^{13}$CH$_3$ SH3 and 500 µM U-$^2$H Hsp70. Peaks from the native (N, red), unfolded (U, green) and Hsp70-bound forms of SH3 (B1-B4, cyan) are labeled. (C,D) $^1$H CEST profiles of the δ1 methyl proton of Ile27 in the native (C) and unfolded (D) states. Only the CEST profile of U shows a minor dip at the chemical shift of the Hsp70-bound peak, while there is no dip at the corresponding position in the CEST profile of N (cyan arrow), demonstrating that Hsp70 selects the unfolded state for binding. (E) The Met-region of the $^{13}$C-$^1$H HMQC spectrum of a sample containing ILVM-$^{13}$CH$_3$ labeled R17 (250 µM) and U-$^2$H Hsp70 (500 µM), showing resonances from Met26 and Met87 in the U and N conformations, as well as a resonance from Hsp70-bound R17. (F) $^{13}$C-$^1$H 2D magnetization exchange spectrum of the same sample showing cross-peaks between the diagonal peak of Met26 in the unfolded state and the diagonal peak from the Hsp70-bound state. Cross-peaks at the corresponding positions for the diagonal peak of Met26 in the native state are absent (cyan arrows), again indicating that only the unfolded conformation is binding-competent (also see panel A). Panels A-F modified with permission from Sekhar et al. [60].
$$\frac{d}{dt} \left[ \begin{array}{c} M_A \\ M_B \end{array} \right] = \left[ \begin{array}{cc} -R_{AA} - k_{AB} & k_{AB} \\ k_{AB} & -R_{BB} - k_{BA} \end{array} \right] \left[ \begin{array}{c} M_A \\ M_B \end{array} \right]$$  \hspace{1cm} (1.10)$$

2.2.4. The R17-Hsp70 interaction proceeds via a conformational selection pathway

Fig. 3E shows the methionine region of a $^{13}$C-$^1$H HMQC spectrum of 250 μM ILVM-$^{13}$CH$_3$ R17 containing 500 μM U-$^2$H Hsp70. Distinct resonances for Met26 and Met87 in the unfolded and native states of R17, and a resonance from Hsp70-bound R17, are seen clearly in the HMQC spectrum. Fig. 3F shows a 2D magnetization transfer spectrum acquired with an exchange duration of 300 ms. There is no observable cross-peak between the native and unfolded state correlations of Met26 and Met87, highlighting that the U-N interconversion time constant is much longer than $T_{1N}$. On the other hand, cross-peaks between the unfolded state correlation of Met26 and the Hsp70-bound correlation are clearly discernible, demonstrating that R17 U is in direct conformational exchange with the R17-Hsp70 complex. Similar cross-peaks between R17 N and the bound state correlation are not detectable, confirming that the formation of the R17-Hsp70 complex occurs primarily via the conformational selection mechanism.

NMR spectroscopic methods such as CEST and magnetization transfer can thus provide unequivocal insights into the mechanisms underlying biomolecular interconversion. The qualitative statements noted here can be quantified by calculating the flux of molecules traversing each pathway [12,60]. These calculations reveal that the flux along the conformational selection pathway is at least 113-fold higher for R17 and 4-fold higher for SH3 compared to the induced fit pathway. When resonances for all the exchanging conformations are visible in the 2D correlation spectrum, the magnetization transfer experiment is generally the method of choice because it takes lesser time to acquire a complete magnetization transfer dataset compared to a CEST experiment. However, the real utility of CEST lies in its ability to characterize ‘invisible’ conformations that cannot be robustly detected above noise level in NMR spectra. In the case of SH3, the CEST experiment was used because there is a degeneracy in $^{13}$C chemical shifts of Hsp70-bound SH3 and the native state resonances of Ile4 and Ile27 (Fig. 3B), which would result in an overlap of potential cross-peaks between the different states in a magnetization transfer spectrum. $^1$H CEST was preferred over $^{13}$C CEST, not only because there are sizeable chemical shift differences in the $^1$H dimension between resonances arising from U, N and Hsp70-bound SH3, but also because the version of the $^1$H CEST pulse sequence used here maintains the methyl-TROSY effect which is essential for acquiring high-sensitivity CEST datasets for such large biomolecular systems. While $^1$H CEST profiles generally contain interfering dips in intensity arising from $^1$H-$^1$H dipolar cross-relaxation [31,82], such artifacts are not present in these profiles because SH3 is perdeuterated and has sparse $^4$H density, with only Ile61 methyl groups and Met residues possessing protons.

2.3. Case study 3: an off-pathway intermediate in the folding free energy landscape of an acyl carrier protein

The strategies employed in case studies 1 and 2 closely resemble each other because NMR spectral resonances belonging to all the relevant conformational states in both the studies could be resolved and quantified reliably. However, the existence of a conformational selection pathway can be easily missed because the conformation that is selected for binding is higher in free energy by a few kcal/mol, and therefore is not sufficiently populated to be visible in NMR spectra. Similarly, on- and off-pathway folding intermediates present in protein free energy landscapes are often invisible in 2D correlation NMR spectra because they are sparsely populated and have μs-ms lifetimes. Therefore, structurally characterizing these intermediates and determining whether they facilitate protein folding or represent off-pathway species remains a challenge.

In this third case study [61], Yang and coworkers exploit CEST to detect an intermediate of the Micromonospora echinospora acyl carrier protein (meACP), present at an equilibrium population of 0.7% and coexisting with a native (p$_N$ = 95.2%) state, as well as an unfolded state (p$_U$ = 4.1%). meACP is a vital part of the fatty acid biosynthesis pathway and is responsible for transferring acyl intermediates between catalytic domains of fatty acid synthase and polyketide synthase [83–85]. It is a 93-residue protein domain that adopts a three-helix bundle topology in the native state [85]. In $^{15}$N CEST data acquired by the authors, 34 residues of meACP had two prominent intensity dips in their CEST profiles. Fitting these data to the Bloch-McConnell equations for two-state exchange gave a population of 4.7% for the minor conformation and the chemical shifts of the minor conformation correlated well with the values expected for an unfolded state.

Intriguingly, 5 residues including Ala997 (Fig. 4) showed three dips in intensity, including one at the chemical shift of the major state (N) and one at the chemical shift expected for the unfolded state (U). The third dip in intensity is assigned to an intermediate, with the key question being whether this intermediate is on-pathway between N and U, or off-pathway. In order to address this question, the authors fit the residues displaying three dips to three different three-state models (I–III, Fig. 4), two where the intermediate is off-pathway (I,II) and one where it is on-pathway between U and N (III). While the two off-pathway models fit the CEST data equally well, fits with the on-pathway model are substantially worse with almost 20-fold higher $\chi^2$ values. In addition, clear systematic deviations are observed between the data and the fit.
(Fig. 4C, cyan boxes) indicating that Model III is a poor representation of the CEST data and confirming that the intermediate is an off-pathway conformation.

Modeling data with the Bloch-McConnell equations can provide unambiguous statements on the mechanism of conformational interconversion, as seen in this case study. However, fitting CEST, CPMG RD and R_{1p} RD data to three-state models is fraught with complications and elaborate grid searches must often be undertaken to ensure that the minimization routines do not get trapped in local minima in the parameter surface [86–93]. In this particular report, fitting to a three-state model is facilitated by the fact that there are several residues with three minor dips in their CEST profile, a clear signature of the co-existence of three conformations in equilibrium.

2.4. Case study 4: Double Resonance Dante CEST (DRD-CEST) as a way to distinguish sequential and parallel exchange mechanisms

One of the limitations of brute force fitting of CEST profiles to three-state models of conformational exchange seen in the case study on meACP is that the analysis is unable to distinguish between the N→U→I and I→N→U models. There is a critical distinction between these two models as seen from the perspective of the major state N; while the N→U→I corresponds to a sequential reaction pathway, I→N→U consists of two parallel reactions with N serving as the branching point. Vallurupalli and coworkers have taken advantage of this distinction to develop the DRD-CEST method (Fig. 5A) as a way to evaluate whether multisite conformational transitions are occurring via sequential or parallel pathways [62].

2.4.1. Methodology: DRD-CEST

DRD-CEST builds upon the multi-frequency irradiation capability afforded by DANTE-CEST (D-CEST). D-CEST was originally developed as a way to reduce CEST measurement times by simultaneously irradiating multiple chemical shift offsets [32]. In D-CEST, the continuous wave irradiation used during the exchange time T_{ex} is replaced by the DANTE selective excitation scheme [94,95], which consists of short high power pulses applied at intervals of τ. The frequency domain excitation profile, which can be inferred from the Fourier Transform of the DANTE pulse train [32,95], consists of excitations at offsets separated in frequency by s w_{CEST} = \frac{1}{τ} and having a bandwidth at each excitation site proportional to 1/T_{ex}.

Consider a protein exchanging between a major state N and two invisible states U and I, with all three states showing distinct dips in the CEST profiles of one or more residues (Fig. 5A,B). The regular CEST or D-CEST is acquired typically by irradiating one of these dips at a time (state I in Fig. 5A and B) and monitoring the intensity of the major state N. The RF field applied at the chemical shift offset of I will result in dephasing of N-state magnetization that jumps to state I during T_{ex} and the extent of dephasing will depend on T_{ex}, B_{1} and the lifetime of state I.

The aim of DRD-CEST is to distinguish between the sequential model N→I→U and the branching model I→N→U (Fig. 5C). As the first step, a sufficiently large B_{1} field is chosen that satisfies the condition 2πB_{1}τ > 1/τ. If this condition is met, magnetization that arrives at I from N is completely dephased before leaving I. Next, the spacing between the DANTE pulses is adjusted so that both U and I are simultaneously irradiated. If conformational interconversion follows the sequential Model I (N→I→U, Fig. 5C top), irradiation at both I and U will not cause a significant decrease in intensity compared to irradiation at only I. This is because dephasing is already complete before N-magnetization leaves state I. On the other hand, if the exchange occurs according to the parallel Model II (I→N→U, Fig. 5C bottom), simultaneous irradiation at I and U will cause additional loss of intensity compared to irradiation at only I. This is because I and U exchange independently with N, so that different sub-ensembles of spins in the N conformation arrive at the I and U states, and dephasing occurring at state I will not deter dephasing at state U. DRD-CEST thus helps in model discrimination by furnishing
data that will be measurably different for the sequential and parallel reaction pathways.

2.4.2. Parallel pathways of global and partial unfolding in the free energy landscape of T4 lysozyme (T4L)

Vallurupalli and coworkers used DRD-CEST to derive mechanistic information on the folding of T4L at 50 °C [62]. CEST profiles of several residues from the N-terminal domain of T4L show three dips in intensity, indicating that the major native state of T4L (N) coexists with at least two other minor conformations (Fig. 5B). Similar to Case Study 3, the chemical shifts of one of the conformations matches very well with random coil chemical shifts, making it possible to assign this conformation as the unfolded state. The other minor conformation is an intermediate (I), with chemical shift changes between N and I localized to residues 20–40 in the N-terminus of T4L.

The fits of CEST data acquired using the DANTE-CEST pulse sequence to the N→U→I model are significantly worse ($\chi^2 = 2.2$) than the fits to I→N→U ($\chi^2 = 1$) or N→I→U ($\chi^2 = 1$) models and rule out the possibility that the native state has to unfold in order to interconvert with I. In order to distinguish between the bifurcating I→N→U and sequential N→I→U models, the authors carried out DRD-CEST by irradiating the sample at chemical shift offsets of both the I and U states. The size of the dip belonging to state I in the DRD-CEST profile increases significantly (I0/I0) from the size in the D-CEST data, confirming that the I→N→U model is the appropriate one (Fig. 5B; also compare CEST profiles in Fig. 5D–G). Consistent with this conclusion, D- and DRD-CEST data together can be fit better to a I→N→U model than a N→I→U model, unequivocally establishing that the unfolding of T4L can happen without populating the intermediate I (Fig. 5D–G).

DRD-CEST is the latest addition to the NMR toolbox for deciphering how biomolecules interconvert between conformations. It has the remarkable capacity to distinguish between different pathways of three-site exchange even in cases where two of the conformers are invisible and cannot directly be observed in NMR spectra. The recent development of this novel DRD-CEST method emphasizes not only the continuing need for innovative new pulse sequences in NMR spectroscopy that can improve our understanding of biomolecular transitions governing function and malfunction, but also the active progress that has consistently pushed the frontiers of this area of biomolecular dynamics.

2.5. Case study 5: recoverin binds rhodopsin kinase via a conformational selection mechanism

The case studies discussed so far have all used NMR spectroscopy as the sole investigative tool because of the potential of NMR methods for providing structural and kinetic information. However, NMR can also be synergistically combined with other experimental methods that can be used to measure rate constants, such as time-resolved FRET, stopped-flow fluorescence or CD spectrophotometry, and hydrogen exchange approaches [96–104].

In this final case study, we will see how CPMG RD NMR (see Methodology section below) is used to establish the existence of an alternate higher energy conformation, while fluorescence-detected stopped-flow data confirm that it is indeed this invisible conformation which is preferentially selected for binding by the partner protein.

2.5.1. Methodology: Carr-Purcell-Meiboom-Gill relaxation dispersion (CPMG RD)

In the CPMG RD experiment [39,105], transverse magnetization is subjected to a $τ_{CP}-180^\circ-τ_{CP}$ pulse train, typically during a constant relaxation time $T_{ex}$. The CPMG frequency, $\nu_{CPMG}$, is defined as:

$$\nu_{CPMG} = \frac{1}{4τ_{CP} + 2pw_{180}}$$

where $pw_{180}$ is the 180° pulse width. The transverse magnetization remaining at the end of the CPMG pulse train is quantified as a function of $\nu_{CPMG}$ by fitting $T_{ex}$, through the residue-specific peak intensity (I) and the effective transverse relaxation rate during the pulse train is calculated as:

$$R_{2,eff} = \frac{1}{T_{ex}} \ln \left( \frac{I_0}{I} \right)$$

where $I_0$ is the peak intensity in a sequence where $T_{ex}$ is absent. In the absence of conformational exchange, chemical shift evolution is refocused by the CPMG pulse sequence and magnetization merely relaxes according to its transverse relaxation rate constant; therefore, there is no dispersion or variation in $R_{2,eff}$ with increasing $\nu_{CPMG}$.

In order to understand the origin of dispersions for a system undergoing conformational exchange, consider the two-state system shown in Fig. 2A. Let us assume without loss of generality that the transmitter is placed at the chemical shift of the ground state, so that there is no time evolution of ground state transverse magnetization in the rotating frame due to its chemical shift ($σ_A = 0$). Stochastic transitions of a molecule from state A to state B will change the chemical shift of the spin to $σ_B$ and the corresponding magnetization vector will undergo phase evolution during the $τ_{CP}$ period (Fig. 6A). Since the conformational trajectory followed by every molecule during $T_{ex}$ is different owing to the stochastic nature of conformational exchange, magnetization vectors at the end of $T_{ex}$ are characterized by a distribution of phases in the transverse plane, giving rise to an effective reduction in magnetization and an increase in $R_2$ originating solely from conformational exchange (exchange broadening) (Fig. 6A).

The 180° pulses placed in the middle of every $τ_{CP}$-180°-τ_{CP} element in the CPMG train refocus this chemical shift evolution. As the frequency of these 180° pulses is increased, refocusing becomes more efficient and phase evolution is suppressed, so that magnetization vectors are more tightly clustered and diverge less compared to their counterparts at low $\nu_{CPMG}$ (Fig. 6A). The CPMG pulse train thus serves to quench exchange broadening; dispersion profiles, which are graphs of $R_{2,eff}$ vs $\nu_{CPMG}$, are sensitive to $κ_{ex}$ $p_B$ and $Δσ$ ($= σ_B − σ_A$) of the conformational exchange process (Fig. 6B).

2.5.2. Dissecting the binding mode of the recoverin-rhodopsin kinase interaction

Rhodopsin kinase (RK) is a Ser/Thr kinase that phosphorylates light-activated rhodopsin molecules in retinal rod cells to terminate the photoactivated signaling cascade [106]. Recoverin is a Ca^{2+}-binding protein which inhibits the kinase activity of RK and helps in maintaining the high sensitivity of rod cells for low ambient light [107]. Recoverin interacts with the N-terminal helix of RK, and the conformations of apo and RK-bound recoverin differ in the arrangement of the backbone as well as in the local RK-binding pocket [108–110].

In this report [63], Kern and coworkers examine whether the conformational changes in recoverin occur before (CS) or after RK binding (IF) (Fig. 6C). The first requirement for CS is the existence of a conformation that structurally resembles the protein-protein complex. The authors surveyed for this complex by acquiring CPMG RD data, which have the potential to pick up sparsely and transiently populated states in equilibrium with the major conformation. The dispersion of $R_2$, $κ_{ex}$...
Fig. 6. A combination of CPMG RD NMR and stopped-flow fluorescence spectrophotometry reveals the operation of the conformational selection mechanism in the rhodopsin kinase-recoverin interaction [63]. (A) The basic principle underlying the CPMG RD method. Phase evolution of individual magnetization vectors in the presence of conformational exchange at five CPMG pulsing frequencies indicated at the top of the plots and increasing from left to right. The schematic of the CPMG pulse train is also shown on the right of each plot. Magnetization vectors are started in state A with a phase of 0 and the transmitter offset is placed at $\Omega_A$. The vertical axis corresponds to time-evolution over 60 ms. Histograms depicting the distribution of the final phase of the magnetization vectors is shown at the bottom of each time-evolution plot. The width of this distribution is a measure of the $R_*^{2,\text{eff}}$, which includes a significant component from exchange broadening. (B) CPMG RD profiles graphing $R_*^{2,\text{eff}}$ as a function of $\nu_{\text{CPMG}}$. The points for which phase evolution is shown in panel A are indicated as colored circles with the same color scheme as panel A. When there is no conformational exchange, the CPMG RD profile is flat. (C) Recoverin adopts different structures in the absence (top left) and presence (bottom right) of rhodopsin kinase. The conformational selection and induced fit pathways are shown along with the intermediates in each pathway. (D) CPMG RD profiles of residues 9, 130, and 201 of free recoverin acquired at 600 MHz (closed squares) and 900 MHz (open squares), field strengths, clearly showing the existence of a minor conformation. (E) CPMG RD profiles of rhodopsin kinase-bound recoverin acquired on a 900 MHz spectrometer are flat, indicating that the binding event quenches the conformational exchange. (F) Rate constants ($k_{\text{obs}}$) derived from stopped-flow fluorescence spectrophotometric measurements reporting on the binding of recoverin with rhodopsin kinase, as a function of the concentration of rhodopsin kinase. Solid red lines and dashed black lines are predictions from a CS or an IF model respectively. The CS model is able to correctly predict the experimentally observed decrease in $k_{\text{obs}}$ at low rhodopsin kinase concentrations, indicating that it is the operative model. Panels C–F modified with permission from Chakrabarti et al. [63].
eff observed in CPMG profiles clearly demonstrates the existence of a minor conformation (Fig. 6D) whose population was determined by fitting the profiles globally to a two-state model of exchange to be 3.2%. Crucially, chemical shifts differences obtained from CPMG data correlate well with the difference between the resonance positions of recoverin in the absence and presence of RK, indicating that a conformation structurally similar to the RK-bound state coexists with native recoverin. In contrast to the CPMG profiles of free recoverin, profiles of the RK-recoverin complex are flat, indicating the absence of conformational exchange (Fig. 6E). If the mechanism of binding was IF, dispersions reporting on the exchange between the free-like and full-bound forms of recoverin (see Fig. 1A, IF pathway, B and B) were not expected. While the absence of such dispersions do not eliminate the IF pathway, they provide support for the CS mechanism.

The second condition for CS to be operative is that binding must occur through this structurally similar conformation. Since evaluating this condition requires a knowledge of the kinetic rate constants for the binding interaction, the authors use the intrinsic tryptophan fluorescence of recoverin in a stopped-flow instrument to follow the time-course of interaction between recoverin and varying concentrations of RK. While stopped-flow kinetics traces are expected to be multi-exponential because both the CS and IF models involve more than one elementary step, data at all RK concentrations could be fit to a single exponential equation, likely because the different rate constants are not distinguishable within the S/N of the stopped-flow datasets. The observed rate constant (kobs) varies non-linearly with ligand concentration, decreasing at sub-stoichiometric and stoichiometric concentrations, and plateauing at high concentrations. This non-linear trend in kobs as a function of [RK] matches well with predictions from a CS model but not with those from the IF mechanism (Fig. 6F). While the IF model predicts an increase in kobs with [RK] at low ligand concentrations, the experimentally observed decrease in kobs is captured well only by the CS model, establishing that RK binds recoverin predominantly through the conformational selection pathway.

Whenever multiple methods are used to evaluate different aspects of the same mechanism, it is important to look for measures that highlight the consistency of experimental data between the various methods. In this report, the rate constant for the conversion of native recoverin into its binding-competent form is determined from CPMG RD NMR to be 35 s⁻¹ at 30 °C and 25 s⁻¹ at 10 °C. These rate constants agree very well with the limiting value of kobs at high ligand concentrations, which will be dominated by the contribution from the rate constant for the conversion between the native and higher energy conformers of recoverin.

3. Conclusions and outlook

The development of cutting-edge NMR methods in the last two decades has enabled the interrogation of mechanism in proteins as large as 100 kDa in molecular weight. In this review, we have used five case studies to pinpoint some of the experimental strategies available for probing how protein molecules transition between different structurally distinct states. These studies reveal the diversity of the NMR repertoire in providing mechanistic insights, as well as the exquisite degree of detail that can be obtained by acquiring NMR data containing both structural and kinetic information.

In addition to the protein-based research described here, elegant mechanistic studies have also been carried out on nucleic acids [111] and for identifying intermediates in chemical reactions [112]. Moreover, while we have focused our discussion on experimental techniques in this review, computational techniques have recently gained traction, not only because computational power has increased significantly in the recent past, but also because of the development of advanced sampling methods that allow for exhaustive search of conformational space during a protein molecular dynamics simulation [18,113–120]. The combination of NMR spectroscopy and molecular dynamics promises to be very fruitful for mechanistic studies on biomolecules.

A number of reports have also emerged on alternate conformations of proteins whose structures resemble functional ligand- or protein-bound states, suggesting that conformational selection may be a widespread mechanism governing biological activity [121–125]. Dihydrofolate reductase has been shown to populate higher energy structures closely resembling successive enzymatically active cofactor-bound states, raising the intriguing hypothesis that its enzymatic cycle operates by stabilizing pre-existing conformations in the enzyme free energy landscape [126]. The mature Zn-bound conformation of human superoxide dismutase already coexists with the metal-free immature form of the protein, indicating that metal-induced maturation occurs via a population shift mechanism [90,127]. The complex between the KIX domain of CREB binding protein and the MLL transcription factor allosterically activates the former for binding c-Myb by promoting the formation of a sparsely populated state which structurally resembles the c-Myb-bound form [128]. On the other hand, cGMP allosterically inhibits the binding of DNA to a mutant of catabolite activator protein (CAP) by destabilizing the higher energy binding-competent conformation of CAP [129].

Anfinsen’s thermodynamic hypothesis postulates that the native structure of a protein is encoded by its amino acid sequence [130]. There is now compelling evidence to show that a number of proteins populate alternate higher energy conformations in addition to the native state and many of these hidden states ‘look’ like functional ligand-bound conformations. These observations raise the question of whether ‘excited’ state structures are also encoded within a protein sequence, as well as whether evolutionary pressure operates to select for these functional higher energy conformations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] J.P. Changeux, S. Edelstein, Conformational selection or induced fit? 50 years of Koshland debate resolved, PloS Biol. Rep. (2011) 5(12) e1000034.

[2] J.P. Changeux, 50th anniversary of the word “allosteric, Protein Sci. 20 (7) (2011) 1119–1124.

[3] J. Monod, J.P. Changeux, F. Jacob, Allosteric proteins and cellular control systems, J. Mol. Biol. 10 (1963) 306–329.

[4] J. Monod, J. Wyman, J. Changeux, On the nature of allosteric transitions: a plausible model, J. Mol. Biol. 12 (1965) 88–118.

[5] J.P. Changeux, J. Thierry, Y. Tung, C. Kittel, On the cooperativity of biological membranes, Proc. Natl. Acad. Sci. U. S. A. 85 (2) (1987) 635–640.

[6] D.D. Boehr, R. Nussinov, P.E. Wright, The role of dynamic conformational ensembles in biomolecular recognition, Nat. Chem. Biol. 5 (11) (2009) 789–796.

[7] D. Koshland, Enzyme flexibility and enzyme action, J. Cell. Comp. Physiol. 54 (1960) 254–258.

[8] D. Koshland, G. Nemethy, D. Filmer, Comparison of experimental binding data and theoretical models in proteins containing subunits, Biochemistry 5 (1966) 365–385 (Mosc.).

[9] S. Jain and A. Sekhar (2022) Journal of Magnetic Resonance Open 10–11 (2022) 100034.

[10] G. Karunanithy, J. Reinstein, D.F. Hansen, Multiquantum chemical exchange saturation transfer NMR to quantify symmetrical exchange: application to Gln and Glu residues in a cavity mutant of T4 lysozyme, J. Am. Chem. Soc. 123 (5) (2001) 967–975.

[11] A.J. Baldwin, L.E. Kay, NMR spectroscopy brings invisible protein states into focus, Nat. Chem. Biol. 5 (2009) 808–814.

[12] A.G. Palmer, C.D. Kroenke, J.P. Loría, Nuclear magnetic resonance methods for quantifying microsecond-to-millisecond motions in biological macromolecules, Methods Enzymol. 339 (2000) 304–328.

[13] D.M. Kozhnev, V.Y. Orelkov, L.E. Kay, Off-resonance R1ρ NMR studies of exchange dynamics in proteins with low spin-lock fields: an application to a Fyn SH3 domain, J. Am. Chem. Soc. 127 (2) (2005) 713–721.

[14] X. Salvatella, C.M. Dobson, M. Vendruscolo, Protein structure and function: a T4 lysozyme mutant, Nature 477 (7362) (2011) 111–116.

[15] N. Greives, H.X. Zhou, Both protein dynamics and ligand concentration can shift data, Proc. Natl. Acad. Sci. U. S. A. 105 (12) (2008) 4685–4690.

[16] Y. Shen, et al., Conserved conformational selection mechanism of Hsp70 family member HtpG in the absence of ligands, J. Mol. Biol. 443 (2015) 13737–13753.

[17] G. Bouvignies, et al., Solution structure of a minor and transiently formed state of 23S rRNA, Nature 426 (2003) 395–399.

[18] J.E. Ollerenshaw, V. Tugarinov, L.E. Kay, Isotope imaging and correlation spectroscopy: Principles and Practice, Academic Press, 1995.

[19] J.P. Loria, M. Rance, A.G. Palmer, A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy, J. Am. Chem. Soc. 121 (10) (1999) 2331–2332.

[20] F.A. Mulder, N.R. Skymnyakov, B. Hon, F.W. Dahlquist, L.E. Kay, Measurement of slow (μs–ms) time scale dynamics in proteins side chains by 15N exchange dispersion NMR spectroscopy: application to Asn and Gln residues in a cavity mutant of T4 lysozyme, J. Am. Chem. Soc. 125 (3) (2001) 967–975.

[21] A.G. Palmer, C.D. Kroenke, J.P. Loría, Nuclear magnetic resonance methods for quantifying microsecond-to-millisecond motions in biological macromolecules, Methods Enzymol. 339 (2000) 304–328.

[22] J. Iwahara, C.D. Schwieters, G.M. Clore, Ensemble approach for NMR structure refinement against 1H paramagnetic relaxation enhancement data arising from a transiently formed macrospin, J. Am. Chem. Soc. 132 (28) (2010) 10197–10202.

[23] Y. Jiang, C.G. Kalodimos, NMR studies of large proteins, J. Mol. Biol. 429 (17) (2010) 3492–3509.

[24] S. Gianni, M. Brunori, C. Travaglini-Allocatelli, Plasticity of the protein folding landscape: switching between on- and off-pathway intermediates, Arch. Biochem. Biophys. 466 (2) (2007) 172–176.

[25] R.L. Baldwin, On-pathway versus off-pathway folding intermediates, Fold. Des. 1 (1) (1996) R1–R8.

[26] G.M. Clore, Practical aspects of paramagnetic relaxation enhancement in biomolecular NMR spectroscopy, J. Magn. Reson. 70 (1987) 335–347.

[27] G.M. Clore, J. Biemann, A. Velyvis, A.M. Ruschak, L.E. Kay, An economical method for production of 2H, 13C, and 15N labeled proteins, Chem. Rev. 106 (5) (2006) 2019–2036.

[28] Y. Shen, et al., Consistent blind protein structure generation from NMR chemical shift data, Proc. Natl. Acad. Sci. U. S. A. 105 (2) (2008) 6085–6089.

[29] A. Cavalli, X. Salvatella, C.M. Dobson, M. Vendruscolo, Protein structure determination from NMR chemical shifts, Proc. Natl. Acad. Sci. U. S. A. 104 (23) (2007) 9613–9620.

[30] K. Chen, N. Tjadra, The use of residual dipolar coupling in studying proteins by NMR. NMR of Proteins and Small Biomolecules, Springer, 2011, pp. 47–67.

[31] N. Tjadra, A. Bax, Solution NMR measurement of amide proton chemical shift anisotropy in 15N-enriched proteins. Correlation with hydrogen bond length, J. Amer. Chem. Soc. 129(34) (2007) 8877–8878.

[32] R.S. Liptiz, N. Tjadra, Carbonyl CSA constraints from solution NMR for protein structure refinement, J. Am. Chem. Soc. 123 (44) (2001) 11065–11066.

[33] A.G. Palmer, N. Tjadra, Use of 15N chemical shift anisotropy in protein structure refinement and comparison with NH residual dipolar couplings, J. Magn. Reson. 164 (1) (2003) 171–176.

[34] J. Iwahara, C.D. Schwieters, G.M. Clore, Ensemble approach for NMR structure refinement against 1H paramagnetic relaxation enhancement data arising from a flexible paramagnetic group attached to a macromolecule, J. Am. Chem. Soc. 126 (18) (2004) 5879–5896.

[35] G.M. Clore, Practical aspects of paramagnetic relaxation enhancement in biomolecular NMR, Methods Enzymol. 364 (2015) 485–497.

[36] A. Sekhar, L.E. Kay, An NMR view of protein dynamics in health and disease, Annu. Rev. Biophys. 48 (2019) 297–319.

[37] P. Vallurupalli, G. Bouvignes, L.E. Kay, Studying “invisible” excited protein states in slow exchange with a major state conformation, J. Am. Chem. Soc. 134 (19) (2012) 8148–8161.

[38] P. Vallurupalli, A. Sekhar, T. Yuwen, L.E. Kay, Probing conformational dynamics in biomolecules via chemical exchange saturation transfer: a primer, J. Biomol. NMR 67 (4) (2017) 243–271.

[39] T. Yuwen, A. Sekhar, L.E. Kay, Separating dipolar and chemical exchange magnetization transfer processes in 1H-15N-CEST, Angew. Chem. Int. Ed. 56 (22) (2017) 6122–6125.

[40] T. Yuwen, L.E. Kay, G. Bouvignes, Dramatic decrease in CEST measurement times using multi-site excitation, ChemPhysChem 19 (14) (2018) 1707–1710.

[41] G. Karamanthy, J. Reinstein, D.F. Hansen, Multiquantum chemical exchange saturation transfer NMR to quantify symmetrical exchange: application to rotational dynamics of the guanidinium group in arginine side chains, J. Phys. Chem. Lett. 11 (14) (2020) 5649–5654.

[42] A. Rangadurai, H. Shi, H.M. Al-Hashimi, Extending the sensitivity of CEST NMR spectroscopy to micro-to-millisecond dynamics in nucleic acids using high-power radio-frequency fields, Angew. Chem. 132 (28) (2020) 11358–11362.
S. Jain and A. Sekhar
Journal of Magnetic Resonance Open 10–11 (2022) 100034

G.A. Morris, R. Freeman, Selective excitation in Fourier transform nuclear magnetic resonance, J. Magn. Reson. 29 (3) (1978) 433–462.

S. Warhaut, et al., Ligated-modulated filling of the full-length adenine riboswitch probed by NMR and single-molecule FRET spectroscopy, Nucleic Acids Res. 45 (9) (2017) 5512–5522.

J.M. Atkinson, et al., Time-resolved FRET and NMR analyses reveal selective binding of peptides containing the LC3-interacting region to ATG8 family proteins, J. Biol. Chem. 294 (38) (2019) 14033–14042.

D. Canet, et al., Rapid identification of non-native contacts during the folding of HPr revealed by real-time photo-CIDNP NMR and stopped-flow fluorescence experiments, J. Mol. Biol. 330 (2) (2003) 397–407.

J.B. Udgondaok, R.L. Baldwin, NMR evidence for an early framework Intermediate on the folding pathway of ribonuclease A, Nature 315 (6028) (1985) 699–699.

H. Roder, G.A. Elowe, S.W. Englander, Structural characterization of folding intermediates in cytochrome b6f by H-exchange labelling and proton NMR, Nature 335 (6192) (1988) 790–794.

S.W. Englander, T.R. Sonnich, J.J. Engleman, L. Mayne, Mechanisms and uses of hydrogen exchange, Curr. Opin. Struct. Biol. 6 (1) (1996) 18–23.

S.W. Englander, Protein folding intermediates and pathways studied by hydrogen exchange, Annu. Rev. Biophys. Biomol. Struct. 29 (1) (2000) 213–238.

R. Moulick, R. Das, J.B. Udgondaok, Partially unfolded forms of the prion protein populated under misfolding-promoting conditions: characterization by hydrogen exchange mass spectrometry and NMR, J. Biol. Chem. 290 (42) (2015) 25227–25240.

T.R. Alderson, C. Chatterji, D.A. Torchia, P. Anfinrud, A. Bax, Monitoring hydrogen exchange during protein folding by fast pressure jump NMR spectroscopy, J. Am. Chem. Soc. 139 (32) (2017) 11036–11039.

A. Mittermaier, L.E. Kay, New tools provide new insights in NMR studies of protein dynamics, Science 312 (5771) (2006) 224–228.

R. Frederiksen, et al., Rhodopsin kinase and arrestin binding control the decay of photoactivated rhodopsin and dark adaptation of mouse rods, J. Gen. Physiol. 148 (1) (2016) 1–11.

C.K. Chen, J. Inglese, R.J. Lefkowitz, J.B. Hurley, Ca2+-dependent interaction of recoverin with rhodopsin kinase, J. Biol. Chem. 270 (30) (1995) 18065–18066.

C. James, K. Levey, G. Ling, L. Kuntz, V. Syk, Structural basis for calcium-induced inhibition of rhodopsin kinase by recoverin, J. Biol. Chem. 281 (48) (2006) 37237–37245.

K.E. Komolov, et al., Mechanism of rhodopsin kinase regulation by recoverin, J. Neurochem. 110 (1) (2009) 72–79.

M.K. Higgins, D.D. Oprian, G.F. Schertler, Recoverin binds exclusively to an amphipathic peptide at the N terminus of rhodopsin kinase, inhibiting rhodopsin phosphorylation after activating catalytic activity of the kinase, J. Biol. Chem. 281 (28) (2006) 19430–19439.

B. Zhao, S.L. Guffy, B. Williams, Q. Zhang, An excited state underlies gene regulation of a transcriptional riboswitch, Nat. Chem. Biol. 13 (9) (2017) 968–974.

N. Lokesh, A. Seegerer, J. Hioe, R.M. Gschwind, Chemical exchange saturation transfer in chemical reactions: a mechanistic tool for NMR detection and characterization of transient intermediates, J. Am. Chem. Soc. 140 (5) (2018) 1855–1862.

N. Platter, S. Doerr, G. De Fabritiis, F. Noé, Complete protein–protein association kinetics in atomic detail revealed by molecular dynamics simulations and Markov modelling, Nat. Chem. 9 (10) (2017) 1005–1011.

L.S. Steld, G. Hummer, J. Hwang, From replica exchange molecular dynamics simulations, J. Chem. Theory Comput. 13 (8) (2017) 3972–3975.

A.C. Pan, H. Xu, T. Palpant, D.E. Shaw, Quantitative characterization of the binding and unbinding of millimolar drug fragments with molecular dynamics simulations, J. Chem. Theory Comput. 13 (7) (2017) 3783–3800.

P. Robustelli, S. Piana, D.E. Shaw, Developing a molecular dynamics force field for both folded and disordered protein states, Proc. Natl. Acad. Sci. 115 (21) (2018) E4758–E4766.

R. Appadurai, J. Nagesh, A. Srivastava, High resolution ensemble description of cancerous and intrinsically disordered proteins using an efficient hybrid parallel tempering scheme, Nat. Commun. 12 (2021) 1–11, 958.

A.C. Pan, et al., Atomic-level characterization of protein–protein association, Proc. Natl. Acad. Sci. 116 (10) (2019) 2424–2429.

P. Robustelli, S. Piana, D.E. Shaw, Mechanism of coupled folding-upon-binding of an intrinsically disordered protein, J. Am. Chem. Soc. 142 (25) (2020) 11092–11101.

P. Vallurupalli, N. Chakrabarti, R. Ponias, L.E. Kay, Atomic picture of conformational exchange in a T4 lysozyme cavity mutant: an experiment-guided molecular dynamics study, Chem. Sci. 7 (6) (2016) 3602–3613.

J.S. Fraser, et al., Hidden alternative structures of proline isomerase essential for catalysis, Science 309 (5741) (2005) 535–538.

M. Kovermann, C. Grundström, A.E. Sauer-Eriksson, U.H. Sauer, M. Wolf-Watz, Structural basis for ligand binding to an enzyme by a conformational selection pathway, Proc. Natl. Acad. Sci. 114 (24) (2017) 6298–6303.

J.A. Hanson, et al., Illuminating the mechanistic roles of enzyme conformational plasticity and dynamics, Proc. Natl. Acad. Sci. 104 (46) (2007) 18055–18060.

D.D. Boehr, D. McElheny, H.J. Dyson, P.E. Wright, The dynamic energy landscape of dihydrofolate reductase catalysis, Science 313 (5793) (2006) 1638–1642.
[127] R.M. Culik, et al., Effects of maturation on the conformational free-energy landscape of SOD1, Proc. Natl. Acad. Sci. U. S. A. 115 (11) (2018) E2546–E2555.
[128] S. Brüschweiler, et al., Direct observation of the dynamic process underlying allosteric signal transmission, J. Am. Chem. Soc. 131 (8) (2009) 3063–3068.

[129] S.R. Tzeng, C.G. Kalodimos, Allosteric inhibition through suppression of transient conformational states, Nat. Chem. Biol. 9 (7) (2013) 462.
[130] C.B. Anfinsen, Principles that govern the folding of protein chains, Science 181 (4096) (1973) 223–230.