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PERSPECTIVE

Using relaxation dispersion NMR spectroscopy to determine structures of excited, invisible protein states

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Abstract Currently the main focus of structural biology is the determination of static three-dimensional representations of biomolecules that for the most part correspond to low energy (ground state) conformations. However, it is becoming increasingly well recognized that higher energy structures often play important roles in function as well. Because these conformers are populated to only low levels and are often only transiently formed their study is not amenable to many of the tools of structural biology. In this perspective we discuss the role of CPMG-based relaxation dispersion NMR spectroscopy in characterizing these low populated, invisible states. It is shown that robust methods for measuring both backbone chemical shifts and residual anisotropic interactions in the excited state are in place and that these data provide valuable restraints for structural studies of invisible conformers.

Introduction

Next year marks the 20th anniversary of a paper that Dennis Torchia, Ad Bax and one of us (LEK) published describing a study of the backbone dynamics of the protein staphylococcal nuclease using ^{15}N spin relaxation NMR spectroscopy (Kay et al. 1989). In this publication ^{15}N R_1 and R_2 relaxation rates were measured, supplemented by ^{1}H–^{15}N steady state NOE values, to study pico-second (ps), nano-second (ns) protein dynamics focused on the entire protein backbone. The relaxation parameters described above were interpreted in terms of the Lipari-Szabo formalism (Lipari and Szabo 1982) whereby the square of an order parameter, that is related to the amplitude of amide-bond vector motions, and an effective correlation time, that provides the time-scale of these motions, are extracted on a per-residue basis. Subsequently, Clore and coworkers (Clore et al. 1990) applied a similar analysis to the study of backbone dynamics in the protein interleukin-1β, and they noted that data from approximately 1/3 of all residues could only be fit using an additional term that takes into account contributions to transverse relaxation from exchange dynamics that are not quenched by the Carr-Purcell-Meiboom-Gill (CPMG) pulse scheme (Carr and Purcell 1954; Meiboom and Gill 1958) that is used to record R_2 rates. These initial papers along with those produced from the group of Gerhard Wagner (Peng and Wagner 1992), all focusing on studies of uniformly ^{15}N labeled proteins that could be readily generated, were the forerunners of many interesting and important studies by a large number of laboratories that considered fast time-scale dynamics in a variety of different protein systems (Jarymowycz and Stone 2006). In these initial applications, in those that predated them involving studies of biomolecules with selectively incorporated labels (Henry et al. 1986; Richarz et al. 1980), and in many applications subsequently, the main goal was to interpret relaxation data in terms of the underlying ps–ns timescale dynamics; contributions due to chemical exchange were considered largely to be an annoyance that interfered with the accurate extraction of 'more interesting' dynamics parameters.

In fact, nothing could be farther from the truth! Since the pioneering studies of McConnell (McConnell 1958), Gutowsky and coworkers (Allerhand et al. 1966) and

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others (Deverell et al. 1970) it has long been recognized that NMR is a powerful technique for investigating chemical exchange phenomena and that detailed information about molecular rearrangements on the millisecond-microsecond timescale can be obtained. Studies of dynamics in this time window date back at least four decades, and initial applications focused on isolated spins and small molecules where the underlying spin physics was well understood (Campbell et al. 1971; Carver and Richards 1972). It proved to be considerably more complicated to study chemical exchange in large, biomolecular systems since the need for resolution necessitated that ^{1}H–X, X = {^{15}N, ^{13}C}, spin-pairs be used as probes, and separating the chemical exchange process of interest from contributions arising from the inherent spin physics of the 'probe' spin system proved to be challenging. This problem has been overcome through the pioneering work of Art Palmer and colleagues and since their seminal paper in 1999 (Loria et al. 1999) there have been many biochemical applications, mostly involving protein-based systems (Palmer et al. 2005).

Relaxation dispersion experiments that quantify the effective line-widths of peaks in spectra as a function of the number of refocusing pulses applied in a CPMG pulse scheme (Palmer et al. 2001), Fig. 1a, are particularly useful because, at least for simple two state exchange processes, Fig. 1b, a complete description of the exchange event is possible. Frequently, the exchange involves the interconversion between a highly populated, ground state ('A'), and a low populated and invisible, excited state ('B') and in these cases detailed information about the excited state can often be obtained that is not available through other techniques. This includes the determination of rate constants of exchange between states, k_{AB} and k_{BA}, Fig. 1b, from which the populations of the interconverting conformers can be obtained, as well as the chemical shifts of the excited state (Palmer et al. 2001), Fig. 1c.

The fact that chemical shifts of the excited state can be isolated is particularly exciting since this provides a possible avenue for the determination of three-dimensional structures of the molecules that describe the excited state. In the past year there have been impressive developments in using backbone chemical shifts as structural restraints in combination with a database of structures of small molecular fragments (Cavalli et al. 2007; Shen et al. 2008) and it is now possible to determine structures of proteins with molecular weights $\leq$15 kDa that have better than 2Å root mean square deviations for the backbone atoms relative to the experimentally determined NMR or X-ray structures. Can such analyses be extended to invisible, excited states for which backbone chemical shift data is available from relaxation dispersion experiments? Here we explore this question in some detail, focusing on new methodology that has been developed in the past year that includes approaches for measuring residual anisotropic interactions in the excited state.

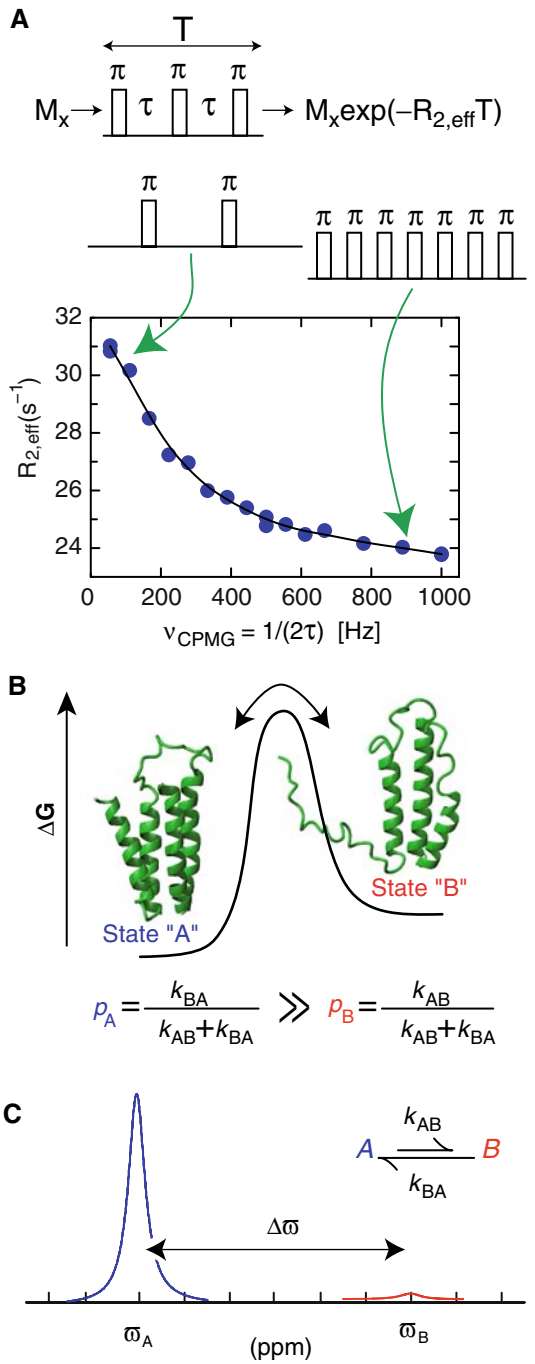

Fig. 1 Schematic overview of CPMG-based relaxation dispersion NMR spectroscopy. (**a**) The effective transverse relaxation rate, $R_{2,eff}$, is calculated as a function of a variable number of 180° refocusing pulses applied during an interval of fixed duration, T. The response of $R_{2,eff}$ to $\nu_{CPMG} = 1/(2\tau)$ can be fitted to extract the parameters that quantify the exchanging system (**b**), including the absolute value of the chemical shift difference, $|\Delta\varpi|$ (ppm), from which the position of the excited state resonance can be determined (**c**) as described in the text and elsewhere (Korzhnev et al. 2005; Skrynnikov et al. 2002; van Ingen et al. 2006)

Towards a new paradigm in structural biology: structures of invisible states

Figure 2 shows a highly schematized one dimensional energy landscape of a protein. Much of structural biology as it is currently practiced focuses on characterizing the conformers that reside in the lowest energy region of the landscape since these are the most populated and since many of the tools of structural biology can only be applied to highly populated ('visible') states. Conformations that are higher in energy than the ground state by only a few k_BT (where k_B is Boltzmann's constant and T is the absolute temperature of the system) are still thermally accessible, and hence in many cases likely to be important for function. Yet these states can be populated to only a few percent or less. For example, the population of an 'excited' state that is $3k_BT$ higher in energy than the ground state is 5% that of the ground state. Moreover, excited states are often only transiently formed so that peaks in NMR spectra that derive from them are broadened, often by hundreds of Hz. As a result such 'high' energy conformations are difficult to study using experiments that are routinely and successfully applied to the study of ground states of proteins.

In cases where the exchange between ground and excited states is on the ms time-scale and where the population(s) of the excited state(s) exceed approximately 0.5% it is possible to use the CPMG relaxation dispersion methods described in detail in the literature (Palmer et al. 2001, 2005) and illustrated in Fig. 1 to quantify the exchange reaction and to obtain qualitative structural information about the excited state(s) that participate in the exchange process. In the past years there have been a steady increase in the number of applications of the methodology, focusing on enzymes and other protein systems (Boehr et al. 2006; Eisenmesser et al. 2005; Grey et al. 2003; Henzler-Wildman et al. 2007; Ishima et al. 1999; Mulder et al. 2001; Vallurupalli and Kay 2006; Watt et al. 2007), protein-ligand interactions (Sugase et al. 2007; Tolkatchev et al. 2003; Vallurupalli et al. 2007) and protein folding (Hill et al. 2000; Korzhnev et al. 2004b; Tollinger et al. 2006; Zeeb and Balbach 2005). A major goal of the field is to extend the qualitative studies of excited states to include a rigorous description of structure. This is particularly relevant when one considers that many biological processes depend on conversions between ground state and higher energy conformers and that in these cases biological function is often not fully explained by high resolution structures of the ground state alone. Recent advances in CPMG relaxation dispersion methodology suggest that the goal of structure determination of invisible conformers may not be as far removed as perhaps one might have thought.

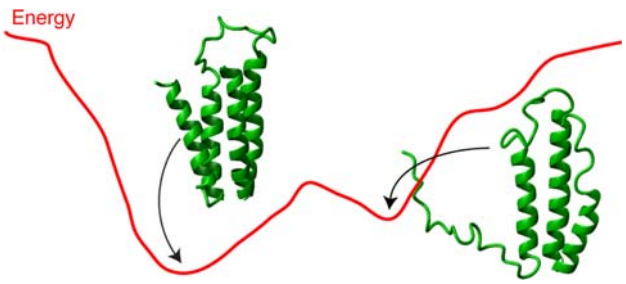

Fig. 2 One dimensional (highly schematized) energy landscape showing the ground state structure, along with a structure of a low-lying excited state that may be amenable to study by CPMG relaxation dispersion NMR spectroscopy

CPMG relaxation dispersion: how well can we do?

Prior to using chemical shifts of the excited state as restraints in structure calculations the accuracy by which one can measure such parameters must first be established. This is far from trivial since in the general case this requires measurements of shifts of invisible states using two orthogonal approaches and then establishing that both methods give the same results. In our experience it is hard enough quantifying such chemical shifts from a single class of measurement! Nevertheless, for at least one type of exchange process there is a simple way to verify the accuracy of the chemical shifts extracted from CPMG-based relaxation dispersion measurements. Consider a simple two-state protein-ligand exchanging system,

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL \qquad (1)$$

where P and L correspond to protein and ligand that bind on a time-scale that is within the window of the dispersion experiment. If a small mole fraction of L $\{[L]/([L] + [PL])\}$ is added, say 1–15%, then the observable protein state in solution is P, while PL is invisible. Further, if the exchange kinetics are favourable (ms) then the chemical shifts of (Hansen et al. 2008b) or residual anisotropic interactions within (Vallurupalli et al. 2008a; Vallurupalli et al. 2007) PL can be measured by CPMG relaxation dispersion and subsequently compared with the corresponding values that are obtained directly on the PL state when excess L is added to shift the equilibrium of Eq. 1 completely to the right.

One such $P \leftrightarrow PL$ exchanging system is available in our laboratory where P corresponds to the SH3 domain from the yeast cytoskeleton protein Abp1p (Drubin et al. 1990) and L is a 17 residue target peptide from the protein Ark1p (Haynes et al. 2007). Studies in our laboratory have established that $K_D = 0.55 \pm 0.5$ μM, $k_{on} = (6.3 \pm 0.7) \times$

10^8 M^{-1} s^{-1} and k_{off} = 350 ± 10 s^{-1}, 25°C (Vallurupalli et al. 2007). The larger than diffusion limited k_{on} reflects the large contribution to binding from electrostatics that follows from the fact that the Abp1p SH3 domain has a net negative charge of 12 and the Ark1p peptide a net positive charge of 6 at pH = 7.

Relaxation dispersion experiments were recorded that monitor backbone ^{15}N, ^{1}HN, $^{13}C^{\alpha}$ and ^{13}CO single-quantum transverse magnetization (Hansen et al. 2008b). The {^{15}N, ^{1}HN, $^{13}C^{\alpha}$, ^{13}CO} chemical shifts of the invisible, 'excited' Abp1p SH3 domain bound state that were extracted from fits of the data sets were subsequently compared with chemical shifts of the *PL* state obtained via direct measurement on a sample where the bound form is the dominant species in solution. Figure 3a shows the correlation between the two independent measurements (Hansen et al. 2008b), emphasizing the excellent level of agreement. It is worth noting that only the absolute value of the difference in chemical shifts between exchanging states is obtained from relaxation dispersion experiments (Palmer et al. 2001) and the missing sign information is clearly important if chemical shifts of the excited state are to be determined. As described previously (Skrynnikov et al. 2002) and illustrated in Fig. 3b the signs are available by comparison of peak positions in HSQC and HMQC data sets recorded at different static magnetic field strengths. In Fig. 3a the points in red correspond to cases where the sign of the shift difference could be obtained, while data points in blue are from residues where sign information was not available (both potential $\Delta\varpi$ values are given in this case). The agreement is excellent and what is most encouraging is that shift differences spanning close to two orders of magnitude could be measured with good accuracy (Hansen et al. 2008b).

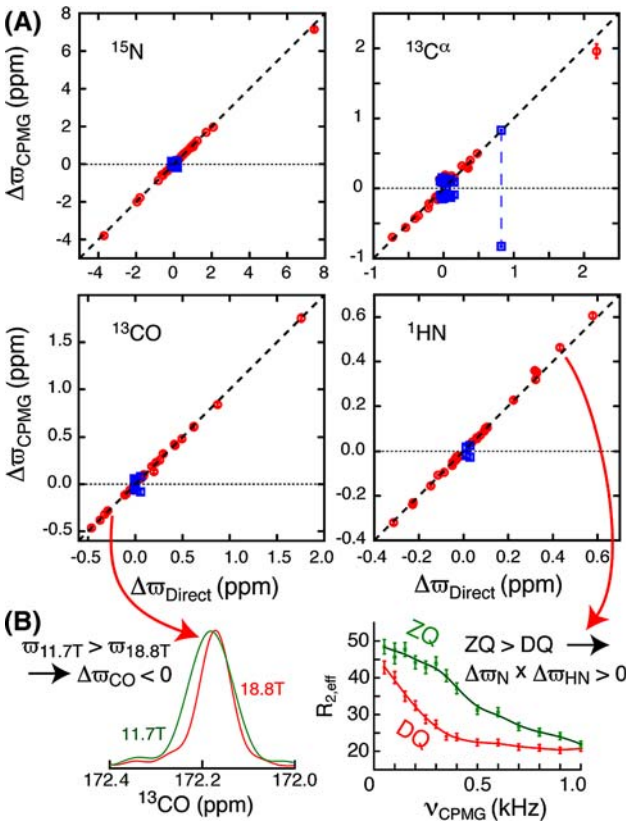

Fig. 3 (**a**) Correlations between changes in chemical shifts between ground (*P*) and excited (*PL*) states in the $P \leftrightarrow PL$ exchanging system described in the text that are measured by CPMG relaxation dispersion NMR ($\Delta\varpi_{CPMG}$, *Y*-axis) and shift changes that are obtained directly from measurements on both *P* and *PL* ($\Delta\varpi_{Direct}$, *X*-axis). Points indicated in red are those for which there was no ambiguity in determination of the sign of $\Delta\varpi$, as described below; points in blue derive from cases where it is not possible to establish the sign of $\Delta\varpi$ and both possible values of $\Delta\varpi$ are given. A less complete correlation of the sort given here was published previously (Hansen et al. 2008b). (**b**) The signs of chemical shift differences are obtained by the method of (Skrynnikov et al. (2002), that compares peak positions in ^{1}HN–X, X = {^{15}N,^{13}CO} correlation spectra. Cross-peaks of the major state are closer to correlations in the excited state at lower field and hence $\Delta\varpi_{CO} = \varpi_{CO}(\text{ground}) - \varpi_{CO}(\text{excited}) < 0$ in the example provided. Signs of $\Delta\varpi_{HN}$ are obtained by comparison of ^{1}HN–^{15}N double- (DQ) and zero- (ZQ) quantum dispersion profiles; the relative sizes of the profiles can be used to obtain the relative signs of $\Delta\varpi_N$ and $\Delta\varpi_{HN}$, with the sign of $\Delta\varpi_{HN}$ following once the sign of $\Delta\varpi_N$ is known (Korzhnev et al. 2005)

Residual anisotropic interactions: a further probe of the excited state

Residual dipolar couplings (RDCs) and chemical shift anisotropies (RCSAs) are particularly valuable restraints for structural studies of biomolecules by NMR because they complement the local structural information that is inherent to chemical shifts, scalar couplings and nuclear Overhauser enhancements (Tjandra and Bax 1997; Tolman et al. 1995). Because RDCs and RCSAs are sensitive to orientations of dipole vectors and tensor components with respect to a molecular frame they provide long range information (Tjandra and Bax 1997; Tolman et al. 1995) that has been exploited in numerous studies over the past decade (Prestegard et al. 2005). In the past year it has become possible to measure residual anisotropic interactions in the invisible state (Igumenova et al. 2007; Vallurupalli et al. 2007) through spin-state selective relaxation dispersion experiments, illustrated schematically for the case of an ^{1}HN–^{15}N spin pair in Fig. 4a. The key to understanding how these experiments work is to realize that the effective chemical shift difference between TROSY multiplet components that derive from the ground and excited states is $|\Delta\nu - \Delta D/2|$, while the corresponding distance between anti-TROSY components is $|\Delta\nu + \Delta D/2|$, where $\Delta\nu$ is the difference in chemical shifts between states (in Hz) and ΔD is the corresponding difference in dipolar couplings

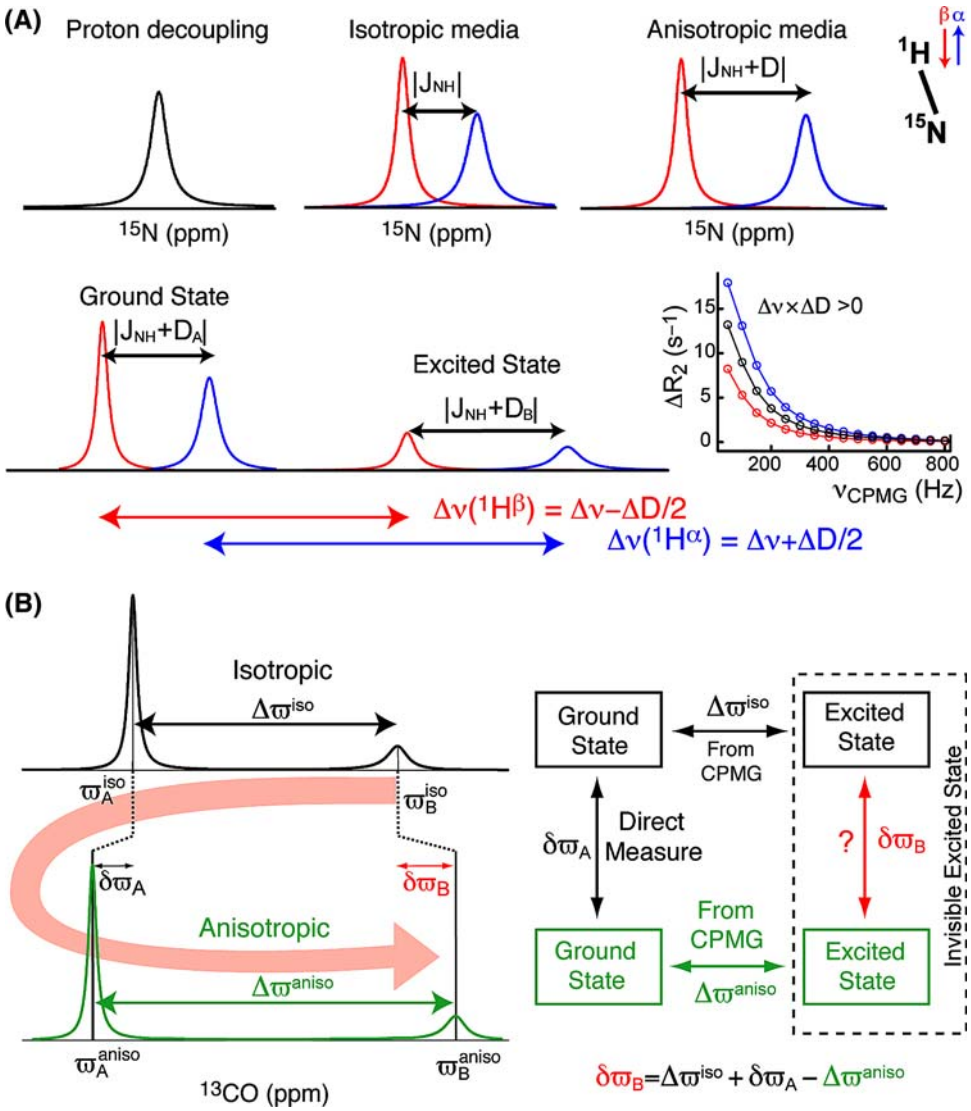

Fig. 4 Measurement of anisotropic interactions in the invisible, excited state (Igumenova et al. 2007; Vallurupalli et al. 2007). (**a**) Changes in $^{1}HN–^{15}N$ dipolar couplings between ground and excited states ($\Delta D = D_A - D_B$) can be measured by recording spin-state selective CPMG relaxation dispersion experiments that quantify exchange between TROSY or anti-TROSY magnetization components. Dispersion profiles ($\Delta R_2 = R_2(\nu_{CPMG}) - R_2(\nu_{CPMG} = \infty)$) reporting on $\Delta\nu$ (black) and $\Delta\nu \pm \Delta D/2$ (blue and red) are obtained where $\Delta\nu$ is the difference in chemical shifts (Hz) between exchanging states (Vallurupalli et al. 2007). (**b**) Changes in ^{13}CO chemical shifts of the excited state resulting from alignment can also be quantified through measurement of dispersion profiles in both isotropic and anisotropic media (Vallurupalli et al. 2008a)

(Vallurupalli et al. 2007). Thus, dispersion profiles that are recorded with the dipolar coupled spin partner in the up or down spin-state will be distinct and fitting the dispersion curves simultaneously allows for the extraction of ΔD (since the sign of $\Delta\nu$ is known). Dipolar couplings in the excited state can be obtained directly from ΔD since RDC values in the visible, ground state are readily measured from a variety of robust experiments (Bax 2003).

Chemical shift changes in the excited state upon alignment can also be measured, although a slightly more elaborate procedure is needed (Vallurupalli et al. 2008a), Fig. 4b. To understand how this is done consider the 'chemical shift cycle' in Fig. 4b, consisting of the four relevant states as shown. It is possible to measure 3 of the 4 chemical shift differences and the fourth difference (the one of interest) can be readily obtained by noting that the sum of the shift differences over the complete cycle must be zero.

Figure 5a plots on a polypeptide chain backbone the $^{1}HN–^{15}N$, $^{1}H^{\alpha}–^{13}C^{\alpha}$, $^{1}HN–^{13}CO$ dipolar vectors and the principal axes of the ^{13}CO CSA tensor, whose orientations are probed by current relaxation dispersion experiments (Hansen et al. 2008a; Vallurupalli et al. 2007, 2008a). Correlations between the RDCs and RCSAs obtained by applying the relaxation dispersion methodology to the $P \leftrightarrow PL$ exchanging system described above and the corresponding values measured directly on the PL 'ground' state are shown in Fig. 5b. As with the chemical shift data (see Fig. 3a) very good agreement is obtained, indicating that, at least in this case, accurate anisotropic restraints can be measured for the invisible state. Not surprisingly, the measured RDCs, RCSAs and chemical shifts form powerful restraints for structure calculations of the invisible state. A combination of RDCs, RCSAs and (φ,ψ) torsion angle restraints that have been generated from backbone chemical shifts with the program TALOS (Cornilescu et al.

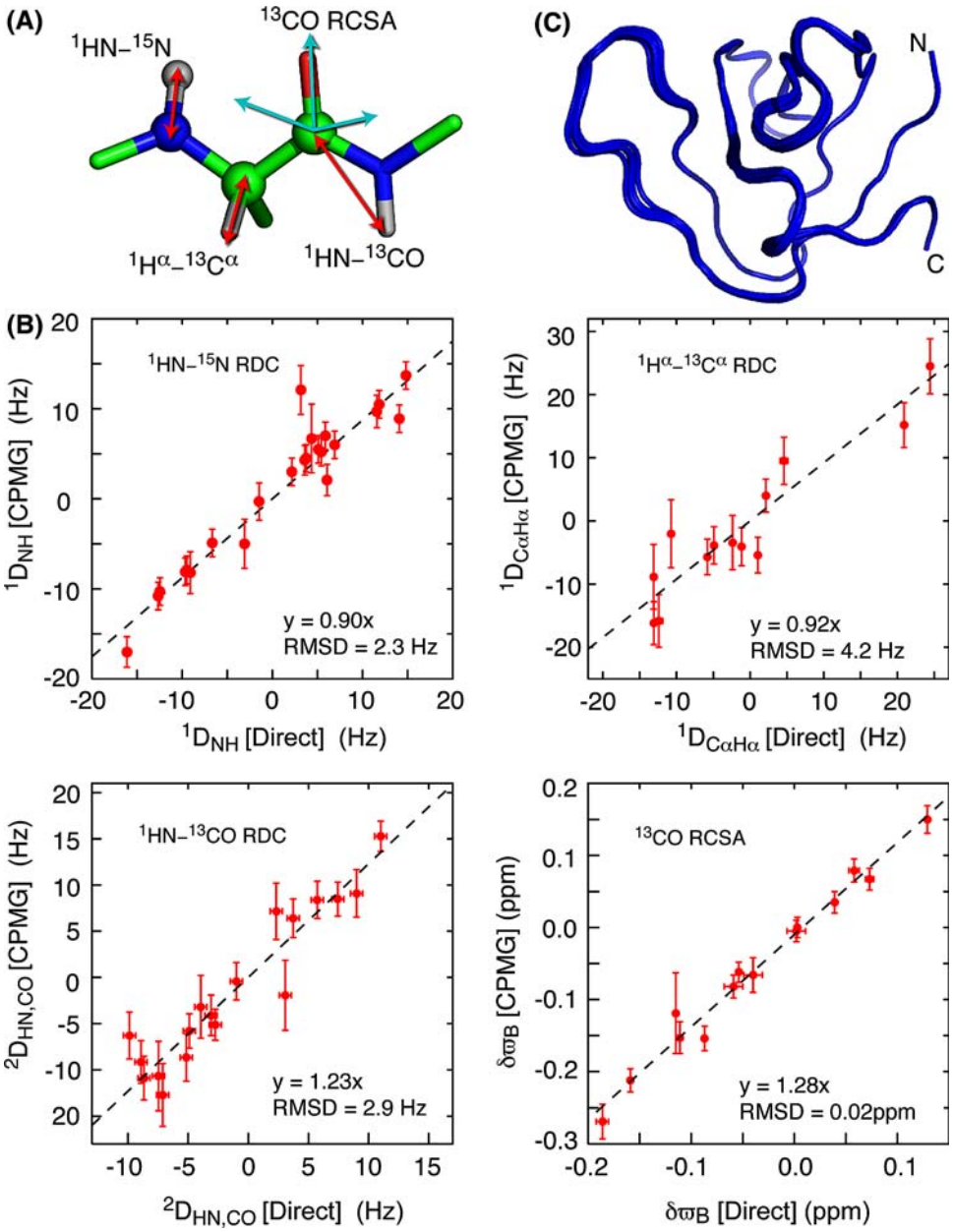

Fig. 5 (**a**) Polypeptide backbone showing the dipolar and CSA interactions that are currently probed from relaxation dispersion NMR experiments. (**b**) Correlation between RDCs and RCSAs extracted from CPMG based methods (*Y*-axis) and measured directly (*X*-axis) using the $P \leftrightarrow PL$ exchanging system described in the text. The best-fit line is illustrated in each case along with the RMSD between CPMG and directly measured values. A non-unity value for the slopes results from slightly different amounts of alignment media in each set of measurements. (**c**) Ensemble of 10 low energy structures of the invisible, excited state (*PL*) calculated from ^{15}N, ^{1}HN, ^{13}C$^{\alpha}$, ^{13}CO chemical shifts, ^{1}HN–^{15}N, ^{1}H$^{\alpha}$–^{13}C$^{\alpha}$, ^{1}HN–^{13}CO RDCs and ^{13}CO RCSAs derived exclusively from relaxation dispersion NMR experiments (Vallurupalli et al. 2008b)

1999) have been used in a torsion angle molecular dynamics protocol that is described elsewhere (Vallurupalli et al. 2008b) to produce well defined backbone folds of the invisible Ark1p ligated form of the Abp1p SH3 domain, Fig. 5c. Although at present this is the only example that makes use of dispersion-based data to determine structures of invisible states the results are encouraging.

Prospects for the future

Since the groundbreaking paper of Loria et al. almost a decade ago showing that complicating effects of spin exchange could be separated from chemical exchange in CPMG relaxation dispersion experiments (Loria et al. 1999) there have been rapid advances in this field. It is now possible to measure relaxation rates of a large set of backbone nuclei (Hansen et al. 2008b; Ishima et al. 2004; Ishima and Torchia 2003; Korzhnev et al. 2005; Loria et al. 1999; Tollinger et al. 2001), side-chain aromatic moieties (Teilum et al. 2006) and methyl groups (Mulder et al. 2001) in suitably labelled proteins. Different types of coherences can be probed to get further insight into a given dynamic process and to cross-validate the extracted exchange information (Dittmer and Bodenhausen 2004; Kloiber and Konrat 2000; Korzhnev et al. 2004a, 2005). Many of the advances in NMR methodology have required the development of new labelling schemes to produce

isolated spin systems that prevent homonuclear magnetization transfer that would otherwise occur during lengthy CPMG pulse trains. For example, to properly record $^{13}C^{\alpha}$ dispersion profiles from which the $^{13}C^{\alpha}$ chemical shifts and $^{1}H^{\alpha}$–$^{13}C^{\alpha}$ RDCs described herein are derived it is necessary to prepare samples with isolated ^{13}C label at the C^{α} backbone position and this can be accomplished using [2-^{13}C]-glucose, as described previously (Lundstrom et al. 2007). Labelling schemes have also emerged for preparing proteins with isolated ^{13}C probes in methyl and aromatic groups (Lundstrom et al. 2007; Teilum et al. 2006). Further developments in this area can be anticipated and will be important for the extension of the methodology to as many protein sites as possible.

In addition to the advances in pulse sequences and labelling that have enabled the practical application of the CPMG methodology to problems in biochemistry, there have also been improvements in computation (Cavalli et al. 2007; Lindorff-Larsen et al. 2005; Shen et al. 2008). These will be critical for the success of structure calculations that are based on the chemical shift and anisotropic restraints that emerge from dispersion data. For example, many of the 'excited' states that are currently probed are highly dynamic (Korzhnev and Kay 2008) and a description of their structural properties can only be accomplished in the context of an ensemble. How to best use the averaged chemical shift information and RDC/RCSA values in this case remains an open question.

It is clear that in the past decade there has been tremendous progress in exploiting the "excess contributions to transverse relaxation" that were initially cast aside as an uninteresting by-product of the analysis of backbone amide transverse relaxation rates. It is equally clear that there are a large number of important biochemical systems that will prove to be amenable to relaxation dispersion studies and that the emerging picture of millisecond time-scale motions and structural transitions that is obtained from such studies will significantly contribute to our understanding of functional molecular dynamics.

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