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NMR spectroscopy brings invisible protein states into focus

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Molecular dynamics are essential for protein function. In some cases these dynamics involve the interconversion between ground state, highly populated conformers and less populated higher energy structures ('excited states') that play critical roles in biochemical processes. Here we describe recent advances in NMR spectroscopy methods that enable studies of these otherwise invisible excited states at an atomic level and that help elucidate their important relation to function. We discuss a range of examples from molecular recognition, ligand binding, enzyme catalysis and protein folding that illustrate the role that motion plays in 'funneling' conformers along preferred pathways that facilitate their biological function.

Much of structural biology so far has focused on studies of ground-state, low-energy conformers of biomolecules, providing detailed atomic descriptions of static three-dimensional structures. Yet pictures of globular protein molecules occupying single rigid conformations have long been known to be insufficient for obtaining a complete understanding of function. Instead, an appreciation of the conformational fluctuations such biomolecules undergo is required to achieve a complete understanding of their many properties[1–3]. Indeed, it was clear in the 1960s that oxygen cannot reach the heme iron in hemoglobin unless the side chains of these molecules undergo rapid conformational rearrangements, creating small crevices that enable penetration of ligand[4]. In addition, early experiments focusing on hydrogen deuterium (HD) exchange in proteins provided strong evidence that these molecules can undergo large-scale fluctuations at equilibrium[5], and both NMR[6–8] and photodissociation[9] studies showed that proteins undergo molecular dynamics over a wide range of timescales and that such motions can involve rearrangements of significant amplitude. Further evidence was provided by early molecular dynamics simulations[10] that showed fluid-like motions of atoms about the average positions in static structures on the picosecond timescale. It is clear that the amino acid sequence of a protein encodes not only structural information, but also the range of dynamics accessible to the molecule, and that the interplay between structure and dynamics determines both the function of a protein and its ability to evolve and adapt to diverse sets of conditions[11].

The amplitudes and the timescales of motion that characterize the dynamics of a protein under a given set of conditions can be understood in terms of an 'energy landscape' that describes the energetic relationship between all possible protein conformations[12–15]. Ground-state conformers that occupy the bottom of the energy basin form the basis of structural studies by NMR and X-ray diffraction, and are separated from each other by very small kinetic barriers that are easily overcome by thermal energy, leading to picosecond to nanosecond timescale dynamics. Such dynamics vary in amplitude, ranging from bond vector vibrations and rotations about unhindered dihedral angles[16], to substantial chain displacements[17]. In some favorable cases such picosecond to nanosecond dynamics can be inferred from temperature factors derived from crystal structures[18] or often more rigorously from NMR spin relaxation properties[19–22]. In addition to these low-energy conformers, higher energy structures are also sampled; these are referred to hereafter as excited protein states. The kinetic barriers between ground and excited states typically lead to their interconversion on the microsecond to millisecond timescale or longer[23,24]. Excited states have been associated with important functional roles in biochemical processes, including molecular recognition and ligand binding[25–33], enzyme catalysis[34–43] and protein folding[44–52] (see below). Hence there is a clear need both to characterize the structural ensembles that describe these functionally important states and to understand the mechanisms by which they interconvert with ground-state conformers.

Yet this is extremely difficult to do. Excited-state conformers are sparsely populated and exist often only transiently. Consider a thermally accessible conformer that is $2k_BT$ (1.2 kcal mol^{-1}; k_B is Boltzmann's constant, T is absolute temperature) higher in free energy than the ground state. From the Boltzmann relationship the fraction of molecules in the excited state is only approximately 13% of those in the ground state, thus challenging the sensitivity of experimental methods. Moreover, the short lifetimes of the excited conformers broadens experimental signals so that they are essentially invisible to many of the tools of modern biophysics. However, recent advances in solution NMR spectroscopy have paved the way for studying such 'invisible' excited states[23,53], and much insight has been obtained from applications of a number of methods, including Carr-Purcell-Meiboom-Gill relaxation dispersion (CPMG RD)[54,55], paramagnetic relaxation enhancement (PRE)[56] and native-state HD exchange[5].

Though the underlying physics of these techniques has been well known for over 50 years, application to studies of complex biomolecules, in the case of the CPMG RD (**Box 1**) and PRE (**Box 2**)

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Box 1 CPMG relaxation dispersion

Consider a molecule undergoing exchange between a pair of conformations denoted by A and B,

$$A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B$$

In what follows we initially explore the exchange process by studying the trajectory of a single NMR spin (probe)[98]. This probe can exist in one of two states (**Fig. 1a**), with stochastic jumps from one state to the next. Critical to the dispersion experiment is that the probe evolves at different frequencies (the chemical shift) when in states A and B, denoted by ω_A and ω_B, respectively. In the absence of this difference, no information on the exchange process can be obtained from this probe, although information may be available from the PRE effect (**Box 2**). If the spin exists in a single state for time T and a 'refocusing' pulse (**Fig. 1b**,**c**, black bars) is applied exactly in the center of this interval, then the overall frequency evolution of the spin for the total T period is reduced to zero. Frequency evolution is said to be 'refocused'. By contrast, if the pulse is applied 'off center' such that the probe is not in a single state during T, then refocusing is not complete. This situation is illustrated in **Figure 1b** where in the first t_1 period the spin is in the 'red' state, with a jump to the 'blue' state occurring during the second t_1 interval; the pulse denoted by '1' in **Figure 1b** is thus not able to refocus the frequency evolution of this spin. Such regions are indicated (gray); they occur during intervals 1, 2, 3 and 4 (**Fig. 1b**) so that by the end of the total time period the spin will have evolved at an effective frequency that depends on the duration in each state and the number of jumps executed. If the rate of application of the pulses increases (**Fig. 1c**), the effects of the jumps are smaller in the sense that refocusing, although not perfect, is improved (less 'gray' intervals). The net NMR signal is given by the sum of all spins in the sample, and since each spin executes a different trajectory (different times in each state, different numbers of jumps), the net frequency distribution will be larger in an experiment involving fewer refocusing pulses, leading to a reduction in peak intensity (**Fig. 1d**). By measuring peak intensities as a function of the number of pulses (CPMG frequency), expressed as a relaxation rate, R_2^{eff}, a 'relaxation-dispersion' curve is obtained (**Fig. 1d**), from which values for $\Delta\omega = \omega_A - \omega_B$ (the chemical shift difference) and the rates k_{AB} and k_{BA} can be obtained (providing $k_{AB}/k_{BA} > \sim 0.5\%$ and $k_{ex} = k_{AB} + k_{BA}$, which is in the millisecond regime). This effect is exploited most remarkably in cases where B is sparsely populated ($k_{BA} > k_{AB}$) and hence otherwise invisible in NMR spectra[23,99].

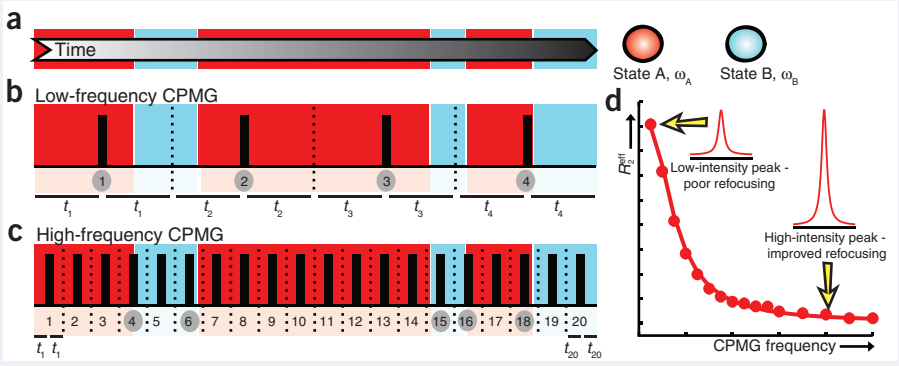

Figure 1 A simple schematic of a CPMG relaxation dispersion experiment. (**a**) A molecule interconverts stochastically between two conformational states, A and B, as a function of time, with states A and B denoted by red and blue colors, respectively. Each molecule will interconvert with its own trajectory, and a single example is shown. (**b**,**c**) During the trajectory, pulses are applied that lead to a modulation of the relaxation rates of NMR probes attached to the molecule. (**d**) A relaxation dispersion profile is obtained from which details of the exchange reaction can be obtained.

approaches, had to await the development of optimized experimental schemes[57], appropriate biochemical techniques that introduce the necessary NMR-active nuclei[58] and refinement of the computational approaches required to interpret the experimental data[59]. Notably, the methods highlighted in this review are very complementary. For example, CPMG RD and PRE are sensitive to the presence of low-lying excited states with populations on the order of 0.5% or higher[23,53]. The CPMG RD experiments are most sensitive to processes occurring on the millisecond timescale, whereas PRE methodology is applied to systems that exchange much more rapidly, typically on a timescale of 100 μs or faster (other differences are highlighted in **Boxes 1** and **2**)[53,60]. By contrast, native-state HD exchange can quantify local and global protein unfolding events that occur on a much slower timescale[61,62]. In addition, HD exchange reports on the existence of excited states that are relatively much higher in energy and whose populations are typically orders of magnitude lower than what can be observed by the CPMG and PRE methods.

The goal of this review is to show how this NMR methodology may be used to obtain detailed insight into the relation between protein dynamics and function. Specifically, we consider the examples of ligand binding, enzyme catalysis and protein folding and highlight the role of sparsely populated states in these important biochemical processes.

Protein-ligand interactions

Biochemical processes are often triggered by the recognition and association of two or more molecules, and such interactions frequently lead to the propagation of signals to downstream targets[63,64]. Owing to their importance, binding events have been probed by a vast range of sophisticated techniques. Many studies have been interpreted in terms of a two-state equilibrium between free and bound conformers; however, the ability to detect sparsely populated states using the CPMG RD and PRE approaches (**Boxes 1** and **2**; **Figs. 1** and **2**) has facilitated more detailed analyses, establishing the presence of unexpected binding intermediates.

One such example emerges from a recent study of the binding of ubiquitin to the C-terminal SH3 domain from the adaptor protein CIN85 (ref. 32). Chemical shift titration data, recording the chemical shift dependencies of probes attached to one molecular partner as a function of the concentration of the second, could be very well fit to a simple two-state binding model. In addition, CPMG RD experiments used to probe the binding were also consistent with a simple on-off binding mechanism. Yet binding constants and chemical shift differences between bound and free states obtained from the two sets of experiments were not in agreement. The combined data could be reconciled only by using more complex binding models, including the one illustrated in **Figure 3a** showing an on-pathway intermediate

Box 2 Paramagnetic relaxation enhancement

The decay of NMR signals from a biomolecule of interest can be enhanced by affixing it to a probe containing an unpaired electron, such as a spin label or a complexed paramagnetic metal ion. Such PRE effects scale with the inverse sixth power of the distance between the unpaired electron and the NMR spin and so can be readily interpreted in terms of distances. PREs are therefore highly useful restraints for structure calculations[53].

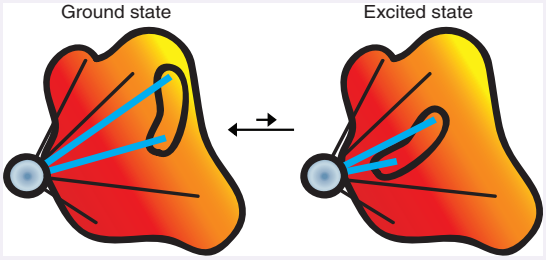

Figure 2 Modulation of spin-relaxation from conformational changes affecting the distance between a paramagnet and NMR probes. Where the molecule of interest undergoes conformational rearrangements that lead to segments transiently moving closer (blue lines) to the paramagnetic label (blue circle), it becomes possible to obtain distance information on the excited state that can be used for structure calculations if a ground-state structure is available[53]. By contrast, if the segment is further from the paramagnet in the excited state, then the PRE gives no information on the sparsely populated state. In this case, it may be possible to obtain the missing information from CPMG experiments if the timescales and populations are amenable to a CPMG analysis (**Box 1**).

with only a limited number of 'hotspot' interactions between the two binding partners.

A combined chemical shift and CPMG RD analysis of the binding of the intrinsically disordered pKID domain of the transcription factor CREB to its partner, the KIX domain of a transcriptional co-activator, showed that this interaction is also more complex than two-state binding (**Fig. 3b**)[30]. In this scheme, a weak encounter complex is initially formed, driven by complementary electrostatic interactions of the two binding partners, followed by the stabilization of the complex by hydrophobic interactions. In the intermediate, one of the two helices of pKID (α_A) is native-like, while the second only folds completely in the final complex. The combined folding and binding of the pKID domain provides a striking example of the 'induced fit' binding mechanism.

'Conformational selection' is an alternative binding mechanism in which the appropriate partner is sought out from an ensemble of conformations that are already populated in solution[65]. A study by Tang *et al.* using PRE (**Box 2** and **Fig. 2**) to investigate the process by which maltose binding protein (MBP) binds maltose establishes that binding can be more complex than either induced fit or conformational selection[31]. MBP was tagged with a paramagnetic label in either the N- or C-terminal domains of the protein, and enhanced relaxation was measured for both holo and apo forms. The PRE data obtained on the holoprotein were consistent with the X-ray structure of the maltose-bound form, and similarly intradomain PRE measurements on the apo state were in agreement with expectations based on the unliganded MBP X-ray structure. However, addition of spin label on the N-domain of the apo protein resulted in enhanced relaxation of probes in the C-domain that could not be explained by the X-ray structure of the apo conformer. The PRE data on apo-MBP could be rationalized, however, by an exchange mechanism in which the ground-state conformation (described by the apo X-ray structure) interconverts with an excited, partially closed state, populated to about 5%, that exposes the sugar binding surface and is thus primed to bind ligand. Binding of maltose then subsequently leads to a fully closed conformation. This example invokes elements of conformational selection (binding to a pre-formed conformer) and induced fit (conformational rearrangements occurring after initial binding), illustrating that in some cases molecular interactions can be complex.

The PRE-based approach described above was used to obtain a well-defined model of the invisible, binding-competent conformation of MBP (**Fig. 3c**)[31]. In some cases it is also possible to obtain models of excited states exclusively from data recorded from CPMG RD measurements (**Fig. 4**). Here chemical shifts of the invisible state are measured (**Box 1** and **Fig. 1**) along with a variety of different magnetic interactions that report on the orientation of bond vectors (**Fig. 4a**). Vallurupalli *et al.* have used a ligand binding exchanging system to develop the necessary methodology and have recently reported an ensemble of structures corresponding to the ligand-bound excited state, produced from dispersion measurements (**Fig. 4b**)[59].

Enzyme catalysis

The ability to perform sophisticated organic chemistry rapidly under ambient conditions is clearly critical for life as we know it, and all known organisms use enzymes to achieve the necessary reaction rate enhancements[66]. Mechanistically, catalysis is accompanied by the enzyme cycling between sets of conformations that facilitate the required chemistry. The role of dynamics has long been known to be crucial to this process[1,2], but determining how specific structural fluctuations are coupled to catalytic function continues to be a challenge[67,68]. Though it is crucial that enzymes provide a pre-organized environment with which to stabilize the transition state of the chemical process under catalysis[69], this step is not necessarily rate limiting; rather, conformational changes required to bind substrate, orient reactants or release products may limit catalytic turnover[37,38,70], as the examples below illustrate. Finally, in one remarkable example it has been shown that a disordered molten globule of the enzyme chorismate mutase can, in the presence of substrate, fold into a structure capable of efficient catalysis[71,72], which indicates at least in this case that it is not even essential for an enzyme to be structured in its unligated state.

NMR methods are particularly powerful for elucidating the relation between dynamics and function in enzyme systems. A major benefit arising from NMR studies of an enzymatic pathway is that the structural consequences of conformational fluctuations are obtained in conjunction with the thermodynamic and kinetic properties of the involved transitions[23]. This is illustrated clearly in a study of the enzyme dihydrofolate reductase (DHFR), which has been shown by analysis of pre-steady state kinetics to cycle through five major states (**Fig. 5a**), whose structures in turn cycle between 'occluded' and 'closed' forms[37]. The rate-limiting step in catalysis involves the dissociation of product THF from the complex—a process shown to be associated with a pronounced 'occluded to closed' transition. Notably, the rate of product dissociation determined by previous stopped-flow measurements[73] is identical to the exchange rate between DHFR–NADPH–THF and an excited state of the product-bound form with a conformation similar to DHFR–NADPH, as established by CPMG RD NMR[74]. Further, although the actual hydride transfer is a very fast process associated with quantum tunneling effects, CPMG RD experiments indicate that the net transfer rate, approximately 1,000 s^{-1}, is limited by structural rearrangements in which a 'closed' DHFR–$NADP^+$–THF excited-state conformation (formed by reduction of DHF to THF in DHFR–NADPH–DHF) relaxes to the 'occluded'

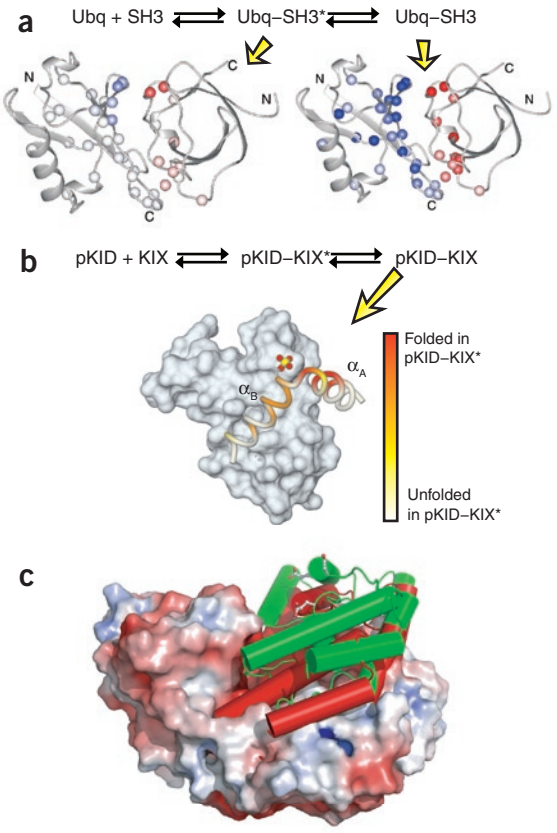

Figure 3 Probing sparsely populated states involved in molecular interactions. (**a**) A binding model for ubiquitin and the C-terminal SH3 domain from the adaptor protein CIN85 involving an intermediate state (SH3*). The structure of the complex is shown with amide nitrogens colored blue (ubiquitin) or red (SH3) to indicate regions showing significant chemical shift changes upon binding, with increasing intensity of color denoting larger shift changes. Adapted from ref. 32. (**b**) The intrinsically disordered protein pKID binds to its target KIX via an intermediate (KIX*) in which helix α_B is only partially formed. Shown is the structure of the fully bound state. Adapted from ref. 30. (**c**) Apo-MBP exchanges with a conformation that is similar but not identical to that of the ligand-bound form. Shown are the C-terminal domains of MBP in the excited, apo state (green) and holoform (red), along with a molecular surface corresponding to the unliganded molecule. Taken from ref. 31.

ground conformation[75]. These observations, coupled with the fact that the other ground states along the reaction pathway interconvert with excited conformers corresponding in structure to states that are either immediately before or after in the cycle, suggest strongly that millisecond-timescale dynamics serve to 'guide' the reaction through an efficient catalytic pathway in which the energy landscape is constantly changing in response to ligand binding, product release and chemistry[74].

Changes in conformation have also been shown to be rate limiting in the function of other enzymes. In an elegant study of dynamics in adenylate kinase (Adk), an enzyme that catalyzes the transfer of one phosphate group from ATP to AMP to form two molecules of ADP (**Fig. 5b**), the rates of lid opening and enzyme turnover were shown to be in agreement, both for the mesophilic and for the thermophilic versions of the protein[38]. Thus, the rate-limiting step in the reaction is lid opening leading to product ADP release, as indicated in the scheme of **Figure 5b**. As in the case of DHFR discussed above, molecular dynamics in Adk funnel the enzyme along a preferred functional path; the exchange between the ground, closed and the excited, open ligand-bound states 'primes' Adk for product release. Finally, even in the absence of substrate, exchange between ground and functionally important low-lying excited states has been quantified in a variety of different enzyme systems, including HIV protease[34], cyclophilin A[39], RNase A[35,41], flavin oxidoreductase[40] and triosphosphate isomerase[42], with rate constants determined by relaxation dispersion on the order of k_{cat}. It thus appears that many enzymes have evolved to the point where the chemistry is rapid, with turnover limited by the time required to undergo often substantial conformational changes. This has particularly significant implications for the design of drugs for key enzymes implicated in disease, for example.

Protein folding

A detailed, quantitative understanding of the mechanism by which globular proteins fold, including the structural properties of the intermediates that (often very transiently) populate the folding landscape, remains an important goal of biophysics. Experimentally, folding rates have been found to range from about 700 ns[76] to the second timescale[77,78] and beyond. On the one hand, this time is much too short for an exhaustive random search for the minimum in the energy landscape, while in the case of slow folders in particular, it is clearly much longer than a 'downhill run' to the lowest free energy structure. Qualitatively, during the folding process favorable intrachain contacts are formed, and this energetic gain is offset partially by a loss of conformational entropy and further modulated by simultaneous rearrangements of the solvent. In general, the changes in these quantities are not perfectly matched, leading to situations where, for example, the energy losses due to changes in conformational entropy occur earlier in the folding process than energetic gains due to the formation of favorable contacts, thus leading to kinetic barriers and a rugged energy landscape. In many cases, folding of small single-domain proteins can be well approximated

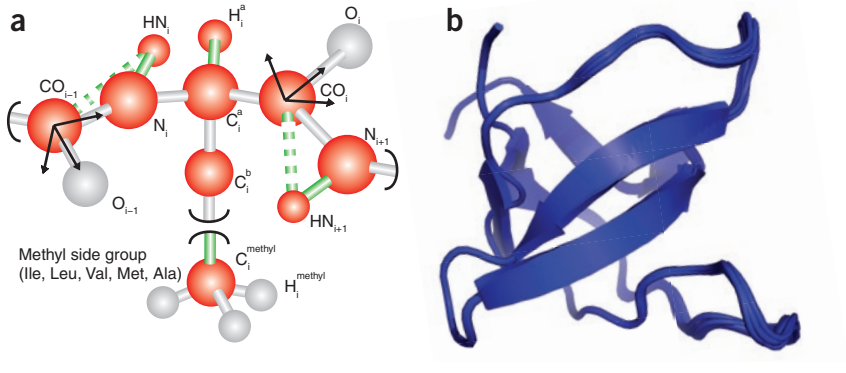

Figure 4 New NMR methods for determining the structures of excited states. (**a**) Polypeptide showing those nuclei for which chemical shifts in the excited state can be obtained using CPMG relaxation dispersion methods (red)[87–92], along with vectors connecting bonded (solid green) and nonbonded nuclei (dashed green) whose orientations can be determined from a special class of CPMG dispersion experiment[93–95]. Shown also are peptide plane fixed coordinate frames linked to carbonyl nuclei that can be oriented using CPMG experiments[96]. (**b**) Structure of an invisible, excited state corresponding to a ligand-bound form of the Abp1p SH3 domain, determined exclusively from CPMG data. Figure adapted from refs. 59 and 97.

by a two-state cooperative process[2]. However, it is clear that as the number of probes increases and the instrumentation used to detect folding becomes sensitive to faster timescale processes, the folding mechanism deduced is often more complex, involving intermediates that despite having 'native-like' characteristics, may also contain significant non-native interactions[79].

CPMG RD NMR spectroscopy has been applied to study the folding pathways of a number of SH3 domains[45,48,51]. Despite the fact that stopped-flow fluorescence folding studies of these molecules can be well interpreted in the context of a two-state cooperative process[80,81], the NMR data clearly establish on-pathway intermediates populated to approximately 1–2% at room temperature. A structural ensemble corresponding to the folding intermediate for a G48V mutant of the Fyn SH3 domain is shown in **Figure 6a**, based exclusively on ^{15}N chemical shifts in the intermediate state that were obtained from the analysis of the dispersion data (**Box 1**). The ensemble of structures, though preliminary, establishes that a central β-sheet that is present in the folded conformer is already formed in the intermediate state[45]. More recently, a study of a second mutant Fyn SH3 domain has shown that non-native interactions are formed in the intermediate state[81]; notably, mutations at the N terminus affect ^{15}N chemical shifts of some residues in the intermediate state but not in the folded conformer, which implies that there are at least a subset of interactions that differ in the two states. Further structural details must await more sophisticated analyses involving the range of chemical shift probes and bond vector orientations that are indicated in **Figure 4a**.

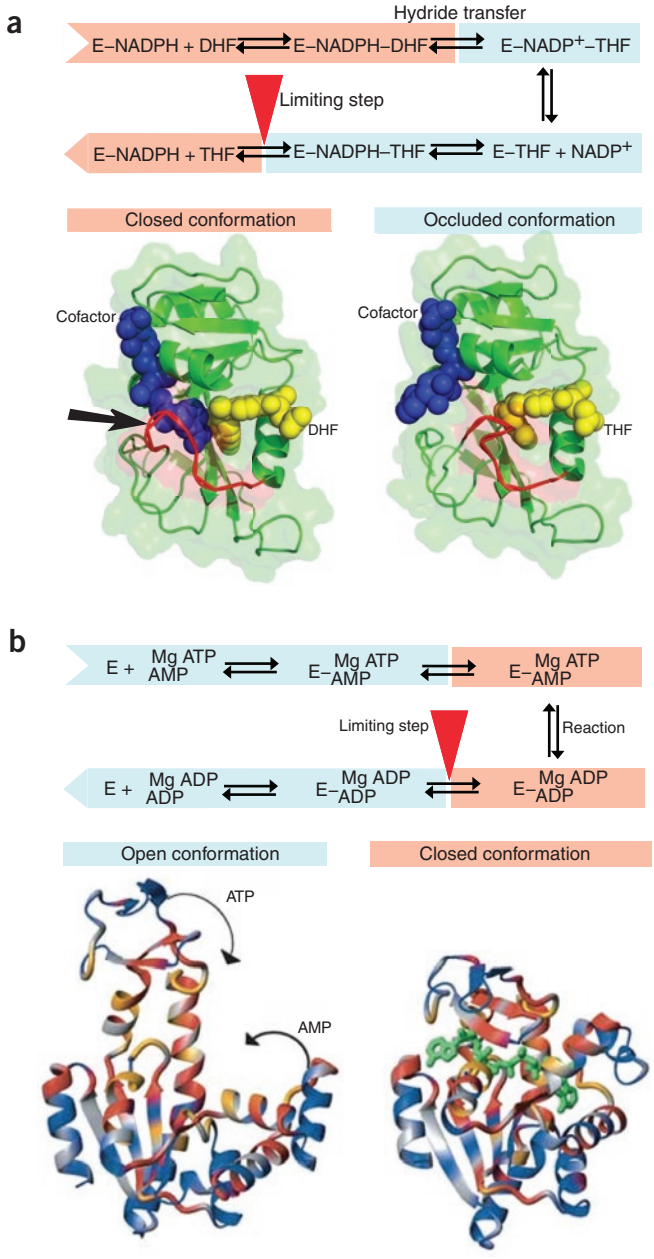

Figure 5 Relaxation dispersion studies of catalysis. (**a**,**b**) Reaction schemes for E = DHFR (**a**) and E = Adk (**b**) with the rate-limiting conformational changes associated with each enzymatic process indicated (red arrowheads). In the catalytic cycles of both enzymes, chemical rearrangements were found to be significantly faster than the conformational change associated with the rate-limiting step. Shown are structures of the closed (left) and occluded (right) conformations of DHFR (**a**) and the open (left) and closed (right) conformers of Adk (**b**), with black arrows indicating the direction of movement required for catalysis. Adapted from refs. 74 and 38.

CPMG RD data are limited in scope to the study of low-lying excited states that are populated to at least 0.5% relative to the ground state. A complementary method that does not have this limitation is native-state HD exchange. HD exchange rates as a function of low concentrations of denaturant, measured on a per-residue basis by sensitive NMR methods, can be used to characterize folding intermediates with miniscule populations[62]. In a seminal series of experiments, a range of excited states of cytochrome *c* have been identified that correspond to the partial unfolding of distinct regions of secondary structure[82], termed 'foldons'. By considering the energetics of the various states, a folding pathway was inferred that describes the progressive formation of foldons that stabilize the formation of additional structural elements (**Fig. 6b**)—a finding later supported by studies on a range of proteins[62]. It is worth noting that the emerging views on protein folding from HD exchange are generally consistent with the picture obtained from relaxation dispersion showing intermediates comprised of folding units (see above), at least for the SH3 and FF (ref. 52) domains that have been studied in detail. Overall, results from both techniques and from a wealth of data produced by the protein engineering method and stopped-flow techniques[2,83,84] indicate that many proteins fold via a network of often high-energy intermediates whose existence is predetermined by the amino acid sequence and the prevalent solution conditions.

Concluding remarks

A large body of evidence using a diverse spectrum of biophysical methods clearly establishes that proteins are dynamic over a broad range of timescales and that such dynamics play critical roles in function. Over the past decade, new and powerful NMR approaches have emerged that have significantly contributed to our understanding of the relationship between dynamics and function. Experiments focusing on invisible excited states have been developed that provide for the first time a detailed atomic description of these sparsely populated yet functionally significant conformers that normally are recalcitrant to study by well-established structural tools. Methods have also emerged for studies of dynamics in high-molecular-weight complexes, which have traditionally been considered to be outside the scope of NMR techniques[85,86]. This review has highlighted some of the new methodologies and provided a number of examples from studies of ligand binding, molecular recognition, enzymology and protein folding illustrating how excited states are used to help navigate complex energy landscapes so as to 'guide' the biochemical processes in question. The utility of this methodology and the promise of future advances suggest that a paradigm shift in structural biology is in the making. It will not be too long before a

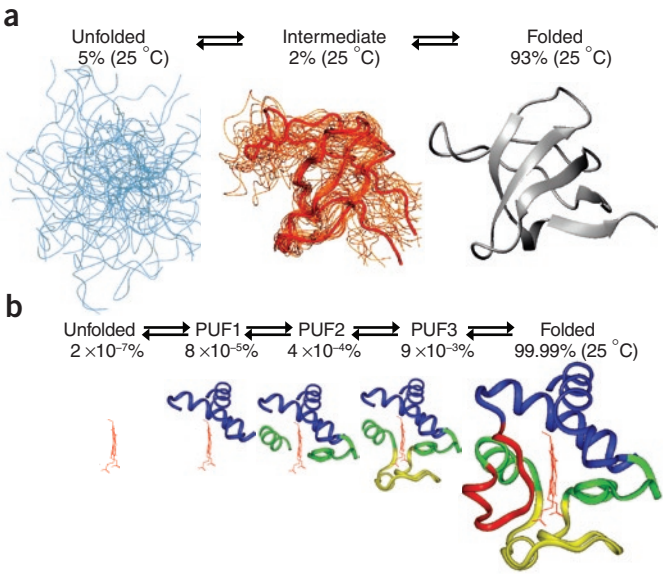

Figure 6 Sparsely populated states along protein folding pathways. (**a**) The G48V Fyn SH3 domain folds via an on-pathway intermediate, as established by CPMG RD. The structural ensemble of the 'invisible' intermediate based on ^{15}N chemical shifts is highlighted. Adapted from ref. 45. (**b**) Folding pathway of cytochrome *c* determined by native-state HD exchange measurements. Shown in color are regions of the protein (foldons) that fold as cooperative units. Regions of the protein not shown in a particular 'partially unfolded intermediate' (PUF) are unfolded. The heme group is shown in red in all structures. Listed are the populations of each intermediate. Figure provided by Y. Bai (US National Cancer Institute).

well-defined 'average' structure is no longer the endpoint goal, but rather the beginning of a larger effort to characterize in detail how changes in such a structure over a broad range of timescales relate to function at the atomic level.

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